Video Article

Implementing Dynamic Clamp with Synaptic and Artificial Conductances in Mouse Retinal Ganglion Cells

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Abstract

Ganglion cells are the output neurons of the retina and their activity reflects the integration of multiple synaptic inputs arising from specific neural circuits. Patch clamp techniques, in voltage clamp and current clamp configurations, are commonly used to study the physiological properties of neurons and to characterize their synaptic inputs. Although the application of these techniques is highly informative, they pose various limitations. For example, it is difficult to quantify how the precise interactions of excitatory and inhibitory inputs determine response output. To address this issue, we used a modified current clamp technique, dynamic clamp, also called conductance clamp [1, 2, 3] and examined the impact of excitatory and inhibitory synaptic inputs on neuronal excitability. This technique requires the injection of current into the cell and is dependent on the real-time feedback of its membrane potential at that time. The injected current is calculated from predetermined excitatory and inhibitory synaptic conductances, their reversal potentials and the cell's instantaneous membrane potential. Details on the experimental procedures, patch clamping cells to achieve a whole-cell configuration and employment of the dynamic clamp technique are illustrated in this video article. Here, we show the responses of mouse retinal ganglion cells to various conductance waveforms obtained from physiological experiments in control conditions or in the presence of drugs. Furthermore, we show the use of artificial excitatory and inhibitory conductances generated using alpha functions to investigate the responses of the cells.

Video Link

The video component of this article can be found at http://www.jove.com/video/50400/

Introduction

The retina is a near-transparent neural tissue lining the back of the eye. Many studies use the retina as the model to investigate the first steps in visual processing and mechanisms of synaptic signaling. Since the retinal network in the whole-mount preparation remains intact after dissection, it represents an ideal system to study synaptic interactions as its physiological responses are very similar to the *in vivo* conditions. Thus, using an isolated retina the properties of its neurons can be studied using patch clamp techniques (for reviews on the technique, see [6,9,13]). Identification of the exact contribution of specific circuits and neurotransmitters to ganglion cell response, however, is usually hindered as pharmacological agents act on various sites.

Physiological responses of retinal neurons to light, the natural stimulus, can be recorded with glass pipettes filled with intracellular fluid. Using patch clamp techniques, neuronal responses to light stimulation can be recorded as membrane potential fluctuations (current clamp) or as currents (voltage clamp). By holding the membrane potential at different voltages and implementing *a posteriori* conductance analysis, it is possible to isolate inhibitory and excitatory synaptic inputs [5,12]. This type of experiments can be carried out in normal bathing medium and in the presence of different pharmacological agents to isolate the contribution of different neurotransmitters and receptors to neuronal responses. A wealth of studies from many laboratories characterized the dependence of spiking output and excitatory and inhibitory inputs on stimulus properties such as size, contrast, spatial and temporal frequencies, direction, orientation and other stimulus variables. Although these experimental approaches provide information about the relationship between spike output and synaptic inputs as a function of stimulus properties, interpretation of the contribution of specific cell types and their synaptic inputs to cell excitability is not straightforward. This is due to the fact that typically both excitatory and inhibitory inputs vary with stimulus properties and thus, it is not possible to assess the precise impact that changes in either of these inputs has on neuronal spiking.

An alternative approach to circumvent these limitations is to carry out dynamic clamp recordings, which allow a critical evaluation of the contribution of individual synaptic inputs to spiking output. The dynamic clamp technique allows direct injection of current into the cell and the amount of current injected at a given time depends on the recorded membrane potential at that time [1,2,3] (for review, see [7,14]). It is a modified current clamp set-up where a real-time, fast feedback interaction between the cell under recording and the equipment comprising specialized

hardware, software and a computer is achieved. The amount of current injected into the cell is computed accordingly. Hence, the advantage of this method is that the cell can be stimulated with different combinations of conductance waveforms, and its response will mimic the activation of receptors that mediate synaptic inputs. For example, comparison of the response to injection of excitatory and inhibitory conductances for a small spot with the response to injection of excitatory conductance for a small spot only provides information about the impact of inhibition on cell response. Likewise, other combinations of physiologically recorded conductances can be co-injected to reveal how stimulus-dependent changes in excitatory and/or inhibitory conductances affect spike output.

In our study, the dynamic clamp technique is used to demonstrate the impact of the relative amplitude and timing of synaptic inputs on the firing properties of retinal ganglion cells. Various conductances obtained from physiological experiments in control conditions or in the presence of pharmacological agents were employed as inputs. In addition, artificial conductances based on alpha functions were also used in order to investigate how synaptic inputs are integrated by neurons. Thus this is a versatile technique that allows various types of conductance generated either physiologically, pharmacologically or computationally to be injected into the same ganglion cell, so comparison of responses to these inputs can be made.

Protocol

1. General Set Up and Tissue Preparation

1. Keep the mouse in darkness for 30 min (aim to reduce its stress level). While waiting, prepare 1 L of extracellular solution. First dissolve 1.9 g of sodium bicarbonate in half a liter of Milli-Q water. Its pH is maintained at 7.4 by bubbling with 95% O_2 and 5% CO_2. Five minutes later, dissolve 8.8 g of Ames Medium in 100 ml of Milli-Q water, add to the sodium bicarbonate solution, top up to 1 L with Milli-Q water and mix well. Keep this solution carboxygenated for the rest of the experiment.
2. Take 250 ml of the carboxygenated Ames Medium and set up the fluid reperfusion system in the electrophysiological set-up. Keep the solution carboxygenated.
3. Uniformly smear a thin layer of vacuum grease onto the bottom of the perfusion chamber. Seal it with a cover slip and make sure it is air-tight. Take a small amount of grease (~ 2 mm in diameter) and position it on the top and bottom ends of the chamber. These balls will hold the grid (platinum frame strung with dental floss) in place and prevent it pressing too hard on the retina. Fix the chamber onto the platform and align both openings.
4. The animal is first anaesthetized with Isoflurane for 2 min, and then sacrificed by cervical dislocation. Quickly remove eyes with a pair of small scissors (curved blades), wash thoroughly with carboxygenated extracellular fluid in a small beaker, and then place in a Petri dish filled with carboxygenated extracellular fluid.
5. Under the dissecting microscope, make an insertion hole in the cornea (~ 2 mm from the edge) using a 19 gauge needle. Repeat for the other eye. This important step should be quick to allow oxygenation of both retinae. Remove one cornea with a small pair of iris scissors by cutting parallel to its edge. Next, remove the iris and lens with a pair of fine forceps. Repeat for the other eye. Transfer one eye cup into a beaker containing carboxygenated extracellular solution.
6. With a scalpel blade, cut one eye cup into half, to be able to flatten the whole-mount and to obtain two whole-mounts per retina. Carefully separate the retina from the pigment epithelium using a blunt dissecting probe or by gently pulling it off. Once detached, the retina is fairly transparent. Note the convex surface is the photoreceptors side. The concave surface is the ganglion cells side and it may stick to itself due to the presence of sticky vitreous humour.
7. With a pair of fine forceps, hold the edge of the detached retinal tissue close to the ora serrata and grab the ora serrata with another pair of fine forceps, pull it towards the center of the retina. This is a delicate process and may take a few attempts. If successful, ora serrata, ciliary body and vitreous humor are removed by this process, and the curvature of the retina is reduced (*i.e.* flatter).
8. Trim the edges, and then transfer it to the perfusion chamber with ganglion cells facing up. Hold the tissue in place by placing the grid onto the grease balls. Place the remaining tissue with the other eye cup for later use. Similar procedures for retinal dissection are described in Middleton and Protti [16] and Pottek *et al.* [17].
9. Mount the recording chamber under an upright microscope. Immediately set up the continuous perfusion of the retina with carboxygenated extracellular solution (35.5 °C) at a rate of 3 - 5 ml/min. Make sure the grounding electrode is in place.
10. Attached to the microscope is a digital camera allowing visualization of the tissue. The whole set up sits above an air table (shock absorption) and inside a Faraday cage (blocks external static and non-static electric fields).
11. Turn on the light source (infrared, > 850 nm) and with a 40X objective, bring its focus onto the cell bodies of ganglion cells. Find the central area of the whole-mount for recording.
12. Defrost a frozen vial of K^+ based intracellular solution (300 µl, pH = 7.4) containing (in mM) K-gluconate: 140, HEPES acid: 10, $MgCl_2$: 4.6, ATP-Na^+: 4, GTP-Na^+: 0.4 and EGTA: 10. All reagents purchased from Sigma-Aldrich.

2. Patching Cell Bodies of Retinal Ganglion Cells

1. First fire polish the ends of borosilicate capillary glass tubing, then pull them with a Glass Microelectrode Puller with two heating steps (resistance: 5 - 8 MΩ).
2. Add 3 µl of Lucifer Yellow (final concentration 0.2%) to the intracellular solution, mix well, and centrifuge it. Next, transfer the top 85% of the solution into a 1 ml syringe, connect it to a 0.22 µm filter unit, then to a 30 G reusable hypodermic needle. Refrigerate.
3. Fix the glass pipette, of similar tip size as those used for recordings, in its holder and move it under the microscope using a micromanipulator. Under a 40X objective, slowly lower the pipette until it makes a small dimple on the surface of the inner limiting membrane. Carefully advance the pipette forward until it catches a small amount of tissue, and then move it upward and/or sideways to tear a small hole to expose the cell bodies of the ganglion cells. The smaller the hole, the higher the degree of integrity of the retinal network is maintained. If desired, sulforhodamine 101 (SR101; 0.5 - 1 µM) can be added to the extracellular medium to label damaged neurons by using fluorescence microscopy [15].
4. Fill up a clean pipette with 8 - 10 µl of intracellular solution. Insert it into the holder attached to the amplifier's head stage and make sure the chlorinated silver chloride electrode is submerged. Discard if dirt and/or air bubbles is/are present.

5. Turn on all equipment. We used an EPC 8 patch clamp amplifier controlled by PatchMaster to achieve whole-cell recordings in voltage clamp configuration. Note that other amplifiers that allow current clamp recordings in fast mode can also be used. Apply and maintain positive pressure in the pipette solution. Select a cell with a large cell body so most likely it is a ganglion cell rather than a displaced amacrine cell or a glial cell. Slowly lower the pipette tip so it is making a small dimple on the surface of the membrane, stop, and release the pressure. Immediately lower the holding potential to - 60 mV. Once a gigaseal (GΩ) is achieved between them, gently suck to break the membrane to achieve a whole-cell patch clamp configuration. This procedure is a delicate step, if too much negative pressure is applied, then the connection becomes leaky; if too little, the membrane remains intact.
6. Over time, Lucifer Yellow will fill the cell, allowing visualization of the cell's morphology. If stable, this set-up should last 30 min or more.

3. Recordings of Ganglion Cells Using Dynamic Clamp

1. First, a current-voltage test (from - 75 to + 35 mV every 10 mV) is executed using PatchMaster. Proceed to later tests only if a large Na^+ current (> 1 nA) is present. Note that in the rare occasion where a displaced amacrine cell is patched, in that case, will not respond with trains of action potentials to subsequent current injections.
2. Open LabVIEW and execute the custom written Neuroakuma program, then load various conductance waveforms. Set repeat number to 8 (6 - 8 for statistical purpose). Set reversal potentials of excitatory and inhibitory conductances at 0 and - 75 mV respectively. Select one matching pair of excitatory and inhibitory conductances for testing. Excitatory conductance trace (in green) and inhibitory conductance trace (in red) will appear in the lower panel.
3. Go back to PatchMaster, select current clamp mode, and immediately switch the amplifier to 'CC + Comm FAST' current clamp mode. This mode allows to accurately follow rapid changes in membrane potential. If a good seal between the membrane and pipette is maintained, little/no compensation is needed; otherwise, the recording will not last long. In Neuroakuma, press the "record" button. The cellular response (usually with a phasic burst of action potentials, **Figure 1A**) will appear on the upper panel. Inject current into the cell with low scaling of conductances first, if little/no response is observed, increase in small increments until it produces a strong response. Excessive current injection can kill the cell. Once tests are completed, select a new pair of conductance waveforms, repeat the above for all pairs of physiological and artificial conductances. The physiological currents (I) injected in real time are based on:

$$I(t) = G_{exc}(t) \times (V_m(t) - V_{exc}) + G_{inh}(t) \times (V_m(t) - V_{inh})$$

Conductance (G in nS) could be synaptic or artificial. Excitatory and inhibitory synaptic conductance waveforms were collected from previous experiments performed by Protti, Di Marco, Huang, Vonhoff, Nguyen and Solomon (unpublished results) in response to different visual stimuli in control conditions and in the presence of tetrodotoxin (TTX, 1 µM). Artificial conductance waveforms were modeled using an alpha function. V_m (in mV) is the recorded membrane potential. G_{exc} and G_{inh} represent excitatory and inhibitory conductances respectively whilst V_{exc} and V_{inh} represent the reversal potentials of excitatory and inhibitory conductances respectively. Time is t in ms. Sampling rate is 40 kHz.

$$I_s = I_0(t/\alpha)e^{-\alpha t}$$

The above equation is an alpha function (I_0 = maximum current; 1/α = time to peak (sec^{-1}) of the current). The rise time and decay time of the synaptic current is dictated by α [4]. The excitatory conductance was unchanged whilst the latency of the inhibitory conductance was modified, reducing its delay relative to the onset of excitation (**Figure 2D**).

4. After recording, carefully retract the pipette so the cell body stays intact. Immediately fix the tissue in 4% paraformaldehyde for 30 min, followed by three 10 min washes with 0.1 M of phosphate buffer. Refrigerate and perform antibody staining the following day or within a week.

4. Antibody Staining Against Lucifer Yellow Filled Retinal Ganglion Cells

1. Antibody staining against Lucifer Yellow filled ganglion cells at room temperature (primary anti-Lucifer Yellow rabbit IgG (dilution = 1:10,000) for five days; on the sixth day, overnight staining with secondary goat anti-rabbit IgG (dilution = 1:500)). Wet mount the retina in Fluorescent Preserving Media. Cover slip and seal with nail polish.
2. Take confocal pictures of the cell morphology using a Leica Spec-II confocal microscope (**Figure 1B**). The presence of an axon allows the confirmation of ganglion cells.

Representative Results

The contribution of different sources of inhibitory inputs to ganglion cell responses is demonstrated through the application of various conductance waveforms. These waveforms were obtained with stimuli of different luminance in normal conditions and in the presence of TTX, a voltage-gated Na^+ channel blocker that blocks action potential generation only in a subset of inhibitory retinal interneurons. **Figure 2A** shows a representative response to injection of excitatory and inhibitory conductance waveforms recorded in response to stimulation with a small black spot on a grey background in normal conditions. When the conductance waveforms obtained with a black spot of different size in the presence of TTX were injected into the same cell, only a weak response was observed (**Figure 2B**).

Figure 2C illustrates the responses of another ganglion cell to injection of various pairs of conductance waveforms in which the ratio between control excitatory and inhibitory conductance was manipulated (G_{exc}:G_{inh} ratios: 1:0 to 1:2). It is clear that the response of the cell decreased as the level of inhibition was increased. Hence, manipulating the balance between excitation and inhibition allows the quantification of their impact on neuronal output.

We also tested the effect of changing the relative timing between excitation and inhibition on neuronal responses. As illustrated in **Figure 2D**, the responses of a different ganglion cell were reduced as the latency of the inhibitory conductance was decreased, demonstrating that the strength of the response depends on the timing of its synaptic inputs.

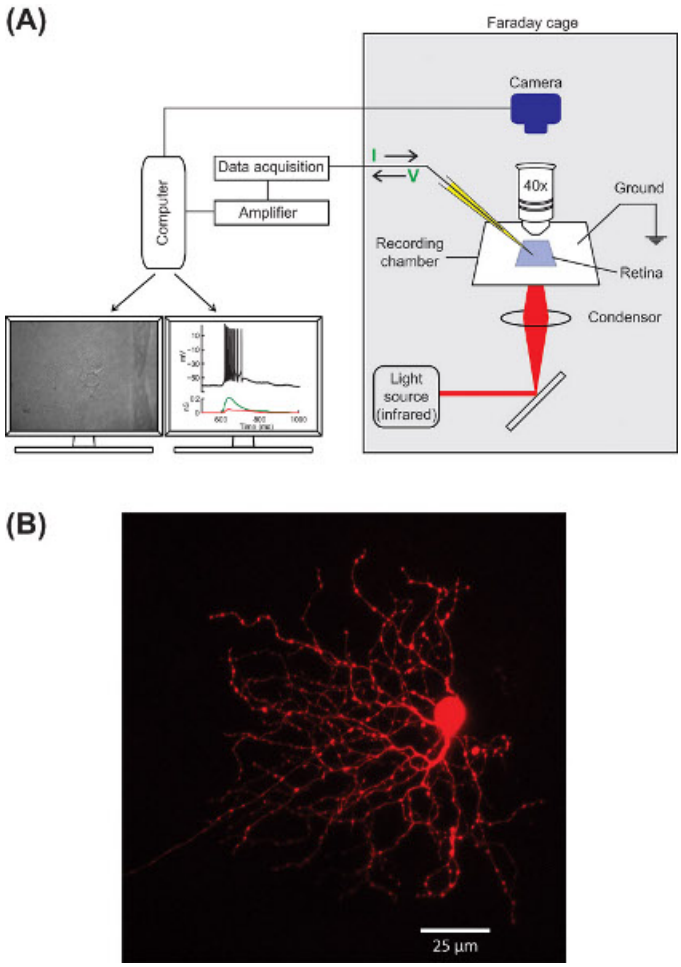

Figure 1. (A) Experimental set-up in schematic form. A digital camera is used to visualize cell bodies of ganglion cells in order to patch clamp them (left monitor). Responses of each cell to various current injections were first recorded, amplified and then displayed on the right monitor. Note that a feedback loop is also set up between the computer and the cell such that membrane potential (V) of the cell at a given instance is used to calculate the current (I) to be injected. (**B**) The morphology of a ganglion cell visualized under a Leica Spec-II confocal microscope after recording and immunocytochemical staining. Final picture was produced using ImageJ after collapsing a stack of images. Click here to view larger figure.

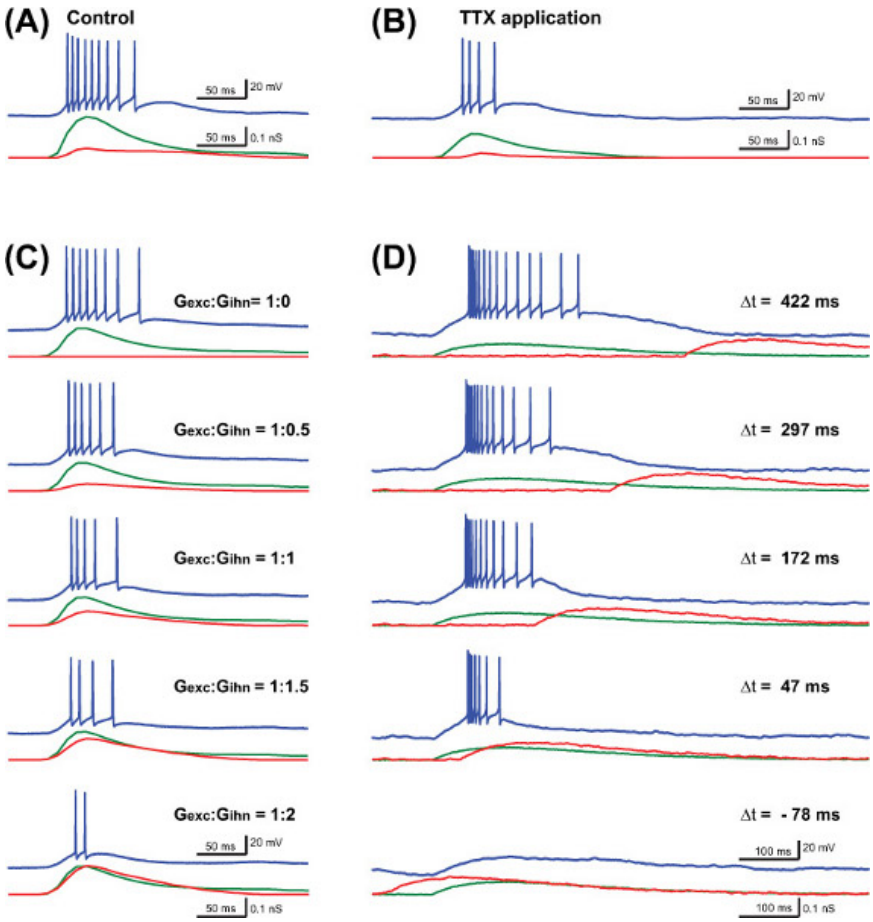

Figure 2. Responses of a ganglion cell to injection of excitatory (green traces) and inhibitory (red traces) conductance waveforms obtained from experiments carried out in control conditions (A) and after bath application of tetrodotoxin (TTX, B). (C) Responses of another ganglion cell to various pairs of conductance waveforms. The ratios between excitatory conductance (G_{exc}) and inhibitory conductance (G_{inh}) were changed from 1:0 to 1:2. As the degree of inhibition increased, the response of the cell was decreased. (**D**) Responses of a ganglion cell to the same level of excitation in which the onset of inhibition was varied. Δt represents the time difference between the onset of inhibitory and excitatory conductances. As the delay of inhibition was reduced, the response of the cell became weaker. Click here to view larger figure

Discussion

Here we show the use of dynamic clamp to assess the influence of the ratio and relative timing of excitation and inhibition on retinal ganglion cell output. Dynamic clamp makes use of computer simulations to introduce physiologically recorded or artificial synaptic conductances into living neurons. This methodology provides an interactive tool by which conductances can be modified and injected into neurons for computing their influence on neuronal responses. Conductance waveforms can be obtained from experiments in which visual stimuli are used to activate photoreceptors in control conditions and in the presence of pharmacological agents to isolate the contribution of specific cell or circuits. Injection of different combinations of these conductance waveforms reveals their contribution to spiking output. The use of dynamic clamp recordings also enables manipulation of the levels of background noise, which may take place under different light-adaptation conditions. Furthermore, conductance waveforms can also be generated from models of synaptic inputs, such as alpha functions, and also for synaptic inputs with voltage dependency (*i.e.* NMDA receptors) as well as for voltage-gated ion channels or electrically coupled cells. Hence, comparisons made between various conditions open up enormous possibilities to understand the contribution of different synaptic mechanisms to neuronal responses and signal processing in neural networks. It is important to remark that even when different types of ganglion cells express distinctly different types and densities of voltage-gated channels, we found that the results of the integration of synaptic conductances were consistent across cell types. Thus, changes in ratio and timing of the excitatory and inhibitory conductances were similarly reflected in the responses although spike frequency, inter-spike intervals and adaptation properties varied according to cell type (data not shown).

Although dynamic clamp offers many advantages, there are some limitations intrinsic to the technique (for review, see [7]). A major one is that conductance injection in the soma may not accurately represent synaptic inputs mediated by receptors and channels that are specifically distributed along the membrane of dendritic processes, and any non-linear interactions that can occur in the dendrites. Thus, conductances distal to the injection site can be simulated only approximately. Although dynamic clamp was successfully applied to dendrites in pyramidal cells doing quadruple whole-cell recordings [8], the morphological complexity of ganglion cell dendritic processes precludes a realistic simulation of the spatial distribution of synapses by doing dendritic recordings. The approach presented here uses conductance waveforms computed from somatic voltage-clamp experiments that also rely on a point-source electrode and assumes linear interactions between excitatory and inhibitory inputs,

an assumption widely accepted in the community. Thus, these studies provide significant information about the effects of changes in synaptic conductances on neuronal integration at the level of the soma and axon hillock.

Our experiments do not replicate physiological responses that also recruit voltage-gated conductances in ganglion cell dendrites. This, however, could also be accomplished by modifying the model equations so that the current injected include dendritic conductances and cable effects based on a multi-compartment model (as described in [7]).

Another limitation is that activity-dependent changes that modify neuronal response, *i.e.* changes in intracellular calcium or second messengers triggered by activation of membrane channels or receptors are not reproduced in these dynamic clamp recordings. Although our model does not deal with that level of complexity which might be specific for some cell types, simulations involving a calcium pool and calcium influx to this pool in real-time based on the voltage fluctuations of the recorded neuron were successfully implemented [10].

Even though a sampling rate of 10 kHz would accurately follow the time course of action potentials, our recordings instead used a sampling rate of 40 kHz. This higher sampling rate is required to maintain stability in the system and to allow realistic and highly accurate recordings (for review see [11]).

The experimental approach described here does not require to keep the retina dark-adapted. Conductance waveforms obtained from dark-adapted retinae in physiological conditions were used to calculate the current to be injected into the cell; in this way the current injected mimicked physiological stimulation with light. Nevertheless, it would still be possible to keep the tissue dark-adapted and thus, be able to compare the responses to conductance injection with those to light stimulation. In this instance, physiological properties of ganglion cells (response polarity, *i.e.* ON, OFF or ON-OFF, spatial and temporal frequency sensitivity, direction selectivity, etc.) could be classified before the application of various conductance waveforms to evaluate whether or not injection of conductances from a particular cell type has different effects depending on the cell type they are applied to.

Overall, we consider that dynamic clamp is a very powerful technique to help reveal the interactions between excitatory and inhibitory synaptic inputs that generate neuronal output, to investigate the role of different voltage-gated conductances in neuronal firing as well as to test hypotheses about synaptic mechanisms. Application of this technique in the retina is particularly useful, as the synaptic inputs onto several ganglion cell types have been thoroughly characterized as a function of the physiological stimulus. Similarly, this approach can provide insights into the function of neuronal circuits in other areas of the central nervous system.

Disclosures

All procedures were first approved by the Animal Care Ethics Committee of The University of Sydney, and then performed in accordance with the guidelines of the Australian Code of Practice for the Care and Use of Animals for Scientific Purposes (National Health and Medical Research Council of Australia).

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