Encoding of light by the cone photoreceptor synapse

by

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Retinal photoreceptors are the primary sensory neurons responsible for vertebrate vision. The photoreceptor converts light into a voltage signal that modulates neurotransmitter release at the photoreceptor synapse. Synaptic release from photoreceptors stimulates downstream cells and ultimately provides our brain with an image of the visual world. Hence, converting the voltage signal into a synaptic signal at the photoreceptor synapse is the crucial first step in visual perception. Here I present research conducted for this dissertation that elucidates multiple mechanisms that shape synaptic transmission from cone photoreceptors.
This thesis is dedicated to my parents, Alan and Jean Jackman. I can never thank them enough for all the support and encouragement they have given me over the years.
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INTRODUCTION: Encoding of light by the cone photoreceptor synapse.

Simply put, the vertebrate retina is an astonishingly clever device. A gossamer wafer of neurons, separated from the rest of the central nervous system, the retina converts electromagnetic radiation into the signal that illuminates our rich visual world. The retina plays the dual role of camera and computer: It captures an image, then filters and compresses that image before sending it to the brain. Amazingly, the neuronal circuits of the retina perform these functions in only a fraction of a second, using just five main neuronal cell types (Dowling, 1987).

Understanding the neuronal connections of the retina proved an early triumph for systems neuroscience. Over a century ago, the prodigious neuroanatomist Santiago Ramon y Cajal recognized that the retina’s accessibility, along with its transparency and layered structure, presented a wonderful platform for studying the connections of a neural circuit (Cajal, 1893). Cajal employed the cell-staining method developed by Golgi to sparsely label retinal neurons. He recorded his observations at the microscope in beautiful hand-drawn images of neurons which he used to classify different cell types by morphology. By overlaying images of multiple cell types based on their position within the retina’s ordered layers, Cajal inferred the synaptic connections between retinal neurons (Cajal, 1899). The synaptic connections that Cajal inferred were confirmed and refined in the 20th century with the advent of modern electrical recording techniques. Recording from each neuronal cell type, electrophysiologists unraveled the functional connections of the retina by examining how the light response is transformed from one retinal neuron to the next (Werblin and Dowling, 1969).

Photoreceptors, the primary sensory neurons, convert light into a voltage signal (Baylor and Fuortes, 1970) that is relayed to the photoreceptor synaptic terminal, where voltage modulates the release of neurotransmitter onto bipolar cells (Schwartz, 1974; Ashmore and Copenhagen, 1983). Bipolar cells in turn synapse onto ganglion cells, whose axons carry information away from the retina towards the brain (Werblin and Dowling, 1969). At the photoreceptor synapse there is tremendous convergence. In the human retina, the output of 100 million photoreceptors is transmitted to the brain by just one million ganglion cells (Osterberg, 1935). Hence it is vital that photoreceptors filter their synaptic output to encode relevant visual information while discarding unimportant information. The vertebrate retina possesses two types of photoreceptors; rods and cones. Rod photoreceptors are highly sensitive to low levels of light, and thus are responsible for our nighttime vision. Cone photoreceptors are less sensitive to light, but provide us with color perception and high-acuity vision during daytime vision. Both types of photoreceptors possess specialized release machinery called synaptic ribbons, which presumably contribute to synaptic filtering (Heidelberger et al., 2005). Additionally, synaptic release from photoreceptors is modulated by horizontal cells, neurons that extend broadly to integrate the output of multiple photoreceptors and provide a feedback signal which accentuates the representation of contrast and color (Baylor et al., 1971; Fuortes et al., 1973). Despite our great understanding of circuit connections in the retina, relatively little is known about how these processes shape synaptic transmission from photoreceptors.

This dissertation examines how visual information is encoded by the cone synaptic terminal. The cone photoreceptor differs markedly from most neurons of the central nervous system. In a typical CNS neuron, synaptic transmission occurs during brief, pronounced depolarizations of
the cell membrane potential, called action potentials. During an action potential, voltage-gated Ca\(^{2+}\) channels open at the synapse. The resulting influx of Ca\(^{2+}\) triggers the transient (phasic) release of neurotransmitter. In contrast, cones are continuously depolarized in darkness. The cone outer segment generates a graded, hyperpolarizing voltage signal over a broad range of light intensities. At the other end of the cell, voltage-gated Ca\(^{2+}\) channels at the synaptic terminal transform the voltage signal into a change in the local Ca\(^{2+}\) concentration. This modulates the rate of continuous (tonic) neurotransmitter release from maximal in darkness to minimal in bright light.

The primary neurotransmitter released by cone terminals, glutamate, is packaged in synaptic vesicles that are exocytosed in a quantal manner. Hence, the dynamic range of vesicle release rates governs how precisely light intensity may be encoded by the synapse, and ultimately limits the capabilities of cone-mediated vision (Choi et al., 2005). Cone terminals possess several specializations that support the tonic exocytosis of synaptic vesicles at exceptionally high rates. First, cone terminals contain an inordinate number of synaptic vesicles, nearly 1000 times more than the number typically found at CNS synapses, which allows the cone terminal to maintain high release rates without depleting (Choi et al., 2005; Sheng et al., 2007). Second, cone terminals express non-inactivating L-type Ca\(^{2+}\) channels, which allow the steady influx of Ca\(^{2+}\) required to maintain the continuous release of neurotransmitter (Taylor and Morgans, 1998; Nachman-Clewner et al., 1999). Finally, cone terminals possess mysterious structures called synaptic ribbons, which tether hundreds of vesicles in close proximity to release sites, and are presumed to facilitate tonic exocytosis (Heidelberger et al., 2005).

The first three chapters of this dissertation examine how the cone light response is transmitted by the cone terminal, with an emphasis on how Ca\(^{2+}\) controls neurotransmitter release. In chapter one we quantify the effect of light and darkness on Ca\(^{2+}\) concentrations at the cone terminal. This seemingly modest achievement is deceptively difficult. Cytoplasmic Ca\(^{2+}\) is traditionally measured using fluorescent Ca\(^{2+}\) indicators. Unfortunately, exciting fluorescent indicators with visible light will also excite the cone phototransduction cascade, which will alter cytoplasmic Ca\(^{2+}\). To overcome this obstacle we employed two-photon microscopy, which uses infrared light that does not excite photoreceptors (Choi et al., 2005). By measuring Ca\(^{2+}\) in the cone terminal in light and darkness, we were able to estimate the dynamic range of the Ca\(^{2+}\) signal that controls synaptic transmission under physiological conditions. However, because this technique measures the average Ca\(^{2+}\) in the cone terminal, it likely underestimates the Ca\(^{2+}\) concentration near sites of neurotransmitter release. Indeed, confocal microscopy of voltage-clamped cones shows a local rise in Ca\(^{2+}\) near release sites. The results presented in chapter one have already been published (Choi et al., 2008).

In chapter two we continue our investigation of Ca\(^{2+}\) in the cone terminal. We again employed two-photon microscopy, but this time we were able to measure local Ca\(^{2+}\) in the cone terminal near release sites under conditions of light and darkness. Our results presented a conundrum: The high Ca\(^{2+}\) concentration near release sites in darkness conflicts with measured rates of synaptic release in darkness. To explain this discrepancy, we investigated the population of synaptic vesicles tethered to the cone synaptic ribbon using electron microscopy. We found that the synaptic ribbon is depleted of vesicles during tonic release in darkness. This challenges the presumption that the synaptic ribbon provides the synapse with an indefatigable supply of releasable vesicles. Surprisingly, depleting the ribbon of releasable vesicles enhances the
encoding capabilities of the synapse, by causing it to high-pass filter the light response to more accurately encode changing light intensity. The results presented in chapter two have already been published (Jackman et al., 2009).

In chapter three we attempt our most definitive measurement of local Ca\textsuperscript{2+} signals at the cone terminal. While chapters one and two measured cone synaptic Ca\textsuperscript{2+} with increasing spatial precision, both these measurements relied on diffusible Ca\textsuperscript{2+} indicators. Diffusible indicators provide poor resolution of local signals, as they can bind Ca\textsuperscript{2+} in regions of high concentration before migrating to regions of low concentration (Nowycky and Pinter, 1993). This “blurs” the resulting fluorescence image, obscuring our ability to measure the local Ca\textsuperscript{2+} signals that are most relevant to synaptic transmission. To overcome this obstacle, we synthesized a new Ca\textsuperscript{2+} indicator that binds to synaptic ribbons where release occurs. We introduced our Ribbon Associated Calcium Indicator (RACI) into cone photoreceptors via whole-cell patch pipette. This allowed us to measure Ca\textsuperscript{2+} exclusively at release sites. Moreover, the architecture of synaptic ribbons that bind RACI allows us to sample Ca\textsuperscript{2+} at a range of distances from the pores of open Ca\textsuperscript{2+} channels. We used the fluorescence signal from RACI to deduce the concentration gradient of Ca\textsuperscript{2+} near open channels. The measured gradient explains synaptic transmission at the cone terminal, but the results can also be extended to explain Ca\textsuperscript{2+} dependent processes at all CNS synapses. The results presented in chapter three are in preparation as a manuscript for publication.

Chapter four departs from the presynaptic mechanisms that modulate release from cone terminals, and focuses instead on the feedback signal from horizontal cells onto cones. Horizontal cells extend laterally across the retina, integrating the synaptic output from many cones to provide an inhibitory feedback signal that mediates center-surround antagonism (Baylor, 1971). This center-surround antagonism constitutes the first step of contrast enhancement in the visual system that ultimately augments edge detection and color perception. While negative feedback from horizontal cells has been well characterized using electrophysiology (Verweij et al., 1996; Rabl et al., 2005), previous studies have not measured the effect of feedback on synaptic release from cones. Furthermore, despite decades of study the mechanism of horizontal cell feedback remains controversial (Verweij et al., 1996). We used two-photon imaging of activity dependent dyes to directly monitor synaptic release from cones in the intact retina. We manipulated horizontal cells pharmacologically, and measured the effect of the feedback signal on cone synaptic release. Our experiments uncovered a new, positive feedback signal from horizontal cells onto cones. The positive feedback signal persists only in the intact retina, and communicates via a different mechanism from negative feedback. Positive feedback increases the gain of the photoreceptor synapse. It boosts synaptic transmission from cones, while preserving the surround antagonism of negative feedback. The results presented in chapter four are in preparation as a manuscript for publication.
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Chapter 1: How light regulates $\text{Ca}^{2+}$ in the cone photoreceptor synaptic terminal

Retinal cones are depolarized in darkness, keeping voltage-gated $\text{Ca}^{2+}$ channels open and sustaining exocytosis of synaptic vesicles. Light hyperpolarizes the membrane potential, closing $\text{Ca}^{2+}$ channels and suppressing exocytosis. In this chapter we quantify the $\text{Ca}^{2+}$ concentration in cone terminals, with $\text{Ca}^{2+}$ indicator dyes. Two-photon ratiometric imaging of fura-2 shows that global $\text{Ca}^{2+}$ averages $\sim 360$ nM in darkness and falls to $\sim 190$ nM in bright light. Depolarizing cones from their light to their dark membrane potential reveals hot spots of $\text{Ca}^{2+}$ that co-label with a fluorescent probe for the synaptic ribbon protein ribeye, consistent with tight localization of $\text{Ca}^{2+}$ channels near ribbons. Measurements with a low affinity $\text{Ca}^{2+}$ indicator show that the local $\text{Ca}^{2+}$ concentration near the ribbon exceeds $4 \, \mu \text{M}$ in darkness. The high level of $\text{Ca}^{2+}$ near the ribbon combined with previous estimates of the $\text{Ca}^{2+}$-sensitivity of release leads to a predicted dark release rate than is much faster than observed, suggesting that the cone synapse operates in a maintained state of synaptic depression in darkness.
INTRODUCTION

The biochemical signaling steps of rod and cone phototransduction are known in quantitative detail, but our understanding of how the light response is transmitted to postsynaptic neurons is more limited. We know that sustained depolarization in darkness activates L-type Ca\(^{2+}\) channels in photoreceptor terminals (Corey et al., 1984; Wilkinson and Barnes, 1996; Baldridge et al., 1998) and that intracellular Ca\(^{2+}\) is required for synaptic vesicle exocytosis, which occurs tonically in darkness (Cervetto and Piccolino, 1974). Intraterminal Ca\(^{2+}\) is known to decrease in the light (Johnson et al., 2007), but the concentrations of Ca\(^{2+}\) that underlie synaptic vesicle release in darkness and light have not been accurately quantified with ratiometric Ca\(^{2+}\) indicators.

Previous studies on isolated photoreceptors (Rieke and Schwartz, 1996) provided estimates of Ca\(^{2+}\) in terminals by utilizing voltage-clamp to hold the membrane potential near the expected dark and light voltages. In cone terminals, the steady-state Ca\(^{2+}\) concentration is a consequence of influx through ion channels (both L-type Ca\(^{2+}\) channels and cyclic nucleotide-gated channels; Savchenko et al., 1997), balanced by cytoplasmic buffering and efflux through the plasma membrane Ca\(^{2+}\)-ATPase (Morgans et al., 1998; Krizaj and Copenhagen 1998). In rods, Ca\(^{2+}\)-dependent Ca\(^{2+}\)-release from intracellular stores also plays a role in setting the intracellular Ca\(^{2+}\) level (Krizaj et al., 1999, 2003; Cadetti et al., 2006; Suryanarayanan and Slaughter, 2006). We have shown previously that the dark Ca\(^{2+}\) concentration is maintained at a lower concentration in rods than in cones, accounting for the slower dark rate of exocytosis in rods than in cones (Sheng et al., 2007).

Here we measure Ca\(^{2+}\) in cone synaptic terminals with the fluorescent ratiometric indicator dye fura-2. To monitor Ca\(^{2+}\) in darkness we use 2-photon microscopy, in which infrared light excites dye fluorescence in cone terminals while minimally exciting phototransduction in outer segments. Infrared light penetrates deeply into tissue, enabling visualization of Ca\(^{2+}\) in cone terminals in the intact retina. To localize subcellular sites of Ca\(^{2+}\) entry, we more closely examine individual cone terminals in retinal slices with low affinity non-ratiometric Ca\(^{2+}\) dyes dialyzed into cells with a patch pipette.

Photoreceptor terminals have synaptic ribbons which play a role in tonic release, but the specific function of the ribbon is unclear (Prescott and Zenisek, 2005). Voltage-gated Ca\(^{2+}\) channels are found adjacent to ribbons (Nachman-Clewner et al., 1999; Morgans, 2001), consistent with local control of Ca\(^{2+}\)-dependent exocytosis at the plasma membrane, but other Ca\(^{2+}\)-dependent events may also occur at the ribbon (Heidelberger et al., 2005). Measuring and localizing light-induced changes in the Ca\(^{2+}\) concentration will help constrain the possible locations and affinities of Ca\(^{2+}\) sensors for vesicle replenishment and exocytosis, bringing us closer to understanding how neurotransmission is regulated at the ribbon synapse.
RESULTS

Illumination causes a decrease in the cone terminal Ca\(^{2+}\) concentration.

We first examined light-regulated changes in synaptic Ca\(^{2+}\) in flat mounts of the cone-only retina of the anole lizard. Cone terminals in the outer plexiform layer (OPL) were identified by their loading with the synaptic vesicle marker dye FM4-64 (Rea et al., 2004). The same terminals also label strongly with the non-ratiometric Ca\(^{2+}\) indicator Oregon Green BAPTA-1 (OGB) (Fig. 1A). Light stimulation results in a decrease in dye fluorescence, indicating a drop in intraterminal Ca\(^{2+}\) concentration (Fig. 1B). A difference image from scans of the OPL before and after exposure to white light reveals a decrease in Ca\(^{2+}\) in nearly all the cone terminals (Fig. 1C). We controlled for possible effects of the laser scan by constructing a difference image from repeated scans in the dark. Repeated scanning alone caused no change in Ca\(^{2+}\) (Fig. 1D-F).

To quantify the change in Ca\(^{2+}\) elicited by light stimulation, we used the ratiometric Ca\(^{2+}\) indicator fura-2. Scans of the OPL were obtained with 700 nm and 760 nm light to minimize photostimulation of cones, and pixel-by-pixel ratio values were converted into Ca\(^{2+}\) concentrations. To enable this conversion, we calibrated the Ca\(^{2+}\) dependence of the 700/760 nm fura-2 excitation ratio in test solutions (Fig. 1G). A complete in situ dye calibration in patch-clamped cones was not possible because high Ca\(^{2+}\) concentrations resulted in cell death. However, we did confirm that dialysis into cones of a solution containing 180 nM free Ca\(^{2+}\) produced an excitation ratio of 1.32 +/- 0.04 (n=8), in good agreement with the ratio predicted from the cell-free calibration (1.31). These measurements indicate that the average Ca\(^{2+}\) concentration in cone terminals was ~359 +/- 24 nM in darkness (n=8), but dropped to ~188 +/- 10 nM after a 5 minute exposure to bright white light (n=8) (Fig. 1H). We observed that the decrease in Ca\(^{2+}\) elicited by light was slow to develop (> 2 min), presumably resulting from slow buffered diffusion of Ca\(^{2+}\) throughout the cytoplasm of the cone terminal. Hence, the steady-state concentration of Ca\(^{2+}\) in bright light is likely to be underestimated. Consistent with the notion that prolonged cessation of Ca\(^{2+}\) entry would lower Ca\(^{2+}\) further, application of Ca\(^{2+}\)-free saline for 15 min resulted in a larger decrease in internal Ca\(^{2+}\) to ~88 +/- 7 nM (n=4).

To investigate the role of phototransduction in mediating the effect of light on intraterminal Ca\(^{2+}\), we applied the phosphodiesterase (PDE) inhibitor isobutyl methylxanthine (IBMX). We found that IBMX eliminated the light-dark difference and also elevated the basal Ca\(^{2+}\) concentration to ~450 nM. IBMX inhibition of PDE leads to a supra-normal accumulation of cGMP, thereby opening cyclic nucleotide-gated (CNG) channels in cone terminals (Savchenko et al., 1997) and outer segments (Fesenko et al, 1985). Enhancement of the CNG current can lead to membrane depolarization and greater Ca\(^{2+}\) influx, which could contribute to elevating basal Ca\(^{2+}\) and blunting the light-dark difference in synaptic Ca\(^{2+}\) concentration.

Ca\(^{2+}\) entry is localized to synaptic ribbons

To localize sites of Ca\(^{2+}\) entry in cone terminals, we imaged Ca\(^{2+}\) changes resulting from depolarizing steps. Ca\(^{2+}\) indicator dyes were introduced through an inner segment-attached patch
electrode. A long axon separates the terminal from the inner segment of anole cones, so to avoid possible space clamp errors, we used tiger salamander cones, which have a synaptic terminal incorporated directly into the inner segment.

The \( \text{Ca}^{2+} \) concentration in the nm-scale region (the “nanodomain”) adjacent to the inner mouth of an open \( \text{Ca}^{2+} \) channels is likely to exceed 100 \( \mu \text{M} \) (Matthews, 1996; Naraghi and Neher, 1997; Demuro and Parker, 2006). Consistent with this, we found that activating the \( \text{Ca}^{2+} \) current by applying depolarizing steps from -70 to -10 mV produced a localized change in \( \text{Ca}^{2+} \) that could be visualized with the low affinity \( \text{Ca}^{2+} \) dye, OGB-5N, which has a \( K_d \) of 20 \( \mu \text{M} \). Subtraction of the image obtained at -70 mV (Fig. 2A1) from that obtained at -10 mV (Fig. 2A2) yields a difference image (Fig. 2A3), revealing a “hot spot” of \( \text{Ca}^{2+} \) in the synaptic terminal region at the base of the cone. We converted fluorescence changes in a \( 2 \mu \text{m}^2 \) region at the center of the hot spot into \( \text{Ca}^{2+} \) levels with equation 1 (see Methods), yielding an estimate of 8.1 \( \pm 1.1 \mu \text{M} \) (N=6).

For the experiment in Fig. 2, we plotted \( \text{Ca}^{2+} \) changes as a function of time. The graph at the left shows \( \text{Ca}^{2+} \) levels measured in the hot spot and the graph at the right shows \( \text{Ca}^{2+} \) levels measured in an adjacent region (just to the upper right of the hot spot) and a region in the center of the soma. The graph shows that \( \text{Ca}^{2+} \) levels rise abruptly during the test step and fall quickly afterwards. Because diffraction limits of light microscopy blur accurate localization of fluorescence, these measurements underestimate the \( \text{Ca}^{2+} \) concentration adjacent to the open pore of a voltage-gated \( \text{Ca}^{2+} \) channel. Unlike what is found in rods (Cadetti et al, 2006; Suryanarayanan and Slaughte, 2006), we did not observe \( \text{Ca}^{2+} \) waves in response to depolarizing steps of 200 ms or longer, even when we used OGB-1, a high affinity indicator (data not shown). This is consistent with other observations suggesting little role for \( \text{Ca}^{2+} \)-induced \( \text{Ca}^{2+} \) release in cones (Krizaj et al, 2003).

We next examined changes in \( \text{Ca}^{2+} \) evoked by depolarization to -35 mV, equivalent to the dark potential of cones. Only a small fraction of the voltage-gated \( \text{Ca}^{2+} \) conductance should be active at -35 mV (Thoreson et al, 2003). Nevertheless, steps to -35 mV elicited a clear increase in fluorescence (Fig. 2B), with \( \text{Ca}^{2+} \) hot spots reaching levels of several micromolar (4.6 \( \pm 1.5 \mu \text{M} \); N=4). Thus, while \( \text{Ca}^{3+} \) levels in the terminal cytoplasm average ~360 nM in darkness, local \( \text{Ca}^{2+} \) levels near the channels are much higher.

We tested whether the sites of \( \text{Ca}^{2+} \) influx revealed with these dyes are close to the ribbon, as predicted by immunohistochemistry (Nachman-Clewner et al., 1999; Morgans, 2001; tom Dieck et al., 2005). To label synaptic ribbons, we used a Rhodamine-tagged Ribbon Binding Peptide (Rh-RBP) that specifically binds to the ribbon protein ribeye (Zenisek et al., 2004). Rh-RBP and the \( \text{Ca}^{2+} \)-sensitive dye, OGB-6F, were introduced into cones through patch pipettes. Depolarization of the cone elicited a rise in OGB-6F fluorescence, indicating a localized rise in \( \text{Ca}^{2+} \). The regions exhibiting the largest change in \( \text{Ca}^{2+} \) are again highlighted by subtracting the -70 mV image (Fig. 3A) from the -10 mV image (Fig. 3B). The resulting difference image shows 3 hot spots of \( \text{Ca}^{2+} \) (Fig. 3C). Consistent with results using OGB-5N (Fig. 2), \( \text{Ca}^{2+} \) levels measured with OGB-6F in the hot spot rose to micromolar levels (Fig. 3D). Visualization of Rh-RBP in the same cell showed selective labeling of 3 spots at the base of the cone (Fig. 3E), where synaptic ribbons are located. Merging the OGB-6F and Rh-RBP images shows that the \( \text{Ca}^{2+} \) hot spots overlap with the ribbon labeling (Fig. 3F). Similar co-localization was seen in 11 photoreceptors (6 rods and 5 cones). This suggests that high levels of \( \text{Ca}^{2+} \) are attained at the ribbon, the principle site of synaptic vesicle exocytosis.
It has been reported that the Ca\textsuperscript{2+} indicator dye Fluo-3 can accumulate on ribbons in hair cell terminals (Issa and Hudspeth, 1996), which could confound identification of local regions having a high Ca\textsuperscript{2+} concentration. However, OGB-6F fluorescence is weak at the hot spot region when the cell is hyperpolarized (Fig. 3A), indicating no local accumulation of the dye. Moreover, elevating Ca\textsuperscript{2+} homogenously by flash photolysis of caged Ca\textsuperscript{2+} (DM-nitrophen) did not produce hot spots (data not shown).
DISCUSSION

Optical measurement of Ca\(^{2+}\) in light and darkness.

The signaling steps that link photoreceptor light responses to suppression of neurotransmitter release have been studied chiefly with electrophysiological techniques. Thus, light-induced hyperpolarization (Baylor and Fuortes, 1970), the consequent decrease in voltage-gated Ca\(^{2+}\) current (Corey et al., 1984), and the resulting change in membrane capacitance resulting from Ca\(^{2+}\)-dependent exocytosis (Rieke and Schwartz, 1996; Thoreson et al., 2004) were all revealed by electrical recording methods. Only recently has optical imaging been applied to measuring the decrease in synaptic vesicle exocytosis in response to light (Choi et al., 2005a). Here we used optical methods to visualize the one crucial signaling step between phototransduction and synaptic release that has remained unmeasured, the light-triggered decrease in synaptic Ca\(^{2+}\) concentration.

There are several technical challenges in optically measuring synaptic Ca\(^{2+}\) while keeping cones dark-adapted. The first challenge is minimizing the inadvertent photoisomerization of cone opsins while eliciting fluorescence from Ca\(^{2+}\) indicator dyes. Our solution is to use 2-photon microscopy, which excites the dyes with long wavelength light (>700 nm), largely outside the spectral range of photoreceptor activation. Even so, we are limited to capturing brief and infrequent “snapshots” of Ca\(^{2+}\), as more prolonged or repeated (>5 sec) illumination with these wavelengths does indeed elicit a photo-response of sufficient magnitude to alter synaptic function (Sheng et al., 2007).

A second challenge is localizing Ca\(^{2+}\) signals with high spatial accuracy. Given our illumination parameters, diffraction limits direct microscopic resolution to ~300 nm (Stelzer, 2000). This blurring of the observed Ca\(^{2+}\) gradient limits the ability to accurately measure highly localized Ca\(^{2+}\) changes, leading to underestimation of their values (Augustine et al., 2003). Nevertheless, our measurements indicate that at a minimum, steady-state levels of Ca\(^{2+}\) at the base of the synaptic ribbon reach several micromolar in darkness. We expect that the local Ca\(^{2+}\) concentration probably reaches much higher levels at release sites, which appear to be located within nm of Ca\(^{2+}\) channels.

Roles of Ca\(^{2+}\) in synaptic transmission

In neurons that fire action potentials, voltage-gated Ca\(^{2+}\) channels are tightly localized near sites of synaptic vesicle fusion. This co-localization enables the high Ca\(^{2+}\) concentration in nanodomains near the mouth of an open channel (>100 µM) to saturate the release machinery on neighboring primed vesicles (Augustine et al., 2003; Schneggenburger and Neher, 2005). This arrangement ensures rapid and reliable phasic release of vesicles to a very brief stimulus, namely the action potential.
Photoreceptors, in contrast, generate graded voltage signals and release neurotransmitter in a tonic manner. Photoreceptor synapses have several biochemical and morphological specializations not found in conventional synapses, but how these specializations contribute to tonic release is not well understood. The Ca\textsuperscript{2+} sensor for neurotransmitter release in rods and cones has an unusually high affinity, enabling release at sub-micromolar Ca\textsuperscript{2+} levels (Rieke and Schwartz, 1996; Thoreson et al., 2004; Sheng et al., 2007). This would seem to alleviate the need for high local Ca\textsuperscript{2+} and therefore close association between Ca\textsuperscript{2+} channels and Ca\textsuperscript{2+}-sensitive release sites. In fact, it has been suggested that the sensor for neurotransmitter release is so far removed from Ca\textsuperscript{2+} channels that vesicle fusion events are not tightly coordinated with individual channel opening events (Rieke and Schwartz, 1996). The present results revealed a twofold change in intraterminal Ca\textsuperscript{2+} levels between light and dark. A twofold change in Ca\textsuperscript{2+} levels should produce only a twofold change in synaptic release from cones (Thoreson et al., 2004; Sheng et al., 2007) but release rates vary tenfold between light and dark (Choi et al., 2005a). These results therefore suggest that larger, local Ca\textsuperscript{2+} changes are more directly responsible for regulating release than average intraterminal Ca\textsuperscript{2+} levels.

We found that Ca\textsuperscript{2+} influx through voltage-gated Ca\textsuperscript{2+} channels in cones produced hot spots that tightly co-localize with synaptic ribbons, similar to results obtained in hair cells and retinal bipolar cells (Issa and Hudspeth, 1996; Zenisek et al., 2003, 2004). Furthermore, our results showed that micromolar dark Ca\textsuperscript{2+} concentrations are achieved near the base of ribbons and probably much higher concentrations near individual open Ca\textsuperscript{2+} channels. Our functional imaging results are also consistent with morphological evidence for Ca\textsuperscript{2+} channel localization near ribbons. Freeze-fracture EM studies show rows of intramembranous particles, proposed to be Ca\textsuperscript{2+} channels, at the base of synaptic ribbons (Raviola and Gilula, 1975), and more recent immuno-EM studies show labeling for \(\alpha_1\) Ca\textsuperscript{2+} channel subunits clustered near synaptic ribbons (Nachman-Clewner et al., 1999; Morgans, 2001; tom Dieck et al., 2005).

Our measurements indicate that the average Ca\textsuperscript{2+} concentration at hot spots in darkness is at least 4 \(\mu\)M and likely to be much higher within 50 nm of voltage-gated Ca\textsuperscript{2+} channels. Studies utilizing paired recordings from cones and bipolar cells show that depolarization to the dark potential of cones triggers a rapid burst of exocytosis with an instantaneous vesicle fusion rate of several thousand vesicles per second (DeVries and Schwartz, 1999; Rabl et al., 2005). However, measurements of FM1-43 release indicate that cones sustain a tonic release rate of only 250 vesicles per second in darkness (Choi et al., 2005a). This discrepancy between abrupt and tonic release rates would be predicted if release sites were quite distant from Ca\textsuperscript{2+} channels. However our results show that Ca\textsuperscript{2+} channels are clustered close to the synaptic ribbon and average intraterminal Ca\textsuperscript{2+} changes are too small to account for light/dark changes in release rates. A more likely explanation is that the cone terminal is in a continual state of synaptic depression in darkness. The high release rates stimulated by membrane depolarization cannot be maintained for more than a few milliseconds because release sites are rapidly depleted of vesicles (Rabl et al., 2006). Thus, release from cones declines to slower tonic rates as they remain depolarized in darkness, despite the presence of locally high Ca\textsuperscript{2+} levels at the ribbon synapse.
Figure 1. Light–elicited decrease in average Ca\textsuperscript{2+} in cone terminals. (A-D) Images from 2-photon scans of OPL of a flat-mounted anole retina loaded with the Ca\textsuperscript{2+} dye OGB. To reveal the decrease in Ca\textsuperscript{2+} resulting from light, scans were taken from dark-adapted retina before (A) and after 5 minutes illumination with white light (B). Pseudocolor difference image (scan in B – scan in A) highlight the drop in Ca\textsuperscript{2+} resulting from illumination (C) Scale bar in A is 10 µm. The blue end of the color scale represents a larger decrease in Ca\textsuperscript{2+}; the red end of the scale represents a smaller decrease. To control for possible effects of scanning, control scans were taken before (D) and after a continued period of 5 minutes in darkness (E). The difference image (scan in D – scan in C) shows no decrease in Ca\textsuperscript{2+} (F). Quantification of spatially averaged Ca\textsuperscript{2+} in cone terminals by 2-photon imaging of fura-2. G) The calibration curve was determined with a fura-2 calcium imaging calibration kit (Invitrogen) and fit with the standard equation (Grynkiewicz et al., 1985) yielding a best fit $K_d$ of 234 nM. For clarity, only the lower concentration portion of the sigmoidal calibration curve is shown (continuous line). H) White light stimulation causes a 50% drop in internal Ca\textsuperscript{2+}. Blocking rod and cone phosphodiesterase with IBMX leads to a rise in Ca\textsuperscript{2+} above the dark level and eliminates the effect of light. Exposing the retina to Ca\textsuperscript{2+}-free saline for 15 minutes causes Ca\textsuperscript{2+} to drop below the light level.
Figure 2. Depolarizing steps to -35 and -10 mV evoke localized Ca\(^{2+}\) increases in voltage clamped cones that can be detected with the low affinity Ca\(^{2+}\) dye, OGB-5N. The figure shows pseudo-color images from a single confocal section of a cone filled with OGB-5N. Fig. 2A1 is a control image obtained prior to the depolarizing test step. Fig. 2A2 shows the image obtained during the last 55 ms of a 200 ms depolarizing test step from -70 to -10 mV which produced a localized fluorescence increase in the synaptic region at the base of the cone. The Ca\(^{2+}\) hot spot can be seen more clearly in the difference image in Fig. 2A3. The graphs show Ca\(^{2+}\) levels plotted as a function of time for regions within the hot spot (left graph), just to the upper right of the hot spot (right graph), and in the soma (right graph). DF/F was converted to [Ca\(^{2+}\)] using equation 1. Images illustrated in the figure were acquired at time points indicated in the left graph. Fig. 2B1 shows a subsequent control image obtained in the same cone, Fig. 2B2 shows an image of the cone obtained during a depolarizing step from -70 to -35 mV, and Fig. 2B3 shows the difference image of the hot spot produced by this modest depolarization. OGB-5N was imaged with 488 nm excitation and 525 nm emission filters. As in panel A, the graphs show [Ca\(^{2+}\)] as a function of time for the hot spot, an adjacent region, and the soma. Image acquisition time: 55 ms. Scale bar = 10 µm.
**Figure 3. Ca\(^{2+}\) hot spots co-localize with synaptic ribbons**  Cone loaded via a patch pipette with the low affinity Ca\(^{2+}\) indicator dye OGB-6F and the ribbon-specific peptide, Rh-RBP. Fluorescent images were taken at -70 mV (A) and during depolarization to -10 mV (B). The voltage clamp stimulus and times at which images A and B were obtained are illustrated below the images. Difference image (C) shows regions at the base of the terminal exhibiting increased fluorescence (green pixels), indicating a higher Ca\(^{2+}\) concentration. Note the three Ca\(^{2+}\) “hot spots”. OGB-6F was imaged with 488 nm excitation and 525 nm emission filters. Duration of each frame during Ca\(^{2+}\) imaging was 48 ms. (D) The graph plots Ca\(^{2+}\) levels measured within the hot spot and soma as a function of time. DF/F was converted to [Ca\(^{2+}\)] using equation 1 and a Kd of 3 mM for OGB-6F. (E) Binding of Rh-RBP to synaptic ribbons in the same cone was visualized by exciting the dye with 568 nm excitation and 607 nm emission filters. Note the 3 spots of bright labeling (red pixels), presumably from 3 distinct synaptic ribbons. (F) Overlap of the change in OGB-6F fluorescence and Rh-RBP binding (yellow pixels), showing co-localization of Ca\(^{2+}\) hot spots and synaptic ribbons. (G) Magnification of the region containing Ca\(^{2+}\) hot spots. Scale bar = 10 µm.
METHODS

Tissue preparation and dye loading

The retina from the lizard Anolis segrei was isolated with the retinal pigment epithelium (RPE) attached (Choi et al., 2005a,b; Sheng et al., 2007) with procedures approved by the UC Berkeley Animal Care and Use Committee. The retina was prepared and imaged in saline containing (in mM): NaCl 149; KCl 4; CaCl$_2$ 1.5; MgCl$_2$ 1.5; HEPES 10; Glucose 10; pH 7.4. First, a membrane-permeant Ca$^{2+}$ indicator dye, either Oregon Green BAPTA-1-AM (OGB-1) or fura-2-AM, was applied at 100 µM for 2 hours to load retinal cells. The saline contained 1% DMSO and 0.2% pluronic acid to enhance dye solubility and cell permeation. Dye-loaded retinas were then mounted flat on nitrocellulose filter paper with the inner retina facing the microscope objective. For dual imaging of synaptic vesicles and Ca$^{2+}$, the retina was double-labeled, first with FM 4-64 (Choi et al., 2005a), and then with fura-2-AM. Retinal preparation and labeling was carried out at 21ºC in complete darkness.

The retina from the larval tiger salamander, Ambystoma tigrinum, was isolated with procedures approved by the UNMC Institutional Animal Care and Use Committee. Retinal slices were prepared (Rabl et al, 2005) and whole-cell voltage-clamp recordings were obtained with an Optopatch patch-clamp amplifier (Cairn Instruments, Faversham, Kent) with 8-15 Mohm borosilicate glass patch electrodes. The pipette solution contained (in mM): 94 CsGluconate, 9.4 TEACl, 1.9 MgCl$_2$, 9.4 MgATP, 0.5 GTP, 0.5 EGTA, 32.9 HEPES (pH 7.2). To label the synaptic ribbon, a rhodamine-conjugated ribeye peptide (Zenisek et al, 2004) was used at 50 µM.

Imaging

Anole cones were imaged with a Zeiss LSM two-photon microscope equipped with a tunable Mai-Tai laser (Spectraphysics). The excitation wavelength was tuned to 800 nm for OGB-1 and 700 nm and 760 nm for ratiometric imaging of fura-2. To stimulate cone phototransduction, we used white light from a halogen lamp with intensity of $10^7$ photons/µm$^2$/s, attenuated with neutral density filters. Images were analyzed with Scion Image software (Scion Corporation, Frederick, MD).

The intraterminal Ca$^{2+}$ concentration of anole cones was calculated from the ratio of emitted light evoked by 2-photon dye excitation of fura-2 with 700 nm and 760 nm light, with standard equations (Grynkiewicz et al., 1985). Calibration constants were determined using a fura-2 calcium imaging calibration kit (Invitrogen) as described previously (Sheng et al, 2007) yielding a $K_d$ of 234 nM (Fig. 1G). A similar midpoint ratio value was obtained in situ by recording from individual lizard cones and dialyzing them with highly-buffered, known concentrations of Ca$^{2+}$. For the midpoint ratio, the pipette solution had a free Ca$^{2+}$ concentration of 180 nM and consisted of (in mM): 132 KCl, 2 NaCl, 10 EGTA, 3.5 CaCl$_2$, 2 MgCl$_2$, 20 HEPES (pH 7.1, N=8 cells). The minimum and maximum ratios were obtained by dialyzing solutions containing 0 CaCl$_2$ (N=4) or 0 EGTA (N=6), respectively.
Salamander cones were imaged with a spinning disk confocal microscope (Perkin Elmer Ultraview LCI) equipped with a cooled CCD camera (Orca ER). Images were acquired at 60 ms intervals with single frame durations of 48-56 ms. Pixel values were binned 2x2. Ca\(^{2+}\) indicator dyes were included in the patch pipette at 100 µM and were dialysed into cells during whole cell recording. We used three dyes: Oregon Green BAPTA 1 (OGB), Oregon Green BAPTA 6F (OGB-6F), and Oregon Green 488 BAPTA-5N (OGB-5N), with K\(_d\) values of 0.17, 3, and 20 µM, respectively, as reported by Molecular Probes (Eugene, OR). We estimated changes in Ca\(^{2+}\) with OGB-5N by using the following equation (Helmchen, 2000):

\[
\Delta [\text{Ca}^{2+}]_i = ([\text{Ca}^{2+}]_{\text{rest}} + K_d (\Delta F/F)_{\text{max}}/1 - (\Delta F/F)/(\Delta F/F)_{\text{max}})
\]

\(\Delta F/F\) represents the fractional change in fluorescence resulting from a brief depolarizing step. \((\Delta F/F)_{\text{max}}\) was determined from the maximal fluorescence change produced by a 500 ms depolarization to -10 mV. Variability in the amount of dye that enters each cell during whole cell recording produces cell to cell differences in absolute fluorescence that prevented \textit{in situ} determination of the K\(_d\) for OGB-5N. We therefore used the K\(_d\) value of 20 mM provided by Molecular Probes. There was no added Ca\(^{2+}\) in the pipette solution and thus the resting Ca\(^{2+}\) concentration ([Ca\(^{2+}\)]\(_{\text{rest}}\)) was assumed to be 10 nM, but varying this value from 1-100 nM had only a small effect (<100 nM) on the calculated value of \(\Delta [\text{Ca}^{2+}]_i\). To validate measurements obtained using equation 1, we measured fluorescence changes produced by the three different Oregon Green dyes under identical stimulation conditions and obtained similar estimates of intraterminal Ca\(^{2+}\) concentration. This comparison was carried out in salamander rods because recordings are more stable and fluorescent hot spots larger and easier to measure than in cones (unpublished observations).
REFERENCES


Chapter 2: The role of the synaptic ribbon in transmitting the cone light response

Cone photoreceptors distinguish small changes in light intensity while operating over a wide dynamic range. The cone synapse encodes intensity by modulating tonic neurotransmitter release, but precise encoding is limited by the quantal nature of synaptic vesicle exocytosis. Cones possess synaptic ribbons, structures thought to accelerate the delivery of vesicles for tonic release. In this chapter we present evidence that suggests the synaptic ribbon actually constrains vesicle delivery, resulting in a maintained state of synaptic depression in darkness. Electron microscopy reveals that depression is caused by depletion of vesicles on the ribbon, indicating that resupply, not fusion, is the rate-limiting step controlling release. Postsynaptic responses show that the ribbon behaves like a capacitor, charging with vesicles in light and discharging in a phasic burst at light offset. Phasic release extends the operating range of the cone synapse to encode more accurately changes in light intensity, accentuating features that are salient to photopic vision.
INTRODUCTION

From the first glimmer of dawn, through the brightest light of midday, to the fading glow of sunset, human vision depends primarily on cone photoreceptors. The cone outer segment generates a voltage signal that is finely graded over the broad range of light intensities encountered during the day, as the number of photons absorbed ranges over ~6 orders of magnitude (Dowling and Ripps, 1973). At the other end of the cell, the cone synaptic terminal possesses voltage-gated Ca\(^{2+}\) channels that transform the voltage signal into a change in the local Ca\(^{2+}\) concentration, which alters the rate of neurotransmitter release from maximal in darkness to minimal in bright light (Baylor et al., 1971; Ashmore and Copenhagen 1983). Since neurotransmitter is packaged in synaptic vesicles that are exocytosed in a quantal manner, the dynamic range of vesicle release rates governs how precisely light intensity is signaled to postsynaptic neurons and ultimately limits the capabilities of cone-mediated vision (Sterling and Freed 2007).

The cone terminal has been thought to serve as a relay that reliably converts light-evoked voltage signals into changes in tonic neurotransmitter release with no time-dependent changes. However, recent studies of rod, cone, and bipolar cell ribbon synapses have revealed that neurotransmitter release is not strictly tonic, but rather occurs through transient (phasic) and sustained (tonic) processes (Mennerick and Matthews 1996; Rabl et al., 2005). The two modes of release may occur at distinct ribbon and non-ribbon locations (Midorikawa et al., 2007) but it has also been suggested that the shift from phasic to tonic results from a progressive change in the availability of vesicles at a single site, the synaptic ribbon (Mennerick and Matthews 1996; DeVries 2000; Singer and Diamond 2006). Although the mechanisms of phasic and tonic release are still uncertain, the two modes of release suggest that ribbon synapses do not faithfully relay voltage. Rather they accentuate dynamic changes in membrane potential, thereby high-pass filtering the synaptic output.

The synaptic ribbon is the primary site of neurotransmitter release from cones. Synaptic ribbons are anchored to the membrane at active zones, where they tether hundreds of vesicles in close proximity to Ca\(^{2+}\) channels, enabling small voltage changes to efficiently control exocytosis (Heidelberger et al., 2005). Vesicles presumably descend along the ribbon from the cytoplasm toward the surface membrane. Whether they diffuse passively or are propelled by a molecular motor is unclear, and whether the ribbon participates in biochemical "priming" of vesicles for membrane docking and fusion is unknown. However, because ribbons are found only in tonically releasing neurons (rods, cones, bipolar cells, and hair cells), there is the notion that the ribbon serves to accelerate the continual delivery of vesicles to meet the demands of tonic release (Parsons et al., 1994; von Kriegstein et al., 1999).
In this report, we examine transmission of the light response by the cone synapse, and we evaluate the role of the synaptic ribbon. By measuring the intraterminal Ca\(^2+\) concentration, thCa\(^2+\)-dependence of neurotransmitter release, and the distribution of synaptic vesicles on the ribbon, we show that the ribbon is depleted of synaptic vesicles in darkness, leading to a maintained state of synaptic depression. In light, the ribbon is re-loaded with vesicles, resulting in a phasic burst of release upon return to darkness. The synaptic ribbon thus operates like a leaky capacitor, generating a transient signal (phasic release) that amplifies changes in illumination, superimposed on a sustained signal (tonic release) that encodes the background level of light.
RESULTS

The concentration of Ca\(^{2+}\) in the cone terminal

To understand how the cone terminal transmits the light response, we started by measuring intraterminal Ca\(^{2+}\) in light and darkness using ratiometric Ca\(^{2+}\) imaging. We monitored Ca\(^{2+}\) in darkness with 2-photon laser-scanning microscopy, employing infrared light to excite dye fluorescence in cone terminals while minimizing phototransduction in outer segments (Sheng et al., 2007). Cone terminals loaded with the Ca\(^{2+}\) indicator dye fura-2 AM were imaged in retinal slices from the all-cone retina of the anole lizard (Rea et al., 2004). We found that the terminals of most cones have a Ca\(^{2+}\) concentration that is higher in darkness (Fig. 1a-c) than after prolonged white-light illumination (Fig. 1d-f), with the highest Ca\(^{2+}\) at the ribbon end of the terminal. Voltage-clamp depolarization of the cone also elicited an increase in Ca\(^{2+}\) at the ribbon end (Fig. 1g-i) consistent with immunolocalization of Ca\(^{2+}\) channels adjacent to synaptic ribbons (Taylor and Morgans, 1998; Nachman-Clewner et al., 1999). Repeated laser scanning of cone terminals in darkness had no effect on Ca\(^{2+}\) concentration, confirming that the imaging procedure did not confound Ca\(^{2+}\) measurements by inadvertently triggering phototransduction. Examples of cone terminals (Fig. 1j) show that some have a steep Ca\(^{2+}\) gradient in darkness. In those terminals, white light stimulation reduced Ca\(^{2+}\) near the membrane, collapsing the gradient (Fig. 1k). Other cone terminals did not have a Ca\(^{2+}\) gradient and showed little change in Ca\(^{2+}\) with illumination, presumably a consequence of cell damage during retinal slicing.

We quantified the dark level of Ca\(^{2+}\) by averaging Ca\(^{2+}\) profiles from those terminals with a substantial light response, excluding terminals that were unresponsive. We used a >5% decline in overall Ca\(^{2+}\) after light stimulation as a criterion for responsiveness. Average profiles showed peak Ca\(^{2+}\) at the ribbon end of the terminal of ~600 nM in darkness and ~250 nM in bright light (Fig. 1l). The measured peak submembrane Ca\(^{2+}\) concentration in darkness is underestimated, owing to the limited spatial resolution of light microscopy. The Ca\(^{2+}\) ionophore ionomycin elevated Ca\(^{2+}\) uniformly throughout the terminal, indicating that influx through Ca\(^{2+}\) channels is necessary for the Ca\(^{2+}\) gradient. As expected, removing extracellular Ca\(^{2+}\) reduced the intraterminal Ca\(^{2+}\) concentration to <100 nM throughout the terminal. The uniform Ca\(^{2+}\) distribution with ionomycin and Ca\(^{2+}\)-free saline suggests that the Ca\(^{2+}\) gradient in darkness results from Ca\(^{2+}\) influx through localized channels and not from Ca\(^{2+}\) removal through localized extrusion mechanisms, such as the plasma membrane Ca\(^{2+}\) ATPase.

Ca\(^{2+}\)-dependence of neurotransmitter release

To investigate the Ca\(^{2+}\)-dependence of neurotransmitter release, we employed paired electrophysiological recordings from cones and retinal bipolar cells, enabling measurement of the excitatory postsynaptic current (EPSC) elicited by a rise in presynaptic Ca\(^{2+}\). For these experiments we used cones from the tiger salamander retina because their compact structure (Barnes and Hille, 1989) allows more effective voltage-clamp of the synaptic terminal than lizard cones, which have a long axon separating the inner segment from the terminal (Savchenko et al., 1997). Depolarizing a salamander cone from -70 to -10 mV evoked an inward Ca\(^{2+}\) current,
which in turn elicited an EPSC in a hyperpolarizing bipolar cell (HBC). Despite the sustained presynaptic Ca\(^{2+}\) current, the evoked EPSC is transient, decaying from a large phasic response to a small tonic response (Fig 2a). Presynaptic capacitance measurements demonstrate that EPSCs accurately reflect cone exocytosis (Rabl et al., 2005). Hence a sustained Ca\(^{2+}\) increase in cones triggers both phasic and tonic release processes.

To quantify the Ca\(^{2+}\)-dependence of each of these processes, we recorded EPSCs while controlling intraterminal Ca\(^{2+}\) with “caged Ca\(^{2+}\)”. The photolyzable Ca\(^{2+}\)-chelator DM-nitrophen (Kaplan and Ellis-Davies, 1988) was delivered via whole cell patch pipette along with the Ca\(^{2+}\) indicator dye Oregon Green BAPTA-6F (OGB-6F). A flash of UV light increased presynaptic Ca\(^{2+}\), evoking an EPSC that we recorded in an HBC (Fig. 2b). We calculated quantal release from the cone synapse (Fig. 2c) by deconvolving the EPSC with the waveform of the averaged mEPSC (Van der Kloot, 1988) (see inset, Fig. 2b). The response triggered by a sustained rise in Ca\(^{2+}\) could be attributed to two components of release, a phasic component (green line) that decayed over a double exponential time course, and a tonic component (blue line) that persisted for hundreds of ms after the uncaging flash. Integrating the release rate over time shows that the abrupt rise in Ca\(^{2+}\) triggers the prompt fusion of a pool of ~120 vesicles while simultaneously enhancing tonic release (Fig. 2d).

We varied the intensity of the uncaging light to evoke a range of abrupt jumps in presynaptic Ca\(^{2+}\) concentration (Fig. 3a, b). We found that the phasic and tonic release processes were regulated similarly by Ca\(^{2+}\). Both had Ca\(^{2+}\) thresholds of <1 µM and the rates of both processes increased linearly over the narrow range of Ca\(^{2+}\) concentrations tested. However, the tonic release rate was ~10-fold slower than the phasic release rate (Fig. 3a).

To provide an independent measure of the Ca\(^{2+}\)-dependence of tonic release, we added ionomycin to anole cones and varied extracellular Ca\(^{2+}\) to “clamp” Ca\(^{2+}\) at different intracellular concentrations (Sheng et al., 2007). The Ca\(^{2+}\) concentration was measured with fura-2 and tonic release rate was determined by measuring the loss of the synaptic vesicle dye FM1-43 (Supplementary Figure S1). The relationship between ionomycin-elevated Ca\(^{2+}\) and release in anole cones (open blue squares, Fig. 3b) was consistent with the relationship between photolytically-elevated Ca\(^{2+}\) and release in salamander cones, suggesting that both measurements reflect the same tonic release process, even though they were obtained with different methods in different species.

It is possible that tonic and phasic release are mediated by distinct biochemical mechanisms, both with a linear dependence on Ca\(^{2+}\) and a low threshold. However, a simpler explanation is that the two processes utilize the same Ca\(^{2+}\) sensor for exocytosis and differ only in the number of vesicles available for release. In this scenario, a readily releasable pool is depleted quickly by the initial burst of phasic release, leaving a smaller pool to mediate tonic release, but nonetheless by the same mechanism. This model contrasts with the Calyx of Held, where synchronous and asynchronous release display different Ca\(^{2+}\)-dependencies because they use different Ca\(^{2+}\) sensors (Sun, Pang et al. 2007).

Which process, phasic or tonic, accounts for transmitter release from cones in darkness? Assuming a submembrane Ca\(^{2+}\) concentration of 600 nM (Fig. 1), the phasic process predicts a dark release rate of ~2,400 vesicles/s and the tonic process predicts ~230 vesicles/s (dotted red
Given that a salamander HBC contacts 2-5 ribbons from each presynaptic cone (Lasansky, 1978) our uncaging experiments predict a dark release rate of 470-1200 vesicles/ribbon/s for the phasic process and 45-110 vesicles/ribbon/s for the tonic process. The measured release rate from salamander cones is ~500 vesicles/s in darkness (Sheng et al., 2007), and cone terminals possess ~13 ribbons (Pang et al., 2008). Thus each ribbon releases ~40 vesicles/s in darkness, consistent with the tonic rather than the phasic process.

**Distribution of synaptic vesicles on the ribbon**

The transition from phasic to tonic release during a depolarizing step could stem from depletion of synaptic vesicles available for release. Since the tonic mechanism appears to govern release in darkness, we would expect that keeping the retina in darkness would result in depletion of synaptic vesicles near release sites. To test this hypothesis, we directly visualized cone synapses with electron microscopy (EM). Retinal slices from anoles were dark-adapted, light-adapted, or exposed to Ca$^{2+}$-free saline for 30 min before EM preparation. Cone terminals possessed synaptic ribbons with closely associated vesicles in all three treatments (Fig 4a and Supplementary Fig. 2). However, a difference emerged when we mapped and superimposed the vesicle positions from many ribbons (Fig. 4b). Ribbons in dark-adapted cones had a clear deficit of vesicles, particularly within 200 nm of the base of the ribbon, near release sites (Fig. 4c). Comparison of 115 ribbons from each treatment showed little difference in the total number of vesicles per ribbon (Fig. 4d) or the average ribbon length (Supplementary Fig. S3), but a highly significant difference ($P<0.0001$) at the base of the ribbon, where dark-adapted cones had 3.7-fold fewer vesicles than light-adapted cones (Fig. 4e). This is less than the 10-fold difference expected from our comparison of tonic and phasic release rates (Fig. 3), but this difference could arise from systematic over-counting of vesicles on depleted ribbons (see Supplementary Fig. S4 for discussion of EM error). Treatment with Ca$^{2+}$-free saline significantly increased vesicle number not only at the ribbon base ($P<0.0001$), but also on the entire ribbon ($P<0.01$). These results confirm that synaptic vesicles are depleted at the base of the ribbon, implying that vesicle release outpaces replenishment. Hence the resupply of vesicles to the base of the ribbon, rather than fusion with the plasma membrane, is the rate-limiting step in tonic neurotransmitter release in darkness.

**Impact of vesicle depletion on synaptic signalling**

To evaluate the impact of vesicle depletion on synaptic transmission of the cone light response we considered a scenario where vesicles first bind to the ribbon, then descend into a release zone at the base of the ribbon, and finally fuse with the plasma membrane in a Ca$^{2+}$-dependent manner. In darkness, when Ca$^{2+}$ is high near the membrane, fusion outpaces vesicle descent and the release zone becomes depleted. In light the Ca$^{2+}$ concentration falls, slowing fusion without affecting descent. This allows the base of the ribbon to repopulate with vesicles. At light offset, the fully populated ribbon is suddenly exposed to elevated Ca$^{2+}$. At first exocytosis resumes at a high rate, but then the base of the ribbon becomes depleted and release subsides to the tonic dark rate.
All neurons receiving input from cones exhibit a transient Off response after a light flash (Yang and Wu 1989). Depolarizing bipolar cells (DBCs) show a hyperpolarizing transient and hyperpolarizing bipolar cells (HBCs) show a depolarizing transient (Fig. 5a), as do horizontal cells (Supplementary Fig. S5). In a subset of HBCs, glutamate receptors recover from desensitization during the light response, and this accounts for the depolarizing overshoot that occurs upon return to darkness when cone glutamate release resumes (DeVries, 2000). However in other HBCs (DeVries, 2000), HCs (Rabl et al., 2006), and DBCs (Snellman et al., 2008) the kinetics of recovery from receptor desensitization cannot account for the Off response, suggesting a presynaptic mechanism.

We propose that the accumulation of vesicles on the ribbon during a light flash generates these Off responses. In darkness, the group of cones contacting the HBC release 300 vesicles/s, as determined by deconvolution of the HBC’s synaptic current (Fig. 5b). Since the dark release rate is limited by vesicle resupply and not fusion, the resupply rate must also be 300 vesicles/s. During illumination release slows to 100 vesicles/s (yellow region in Fig. 5b). Hence the ribbons should have amassed 400 extra vesicles during the 2 s light flash. Matching this prediction, we find that 390 vesicles were released within 200 ms of light offset (blue region in Fig. 5b).

To further test whether accumulation of vesicles on the ribbon underlies the Off transient response, we measured how light flash duration affects the magnitude of the response. The depleted ribbon model predicts that more vesicles will accumulate on the ribbon with longer flashes of light, until the ribbon is saturated with vesicles. We find that the Off transient response increases with flash duration and then saturates (Fig. 6a). On average, the Off response increased with a time constant of 1.7 s (circles in Fig. 6b), although even a 100 ms flash generated a prominent Off response. The Off response reached a plateau at ~5 s, consistent with saturation of vesicles on ribbons (line in Fig. 6b).

To confirm that the Off response is mediated by a change in presynaptic release, we measured capacitance changes in the cone terminal resulting from the increase in membrane surface area that occurs during exocytosis. The cone was initially held at the dark membrane potential of -35 mV, then stepped to -70 mV to simulate light-induced hyperpolarization, and finally stepped to -10 mV to trigger exocytosis of the releasable pool of vesicles (Fig 6c). Increasing the duration of the hyperpolarizing step increased the magnitude of the capacitance response, but responses began to saturate after several seconds. We calculated the number of vesicles released using a capacitance value of 57 aF per vesicle (Thoreson et al., 2004). The presynaptic capacitance signal (Fig. 6d) and the postsynaptic Off response (Fig 6b) grew at a similar rates with increasing flash duration and both signals could be fit with our model of vesicle depletion and resupply (Supplementary Fig. S6). However, a fast component of recovery is evident in the capacitance measurement but not the postsynaptic response. This could reflect a small contribution from presynaptic release at non-ribbon sites (Midorikawa et al., 2007) or recovery from postsynaptic desensitization (DeVries, 2000). The kinetics of vesicle accumulation resembles recovery from paired-pulse depression by the cone synapse (Rabl et al., 2006), an indicator of changes in the size of the readily-releasable pool. The total number of vesicles that accumulate on a ribbon during a prolonged hyperpolarizing step (56 vesicles) is consistent with the capacity of the base of a fully populated ribbon (65 vesicles), determined from our EM data and morphological analysis of the salamander cone synapse (Pang et al., 2008) (for calculations see Tables 1, 2 in Methods).
Other possible mechanisms for the Off response have been proposed. For example, inhibition of cone Ca\(^{2+}\) channels by exocytosed protons (DeVries, 2001) or feedback from horizontal cells (Hirasawa and Kaneko, 2003) could decrease during the light response, enhancing release upon return to darkness. However, prominent Off responses are seen with HEPES-buffered saline (Fig. 5b) which blocks both of these effects (DeVries, 2001; Hirasawa and Kaneko, 2003). Some cones exhibit a depolarizing afterpotential at light offset (Yang and Wu, 1989), but this response is too small and too slow to account for the postsynaptic Off response (Supplementary Fig. S5).

**Phasic release improves the encoding of light intensity.**

Cones generate a graded response to increments of light and this causes a graded decrease in synaptic vesicle release. Brighter light more effectively slows release, resulting in the accumulation of more synaptic vesicles on the synaptic ribbon. This suggests that a brighter light flash will produce a larger Off response in post-synaptic cells. To test this prediction we recorded responses to various flash intensities in HBCs. We found that the peak Off response following a 2s flash increases over ~4 log units of intensity before saturating with very bright light (Fig. 7A). Furthermore, the magnitude of the Off response after the light flash increased over the same intensity range as the response during the light flash (i.e. the “On response”). This is consistent with graded suppression of tonic release underlying the On response and phasic release of accumulated vesicles underlying the Off response. However, throughout this intensity range the magnitude of the Off response was always 3-4 fold larger than the On response. Phasic release at light offset employs a larger range of synaptic vesicle releases rates and could allow more accurate encoding of light intensity.

We compared the encoding capabilities of tonic and phasic release rates. Assuming that vesicle release is stochastic and therefore obeys Poisson statistics, a salamander cone tonically releasing 500 vesicles/s in darkness (Sheng et al., 2007) can transmit only ~13 distinguishable levels of steady light before release is suppressed completely (Fig. 7b; for calculations, see Methods). By increasing the release rate to ~3,350 vesicles/s (the phasic release rate after a 2 s light flash, average response from 6 HBCs), phasic release improves the encoding of fine gradations of light, allowing ~35 distinguishable decrements in light (Fig. 7c).
DISCUSSION

Function of the synaptic ribbon in cones

At each cone active zone, hundreds of voltage-gated Ca$^{2+}$ channels, an unknown number of vesicle fusion sites, and a single synaptic ribbon assemble in a stereotypic manner to control neurotransmitter release (Morgans, 2000; Heidelberger et al., 2005; Sterling and Matthews, 2005). In darkness, the depolarized membrane potential opens Ca$^{2+}$ channels, elevating the Ca$^{2+}$ concentration at the active zone and supporting exocytosis of synaptic vesicles at fusion sites. Diffusion of Ca$^{2+}$ away from the mouth of the channels and buffering by Ca$^{2+}$-binding proteins establish a Ca$^{2+}$ gradient in the cytoplasm that diminishes with distance from the membrane. Thus, any Ca$^{2+}$-regulated process that occurs should be affected differentially along the ribbon surface.

Studies on the bipolar cell ribbon synapse showed that elevated Ca$^{2+}$ accelerates vesicle resupply (Gomis et al., 1999). This implies that Ca$^{2+}$ regulates the interaction of vesicles with the ribbon in addition to regulating fusion with the plasma membrane. Our studies in cones suggest that resupply is unaffected by physiological levels of Ca$^{2+}$ because the rate of vesicle accumulation in the light, when cytoplasmic Ca$^{2+}$ is low, is nearly identical to the tonic release rate in darkness, when cytoplasmic Ca$^{2+}$ is high. It is possible that this reflects a fundamental difference in the resupply process in cones and bipolar cells. Alternatively, the modest Ca$^{2+}$ elevation in cones in darkness may be too localized to the surface membrane to influence Ca$^{2+}$ sensors that might be located further up the ribbon.

The synaptic ribbon has been portrayed as a "conveyor belt" for transporting vesicles from the cytoplasm to release sites (Lenzi and Gersdorff, 2001; Parsons and Sterling, 2003). Vesicles appear to exit the synaptic ribbon exclusively at the membrane end (LoGiudice et al., 2008). Depletion on the ribbon in darkness indicates that vesicles near the base have either descended more rapidly than those at the top, fused directly to the plasma membrane along the flanks of the synaptic ridge, or have detached before reaching the plasma membrane. The ribbon has also been thought to accelerate delivery of vesicles to release sites (Parsons, Lenzi et al. 1994; von Kriegstein et al., 1999). However, our calculations suggest that in comparison to vesicle delivery by free diffusion through the cytoplasm, the ribbon actually slows delivery, thereby restraining release. In darkness vesicles are released from anole cones at 250 vesicles/s, or 10 vesicle/ribbon/s (Choi et al., 2005). Given the concentration of vesicles in cone terminals (~2,000 vesicles/µm$^2$) and their cytoplasmic diffusion coefficient (0.11 µm$^2$/s) (Rea et al., 2004), freely diffusing vesicles should collide with an area the size of a ribbon-associated active zone (200 nm wide by 500 nm long) at 75 vesicles/s, faster than the observed release rate at individual ribbons. Unlike cytoplasmic vesicles that move in 3 dimensions, ribbon-bound vesicles are constrained to move in 2 dimensions, predicting an even faster active zone encounter rate of 180 vesicles/s. Hence, while the ribbon may prime vesicles to make them fusion-competent (Heidelberger, 1998), the calculations suggest that it slows rather than speeds the delivery of vesicles to release sites and limits the fusion rate. We speculate that slowing delivery enables the ribbon to serve as a "timing belt", making the interval between sequential fusion events more regular than if vesicles were resupplied directly from cytoplasm. Theoretical considerations suggest that small sensory stimuli would be undetectable to the visual system.
unless the photoreceptor terminal possessed a mechanism for regularizing release (Schein and Ahmad, 2005).

Recent studies have shown that in bipolar cells, the synaptic ribbon serves as a platform for promoting compound fusion during strong depolarizations (Matthews and Sterling, 2008). Multivesicular release, possibly mediated by compound fusion has been observed in a variety of ribbon synapses (Glowatzki and Fuchs, 2002; Singer et al., 2004). None of our EM images from cones in darkness or in steady light show structures indicative of compound fusion. However, it is possible that compound fusion might be more prevalent during the phasic burst of release that occurs upon transition from light to darkness. It has also been shown that release at ribbon synapses can occur at extra-ribbon locations (Midorikawa et al., 2007). The quantitative agreement between the vesicle storage capacity of the ribbon and the magnitude of postsynaptic responses suggests that the ribbon mediates release in response to our light and dark stimuli, but it possible that larger depolarizations might recruit extra-ribbon release.

**Synaptic encoding of steady and changing light**

At most conventional synapses vesicle release is triggered by the intermittent rise in Ca\(^{2+}\) that occurs during action potentials. The cone synapse also uses Ca\(^{2+}\) as a trigger for release, but adds a second layer of control by dynamically varying the number of vesicles on the ribbon available for release. The release rate is minimal when a *depleted ribbon* is exposed to *low* Ca\(^{2+}\), i.e., during a step from darkness to bright light. The release rate is maximal when a *fully populated ribbon* is exposed to *high* Ca\(^{2+}\), during a step from bright light to darkness. Between these two extremes, the synapse can finely tune its release rate by varying the Ca\(^{2+}\) signal and the available vesicle pool, optimizing the encoding of fine differences in light intensity. Studies of other ribbon synapses show that strong depolarization decreases the size of the readily releasable pool of vesicles, leading to synaptic depression (von Gersdorff and Matthews, 1997; Moser and Beutner, 2000; Holt et al., 2004). Here we have explained the physiological importance of this phenomenon in cones and demonstrate directly that it is mediated by depletion of synaptic vesicles on the ribbon.

Our results show that the rate-limiting step that controls cone neurotransmitter release switches from vesicle resupply during tonic transmission to Ca\(^{2+}\)-dependent fusion during phasic transmission. We propose that this switch also improves the synaptic encoding of light intensity. Control of the release rate by vesicle resupply distances tonic transmission from noisy signals generated by the stochastic opening of Ca\(^{2+}\) channels. Also, controlling tonic release via resupply rather than fusion probably reduces the intrinsic noise of release. Vesicle fusion is a stochastic, all-or-none event, whereas descent along the ribbon and docking to the membrane involves multiple steps, which should make the overall rate more consistent by averaging the fluctuations of individual steps (Doan et al., 2006). In contrast, controlling phasic release at the fusion step ensures that the synaptic signal representing changing light is transmitted as rapidly as possible.
The consequence of phasic release is a large and rapid Off response in all neurons that are postsynaptic to cones. Most bipolar cells receive either direct or indirect input from both rods and cones (Lasansky, 1978). Synaptic input driven by the rod light response, which decays very slowly after light offset, can obscure the cone-mediated Off response (Yang and Wu, 1996). However, during daylight hours, the retina is exposed to background illumination that often saturates rod phototransduction. Under these photopic conditions, the cone-mediated Off response becomes a prominent feature of the bipolar cell light response, contributing strongly to transient responses in downstream neurons in the visual system.

The response in postsynaptic cells increases as a function of both flash intensity and duration. Hence rather than encoding absolute intensity, the Off response is a history-dependent representation of the change in intensity with respect to background. The sequence of opposite polarity On and Off responses increases the contrast gain of the synapse. Interestingly, the phasic behavior of the cone ribbon synapse augments the signaling of light decrements, but the synapse displays no equivalent transient behavior at light onset. Other processes such as cone light adaptation (Burkhardt, 1994), horizontal cell-mediated synaptic feedback (Schwartz, 1974), and glutamate receptor desensitization (DeVries, 2000), can generate transient responses to light increments in bipolar cells. However, our results are consistent with previous studies (Yang and Wu, 1996) showing that under photopic conditions, the change in voltage in postsynaptic neurons is larger at light offset than light onset. This asymmetry enables the cone synapse to encode light decrements more accurately than light increments. This is in accord with the observation that natural images display a statistical overabundance of decrements with respect to the mean brightness of a scene (Ruderman and Bialek, 1994). Hence the phasic properties of the cone synapse may help detection of dark objects silhouetted against a bright background. Indeed, psychophysical studies show that human observers are more sensitive to decrements than increments of light. The locus of this asymmetry has been tracked to early retinal processing (Bowen et al., 1989) and may result from the properties of cone vesicle release elucidated in this study.
Figure 1. Light stimulation collapses a Ca$^{2+}$ gradient maintained in cone terminals in darkness. (a-f) Fluorescence images of a cone terminal in a retinal slice loaded with the ratiometric Ca$^{2+}$ dye fura-2, collected after >10 min of dark adaptation (a-c) and subsequent 10 min exposure to bright white light (d-f). Images from 2-photon dye excitation with 700 nm light (a,d), 760 nm light (b,e), and the 700/760 nm ratio, which indicates Ca$^{2+}$ concentration (c,f). The black box shows 3.0 µm X 0.5 µm area for measuring the spatial profile of Ca$^{2+}$. Bottom is the ribbon end of the terminal, top is the axon end. (g-i) Images of another cone terminal showing a local increase in Ca$^{2+}$ evoked by depolarization, measured with the non-ratiometric Ca$^{2+}$ dye OGB-5N (excitation wavelength 488 nm). Intraterminal Ca$^{2+}$ at -70 mV (g) after 100 ms depolarization to -10 mV (h), and the difference in Ca$^{2+}$ induced by depolarization (i). Representative Ca$^{2+}$ profiles from 4 terminals in darkness (j) and after light stimulation (k) measured from 2-photon ratiometric fura-2 images as in c and f. Data is color-coded to allow comparison of the same terminals in darkness and light. Data points are running averages of 30 pixels binned over 500 nm. (l), Average Ca$^{2+}$ profiles in cone terminals in retinal slices exposed to darkness (black), light (red), ionomycin (orange) and Ca$^{2+}$-free saline (blue). N=5-12 terminals for each condition.
Figure 2. Elevation of cone terminal Ca\(^{2+}\) reveals two kinetic components of release. (a) Simultaneous whole-cell recordings from a salamander cone photoreceptor (Cone I\(_{Ca}\)) and a post-synaptic HBC (EPSC). Depolarizing cones from -70 to -10 mV elicits a sustained inward Ca\(^{2+}\) current and triggers an EPSC with phasic and tonic components. (b) Flash photolysis of DM-nitrophen raised Ca\(^{2+}\) levels in a different cone terminal (top), producing an EPSC in a HBC (bottom) with phasic and tonic components. Inset shows the average mEPSC for this cone-HBC pair (scale bars 1 pA, 10 ms). (c) Deconvolution of the EPSC from b with the waveform of the mEPSC (inset, b) provides an estimate of the presynaptic release rate (black trace). Release is fit by the sum of two components (red line), phasic release (the transient release after the flash; green line), and tonic release (the average release rate occurring 0.5-1.0 s after the flash; blue line). (d) Integration of the release rate from panel c yields the cumulative number of vesicles released after the uncaging flash. The pre-flash baseline release was subtracted to remove input from all but the DM-nitrophen loaded cone.
Figure 3. Ca\textsuperscript{2+}-dependence of phasic and tonic release. (a) Phasic (green circles) and tonic (blue circles) release triggered by photolytic uncaging of Ca\textsuperscript{2+} in cone terminals. Cone Ca\textsuperscript{2+} concentration as measured with OGB-6F, and release calculated by deconvolving the EPSC (see Fig. 2). (b) Tonic release rates determined from flash photolysis experiments (blue circles; same data points as in Fig. 3a) compared to release rates measured in anole cone terminals where Ca\textsuperscript{2+} was raised slowly with ionomycin and tracked over many minutes (open blue squares). Release in anole terminals was monitored with the synaptic vesicle marker FM1-43 while simultaneously measuring Ca\textsuperscript{2+} with fura-2. The red dotted lines show the expected rates for phasic release (panel a) and tonic release (panel b) predicted by the dark Ca\textsuperscript{2+} concentration (600 nM).
Figure 4. Electron microscopy of ribbon-associated vesicles. (a) EM pictures of cone ribbons from retina fixed in dark, light, and in Ca\textsuperscript{2+}-free saline. (b) The positions of ribbon-associated vesicles from 115 ribbons were mapped and overlaid for each condition (inset rectangle=200 nm X 400 nm). (c, magnification of inset from b) The resultant cloud of vesicles reveals depletion near the base of the ribbon in darkness. Vesicle occupancy of the entire ribbon (d) and the base of the ribbon (e). Average values ± SEM. N=115 ribbons for each condition.
Figure 5. Phasic release of resupplied vesicles on ribbons underlies the Off-response of post-synaptic neurons. (a) Light-evoked voltage responses from a cone, an HBC, and a DBC recorded from salamander retinal slices. Post-synaptic cells, but not the cone, display an marked overshoot (arrows) of the dark membrane potential at light offset. (b) Predicted changes in vesicle occupancy on the ribbon before, during, and after a light flash (top). Synaptic current response to a light flash in a voltage-clamped HBC (middle). Time course of presynaptic release from the group of cones driving this HBC (bottom). Release was calculated by deconvolving the EPSC with the waveform of the averaged mEPSC. Dotted line is fit to tonic release before the light flash.
Figure 6. The kinetics of vesicle resupply on the ribbon. (a) Postsynaptic responses (EPSCs) of a voltage-clamped HBC to light flashes of 0.5, 1, 5, and 10 s (top). Presynaptic release from cones calculated by deconvolving EPSCs. Release was integrated to reveal changes in cumulative release during and after the light flashes (bottom). Diagonal dotted lines show linear fits to tonic release before the light flash. (b) Vesicles released during the Off response, following light flashes of various durations (black circles, n=5), and the growth of the releasable pool predicted by the resupply model (solid line). (c) Quantification of exocytosis from cones using capacitance measurements. Cones were subjected to a "simulated light flash" voltage protocol and then depolarized to elicit fusion of the releasable pool of vesicles. (d) Growth of the releasable pool of vesicles during the "simulated light flash" (squares, n=9-10), and the growth of the releasable pool predicted by the resupply model (solid line).
Figure 7. **Phasic release improves the encoding of a change in luminance.** (a) Postsynaptic responses were recorded in voltage-clamped HBCs for a range of flash intensities. For both the On response (open circles) and the Off response that follows a 2s flash (solid triangles), the postsynaptic response increases over ~4 log units before saturating with very bright light. (b) Tonic release from cone synapses could encode 13 distinguishable levels of brightness, given stochastic dark release of 500 vesicles/s (100 vesicles in the 200 ms integration time of postsynaptic bipolar cells). (c) Phasic release could encode 33 distinguishable levels of brightness, given a phasic release rate of ~3,350 vesicles per second (670 vesicles in the 200 ms bipolar cell integration time).
METHODS

Ca\(^{2+}\) imaging

All experiments were performed at room temperature (21°-24° C). For Fura-2 AM loading, retinas from the lizard *Anolis segrei* were isolated as described previously (Choi et al., 2005; Sheng et al., 2007) using procedures approved by the UC Berkeley Animal Care and Use Committee. The entire retina with the retinal pigment epithelium attached was flat mounted onto nitrocellulose filter paper and sliced to a thickness of ~300 µm. Slices were dye-loaded for 30 minutes in a lizard saline solution containing 100 µM Fura-2-AM, 1% DMSO, and 0.2% pluronic acid. Images were acquired using a Zeiss LSM two-photon confocal microscope with a Tsunami Ti-Saphire laser (Coherent). Excitation wavelengths of 700 and 760 nm were used for excitation and ratiometric imaging of Fura-2 (Sheng et al., 2007). The laser was calibrated to use an equivalent power for each excitation wavelength. Images were analyzed using Scion Image software (Scion Corporation, Frederick, MD). Retinas were stimulated with white light from a halogen lamp attenuated with a neutral density filter to an intensity of 10\(^7\) photons s\(^{-1}\) µm\(^{-2}\).

To analyze Ca\(^{2+}\) profiles from fura-2 ratio images, a rectangle 0.5 µm wide x 3.0 µm long was drawn along the ribbon-axon axis of the cone terminal, abutting the ribbon end of the terminal. Pixel values were averaged along the 0.5 µm width of the rectangle (~30 pixels). For each individual trace, the running average of 30 pixels was plotted from the values obtained along the 3 µm-long ribbon-axon axis. For the average traces of different experimental conditions, each data point represents an average of 5 pixel values ± SEM (n=5-8).

Intraterminal Ca\(^{2+}\) was calculated from the ratio of light emitted at fura-2 two-photon excitation wavelengths of 700 nm and 760 nm (Sheng et al., 2007) using calibration constants determined in lizard cones by dialyzing cells with highly-buffered, known concentrations of Ca\(^{2+}\). The K\(_{eff}\) of Fura-2 was verified in situ by dialyzing cones with an EGTA-buffered solution with a free Ca\(^{2+}\) concentration of 180 nM determined using MaxChelator. The solution contained (in mM): 132 KCl, 2 NaCl, 10 EGTA, 3.5 CaCl\(_2\), 2 MgCl\(_2\), 20 HEPES (pH 7.1). The minimum and maximum ratios were obtained using similar pipette solutions but with 0 CaCl\(_2\) or 0 EGTA, respectively.

For non-ratiometric Oregon Green Ca\(^{2+}\) imaging, lizard retinal slices were prepared using techniques described for salamander retinal slices (Rabl et al., 2005). Whole-cell voltage-clamp recordings were obtained with an Optopatch patch-clamp amplifier (Cairn Instruments, Faversham, Kent) using 8-15 MΩ borosilicate glass patch electrodes. The pipette solution contained (in mM): 132 CsGluconate, 10 TEACl, 2 MgCl\(_2\), 1 CaCl\(_2\), 10 MgATP, 2 glucose, 0.5 mM GTP, 5 EGTA, 20 HEPES (pH 7.2). Cones were viewed through an upright, fixed-stage microscope (Nikon E600 FN) mounted with a laser confocal scanhead (Perkin Elmer Ultraview LCI) equipped with a filter wheel (Sutter Lambda 10-2) for switching excitation and emission wavelengths. Images were acquired with a cooled CCD camera (Orca ER) at 60 ms intervals with single frame durations of 57 ms and pixel values were binned 2x2. Images were analyzed with UltraView Imaging Suite software. The Ca\(^{2+}\) indicator dyes Oregon Green 488 BAPTA-5N (OGB-5N, K\(_d\) = 20 µM) was added to the patch pipette solution at 200 µM and dialyzed into the cell during whole cell recording.
**Electron microscopy**

Lizard retinas were isolated as above in darkness and maintained for 30 minutes either in darkness, bright white light, or in the light with saline containing no added Ca\textsuperscript{2+} and 5 mM EGTA. The retinas were then fixed with 2% paraformaldehyde plus 2% glutaraldehyde in 0.1 M phosphate buffer (pH 7.4), overnight at 4°C. Fixed tissue was postfixed with 1% osmium tetroxide and 1.5% potassium ferrocyanide, and dehydrated with a graded series of ethanol solutions. Prepared retinas were embedded in Epon 812 for transmission EM, sectioned into 70 nm thickness with a Reichert-Jung Ultracut E using a diamond knife. The sections were examined with the FEI Tecnai 12 electron microscope. Resulting images were contrast enhanced using Scion Image software (Scion Corporation, Frederick, MD).

To minimize circadian fluctuations in ribbon characteristics (Hull et al., 2006), anoles were maintained on a 12:12 light:dark cycle and all experiments were performed within a 4 hour period during the daytime portion of the cycle.

We measured the number and distribution of vesicles on ribbons using the following procedure: We mapped the shape of individual ribbons and the positions of vesicles lying within 120 nm of the edge of ribbons. Images of ribbons were aligned at their bases near the arciform density, just above the plasma membrane, with ribbons oriented vertically. To produce the vesicle densities in Fig. 4b, we superimposed vesicles from 115 ribbons for each of the three experimental conditions (darkness, light, 0 Ca\textsuperscript{2+}; 4 retinas for each condition).

We measured the number of vesicles up the height of the ribbon (perpendicular to the membrane). This value was multiplied by a scaling factor reflecting the depth of the ribbon (parallel to the membrane; 0.5 µm for anoles (Choi et al., 2005)), and the density of vesicle packing on the ribbon (17.65 vesicles/µm; Thoreson et al., 2004). This scaling factor is used to convert two-dimensional measurement of vesicles on a section of ribbon into a three-dimensional estimate of vesicles on the entire ribbon.

The EM sectioning may result in a vesicle counting error caused by superposition of vesicles within a section. Visualization of vesicles deeper into a section could be obscured by vesicles lying in front of them, leading to an under-count of vesicles. The under-estimate should increase with vesicle occupancy on the ribbon; hence the under-count should be greater in light-adapted than dark-adapted cones, enlarging the difference between the two conditions. The maximum error caused by superposition (i.e. in a fully loaded ribbon) is limited by the average diameter of vesicles (50 nm; Rea et al., 2004) and the thickness of the EM sections (70 nm). Our estimate of the maximal vesicle packing density in anoles exposed to Ca\textsuperscript{2+}-free saline is 19.75 vesicles per µm, quite similar to the value reported previously for salamander rods (Thoreson et al., 2004) (17.65 vesicles per µm), adding confidence to our estimates.
Modeling vesicle counting errors in EM sections

We constructed a linear array of vesicles separated by 70 nm. 50 nm diameter vesicles were independently assigned to a row of four lattice points according to the fractional occupancy of the row (probability =1 for a fully populated ribbon, probability = 0 for a fully depleted ribbon). A virtual section was cut from the lattice, with the center of the section randomly placed between the second and third lattice point. If the resulting section enclosed at least one half of a single vesicle, a vesicle would appear in the 2-dimensional projection of the section, and the row was considered occupied. We performed $10^6$ simulations for each value of fraction occupancy, which was varied from 0 to 1 in steps of 0.01.

Electrophysiology

Retinas from the larval tiger salamander *Ambystoma tigrinum* were isolated and prepared slices as described previously (Rabl et al., 2005) using procedures approved by the UNMC Institutional Animal Care and Use Committee. Whole-cell voltage-clamp recordings were obtained from cones using 10 -15 MΩ patch pipettes pulled from borosilicate glass. The pipette solution contained (in mM): 42 CsCl2, 48 Cs Gluconate, 1.9 MgCl2, 32.9 HEPES, 9.4 TEACL, 9.4 MgATP, 0.5 GTP, 5 EGTA (pH 7.2). Salamander cones were voltage clamped at a steady holding potential of -70 mV using an Optopatch (Cairn Instruments, Faversham, Kent) patch-clamp amplifier and stimulated with depolarizing test steps. For current clamp recordings from cones, perforated patch recording techniques were used by adding the perforating agent beta escin to the pipette solution. Currents were acquired using a Digidata 1322 interface and pClamp 9.2 software (Molecular Devices). Unless noted otherwise, current records were filtered with a 5 kHz low-pass filter.

For caged Ca$^{2+}$ experiments, the pipette solution consisted of (in mM): 10 DM-nitrophen, 5 CaCl2, 4 DPTA, 4 MgCl2, 26 Cs Gluconate, 78 HEPES, 6.5 TEACL, 11 Na2ATP, 0.5 GTP, Oregon Green BAPTA 6F (0.5 mM, Kd = 3 µM) (pH 7.2). Calcium levels in flash photolysis experiments were determined from the formula:

$$[Ca^{2+}] = [Ca^{2+}]_{rest} + K_d \frac{\Omega}{(1- \Omega)}$$

where $$\Omega = (\Delta F/F)/(\Delta F/F)_{max}$$

$[Ca^{2+}]_{rest}$ was determined from aliquots of each solution using Fura-2. $(\Delta F/F)_{max}$ was determined from the maximum fluorescence increase evoked by strong depolarizing steps.

For light stimulation experiments, stimuli were generated using a 50 W halogen lamp focused on the retina. To minimize the contribution of rods to post-synaptic light responses, 480 nm background light of ~$1.0\times10^3$ photons s$^{-1}$ µm$^{-2}$ was used to light-adapt rods and the slice was stimulated with superimposed flashes of 680 nm light (unattenuated intensity $1.1\times10^6$ photons s$^{-1}$ µm$^{-2}$). Light intensity was adjusted using Wratten neutral density filters.
Calculating release rates

Transmitter release from salamander cones was calculated by deconvolution of the bipolar cell EPSC with the idealized waveform of the averaged miniature EPSC (Van der Kloot, 1988). For each cone-HBC pair, the averaged mEPSC was fit to a waveform with amplitude i₁, an instantaneous rise time, and exponential decay constant τ. The EPSC with waveform I(t) can be deconvolved according to the equation,

\[ n(t) = \Delta t(dI(t)/dt + I(t)/(\tau)/i_1) \]

where n(t) is the number of quanta released in the time period Δt (0.1 ms in our experiments) centered at time t.

Transmitter release from lizard cones was calculated from the rate of FM1-43 unloading from cone synapses in retinal slices (Sheng et al., 2007). Lizard retinas were removed in darkness and mounted onto nitrocellulose paper, then loaded for 2 hr in lizard ringer solution containing 30 µM FM1-43 (Invitrogen, Eugene, OR). FM1-43 loaded retinas were sliced in Ca²⁺-free saline and transferred to Ca²⁺-free saline containing 5 µM fura-2 AM (Invitrogen) for 30 minutes. We then treated the slices with ionomycin and added various concentrations of extracellular Ca²⁺ (from 0.1 to 10 uM) to elevate internal Ca²⁺ to different levels and trigger different rates of synaptic vesicle release, as described previously (Sheng et al., 2007). Retinal slices were imaged with an upright microscope Nikon (Tokyo, Japan). Fluorescence was detected with an Imago Sensicam (TILL Photonics, Planegg, Germany) via a 40 × 0.7 NA water immersion objective (Olympus, Tokyo, Japan). The wavelength of excitation light was controlled with a Lambda 10–2 filter wheel (Sutter Instruments, Novato, CA). FM1-43 was excited with 490 nm light. Fura-2 was excited with 350 and 380 nm light for ratiometric measurement. A 510±10 nm emission filter was used for both dyes. Images were acquired and analyzed with Imaging Workbench software (Indec BioSystems, Santa Clara, CA). Images of fura-2 and FM1-43 were acquired every 10 s and binned 4 × 4. The in vitro fura-2 calibration of the photometric system was performed with a fura-2 Ca²⁺ imaging calibration kit. Rates of FM1-43 unloading were converted to rates of vesicular exocytosis by methods previously described (Choi et al., 2005).

Our measurements of the Ca²⁺-dependence of phasic and tonic release (Fig. 3) include data from both salamander and lizard cones. Anole lizard cones possess about twice as many ribbons as salamander cones (Choi et al., 2005; Pang et al., 2008), but ribbons in salamanders extend roughly twice as far along the presynaptic membrane as do lizard ribbons (Peter Sterling, personal communication). Thus the total membrane contact area of cone ribbons is equal in the two species, consistent with our measurements showing comparable release rates.

Modeling vesicle resupply

Vesicle resupply was modeled using Mathematica (Wolfram Research). The size of the readily releasable pool (RRP) was estimated from EM data as the number of ribbon-associated vesicles located within the depletion zone (within 100 nm of the base of the ribbon). For retina fixed in Ca²⁺-free saline, when fusion is minimal and the RRP is expected to reach full capacity, our EM sections show an average of 3.7 vesicles/ribbon located within 100 nm of the base of the ribbon.
To convert this number to an estimate of the maximal releasable pool of vesicles ($n_{\text{max}}$) this value was multiplied by a scaling factor reflecting the depth of the ribbon (parallel to the membrane; 1 µm for salamander; Peter Sterling, personal communication), and the 17.65 vesicles µm$^{-1}$ density of vesicle packing on the ribbon (Thoreson et al., 2004). This scaling factor was used to convert two-dimensional measurements of vesicles on a section of ribbon into a three-dimensional estimate of vesicles on the entire ribbon. We estimated the number of ribbons contributing to the presynaptic capacitance signal as the average number of ribbons in salamander cone terminals (13.4) (Pang et al., 2008), and the number of ribbons contacting a post-synaptic bipolar cell from morphological analysis (15.2) (Pang, Gao et al., 2008). Release during the light flash was calculated using parameters determined from Ca$^{2+}$ imaging and flash uncaging experiments. We assumed the rate of resupply was a function of the RRP size and that the maximal resupply rate was equal to the dark rate of exocytosis $R_{\text{Dark}}$.

\[ \text{Resupply rate} = n_{\text{ribbons}} \times R_{\text{dark}} \times (1-n_{\text{vesicles}}/n_{\text{max}}) \]
Table 1: Model parameters for the post-synaptic Off response (Fig. 6b)

<table>
<thead>
<tr>
<th>Parameter</th>
<th>Value</th>
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<tr>
<td>Number of presynaptic cone ribbons contacting bipolar cell</td>
<td>15.2 = 17 (Number of cones in HBC dendritic field)</td>
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<tr>
<td></td>
<td>…X 13.4 (Number of ribbons per cone terminal)</td>
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<tr>
<td></td>
<td>…X 0.8 (80% of HBC contacts are at ribbons)</td>
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<tr>
<td></td>
<td>…X 0.25 (HBCs receive 25% input from cones)</td>
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<td></td>
<td>…/3 (coverage factor) (Pang et al. 2008)</td>
</tr>
<tr>
<td>Releasable vesicles per ribbon (n_{max})</td>
<td>3.7 vesicles (EM data)</td>
</tr>
<tr>
<td></td>
<td>…X 17.65 vesicles/µm (Thoreson et al. 2004)</td>
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<td></td>
<td>…X 1 µm ribbon length (Peter Sterling, personal communication)</td>
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<tr>
<td>Resupply rate</td>
<td>Rate of release onto postsynaptic HBCs in darkness measured from deconvolution of synaptic current: 338 vesicles/s (N=6 HBCs)</td>
</tr>
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Table 2: Model parameters for presynaptic capacitance response (Fig. 6c)

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<th>Parameter</th>
<th>Value</th>
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<tr>
<td>Number of ribbons contributing to capacitance response</td>
<td>13.4 ribbons/terminal (Salamander cones, Pang et al. 2008)</td>
</tr>
<tr>
<td>Releasable vesicles per ribbon</td>
<td>3.7 vesicles (EM data Fig4)</td>
</tr>
<tr>
<td></td>
<td>…X 17.65 vesicles/µm (Thoreson et al. 2004)</td>
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<tr>
<td></td>
<td>…X 1 µm ribbon length (Peter Sterling, personal communication)</td>
</tr>
<tr>
<td>Resupply rate</td>
<td>Dark release rate measured presynaptically by dye loss from FM1-43 stained vesicles 500 vesicles/s: (Sheng et al. 2007)</td>
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Using the measured value for the diffusion coefficient $D$ of cytoplasmic vesicles in the cone synapse (Rea et al., 2004) we estimated the rate at which vesicles would be supplied to the active zone by diffusional collision. Assuming a cytoplasmic concentration $C$ of freely diffusing vesicles, the rate at which vesicles should be resupplied by random collision with an area the size of the active zone $A$ is given by the equation (Holt et al., 2004)

$$\text{Collision rate} = 2 A C \sqrt{\frac{D}{\pi}}$$

In anole cones, cytoplasmic vesicles are present at a concentration of 2000 vesicles $\mu m^{-3}$, and diffuse freely with a diffusion coefficient of $D = 0.11 \mu m^2 s^{-1}$. Given 25 ribbons per cone terminal, each with a active zone of 500 nm by 200 nm (0.1 $\mu m^2$), the expected collision rate is $\sim 1,900$ vesicles $s^{-1}$, far higher than the measured dark rate of release of 250 vesicles $s^{-1}$. (Choi et al., 2005)

It is similarly possible to estimate the rate at which vesicles bound to the ribbon would supplied to the active zone by 2-dimensional diffusion along the ribbon face using the equation,

$$r^2 = 2 D t$$

where $r$ is the mean displacement of a diffusing vesicle within a time $t$. A vesicle located above the depletion zone is expected to traverse the 200 nm distance to the active zone in a time of 180 ms. Given a row of vesicles located above the depletion zone with a density of 17.65 vesicles $\mu m^{-1}$, on ribbon that is 0.5 $\mu m$ long, 2-dimensional diffusion would supply the active zone at a rate of $\sim 2500$ vesicles $s^{-1}$ in an anole terminal, again far exceeding the measured rate of release from anole cones in the dark.

If vesicles are resupplied to the release zone by descent down the ribbon face, it is possible to estimate the descent speed of vesicles, assuming vesicles descend *en masse* along the ribbon face, by using the density of vesicles bound to the ribbon $\rho_v$, the length of the ribbon $L$, and the dark release rate $R_{dark}$. The density of hexagonally packed vesicles on photoreceptor ribbons (Thoreson et al., 2004) is 339.6 vesicles $\mu m^{-2}$. Given a ribbon length of 0.5 $\mu m$ and a dark release rate of 250 vesicles $s^{-1}$.

$$\text{Descent speed} = \frac{R_{dark}}{2 \rho_v L} = 29 nm/s$$
Encoding of light intensity by release

Assuming that release by the cone synapse obeys Poisson statistics, the number of distinguishable light levels represented by the synapse follows from

\[ R_{i+1} = R_i - (1.4 \times \sqrt{R_i}) \]

where \( R_i \) is the release rate at a given intensity, divided by a factor of 5 to reflect the 200 ms bipolar cell integration, and \( R_{i+1} \) is the number of vesicles released at the smallest reliably detected intensity change. Threshold was set at 1.4 standard deviations between probability density functions for the two stimuli, as in equivalent psychophysical experiments.

Contrast sensitivity was computed with an ideal observer model (Banks et al., 1987; Geisler, 1989) that detected intensity increments (positive contrast) or decrements (negative contrast) in a single-interval two-alternative forced choice paradigm. By numerical simulation, contrast sensitivity was computed from the ratio of the background intensity and the smallest intensity increment and decrement that could be detected reliably (68% correct).
SUPPLEMENTARY FIGURES
S1-S6

The following figures are designated as supplementary material because they are not considered absolutely vital to the arguments of this chapter. However, the information in these figures is alluded to within the main body of the text, and hence these figures are included as explanatory aids.
Supplementary Figure S1. Measuring tonic release from cones with FM1-43. (a) Lizard retinal slice treated with the Ca\(^{2+}\)-ionophore ionomycin, loaded with the ratiometric Ca\(^{2+}\)-indicator fura-2 AM (top) and the synaptic vesicle marker FM1-43 (bottom). (b) Measuring the Ca\(^{2+}\) dependence of tonic release from cones by monitoring Ca\(^{2+}\) in the outer plexiform layer with fura-2 (top), while simultaneously tracking the rate of FM1-43 destaining from cone terminals (bottom). Terminal Ca\(^{2+}\) was elevated by adding Ca\(^{2+}\) to the perfusion solution. When Ca\(^{2+}\) achieved a steady value, the rate of decrease in the FM1-43 signal was evaluated and converted to a rate of exocytosis\(^1\).
Supplementary Figure S2. Counting ribbon-associated vesicles in dark, light, and 0 Ca\(^{2+}\). 20 cone ribbons (black objects), their associated vesicles (green circles), and the position of the presynaptic cone membrane (dashed lines) from retinas fixed in (a) dark, (b) light, and (c) Ca\(^{2+}\)-free saline. Above each ribbon is the number of vesicles associated with that ribbon (lying within 120 nm of the ribbon surface). Although ribbons varied morphologically, most ribbons appeared in our EM images as thin bars extending into the cytoplasm from the base of a synaptic ridge. To quantify the position of vesicles along the ribbon face, a metric axis was fixed to the ribbon base and directed along the long axis of the ribbon. Because some ribbons were curved, a second metric axis was included if the curvature of the ribbon exceeded \(\sim 20^\circ\). To qualify for analysis, a ribbon had to be located within 200 nm of the plasma membrane, and be continuous along its length, with no gaps greater than 100 nm.
Supplementary Figure S3. Length of cone ribbons fixed under different conditions. 4 retinas for each condition were fixed after 30 min exposure to either darkness, saturating light (10^7 photons s^{-1} µm^{-2}), or Ca^{2+}-free saline. Values represent the mean for 115 ribbons from each condition, ± S.E.M.
Supplementary Figure S4. Stereological considerations for quantifying vesicles on ribbons. (a) We evaluated ribbon occupancy by counting vesicles along the height of the ribbon because most synaptic ribbons (>95%) appeared as cross sections in our EM images. The number of observed vesicles was multiplied by a factor to account for the depth of the ribbon and linear density of vesicles arranged in rows. (b) A small number of ribbons in our EM images (<5%) were captured en face, with the plane of the ribbon parallel to the plane of the section. In principle, an en face ribbon provides a direct view of the 2-dimensional array of vesicles on the ribbon surface. However, we found that the electron dense structure of the ribbon obscured the vesicles tethered to its surface. (c) In a single case out of ~400 ribbons the section cut immediately in front of an en face ribbon, providing a view of the tethered vesicles without showing the underlying ribbon. (d) Higher resolution of the image shown in c. Vesicles are arranged on the ribbon in a hexagonal array. Images a-d came from light-adapted retinas. Scale bars, 200 nm. (e,f) Illustration of 3-dimensional ultrathin sections (top) and the resulting 2-dimensional projections (bottom). With transmission electron microscopy, vesicles located anywhere within the 70 nm-thick section will appear on a single 2-dimensional plane. Vesicles
that penetrate at least halfway into a section (outlined in yellow) will appear to have a full diameter. (e) A fully occupied ribbon will always appear fully populated in cross section (bottom). If two vesicles in the same row are contained within a section their 2-dimensional projections overlap and they are not counted twice. (f) A depleted ribbon is more likely to appear fully occupied because only half a vesicle must penetrate a section to be counted (black arrow points to a half-vesicle that will appear in cross section). (g) Monte Carlo modeling of vesicle over-counting. Sections were cut from an array of vesicles with 70 nm spacing at different occupancy levels (see Supplementary Methods). A row appeared occupied if >50% of at least one vesicle fell within the 70 nm-thick section. Depleted ribbons yielded an over-estimate of vesicle occupancy. (h) Output from the same model, plotted as the percentage of over-counting. The dotted blue lines in g and h depict errorless counting.
Supplementary Figure S5. Postsynaptic Off responses precede cone depolarizing afterpotentials at light offset. Paired whole-cell recordings from a current-clamped cone and a voltage-clamped horizontal cell (a) or a hyperpolarizing bipolar cell (b). Both the cone and the postsynaptic cell show an Off response at the end of the light flash. However, the postsynaptic Off response reaches a peak (vertical dotted line) while the cone is still hyperpolarized, >200 ms before the peak of the cone depolarizing afterpotential (arrows). (c) Comparison of Off response latencies in presynaptic cones (N=5) and postsynaptic bipolar cells (N=7). The latency was significantly shorter in the bipolar cell (p> 0.0002, paired t-test). Off responses were measured at the end of 10 s light flash, superimposed on a dim background to minimize the contribution of rod synaptic inputs. Voltage-clamp current records were filtered with a 5 kHz low-pass filter. Current-clamp voltage records are naturally filtered by the membrane properties of cones and no additional electronic filtering was used.
Supplementary Figure S6. Modeling the dynamics of synaptic release during and after a light flash. (a) The release rate $R$ is influenced by the rate constants of several sequential events, including binding and descent along the ribbon (lumped as $k_{\text{resupply}}$), followed by membrane fusion ($k_{\text{fusion}}$). We varied the rate-limiting step by keeping $k_{\text{resupply}}$ constant while varying $k_{\text{fusion}}$ over 3 orders of magnitude. (b-e) Simulated release rates in response to a light flash that decreases $k_{\text{fusion}}$ by a factor of 20 during the 1 s flash. (b) If $k_{\text{fusion}}$ is very low, the synapse supports little tonic release in darkness. (c) Setting $k_{\text{resupply}}$ equal to $k_{\text{fusion}}$ results in tonic release and a robust On response but no phasic Off response. (d) Phasic Off responses appear only when the fusion rate constant exceeds the resupply rate constant. (e) If $k_{\text{fusion}}$ is very high, the synapse fails to lower the rate of tonic release throughout the light response.
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Chapter 3: Imaging synaptic Ca\textsuperscript{2+} nanodomains in the cone terminal with a ribbon-associated calcium indicator

In the previous two chapters, we attempted to measure the Ca\textsuperscript{2+} signal that regulates neurotransmitter release at the cone synapse. We showed that Ca\textsuperscript{2+} influx elevates Ca\textsuperscript{2+} locally near synaptic ribbons. However, we were limited in our ability to resolve Ca\textsuperscript{2+} below a length scale of roughly one micron. Meanwhile, synaptic physiologists have postulated that open Ca\textsuperscript{2+} channels produce nanometer-scale intracellular Ca\textsuperscript{2+} gradients. Attempts to directly image nanodomains using fluorescent probes have been frustrated by the resolution limits of conventional microscopy. In this chapter we describe a novel approach to measuring nanometer-scale Ca\textsuperscript{2+} gradients, using synaptic ribbons to tether Ca\textsuperscript{2+} sensors in close proximity to Ca\textsuperscript{2+} channels. We have designed a new ribbon-associated calcium indicator (RACI) to exploit the architecture of photoreceptor synapses, where synaptic ribbons associate closely with Ca\textsuperscript{2+} channels and provide a unique platform for sensing Ca\textsuperscript{2+} gradients. RACI was introduced into tiger salamander cone photoreceptors via patch pipette, and RACI fluorescence was recorded while the Ca\textsuperscript{2+} current was manipulated. Analyses of the relationship between Ca\textsuperscript{2+} current and fluorescence provided an estimate for the spatial profile of Ca\textsuperscript{2+} nanodomains. The results of this experiment show close agreement with theoretical models for Ca\textsuperscript{2+} gradients, and can be used to explain the dynamics of vesicle release at the ribbon synapse.
INTRODUCTION

Localized Ca\(^{2+}\) signaling in neurons

Calcium regulates a myriad of intracellular signals in neurons, from synaptic vesicle fusion to gene transcription and apoptosis. Due to the ubiquity of Ca\(^{2+}\)-sensitive pathways, it is crucial that neurons constrain the spread of unbound Ca\(^{2+}\) ions that enter during depolarizations of the cell membrane. Neurons restrict the spatial spread of Ca\(^{2+}\) signals through the use of cytoplasmic buffers and membrane pumps, which quickly capture and extrude free Ca\(^{2+}\) as it enters through voltage-gated Ca\(^{2+}\) channels. For several decades synaptic physiologists have postulated that open Ca\(^{2+}\) channels produce nanometer-scale Ca\(^{2+}\) gradients (often termed “nanodomains” or “microdomains”) (For reviews, see Augustine et al., 2003, Oheim et al., 2006). The shapes of these concentration gradients are of broad interest because every Ca\(^{2+}\)-dependent process will be modulated differentially depending on its distance from channels. As a result, a host of analytical models (Neher 1986; Smith 2001) and numerical simulations (Chad & Eckert, 1984; Zucker & Fogelson, 1986) have attempted to describe these gradients. Unfortunately, attempts to characterize Ca\(^{2+}\) nanodomains using imaging techniques have been frustrated by the inability of conventional microscopy to resolve nanometer-scale features (Llinas et al., 1992; DiGregorio et al. 1999). Furthermore, resolution of Ca\(^{2+}\) gradients is inhibited by the mobility of cytoplasmic Ca\(^{2+}\) sensors, which bind Ca\(^{2+}\) and then diffuse throughout cells causing a “blurring” of gradients.

A more recent attempt to resolve nanodomains in synapses involved monitoring intracellular Ca\(^{2+}\) near the site of Ca\(^{2+}\) entry by tethering a fluorescent Ca\(^{2+}\) sensor directly to the intracellular domain of voltage gated Ca\(^{2+}\) channels (Tour et al, 2007). This approach yielded excellent temporal resolution of Ca\(^{2+}\) signaling within nanodomains. However, because the Ca\(^{2+}\) sensor was restricted to a single site relative to the open channel pore, this approach offered no spatial resolution of the Ca\(^{2+}\) gradient. In order to characterize the gradient, it is important to sample the Ca\(^{2+}\) signal at multiple distances from open channels.

Synaptic Ribbons and RIBEYE

Here we extend the approach of Tour et al. by using synaptic ribbons as scaffolds to bind Ca\(^{2+}\) sensors across the entire synaptic Ca\(^{2+}\) gradient. Sensory neurons such as photoreceptors, hair cells and retinal bipolar cells posses synapses that release neurotransmitter tonically. The synapses of these cells contain specialized organelles called synaptic ribbons which are believed to facilitate the tonic release of neurotransmitter. Synaptic ribbons appear in electron microscopy as electron-dense structures which anchor to the plasma membrane at active zones (Fig. 1a), tethering large numbers of vesicles in close proximity to voltage gated Ca\(^{2+}\) channels (Zenisek et al., 2003, Heidelberger et al., 2005). The primary constituent of synaptic ribbons is RIBEYE, a protein which is found exclusively at ribbon synapses and which has a B domain that is nearly identical to the transcription factor C-terminal binding protein 2 (CtBP-2) (Schmitz et al., 2000). CtBP proteins are known to bind peptides bearing a PXDLS motif. Several labs have
recently exploited this interaction by using fluorescently-tagged RIBEYE-binding peptides bearing the PXDLS motif to label synaptic ribbons in vivo.

Experiments conducted on goldfish bipolar cells show that fluorescently tagged RIBEYE-binding peptides introduced via patch pipette enter neurons readily and label synaptic ribbons selectively (Zenisek et al., 2004). Fluorescence measurements indicated that thousands of dye-labeled peptides attach to each synaptic ribbon. Immunogold labeling reveals RIBEYE to be uniformly distributed across the length of ribbons (Schmitz et al., 2000). Cells simultaneously loaded with RIBEYE-binding peptide and Calcium indicators show colocalization of synaptic ribbons with sites of Ca\textsuperscript{2+} entry. Interestingly, depolarization of cells injected with RIBEYE-binding peptide evokes normal capacitance changes and Ca\textsuperscript{2+} currents, suggesting that RIBEYE-binding peptides do not interfere with normal synaptic function (Zenisek et al., 2004).

Recent work by our lab has shown that in the tiger salamander, ribbon synapses of cone photoreceptors assemble into orderly, predictable complexes. Electron microscopy reveals synaptic ribbons as flat rectangular “wall-like” structures that extend ~1 µm along the plasma membrane and ~0.5 µm upwards into the cytoplasm (Sheng et al., 2007). Ca\textsuperscript{2+} channels line the base of the ribbon (Raviola, 1975; tom Dieck et al., 2005), and open Ca\textsuperscript{2+} channels establish a concentration gradient that diminishes up the height of the ribbon (Jackman et al., 2009). Analytical and numerical modeling suggest that this concentration gradient decays exponentially as a function of distance from Ca\textsuperscript{2+} channels (Fig. 1b), with a exponential decay constant of <100 nm (Neher 1986; Chad & Eckert, 1984). Thus the architecture of synaptic ribbons provides an ideal scaffold onto which sensors may be attached to detect Ca\textsuperscript{2+} concentrations over the full extent of the Ca\textsuperscript{2+} gradient.
RESULTS

Synthesis and imaging of RACI

We synthesized a molecule we call RACI (Ribbon-Associated Calcium Indicator) designed to tether a fluorescent calcium indicator to ribbons. The functional components of RACI include the RIBEYE-binding peptide (which binds the CtBP domain of RIBEYE with an estimated $K_d$ of 27 uM; Zenisek et al., 2004) linked to 2 molecules of fluo-4 (which binds Ca$^{2+}$ with $K_d$ of 345 nM, and exhibits a 100-fold increase in fluorescence upon binding; Gee et al., 2000). The Ca$^{2+}$-indicator fluo-4 cadaverine (an amine-containing fluo-4; available from Invitrogen) was linked with a 16-residue peptide containing the CtBP binding motif via a transglutaminase-catalyzed reaction (Fig 1c). We designed a second version of RACI (Fig. 1d), in which the Ca$^{2+}$-indicator is not covalently attached to the RIBEYE binding sequence, but instead associates with a second, 35-amino acid sequence, which binds the xanthene core of rhodamine family dyes with picomolar affinity (Marks et al., 2004).

This second version of RACI (synthesized by Elim Biopharmaceuticals) holds several possible advantages. Because the peptide requires no covalent attachment with the calcium indicator, it may be genetically expressed in vivo, and tissue may then be loaded with an AM form of calcium indicator. Furthermore, this peptide binds to a library of dyes with affinities for Ca$^{2+}$ ranging from 700 nm (X-rhod 1) to 350 µM (Rhod 5N). Unfortunately, this version of RACI does not load into cells through patch-pipettes, for reasons we do not understand. Further experiments will attempt to improve our ability to load this version of RACI.

RACI was introduced into synapses of tiger salamander cone photoreceptors via patch pipette. Fluorescence from RACI was monitored with a high-speed EMCCD camera mounted on an upright fluorescence microscope, while the Ca$^{2+}$ current was simultaneously modulated by changing the cell holding potential. Salamander cones are ideal for this experiment because their synaptic terminal is continuous with the cell body, and this compact axon-less structure facilitates the diffusion of dye throughout the synapse and allows effective voltage-clamp of the synaptic terminal. Furthermore, salamander cones possess non-inactivating L-type Ca$^{2+}$ channels, and the voltage-activation and single-channel conductance of these channels is well characterized (Thoreson et al., 2000).

To determine whether RACI binds to synaptic ribbons, we dialyzed RACI into patch-clamped rod and cone photoreceptors. To ensure that localized fluorescence reflected binding to ribbons, and was not the result of local Ca$^{2+}$ hot spots, cells were held at -70 mV to keep Ca$^{2+}$ channels inactive, and the pipette solution contained 1 µM free Ca$^{2+}$ to make RACI fluoresce uniformly. Upon achieving whole-cell patch, RACI quickly spread through the cell. Fluorescence appeared as bright puncta at the synapse of both rods (Fig. 2a) and cones (Fig. 2b), presumably reflecting binding of RACI to synaptic ribbons. Additional dim, mottled labeling was observed at the inner segment of cells. The mottled pattern of fluorescence suggests that RACI in this region was bound, and not freely diffusing through the cytoplasm. Yet the location the mottled labeling in the inner segment suggests that it does not represent ribbon-bound RACI. What accounts for the labeling of the inner segment? The RIBEYE-binding sequence of RACI was originally discovered as a transcription factor-binding peptide. The photoreceptor nucleus is located in the
inner segment, hence it is likely that the mottled labeling represents RACI bound at the nucleus. In a sense, the binding of RACI at the nucleus may be viewed as confirmation of the efficacy of RACI binding.

When free Ca\(^{2+}\) was removed from the pipette solution, the synaptic puncta disappeared, and cells generally exhibited less fluorescence. Fluorescence puncta returned at the synapse when cells were depolarized to -10 mV to open voltage-gated Ca\(^{2+}\) channels. Fluorescence did not increase uniformly throughout the cell, indicating that depolarization caused only a local increase in Ca\(^{2+}\) only at the synapse. The fluorescent puncta at the synapse were most striking when difference images were taken between -70 mV (closed Ca\(^{2+}\)-channels) and -10 mV (open channels) (Fig. 2c). To capture information about the local Ca\(^{2+}\) signal, analyses of the RACI signal was conducted by placing a region of interest around synaptic puncta which appeared in difference images, under the presumption that RACI in this region was bound to ribbons.

**Extracting the Ca\(^{2+}\) gradient from RACI**

To manipulate the synaptic Ca\(^{2+}\) current, and hence the Ca\(^{2+}\) gradient, cones loaded with RACI were subjected to a voltage ramp (Fig. 3a). Cells were depolarized to +100 mV, a voltage that opens L-type channels, but which is above the Ca\(^{2+}\) reversal potential. The voltage was then ramped to more negative potentials to increase the driving force for Ca\(^{2+}\) ions through open channels. The inward current increased linearly with the driving force until -10 mV, the range where L-type voltage-gated Ca\(^{2+}\) channels begin to close (Fig. 3b). Subtracting the slope of the leak current between -100 mV and -50 mV revealed the Ca\(^{2+}\) current (Fig. 3c). To remove voltage-gated K\(^+\), and Ca\(^{2+}\)-gated Cl\(^-\) currents, CsCl, TEA-Cl, and niflumic acid were added to the pipette solution. RACI fluorescence, imaged at 100 Hz, increased along with the Ca\(^{2+}\) current (Fig. 3d). Notably, RACI fluorescence did not decrease as abruptly as the current, due to the slow off-rate of the fluo-4 dye used in this experiment. Hence this fluo-4 form of RACI can only be used to evaluate increases in the Ca\(^{2+}\) signal, during the rising phase of the Ca\(^{2+}\) current.

We next considered how increasing Ca\(^{2+}\) current would affect RACI fluorescence. The predominant theoretical model, of the Ca\(^{2+}\) gradient, first derived by Neher, predicts that the gradient scales linearly with the flux of ions through channels (Neher 1986). According to this model, increasing the driving force for Ca\(^{2+}\) during the voltage ramp should proportionally increase the concentration of Ca\(^{2+}\) ions at every point along the height of the ribbon. While published analytical models of the Ca\(^{2+}\) gradient take on different mathematical forms, they are all approximated by decaying exponentials. Hence, to analyze the RACI fluorescence, it becomes convenient to fit the data to a gradient of the form,

\[ [\text{Ca}^{2+}] \propto j c_0 e^{-x/\lambda} \]

where \(j\) is the flux through channels, \(c_0\) the concentration at the mouth of the channel, \(x\) the distance along the ribbon, and \(\lambda\) the exponential decay constant of the gradient. A peak concentration value of 1 mM was arbitrarily assigned to \(c_0\) for the purposes of fitting. While this might seem a high concentration for intracellular Ca\(^{2+}\), theoretical models predict such
concentrations within nanometers of the channel pore. Future work will attempt to fit \( c_0 \) as a parameter.

The fluorescence from RACI, when integrated over the length of the ribbon, becomes,

\[
\Delta F \propto \int \frac{[Ca^{2+}]/(k_D + [Ca^{2+}])}{dx} = \int j \ c_0 \ e^{-x/\lambda}/(k_D + j \ c_0 \ e^{-x/\lambda}) \ dx = \left[ x - k_D \ Ln(k_D e^{-x/\lambda} + j \ c_0) \right]
\]

where \( k_D \) is fluo-4 the \( Ca^{2+} \) affinity. This function was fit to the slope of the RACI fluorescence signal over the linear range of the \( Ca^{2+} \) current, with the decay constant \( \lambda \) as the fit parameter (Fig. 4a). Averaging the decay constants from 11 cells yielded a \( \lambda \) value of \( 25 \pm 2 \) nm (SEM) (Fig. 4b). This fit yields an experimentally determined concentration curve (Fig. 4c, blue line) which is strikingly similar to the predictions of Neher’s model, given the 0.5 mM EGTA buffering conditions present in the patch pipette (Fig. 4c, green line). Both model and data predict that \( Ca^{2+} \) exceeds hundreds of micromolar near the mouth of channels, but drops precipitously to near background levels at a distance of 100 nm.

To test the validity of these results, we used the concentration gradient to make predictions about vesicle release at the cone ribbon synapse. Photoreceptor synaptic vesicles are known to have a submicromolar \( Ca^{2+} \) threshold for exocytosis (Thoreson et al., 2004; Jackman et al., 2009). The steep profile of the \( Ca^{2+} \) gradient should cause ribbon-tethered vesicles to experience vastly different \( Ca^{2+} \) concentrations depending on their location on the ribbon (Fig. 5a). Electron microscopy of cone ribbons fixed in the presence or absence of extracellular \( Ca^{2+} \) reveals that influx of \( Ca^{2+} \) through channels results in depletion of synaptic vesicles at the base of ribbons (Fig. 5b; Jackman et al., 2009). Removal of extracellular \( Ca^{2+} \) reverses this depletion, yielding an estimate for the maximum vesicle occupancy (Fig. 5c). Given the \( Ca^{2+} \)-threshold for exocytosis, it is possible to use the experimentally determined gradient to predict the probability of release for vesicles as a function of position on the ribbon. The resulting prediction of depletion agrees with observed vesicle occupancy, defined as the ratio of vesicles in the presence and absence of \( Ca^{2+} \) with electron microscopy (Fig. 5d).

**DISCUSSION**

These results provided by RACI offer tantalizing experimental confirmation of long-standing theoretical models for synaptic \( Ca^{2+} \) nanodomains. However, both data and analysis are incomplete pending confirmation that the RACI present at the synapse is in fact bound to the surface of ribbons, and not freely diffusing through the cytoplasm. Because some RACI will always be unbound, a more sophisticated analysis might account for both bound and freely diffusing RACI. Future experiments could include photobleaching assays to determine the extent of RACI binding, use of the second form of RACI to test calcium indicators with differing affinities, and control experiments using calcium indicators lacking the RIBEYE-binding peptide. Although more work is needed, hopefully this technique may offer the first experimental measure of nanometer-scale cytoplasmic gradients.
**Figure 1. Design of a ribbon-associated calcium indicator.** a) EM image of a salamander cone synapse showing the electron dense synaptic ribbon anchored to the plasma membrane and extending into the cytoplasm. b) The RIBEYE protein that composes the bulk of the synaptic ribbon binds to RACI, coating the ribbon surface with a fluorescent Ca$^{2+}$ indicator. This indicator samples the entire Ca$^{2+}$ gradient (shaded blue circles), which radiates into the cytoplasm from the Ca$^{2+}$ channels located at the base of the ribbon. c) One version of RACI is synthesized by covalently coupling the 14-residue RIBEYE-binding sequence (green line) with fluo-4 cadaverine. d) A second version of RACI adds a 35-residue self-interacting peptide (red loop), which forms a pocket that binds to rhodamine dyes with picomolar affinity.
Figure 2. RACI illuminates synaptic ribbons. a-b) Fluorescence image of a patch-clamped salamander rod (a), and cone (b) dialyzed with and intracellular solution containing 32 µM RACI and buffered to contain 1 µM free Ca$^{2+}$, which renders the Ca2+ indicator dye fluorescent. Both types of photoreceptor show bright labeling at the base of terminal, as well as the more mottled fluorescence at the inner segment in the region of the cell nucleus. (c) Fluorescence increase in a cone patched with normal intracellular solution in response to a depolarizing step. Cells show less overall fluorescence when free Ca$^{2+}$ is omitted from the pipette solution. However the fluorescence increases at the synapse when the cell holding potential is changed from -70 mV to -10 mV.
Figure 3. Monitoring RACI fluorescence while manipulating the cone Ca\textsuperscript{2+} current. a) Voltage ramp protocol applied to cones during RACI imaging. Cones were stepped from the -70 mV holding potential to +100 mV, a voltage which maximally opens Ca\textsuperscript{2+} channels but which is above the effective reversal potential for Ca\textsuperscript{2+}. Membrane potential was then ramped downwards at a rate of -0.2 mV/ms to increase the driving current for Ca\textsuperscript{2+}. b) Holding current in a cone subjected to the voltage ramp in (a). On top of the voltage-independent leak current, a voltage-dependent inward current developed. c) Subtracting the voltage-independent leak current from the trace in (b) reveals the cone Ca\textsuperscript{2+} current. The Ca\textsuperscript{2+} current peaks at around -10 mV, then begins to decrease as L-type Ca\textsuperscript{2+} channels close at more negative potentials. d) RACI fluorescence from the synapse of the same cell as (a-c).
Figure 4. Extracting the Ca^{2+} gradient from the dynamic RACI signal. a) RACI fluorescence vs. Ca^{2+} current (black circles) fit with the equation given in the text (red line). b) Values of the decay constant fit for 11 cells, and the average (gray bar). c) Comparison of the experimentally determined gradient (blue line) and modeled gradient proposed by Neher (green line).
Figure 5. The Ca$^{2+}$ gradient predicts vesicle occupancy on cone ribbons. a) Vesicles at different locations on the ribbon will experience different Ca$^{2+}$ concentrations. This will cause vesicles at the bottom of the ribbon to exocytose more quickly than vesicles at the top, and thus deplete the bottom of the ribbon of vesicles. b) Electron micrographs showing ribbon-tethered vesicles (green circles) in the absence and presence of extracellular Ca$^{2+}$. c) Quantification of the number of vesicles at different heights along the ribbon, observed with EM (average of 115 ribbons for each condition, Jackman et al., 2009). d) Observed fractional vesicle occupancy of the ribbon (black circles), compared to the prediction given by applying the experimentally determined Ca$^{2+}$ gradient to the measured Ca$^{2+}$-dependence of release for photoreceptor synaptic vesicles.
METHODS

Imaging

Retinas from the larval tiger salamander *Ambystoma tigrinum* were isolated and prepared as slices as described previously (Rabl et al., 2005) using procedures approved by the Marine Biological Laboratory Animal Care and Use Committee. Retinal slices were mounted in a gravity fed perfusion chamber (Warner Instruments) and perfused with oxygenated saline solution. Salamander cones were imaged with an upright fluorescence microscope (Olympus BX51W) equipped with a plasma light source (Lambda XL, Sutter Instruments). Images were acquired at 100 Hz (exposure duration ~9 ms) with a cooled back-illuminated EMCCD camera (Andor iXon 860). RACI was included in the patch pipette at 32 μM and dialyzed into cells during whole cell recording. Images were analyzed with ImageJ (http://rsbweb.nih.gov).

Electrophysiology

Whole-cell voltage-clamp recordings were obtained from cones using 10 - 15 MΩ patch pipettes pulled from borosilicate glass. The pipette solution contained (in mM): 90 CsCl2, 2 MgCl2, 20 HEPES, 10 TEACl, 2 NaATP, 0.5 GTP, 0.5 EGTA (pH 7.2). Salamander cones were voltage clamped at a steady holding potential of -70 mV using an Multiclamp 700B (Axon Instruments) patch-clamp amplifier and stimulated with depolarizing ramps. Currents were acquired using a Digidata 1440A interface and pClamp 10.2 software (Molecular Devices). Current records were filtered with a 2 kHz low-pass filter. Leak subtraction was performed offline using pClamp software.
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Chapter 4: A positive feedback signal from horizontal cells to cones

The previous three chapters have examined the presynaptic mechanisms that regulate release by the cone synapse. However, this is only half the story. Release from cones is also controlled by a feedback circuit. Cone photoreceptors and horizontal cells (HCs) have a reciprocal synapse that underlies lateral inhibition and establishes the antagonistic center-surround organization of the visual system. Cones transmit to HCs through an excitatory synapse and HCs feedback to cones through an inhibitory synapse. In this chapter we report that HCs also transmit a positive feedback signal to cone terminals that elevates intracellular Ca\(^{2+}\) and accelerates neurotransmitter release. Positive and negative feedback are both initiated by AMPA receptors on HCs, but positive feedback is mediated by an increase in HC Ca\(^{2+}\), whereas negative feedback is mediated by a change in HC membrane potential. Positive feedback is spatially constrained to active HC-cone synapses, whereas the negative feedback signal spreads through HCs to affect release from surrounding cones. By locally offsetting the effects of negative feedback, positive feedback amplifies photoreceptor synaptic release without sacrificing HC-mediated contrast enhancement.
INTRODUCTION

The retina is an exceptionally approachable part of the brain, hence deciphering the retinal neural circuit was one of the earliest triumphs of systems neuroscience (Dowling, 1987). The basic wiring diagram of the retina was determined largely from electrical recordings from each of the main neuronal cells types. Synaptic connections were first deduced by examining how the light response is transformed from one retinal cell type to the next (Werblin and Dowling, 1969). Paired recordings from different cell types and anatomical studies confirmed the occurrence of these connections and helped define their functional properties.

The synaptic connection between HCs and cone photoreceptors attracted particular interest right from the beginning (Baylor et al., 1971). Voltage changes in HCs result in sign-inverted voltage changes in cone photoreceptors, a negative feedback connection. HCs project laterally in the retina over hundreds of microns and integrate inputs from many rods and cones, so negative feedback causes cones (Baylor et al., 1971) and rods (Thoreson et al., 2008) to have an antagonistic center-surround receptive field. This receptive field organization is reflected postsynaptically first in bipolar cells (Werblin and Dowling, 1969) and again in subsequent neuronal layers of the visual system (Hubel and Weisel, 1959), enhancing the neural representation of spatial contrast and sharpening visual edge detection.

Despite decades of study, the mechanism of negative feedback from HCs remains controversial. Three main hypotheses have been advanced to explain how this sign-inverting synapse works; i.e., how depolarization of the HC inhibits neurotransmitter release from cones. First, it was proposed that HCs release the neurotransmitter GABA, hyperpolarizing the cone membrane potential (Wu, 1992). Second, an ephaptic mechanism was proposed, in which electrical current through channels in HC dendrites locally changes the transmembrane potential of the cone terminal (Byzov and Shura-Bura, 1986; Kamermans et al., 2001). Third, it was proposed that depolarization of HCs causes the efflux of protons, which acidifies the extracellular space and inhibits cone voltage-gated Ca^{2+} channels (Hirasawa and Kaneko, 2003; Vessey et al., 2005). The debate continues over which of these mechanisms predominate in generating negative feedback to cones.

Here we report the surprising discovery that HCs also generate positive feedback onto cones, distinct from the negative feedback signal that has been studied for the past 40 years. Optical imaging methods reveal that the cone neurotransmitter glutamate triggers a retrograde signal from HCs, which elevates intracellular Ca^{2+} in cones and enhances neurotransmitter release. This signaling system is robust in the intact retina, but disrupted in retinal slices, which are often used for investigating the HC synapse. The positive feedback synapse between HCs and photoreceptors functions to locally offset the effect of negative feedback and boost photoreceptor transmission, preserving signal strength in the visual system without sacrificing HC-mediated contrast enhancement.
RESULTS

Glutamate increases synaptic release from cone terminals

To investigate feedback at the cone-HC synapse we monitored synaptic vesicle release from cone terminals using fluorescence microscopy. As previously described, the all-cone retina of the anole lizard was dark adapted in physiological saline containing the amphipathic dye FM1-43 (Rea et al., 2004). In darkness, cone terminals support continuous exocytosis and compensatory endocytosis. Vesicles internalized during endocytosis incorporate the dye, producing brightly labeled cone synaptic terminals. Washing the retina with a solution containing Advasep-7 removes FM1-43 from the surface membranes of cells, but spares the dye in internalized vesicles. Subsequent loss of dye from synapses results from the exocytosis of labeled vesicles (Rea et al., 2004; Choi et al., 2005), and this can be monitored with an infrared 2-photon laser-scanning microscope (Figure 1A).

To elicit feedback from HCs onto cones we added glutamate to the bath solution. HCs become depolarized when glutamate activates ionotropic glutamate receptors on their dendrites (Lasater and Dowling, 1982; O’Dell and Christensen, 1989). Depolarized HCs feedback onto cones by inhibiting the voltage-gated Ca\textsuperscript{2+} channels that support exocytosis (Verweij et al., 1996). Hence, the predicted effect of HC depolarization is a decrease in the rate of synaptic release from cones. Remarkably, the addition of glutamate increased release from cone terminals (Figure 1B), with the rate of exocytosis increasing about 4-fold over the rate in darkness (Figure 1C). The increase caused by glutamate exceeded the more modest 2-fold increase observed when cones were directly depolarized by a bath solution containing high K\textsuperscript{+} (50 mM) (Figure 1D).

Ionotropic glutamate receptors are responsible for accelerating cone release

Glutamate activates ionotropic receptors (iGluRs) and metabotropic receptors (mGluRs). While there is no evidence that cones express iGluRs, cone terminals do possess group III mGluRs that can regulate synaptic Ca\textsuperscript{2+} (Koulen et al., 1999). However, mGluR agonists reportedly decrease synaptic release (Hirasawa et al., 2002). While this is inconsistent with a positive feedback role, mGluR autoreceptors might play a role in negative feedback by allowing cones to become inhibited by their own synaptically-released glutamate.

To better understand the role of mGluRs, we used pharmacology to manipulate the cone mGluR pathway. As expected, the mGluR group III-selective agonist L-APB caused a decrease in the rate of exocytosis from dark-adapted cones (Figure 2A). However, blocking cone mGluRs with the group II/III antagonist MSPG failed to increase release (Figure 2A). This indicates that cone mGluRs are not continually activated in darkness, when glutamate release is high. However, cones release a transient spike of glutamate at the end of a light flash (Jackman et al, 2009), and it is possible that this activates mGluR autoreceptors to inhibit cone release.

Glutamate also binds to and activates plasma membrane transporters in cones, triggering a Cl\textsuperscript{-} current (Picaud et al, 1996). The current is usually hyperpolarizing, but under some conditions it might depolarize the cone and activate voltage-gated Ca\textsuperscript{2+} channels, increasing the release rate.
To preclude activation of transporters, we used the iGluR-selective agonist AMPA, which depolarizes HCs but does not affect the glutamate transporter (Rabl et al., 2003) and has no detectable direct action on photoreceptors (Koulen et al., 1999; O’Dell and Christensen, 1989). Similar to glutamate, AMPA caused a large (~6-fold) increase in the release rate from cone terminals (Figure 2B). The AMPA/kainate receptor antagonist DNQX blocked the effect of AMPA. A dose-response curve for AMPA reveals an EC$_{50}$ of 13 µM (Figure 2C), similar to the EC$_{50}$ of 15 µM for AMPA receptors in isolated catfish HCs (O’Dell and Christensen, 1989). Taken together, these results establish that iGluRs are responsible for triggering the increase in vesicular release from cone terminals. In contrast to mGluRs and glutamate transporters, which might play a negative feedback role in regulating cone release, iGluRs play a positive feedback role in augmenting the release rate.

To test whether there is sufficient glutamate released in darkness to activate the positive feedback mechanism, we applied DNQX. DNQX decreased the FM1-43 release rate by 39±11% (Figure 2D and E), a significant decrease (two-tailed t-test; p< 0.5). Hence the positive feedback system is active in darkness, and it may be even more active with rapidly changing light conditions that trigger strong phasic glutamate release (Jackman et al., 2009). These results indicate that positive feedback is a physiologically relevant process that determines the ambient release rate from cones and sets the gain of the first synapse in the retina.

We found that AMPA increased vesicular release from retinal photoreceptors in species across several phyla, including zebrafish (*Danio rerio*), tiger salamander (*Ambystoma tigrinum*), anole lizard (*Anolis carolinensis*), and rabbit (*Oryctolagus cuniculus*) (data not shown). In each species AMPA increased the release rate by >2-fold as compared to darkness. The enhancement of release was uniform over the variety of cone and rod terminals found in the outer plexiform layer of these retinas, indicating that AMPA augments release from both rods and cones. In the rod-only retina of the gecko (*Gecko gecko*) AMPA also increases release from rods. In anole retina, AMPA increased the release rate to about the same final value whether the retina was dark-adapted or light-adapted (data not shown). This result shows that AMPA is dominant in increasing release when photoreceptors are hyperpolarized by light.

**Narrowing down the source of the positive feedback signal**

Our results indicate that iGluRs are responsible for augmenting cone release, yet studies show that cone photoreceptors do not possess iGluRs (Eliasof and Werblin, 1993). To confirm that functional AMPA receptors are absent from cones, we examined release from cones acutely isolated from the retina. Retinas loaded with FM1-43 were treated with papain and mechanically triturated to isolate individual cones. The dissociated cones retained bright FM1-43 fluorescence at their terminals (Figure 3A, top) and spontaneously released the dye at a rate similar to that measured in the intact retina. However, treatment of dissociated cones with AMPA had no effect on release (Figure 3A, bottom). This result is consistent with previous studies showing that dissociated photoreceptors lack iGluR-mediated ionic currents (Eliasof and Werblin, 1993).

We next tested the effect of AMPA on retinal slices. The transverse retinal slice is a popular preparation for studying the synapse between HCs and photoreceptors because it provides
unimpeded access for patch-clamping recordings. However, slicing can damage HCs, whose processes extend laterally over hundreds of microns and thus might compromise HC feedback. Indeed, when we prepared 200 μm-thick slices from FM1-43 loaded retinas, AMPA failed to accelerate release from cones. When we prepared larger 500 μm-width slices, cones retained a response to AMPA, but release accelerated only half as much as in the flatmount retina (Figure 3B). Because the width of these slices should not affect the health of the cones whose diameter is ~10 μm, these results suggest that AMPA-induced feedback operates through larger cells (>200 μm).

To further investigate the source of positive feedback to cones, we used laser ablation to disrupt various neuronal layers in 500 μm-thick slices of the anole retina. Prior to AMPA application, the power of the imaging laser was increased from ~10 mW to ~2 W. We scanned along the slice to induce cell damage in either the portion of the inner nuclear layer (INL) where HC somata reside, or in the inner plexiform layer (IPL), which contains processes of amacrine, bipolar, ganglion, and interplexiform cells (IPCs), but not HCs (Figure 3C, top). Laser ablation produced immediate cell damage, which was apparent from cellular blebbing and the loss of dye in the scanned region. When the laser was targeted to the INL to ablate HCs, AMPA failed to accelerate FM1-43 release from cone terminals. However, when the laser was targeted to the IPL, the effect of AMPA on cone release was the same as in slices without laser ablation (Figure 3C, bottom). These results implicate cells with processes in the INL, but not in the IPL, as the source of positive feedback.

HCs and IPCs are the only two laterally-projecting neurons in the retina that are known to contact cones. There are several different types of IPCs containing different neurotransmitters including dopamine (Dowling and Ehinger, 1978) and glycine (Pow and Hendrickson, 1999) and receptors for these transmitters are found on cone terminals (Witkovsky and Daryy, 1991; Shen and Slaughter, 2002). To ascertain whether IPCs might be the source of positive feedback onto cones, we asked whether AMPA could still accelerate the cone release rate after applying agonists or antagonists of dopamine or glycine receptors. AMPA acceleration of cone release was unaffected by dopamine (100 μM) or glycine (1 mM), and the glycine receptor antagonist strychnine (1 μM) also had no effect (data not shown). Hence it seems unlikely that IPCs are the source of AMPA-elicited positive feedback, focusing our attention on HCs.

Putative negative feedback mechanisms have no effect on positive feedback

Three mechanisms have been proposed to account for negative feedback regulation of cone neurotransmitter release by HCs: 1) GABA-ergic feedback, 2) electrical (ephaptic) feedback and 3) proton-mediated feedback. To evaluate whether any of these mechanisms is involved in AMPA-elicited positive feedback, we manipulated each of these systems with pharmacological agents while monitoring FM1-43 release from flat-mount anole retinas.

For many years, GABA was the leading candidate as the HC negative feedback signal (Wu, 1992). In this scenario, depolarization-elicited GABA release from HCs activates GABA_A receptors on cone terminals, hyperpolarizing the membrane potential and suppressing neurotransmitter release. However, GABA_A antagonists fail to prevent negative feedback
(Perlman and Normann, 1990) and rather than regulating a Cl⁻ conductance in cones as predicted by the GABA hypothesis, HC feedback appears to regulate a voltage-gated Ca²⁺ conductance (Verweij et al., 1996; Tatsukawa et al., 2005). These and other studies challenge the role of GABA as the mediator of negative feedback, but we considered the possibility that GABA could be involved in positive feedback. We found that neither GABA nor bicuculline, a GABA_A antagonist, had a significant effect on the rate of FM1-43 release from cone terminals (Figure 4A). Moreover, applying GABA or bicuculline for 20 minutes prior to AMPA did not block the AMPA-induced increase in FM1-43 release (Figure 4A). These results suggest that GABA is not the positive feedback signal.

The second hypothesis is that negative feedback from HCs is electrical in nature. This “ephaptic” hypothesis states that electrical current through ion channels in the tips of HC dendrites causes a local change in the extracellular voltage, such that a larger depolarization is needed to activate cone voltage-gated Ca²⁺ channels (Byzov and Shura-Bura, 1986; Kamermans et al., 2001). HCs possess connexin hemichannels and HC-mediated negative feedback can be blocked with the hemichannel blockers Co²⁺ (Thoreson and Burkhardt, 1990) or carbenoxolone (CBO) (Kamermans et al., 2001). We used these blockers to test the possible involvement of hemichannels in AMPA-elicited positive feedback. Both Co²⁺ and CBO caused a small but significant decrease in the dark rate of release (Figure 4B). However, both reagents reportedly inhibit cone voltage-gated Ca²⁺ channels (Barnes and Hille, 1989; Vessey et al., 2004), which may account for this effect. More to the point, application of either Co²⁺ or CBO for 20 minutes prior to AMPA had no effect on the AMPA-induced increase in release (Figure 4B). These results rule out hemichannels as mediating AMPA-elicited positive feedback and argue against an ephaptic mechanism.

The third hypothesis is that depolarization of HCs leads to the extrusion of protons through pumps or channels, acidifying the extracellular space and inhibiting the activation of voltage-gated Ca²⁺ channels in the cone terminal. Supporting this hypothesis, HC-mediated negative feedback is blocked by adding high concentrations of strong pH buffers (Hirasawa and Kaneko, 2003; Vessey et al., 2005; Davenport et al., 2008). To test whether protons play a role in AMPA-elicited positive feedback, we performed similar experiments, comparing the effect of AMPA on cone release with bath solutions that contained either HEPES, a strong buffer that blocks negative feedback, or HCO₃⁻ (bicarbonate), a weak buffer that preserves negative feedback. We found that a high concentration of HEPES slightly increased the release rate of cones in darkness as compared to HCO₃⁻, consistent with inhibition of negative feedback (Figure 4C). However, the AMPA-elicited increase in release was the same in HCO₃⁻ and HEPES, inconsistent with positive feedback being mediated by a change in pH (Figure 4C).

**Glutamate receptor activation triggers an increase in Ca²⁺ in cone terminals**

Neurotransmitter release from cones is Ca²⁺-dependent, so we next asked whether AMPA leads to a rise in intracellular Ca²⁺ in the cone terminal. Previous studies showed that iGluR agonists fail to elevate cone terminal Ca²⁺ in retinal slices (Vessey et al., 2005; Babai and Thoreson, 2009), but we know that this impairs positive feedback. Flat-mounted anole retinas were incubated with the AM-ester form of Oregon Green BAPTA (OGB), resulting in incorporation of
Ca\(^{2+}\) indicator dye into cone terminals. Application of AMPA triggered a large increase of Ca\(^{2+}\) (Figure 5A, C). In contrast, DNQX caused a small decrease in Ca\(^{2+}\) (Figure 5B, C). This result indicates that the ambient activation of AMPA receptors is sufficient to keep intracellular Ca\(^{2+}\) elevated, again suggesting that the positive feedback system is operating in darkness.

AMPA triggered a persistent rise in intracellular Ca\(^{2+}\) in cone terminals that was difficult to reverse, even with prolonged washing. To confirm that the rise in Ca\(^{2+}\) is reversible, we needed a faster and more precisely targeted method for activating AMPA receptors. A particularly powerful approach involves the photolysis of a caged neurotransmitter agonist, for example 4-methoxy-7-nitroindolinyln (MNI)-glutamate (MNI-glutamate; Ellis-Davies, 2007). However, glutamate acts on many receptor types in the retina, and while iGluRs and mGluRs can be blocked selectively, blockade of glutamate transporters will lead to an increase in the ambient level of glutamate, confounding our results. In fact, uncaging of MNI-glutamate triggered oscillations of Ca\(^{2+}\) in cone terminals that may have been caused by activation of glutamate transporters (data not shown).

To circumvent this problem, we chemically synthesized a form of caged AMPA, which upon photolysis, should activate AMPA receptors but not glutamate transporters. We synthesized the nitroveratryl carbamate derivative of AMPA (NVOC-AMPA) (Figure 5D), which contains the photolabile NVOC protecting group that can be removed with exposure to 365 nm light. Using NVOC-AMPA on the OGB-loaded anole retina, we found that brief flashes of UV light could trigger a repeated transient rise in Ca\(^{2+}\) in the cone terminals (Figure 5E), but only if the caged molecule was present. These experiments demonstrate that AMPA receptor activation causes a reversible rise in Ca\(^{2+}\) in cone terminals.

To evaluate the spatial spread of the positive feedback signal, we uncaged AMPA in a small circular region of the OPL (diameter \~100 \(\mu\)m) and measured the resulting increase in cone terminal Ca\(^{2+}\) (Figure 5F). We found that the spatial profile of Ca\(^{2+}\) elevation in the underlying array of cone terminals closely matched the area of AMPA uncaging (Figure 5G). Hence the signal for positive feedback remains tightly localized to the AMPA-activated region, in contrast to negative feedback, where the signal that influences cone release can spread widely not only within an individual HC, but between coupled networks of HCs connected through gap junctions (Dowling, 1987).

**The positive feedback signal activates a voltage-independent conductance in cone terminals**

We next turned to electrophysiology to compare positive and negative feedback. Previous patch clamp studies showed that depolarization of HCs leads to inhibition of voltage-gated Ca\(^{2+}\) channels in cones (Verweij et al., 1996), a key consequence of negative feedback. We confirmed this effect by recording from synaptically-connected HCs and cones in a retinal slice. For this and other patch clamp experiments we used the retina from tiger salamander because the compact structure of their cones allows for more effective voltage-clamp of the synaptic terminal than lizard cones, which have a long axon separating the terminal from the cell body.
We used a ramp depolarization to activate voltage-gated Ca\(^{2+}\) channels, which generated an inward current at potentials more positive than ~40 mV (Figure 6A, top). To quantify voltage-dependent activation of the Ca\(^{2+}\) channels, we computed the activation curve, by subtracting the linear leak current from the total current and normalizing the resulting voltage-gated current to its peak value (Figure 6A, bottom). As shown previously (Cadetti et al., 2006), hyperpolarizing the HC increases the cone Ca\(^{2+}\) current and shifts the activation curve to more negative potentials, whereas depolarizing the HC decreases the current and shifts the curve to more positive potentials.

To examine the effects of AMPA on the Ca\(^{2+}\) activation curve, we used flat-mounted retinas rather than slices to preserve both positive and negative feedback. Bath application of AMPA reduced the Ca\(^{2+}\) current and shifted the activation curve to more positive potentials, which was expected because HCs depolarize in response to AMPA (Figure 6B). Conversely, the iGluR antagonist NBQX increased the Ca\(^{2+}\) current and shifted the activation curve negatively (Figure 6C). Both of these effects are consistent with HC-mediated negative feedback.

Superimposed on regulation of voltage-gated Ca\(^{2+}\) channels, we found that AMPA and NBQX had a second effect that involves channels that are apparently not voltage-gated. At negative potentials, AMPA elicited an inward current while NBQX elicited a small, but significant outward current and the current vs. voltage relationships of both of these responses were linear (Figure 6D), suggesting that voltage-gated channels are not involved. The average AMPA and NBQX responses exhibited extrapolated reversal potential of 0 ± 9 mV (n=6) and -19 ± 7 mV (n=4), respectively, with AMPA causing an increase in membrane conductance and NBQX causing a small decrease (Figure 6E). The estimated reversal potentials are slightly different, but the simplest interpretation of these results is that AMPA and NBQX regulate the same population of voltage-independent ion channels in cones, with AMPA opening and NBQX closing the channels. The nature of the channels is still unknown, but the observation that AMPA application leads to rise in Ca\(^{2+}\) in the cone terminal (Figure 5) is consistent with activation of a Ca\(^{2+}\)-permeation pathway, perhaps a non-selective cation channel.

We propose that the voltage-independent conductance in cones is responsible for positive feedback whereas modulation of the voltage-gated Ca\(^{2+}\) conductance is responsible for negative feedback. Consistent with this proposal, we found that application of HEPES, which blocks negative feedback, also prevents AMPA and NBQX from shifting the Ca\(^{2+}\) activation curve (Figure 6F-H). HEPES, however, does not prevent positive feedback regulation of cone transmitter release (Figure 4) and does not block the regulation of the voltage-independent conductance by AMPA or NBQX (Figure 6I,J).

**Raising Ca\(^{2+}\) in HCs triggers positive feedback.**

Our results thus far suggest that the effect of AMPA on cone release is mediated by HCs. However, the results of Figure 6 suggest that manipulation of HC voltage cannot completely recapitulate the effects of iGluR agonists and antagonists on cone release. Assuming that HCs are the source of positive feedback, something other than voltage must be triggering retrograde signaling to photoreceptors.
Ca$^{2+}$ seems a likely candidate. There is evidence that HCs contain Ca$^{2+}$-permeant glutamate receptors (Schultz et al., 2001) and glutamate application has been shown to elicit a rise in internal Ca$^{2+}$ in HCs that does not involve influx through voltage-gated Ca$^{2+}$ channels or release from internal stores (Hayashida et al., 1998; Rivera et al., 2001). Moreover, glutamate receptors on HC dendrites are located adjacent to the cone terminals (Calkins, 2005), ideally positioned to trigger a Ca$^{2+}$-dependent feedback signal.

To confirm that HCs are the source of positive feedback and to determine whether Ca$^{2+}$ can trigger retrograde signaling, we elevated Ca$^{2+}$ exclusively in a single HC and asked whether this could increase neurotransmitter release from photoreceptors. To target a single HC, we introduced photolyzable caged Ca$^{2+}$ (DM-nitrophen) via a patch pipette and used UV light to trigger photolysis and elevate internal Ca$^{2+}$. A brief (1 msec) uncaging light flash elicited a series of currents in the HC. Following a transient outward current, the light flash triggered a sustained inward current that persisted for >1 sec (Figure 7A). The sustained inward current was greatly reduced by applying NBQX (Figure 7B), which suggests that the inward current reflects an increase in glutamatergic synaptic transmission from photoreceptors. A fraction of the flash-induced current was not blocked by NBQX. This may result from the elevated Ca$^{2+}$ activating Ca$^{2+}$-dependent ion channels that are endogenous to the HC. The transient outward current could be observed even if the HC did not contain DM-nitrophen (Figure 7C), suggesting that it results from synaptic transmission of the endogenous light response of photoreceptors, which occurred even though the retina was light-adapted.

Average data collected from several HCs for each condition (Figure 7D) showed that the sustained inward current only appeared when intracellular Ca$^{2+}$ was liberated by DM-nitrophen uncaging. Moreover, they show that the inward current was significantly reduced by blocking glutamatergic synaptic transmission. These results suggest that Ca$^{2+}$ is sufficient for triggering the retrograde signal that accelerates cone neurotransmitter release. Moreover, because Ca$^{2+}$ uncaging is restricted to a single HC, these results firmly establish HCs as the source of positive feedback.
DISCUSSION

**HCs transmit a positive feedback signal to photoreceptors**

The findings presented in this paper reveal a previously unknown positive feedback synapse between retinal HCs and cone photoreceptors. Several special features of this circuit connection may help explain why it has evaded notice over the past 4 decades. First, positive feedback onto a cone cannot be evoked simply by depolarizing a synaptically-connected HC, yet this is the standard test for synaptic connectivity. Second, positive feedback is compromised by making transverse slices of the retina, a procedure that is a near-necessity for making electrophysiological recordings between HCs and other retinal neurons. Without a means for selectively eliminating positive feedback, the synaptic transfer function of cones could be erroneously attributed to a higher intrinsic gain of the synaptic release machinery.

Our results suggest that positive feedback applies not only to cones, but also to rods. Most of our optical studies utilized the all-cone retina from anoles, but in retinas containing both rods and cones (including zebrafish, salamander, and rabbit) we noticed no difference in the AMPA-elicited acceleration of neurotransmitter release in roads and cone terminals interspersed in the OPL. Rods and cones are electrically coupled through gap junctions (Raviola and Gilula, 1973), so it is possible that AMPA-elicited enhancement of release from rods is an indirect consequence of signals originating in cones. AMPA accelerates release from rod photoreceptors in the gecko retina, which has no true cones. However, gecko photoreceptors have shared features of rods and cones (Taniguchi, 1999), perhaps making them a special case. HC-mediated negative feedback has recently been demonstrated to occur in rods as well as cones (Thoreson et al., 2008), so it seems likely that the positive feedback signal is also communicated to both photoreceptors.

Our results indicate that HCs are the source of the positive feedback signal. HCs possess AMPA receptors that are implicated in positive feedback. The AMPA receptors are located on HC dendrites that invaginate the cone terminal, the presumed site of negative and positive feedback signaling. Selective laser ablation indicates that cells in the HC layer of the INL are required for positive feedback. Retinal slice experiments suggest that cells that project laterally for >200 µm are required, also consistent with a crucial role for HCs. Finally, cone neurotransmitter release can be evoked by uncaging Ca$^{2+}$ within an individual HC, definitive evidence for positive feedback regulation of cone neurotransmission.

The discovery of positive feedback helps explain a long-standing puzzle about synaptic signaling in the outer retina. Kainate and other selective iGluR agonists hyperpolarize On-bipolar cells in the intact retina (Sheills et al., 1981; Bloomfield and Dowling, 1985), but not in retinal slices (Euler et al., 1996; Hartveit, 1996; McGillem and Dacheaux, 2001). On–bipolar cells in slices continue to exhibit a robust response to glutamate, but this can be completely attributed to metabotropic glutamate receptors (mGluRs) (de la Villa et al., 1995), which are unaffected by kainate. Our results help explain the indirect action of iGluR agonists; they trigger HC-mediated positive feedback onto rods and cones, increasing their release of glutamate, which leads to hyperpolarization of the On-bipolar cell. An iGluR-elicited signal in amacrine cells may be
communicated to On-bipolar cells through GABA receptors, also contributing to the hyperpolarization (Hartveit, 1996).

**The mechanism of negative and positive feedback**

Our results indicate that HCs communicate to photoreceptors via distinct negative and positive feedback signals. Negative feedback was discovered decades ago, but there is still considerable disagreement about which of the proposed mechanisms is responsible. Our results are consistent with previous studies showing that negative feedback can be blocked by increasing extracellular pH buffering with HEPES (Hirasawa and Kaneko, 2003; Vessey et al., 2005; Davenport et al., 2008). This would seem to support the pH hypothesis, but a recent report challenges the interpretation of HEPES experiments, claiming that extracellular HEPES can directly block connexin hemichannels (Fahrenfort et al., 2009). Given sufficient time, HEPES and other buffers also can alter the intracellular pH of HCs (Fahrenfort et al., 2009), and internal acidification is known to close hemichannels (Malchow et al., 1993; Trexler et al., 1999). These findings raise the possibility that HEPES blocks HC feedback by interfering with ephaptic signaling. Hence the debate continues.

Our studies reveal the outline of the positive feedback mechanism (Figure 8A), but there are gaps in our knowledge, including the nature of the feedback messenger itself. Glutamate released from photoreceptors activates AMPA receptors on the HC, leading to Ca\(^{2+}\) influx (Hayashida et al., 1998; Rivera et al., 2001). AMPA receptors containing Ca\(^{2+}\)-permeant subunits are found on HC dendrites (Schultz et al., 2001). Hence it is likely that the AMPA receptors themselves serve as the conduit for Ca\(^{2+}\) entry.

Our results suggest that intracellular Ca\(^{2+}\) is the key factor that elicits positive feedback from the HC. Negative feedback requires a change in HC membrane potential, but positive feedback can be elicited by uncaging Ca\(^{2+}\) in a HC that is voltage-clamped to prevent changes in membrane potential. Intracellular Ca\(^{2+}\) must trigger the generation and/or release of a chemical messenger that acts on photoreceptor terminals. Our evidence suggests that this messenger activates a voltage-independent conductance in cones, giving rise to the increase in intracellular Ca\(^{2+}\) that causes accelerated neurotransmitter release. The voltage-independent conductance could conceivably arise from several types of non-selective cation channels that are found in photoreceptor terminals, including cyclic nucleotide-gated (CNG) (Rieke and Schwartz, 1994; Savchenko et al, 1997) and TRPC channels (Szikra et al., 2008), both of which are Ca\(^{2+}\)-permeant.

Our experiments rule out many putative neurotransmitters as being responsible for positive feedback from HCs. Positive feedback persists in the presence of GABA, glycine, and dopamine, eliminating these conventional neurotransmitters. In fact, EM studies show that HC dendrites lack accumulations of synaptic vesicles and plasma membrane specializations found at active zones (Raviola and Gilula, 1975), making it unlikely that a conventionally secreted neurotransmitter is involved.
However, neurotransmitters can be released by means other than synaptic vesicle exocytosis. Activation of plasma membrane transporters on HCs can lead to the efflux and extracellular accumulation of GABA (Schwartz, 2002) and protons (Kreitzer et al., 2007; Jouhou et al., 2007), but our experiments rule out either of these in mediating positive feedback. Nitric oxide (NO) diffuses across biological membranes and is thought to serve as a retrograde synaptic messenger in the brain (Hawkins et al., 1998). There is immunocytochemical evidence in HCs for NO synthase (NOS) (Cao and Eldred, 2001; Blom et al., 2009) and in photoreceptors for soluble guanylate cyclase (sGC) (Koch et al., 2002; Haberecht et al., 1998), a major effector for NO signaling. Cone terminals possess a high abundance of CNG channels (Savchenko et al., 1997). NO donors increase exocytosis from isolated cones (Rieke and Schwartz, 1994) and NOS or sGC inhibitors alter HC light responses in retinal slices (Savchenko et al., 1997).

These observations would seem to make NO a leading candidate. However, we have performed a series of pharmacological experiments that seem to exclude the NO/cGMP pathway as mediating positive feedback. Positive feedback persists after extracellular application of NO donors or inhibitors of NOS or sGC (data not shown). It is possible that diffusion of these reagents into the invaginating synapse is slow and ineffective. However, we find that positive feedback persists even when blockers of sGC or CNG channels are introduced directly into the cytoplasm of a cone via the patch pipette (data not shown). Hence it’s no for NO.

Phospholipid-derived molecules are another class of potential positive feedback messengers. Arachidonic acid and other polyunsaturated fatty acids are released by the retina in response to light (Jung and Reme, 1994; Reinboth et al., 1996), but these compounds cause profound inhibition of voltage-gated Ca\textsuperscript{2+} channels in photoreceptors, different from the actions of the positive feedback transmitter. Endocannabinoids, including anandamide and 2-arachidonoyl glycerol (2-AG), are also found in the retina, and there is evidence that they modulate voltage-gated conductances in cones (Yazulla, 2008). We find that anandamide does not activate a voltage-independent conductance in cones (data not shown). Hence, at least for now, the transmitter that mediates positive feedback from HCs to cones is unknown. Hopefully this mystery will be solved more quickly than the negative feedback mechanism.

**The functional significance of positive feedback**

The cone synapse encodes information about light intensity by modulating its rate of vesicular neurotransmitter release. Because vesicular release is quantal, the encoding capacity of the cone synapse is limited by the maximal release rate (i.e. in darkness) (Choi et al., 2005). Any process that decreases the maximal release rate will degrade the representation of light intensity by the cone synapse, which in turn will degrade the performance of the visual system as a whole.

Negative feedback from HCs enhances the neural representation of spatial contrast as an array of photoreceptors transmits a visual image to bipolar cells. However, the benefit of contrast enhancement comes at a cost: negative feedback lowers the maximal release rate and therefore reduces the dynamic range of the cone synapse. This will diminish the neural representation of a visual image, counteracting enhanced contrast sensitivity. By boosting neurotransmitter release from cones, positive feedback may recoup the dynamic range lost to negative feedback.
The signals that give rise to positive and negative feedback spread differently through an HC, which is why the signals do not simply cancel out. Positive feedback is local, occurring only at where HC dendrites receive direct excitatory input. We propose that Ca$^{2+}$ entry through AMPA receptors triggers positive feedback. Immunocytochemistry shows that HCs express the Ca$^{2+}$-binding proteins parvalbumin and CaBP-28K (Rohrenbeck et al., 1987), which could limit the spread of Ca$^{2+}$ to individual HC dendrites, making positive feedback local (Figure 8A). In contrast, negative feedback does not appear to be Ca$^{2+}$ dependent, but rather is controlled by the HC voltage. The voltage signal generated by activating AMPA receptors will spread electrotonically across dendrites in a single HC, as well as across the network of HCs, which are electrically coupled though gap junctions. Thus negative feedback spreads much further than negative feedback.

The interaction of negative and positive feedback is illustrated in Figure 8B. When the retina is stimulated by an annulus of light, photoreceptors within the dark center will be depolarized, maintaining glutamate release, while those in the annulus will be hyperpolarized, decreasing release. In the absence of both forms of feedback, the spatial profile of release will mirror the profile of the stimulus. Addition of negative feedback will enhance the representation of contrast. HCs within the dark center will be depolarized by glutamate and will feedback to decrease release from photoreceptors, while those in the annulus will be hyperpolarized, providing minimal negative feedback. HCs at the border between light and dark will be partially hyperpolarized, and will provide a partial negative feedback signal to all the photoreceptors they contact. The resulting profile of release is often described as a “Mexican hat”. While the Mexican hat profile enhances the difference in release rates at the border between dark and light, negative feedback has the overall effect of decreasing the maximal release rate from photoreceptors, which will degrade the encoding capacity of photoreceptor synapses. Positive feedback restores the maximal release rate from photoreceptors releasing glutamate in the dark, but has little effect in the light, where the amount of glutamate released from photoreceptors is insufficient to activate Ca$^{2+}$ dependent feedback in the dendrites of postsynaptic HCs. As a result, positive feedback acts as an amplifier which increases release from photoreceptors without destroying the contrast enhancement provided by negative feedback.
Figure 1. Glutamate accelerates synaptic release from cone terminals. (A) Fluorescence images of cone terminals in the outer plexiform layer of a flatmount anole retina loaded with FM1-43. Terminals continuously release FM1-43 in darkness as a result of tonic vesicle release. (B) Addition of 2 mM glutamate to the bath solution accelerates release from cone terminals. (C) Time-course of FM1-43 fluorescence decrease from cone terminals in darkness (circles) and 2 mM glutamate (squares). (D) Average rates of FM1-43 release ($\Delta F/\Delta t$). Release in glutamate is 4-fold faster than the rate in darkness (control), and ~2-fold faster than in high $K^+$ (50 mM), which depolarizes cone terminals.
Figure 2. AMPA receptors mediate the acceleration of cone synaptic release. (A) Bath addition of 20 μM L-APB (a mGluR group III agonist) significantly slows release from cones in darkness (p<.05). The mGluR antagonist MSPG (100 μM) has no effect on the dark release rate. (B) 20 μM AMPA markedly increases the release rate from cones. The effect of AMPA is blocked by 10 μM DNQX. (C) Dose-response curve for release rate as a function of AMPA concentration. (D-E) Bath addition of 10 μM DNQX significantly slows release from cone terminals in darkness (p<0.1), suggesting that the physiological level of ambient glutamate boosts release.
Figure 3. Determining the cellular locus of AMPA-accelerated release. (A) AMPA has no effect on release from isolated cones. **Top:** Images of a dissociated cone photoreceptor from a FM1-43 loaded anole retina, brightfield (left), fluorescence (right), with the outline of the cell shown with a dashed line. FM1-43 fluorescence is localized to the synaptic terminal (arrow). Scale bar = 10 µm. **Bottom:** Release rate from cones in 20 µM AMPA is the same as in control cones without AMPA. (B) AMPA accelerates release in thick, but not thin, retinal slices. **Top:** Fluorescence image of a retinal slice, loaded with FM1-43 prior to slicing. **Bottom:** 20 µM AMPA has no effect on release in thin (200 µm) slices, but accelerates release by ~2.5-fold in thick (500 µm) slices. (C) Specific laser ablation of the HC layer disrupts AMPA-accelerated release. **Top:** Fluorescence image of a 500 µm-thick slice after laser ablation of either the region of the INL containing HC bodies (left), or the center of the IPL containing processes of other retinal neurons (right). **Bottom:** Ablation of the INL, but not the IPL, disrupts AMPA-induced release. Scale bar 100 µm in (A) and (B). Laser-ablated regions extend 600 µm laterally along the surface of the slice.
Figure 4. Blockers of hypothesized mechanisms of negative feedback have no effect on AMPA-accelerated release from cone terminals. (A) Tonic release in darkness is unaffected by GABA (500 µM) or bicuculline (100 µM) and neither reagent blocks AMPA-accelerated release. (B) Tonic release is slightly reduced by the hemichannel blockers Co²⁺ (100 µM) and carbenoxolone (CBO; 100 µM), but neither reagent blocks AMPA-accelerated release. (C) Tonic release is slightly increased by increasing the pH buffer concentration from 10 to 30 mM HEPES, but unaffected by substituting HEPES with the weaker pH buffer HCO₃⁻ (20 mM). However, none of these pH buffering conditions blocks AMPA-accelerated release.
Figure 5. AMPA triggers a rise in Ca\(^{2+}\) in the cone synaptic terminal. (A) Bath perfusion of 20 µM AMPA triggers a rise in Ca\(^{2+}\) in cone terminals, determining by measuring fluorescence of the Ca\(^{2+}\)-indicator OGB-1. (B) Bath perfusion of the AMPA receptor antagonist DNQX (10 µM) lowers cone synaptic Ca\(^{2+}\) in darkness. (C) Average peak OGB-1 fluorescence change caused by AMPA and DNQX (N=4-5 for each condition). (D) NVOC-AMPA, a photolyzable agonist for selective optical activation of AMPA receptors. (E) Uncaging NVOC-AMPA with brief flashes of UV light triggers rapid, reversible increases in cone terminal Ca\(^{2+}\). (F) OGB-1 fluorescence increase in a flatmount retina triggered by flash uncaging of NVOC-AMPA. The dotted circle represents the full-width half-maximal of the Gaussian region of uncaging. (G) Profile of the OGB-1 fluorescence increase and the UV spot.
Figure 6. Ionic currents in cones modulated by negative and positive feedback. (A) In simultaneous patch clamp recordings from a synaptically-connected HC and cone in a slice from salamander retina, depolarizing the HC ($V_{HC}$) inhibits the activation of the voltage-gated Ca$^{2+}$ current ($I_{Ca}$) in a cone, indicative of negative feedback. **Top**: Current-voltage (I-V) curves at 3 different values of $V_{HC}$. **Bottom**: $I_{Ca}$ activation curves derived from these I-V curves. (B) Depolarizing HCs with AMPA (20 µM) reduces the magnitude and shifts the activation of cone $I_{Ca}$ to more positive potentials, consistent with negative feedback, but also elicits a voltage-independent conductance that is most apparent at -70 mV, indicative of positive feedback. Data for panels (B-E), (G-J) were from patch clamp recordings from cones in flat-mounted salamander retinas. (C) NBQX (10 µM) increases the magnitude and shifts the activation of cone $I_{Ca}$ to more negative potentials, consistent with negative feedback, but also suppresses a voltage-independent conductance that is most apparent at -70 mV, indicative of positive feedback. (D) I-V curves of the cone voltage-independent conductance that is either activated by AMPA or suppressed by NBQX. Curves were extrapolated to yield a reversal potential of 0 mV, and -18 mV respectively. (E) Quantification of the effect of AMPA and NBQX on the voltage-independent conductance.
(F-J) Same as (A-E), except that instead of HCO₃ (20 mM), the bathing solution contained HEPES (10 mM) to block negative feedback. (G) HEPES (10 mM) prevents AMPA from shifting activation of I_{Ca} consistent with blockade of negative feedback. However, AMPA still elicits the voltage-independent conductance, indicative of positive feedback. (H) HEPES also prevents NBQX from shifting I_{Ca} activation, but does not inhibit NBQX from suppressing the voltage-independent conductance. (I,J) Quantification of the effect of AMPA and NBQX on the voltage-independent conductance, in the presence of HCO₃, to preserve negative feedback, or HEPES to inhibit negative feedback. Note that curves obtained in HCO₃ (D) or HEPES (I) are very similar. I-V curves in all panels were generated by ramp depolarizations from -90 to 0 mV (0.5 mV/msec). I_{Ca} activation curves were obtained by subtracting the linear leak current from the total membrane current during the ramp. The resulting leak-subtracted I_{Ca} was normalized to the maximal I_{Ca} and plotted only over the voltage range where channels are activating (e.g., from -70 to -20 mV). Data are reported as mean ± SEM.
Figure 7. Uncaging Ca\textsuperscript{2+} in HCs increases cone glutamate release. HCs in a slice from salamander retina were patch-clamped with a pipette solution that contained caged Ca\textsuperscript{2+} (DM-nitrophen), allowing abrupt increases in Ca\textsuperscript{2+} with flashes of UV light. (A) Average current from 9 HCs. The uncaging flash produced a transient outward current, followed by a sustained inward current. (B) Addition of NBQX to the bath solution eliminates the outward current, and reduces the inward current by ~60%. (C) When HCs were patched without DM-nitrophen in the pipette solution, the UV flash produced only a transient outward current. (D) Average magnitude of the sustained inward current, measured 2 seconds after the uncaging flash. Holding potential= -70 mV.
Figure 8. Spatial spread of negative and positive feedback from HCs to cones. (A) Diagram depicting the differential spread of positive and negative feedback. A cone depolarized in darkness will release glutamate, activating AMPA receptors, causing Ca\textsuperscript{2+} influx and depolarization. The rise in Ca\textsuperscript{2+} is restricted to specific dendritic branch containing the AMPA receptors, resulting in localized positive feedback. The depolarization spreads electrotonically through the HC, resulting in extensive negative feedback. (B) Synaptic release from an array of photoreceptors of modeled with no feedback from horizontal cells (black dotted line), with negative feedback (red line), and with both global negative feedback and local positive feedback (blue line).
METHODS

Retinal Preparation and Dye Loading

All procedures were approved by the UC Berkeley Animal Care and Use Committee. Retinas were isolated from the lizard *Anolis segrei* maintained on a 12:12 light:dark cycle. Retinas were isolated at 21°C in complete darkness with the aid of an IR converter. The retina was prepared and imaged in saline containing (in mM): NaCl 149; KCl 4; CaCl$_2$ 1.5; MgCl$_2$ 1.5; HEPES 10; Glucose 10; pH 7.5.

For FM1-43 unloading experiments, the retina was mounted onto filter paper RPE-side down and bathed in lizard saline containing 30 µM FM1-43 for 1-3 hr followed by a 5 min wash with 1 mM Advasep-7, as described previously (Rea et al., 2004). Retinas were mounted in a gravity fed perfusion chamber with a bath volume of ~0.5 ml (Warner Instruments) and perfused with solution at the rate of 1 ml/min. Drugs were bath applied by perfusion. High K$^+$ saline contained 50 mM KCl, iso-osmotically replacing NaCl.

For Ca$^{2+}$ indicator dye loading, retinas were bathed for 2 hours in 100 µM of either Oregon Green BAPTA-1 AM (OGB-1 AM) or X-Rhod-1 AM (Molecular Probes). The loading solution contained 1% DMSO and 0.2% pluronic acid to enhance dye solubility and cell permeation. Dye-loaded retinas were then mounted flat on nitrocellulose filter paper with the inner retina facing the microscope objective.

For experiments involving bicarbonate buffered solution, isolation and loading took place exclusively in bicarbonate buffered solution, containing (in mM): NaCl 129; KCl 4; CaCl$_2$ 1.5; MgCl$_2$ 1.5; NaHCO$_3$ 20; Glucose 10; pH 7.5, adjusted after bubbling with 95%/5% O$_2$/CO$_2$.

Imaging

We used a Zeiss 510 confocal system equipped with a MaiTai (Spectra Physics, Mountain View, CA) mode-locked Ti:sapphire laser and a 40× achroplan, 0.8 NA water-immersion objective. Images were acquired with Zeiss LSM software. ImageJ software was used to analyze the average fluorescence of the OPL over square regions 230 × 230 µm. Fluorescence was normalized to 1.0 at the onset of experiments.

Uncaging and Ablation

For experiments involving NVOC-AMPA uncaging, light from a mercury lamp (Zeiss) was focused through the microscope objective onto the plane of the OPL. Light was filtered through a Zeiss FS02 filter set (EM$_{max}$ 360 nm) and controlled by an electronic shutter. To uncage in a
small spot, the aperture stop was closed. This produced a Gaussian spot profile with a FWHM diameter of 100 µm, determined by photobleaching of FM1-43 loaded filter paper.

To ablate cells in retinal slices, the Ti:sapphire laser power was increased to maximal (~2 W average output) and scanned in a line over the width of the field of view (600 µm). This process was repeated at depth intervals of 2 µm, to a final depth of 50 µm into the slice.

Electrophysiology

The retina from the larval tiger salamander, *Ambystoma tigrinum*, was isolated with procedures approved by the UNMC Institutional Animal Care and Use Committee. Retinal slices were prepared (Rabl et al, 2005) and whole-cell voltage-clamp recordings were obtained with an MultiClamp 700A amplifier (Axon Instruments) with 8-15 Mohm borosilicate glass patch electrodes. The pipette solution contained (in mM): CsGluconate 94; TEACl 9.4; MgCl₂ 1.9; MgATP 9.4; GTP 0.5; EGTA 0.5; HEPES 32.9; pH 7.2. For caged-Ca²⁺ experiments, the pipette solution consisted of (in mM): 10 DM-nitrophen, 5 CaCl₂, 4 DPTA, 4 MgCl₂, 26 Cs Gluconate, 78 HEPES, 6.5 TEACl, 11 Na₂ATP, 0.5 GTP,
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