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Imaging light-modulated release of synaptic vesicles in the intact retina: Retinal physiology at the dawn of the post-electrode era

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Abstract

Here, we illustrate an optical method for directly measuring the light-regulated synaptic output of neurons in the retina. The method allows simultaneous recording from many retinal neurons in intact flat-mount preparations of the vertebrate retina. These recordings depend on the use of FM1-43, an activity-dependent fluorescent dye that selectively labels synaptic vesicles. Release of the dye, which occurs upon vesicle exocytosis, is detected with 2-photon microscopy. This utilizes an infrared laser to trigger fluorescence excitation of the dye, while minimally perturbing retinal activity by activating phototransduction in rods and cones. Using this approach, one can measure activity of single neurons in the intact retinal network and populations of neurons in different layers of the retina, providing a new way to examine the function of retinal synapses and how visual information is processed.
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1. Introduction

Over the past 40 years, electrophysiology has been the main experimental tool for understanding how neurons in the retina respond to light and communicate with one another to process visual information. These recordings have elucidated the basic neural circuit of the retina and have explained the biophysical mechanisms underlying the initial phototransduction event in rods and cones and the subsequent synaptic events in downstream retinal neurons. Microelectrode and patch electrode recordings have provided a temporally accurate glimpse of the individual activities of retinal neurons in response to light. However, recent advances in optical recording methods provide new opportunities for functional studies of the retina, with several potential advantages. Optical recordings are relatively non-invasive, and depending on the type of indicator dye, can reveal different aspects of neuronal activity, including changes in intracellular ion concentrations, changes in membrane potential, and release of synaptic vesicles. Unlike single cell electrical measurements, optical recordings can be made from many neurons at once, revealing the 'big picture' of how the retina responds to light.

Our approach to functional imaging of the retina has utilized the fluorescent dye FM1-43 as an activity-dependent marker of vesicle exocytosis and endocytosis. FM1-43 is a lipophilic, styryl dye that increases in fluorescence when it partitions into biological membranes, thereby labeling the surface of cells (Cochilla, Angleson, & Betz, 1999; Ryan, 2001). Since it is amphipathic, FM1-43 inserts into the outer leaflet of the plasma membrane and cannot 'flip' into the inner leaflet. However, if a cell exposed to FM1-43 happens to be endocytosing membrane from the cell surface, the dye will become trapped inside internalized vesicles. By washing away the surface dye,

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one can selectively see and measure the label in these vesicles and thereby measure the rate of endocytosis. Subsequent exocytosis of labeled vesicles will allow the dye to be released from the terminal. Thus, by measuring the decrease in FM1-43 fluorescence from pre-loaded synaptic terminals, the rate of exocytosis can also be estimated.

FM1-43 and related dyes have been used for many years to study synaptic vesicle cycling in presynaptic terminals at the frog neuromuscular junction (Betz & Bewick, 1992; Betz, Mao, & Smith, 1996) and in synapses formed between dissociated hippocampal neurons in culture (Ryan, 2001). However, FM1-43 has been used very little to study synapses in more complex neural circuits (but see, Kay et al., 1999). At least in part, this results from technical difficulties. When added to freshly-obtained slices of the brain or retina, FM1-43 stains not only the presynaptic terminals, but other structures, including cell bodies and dendrites (Rea et al., 2004). This non-specific labeling obscures the synaptic labeling, decreasing the dye's usefulness as a selective marker of synaptic release. For reasons that are not completely explained, making retinal slices or dissociating individual retinal neurons dramatically alters the dye uptake pattern. We have discovered that simply by carrying out the loading procedure in intact retina, we can obtain synapse-specific labeling (Rea et al., 2004). Subsequent imaging of presynaptic terminals in dissociated cells, retinal slices, or retinal flat-mounts has provided new opportunities for studying how physiological stimuli, including light, and regulate synaptic vesicle release.

The key optical tool that we use to measure light-regulated FM1-43 release is 2-photon microscopy. FM1-43 release can be visualized with conventional one-photon microscopy, using visible light (488 nm) to excite the FM1-43 and produce a fluorescence signal. However, illumination with visible light will trigger phototransduction in retinal photoreceptors and elicit synaptic responses in downstream neurons, thereby perturbing the very process that we want to study. Two-photon microscopy utilizes intense pulses of infrared light. When used judiciously, this can excite FM1-43 fluorescence, while minimally triggering phototransduction in the photoreceptors (Choi et al., in press). Hence, 2-photon microscopy is like a '2-way mirror,' allowing the investigator to observe synaptic activity without the retina 'seeing' that it is being monitored. An additional advantage of 2-photon microscopy is the ability to obtain images at deep planes of focus through the entire thickness of the retina (~200 μm), because infrared light penetrates far into tissue.

Here, we combine the use of FM1-43 and 2-photon microscopy to reveal how light regulates exocytosis at two synapses in the retina. First, by imaging the outer plexiform layer (OPL) in retinas from various species, we show that release from terminals of cone photoreceptors is influenced by exposure to white light. Second, by imaging the inner plexiform layer (IPL) of retina from goldfish, we can detect how illumination affects vesicle release from ON bipolar cells, which have giant (~10 μm diameter) synaptic terminals. Isolated ON-bipolar cells from goldfish have become a classic system for studying the subcellular function of ribbon synapses (von Gersdorff & Matthews, 1999). The combination of FM1-43 and 2-photon microscopy now enables study of how this synapse responds to light in the intact system, putting the wealth of biophysical information obtained previously into physiological context.

2. Methods

2.1. Retinal preparation and dye loading

All procedures were approved by the UC Berkeley Animal Care and Use Committee. All animals were maintained on a 12:12 light:dark cycle.

Eyes were obtained from the lizard *Anolis segrei*. After 1 h of dark adaptation, the eye was hemisected and the retina was removed in the dark, with the retinal pigment epithelium (RPE) attached. The retina was cut into thirds and each piece was mounted RPE-side down, onto filter paper. Retinal flat-mounts were bathed in normal lizard saline containing 30 μM FM1-43 from 45 min to 1.5 h, followed by a 5 min wash with 1 mM ADV-ASEP-7, as described previously (Rea et al., 2004). All stages of retinal preparation were carried out at 21 °C in complete darkness, with the aid of an infrared converter. After the dye loading, the retinal flat-mounts were transferred to dye-free normal lizard saline and kept in darkness, or exposed to white light of intensity 10^7 photons/μm^2/s for 20–40 min before imaging. For slice preparation, FM1-43-loaded flat-mounts were transferred to Ca^{2+}-free lizard saline, and using a tissue slicer, cut into ~300 μm thick slices for imaging.

Eyes were obtained from the common goldfish *Carassius auratus* of 4–5 in. in length. Dissection was carried out in darkness as above for the lizard. Retinal flat-mounts were labeled with FM1-43 in goldfish saline in darkness, or in room light for 45 min to 1 h. After dye loading, the retinas were transferred to Ca^{2+}-free saline for imaging.

Eyes from larval tiger salamander *Ambystoma tigrium*, gecko *Gekko gecko*, and mouse (1-month-old) were obtained after dark adapting the animals overnight before the day of the experiment. Dissection and FM1-43 labeling of these tissues were carried out in darkness as above for the lizard. After dye loading, the retinas were transferred to Ca^{2+}-free saline for imaging.

2.2. Two-photon imaging and image analysis

The retina was imaged with a commercially available Zeiss 510 confocal system equipped with a Maitai

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