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Synaptic transmission at retinal ribbon synapses

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Abstract

The molecular organization of ribbon synapses in photoreceptors and ON bipolar cells is reviewed in relation to the process of neurotransmitter release. The interactions between ribbon synapse-associated proteins, synaptic vesicle fusion machinery and the voltage-gated calcium channels that gate transmitter release at ribbon synapses are discussed in relation to the process of synaptic vesicle exocytosis. We describe structural and mechanistic specializations that permit the ON bipolar cell to release transmitter at a much higher rate than the photoreceptor does, under in vivo conditions. We also consider the modulation of exocytosis at photoreceptor synapses, with an emphasis on the regulation of calcium channels.

1. Introduction

The first step in vision is the capture of light by photoreceptors and its transduction into an electrical signal. Transduction occurs in the outer segments of rod and cone photoreceptors; the resultant change in membrane voltage is filtered by the electrical properties of the photoreceptor inner segments, ultimately producing a change in the membrane potential of their synaptic terminals. The final result is a change in the rate at which photoreceptors release their transmitter, glutamate.

Although the general scheme just outlined—transduction of some external energy into a voltage that then gates transmitter release—is common to all sensory systems, photoreceptors have special properties which shape their responses. The transduction cascade initiated by light capture leads to a reduction in a steady inward cation current (the dark current) resulting in a hyperpolarization of the rod or cone. The mechanism of neurotransmitter release by photoreceptors is conventional in the sense that it depends on calcium entry, which in turn is increased by depolarization, but it is unconventional in that transduction-mediated hyperpolarization reduces transmitter release. Thus, photoreceptors release glutamate at a steady rate in darkness, a rate that is slowed to a variable degree as a function of the intensity of the incident light. Rod photoreceptors reliably signal the capture of a single photon, whereas cones require higher rates of quantum capture to alter significantly the rate of glutamate release in darkness. These sensitivity differences notwithstanding, both types of photoreceptor utilize a special synaptic apparatus, the ribbon synapse, and a similar subtype of calcium channel to initiate transmitter release.

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The recipients of photoreceptor signaling are the horizontal and bipolar cells, the two classes of second-order retinal neuron. Like the photoreceptors, their light responses are unusual in being non-spiking, relatively slow changes of membrane potential. Among the second-order neurons, the depolarizing (ON) bipolar cell has received special attention, in part because in the goldfish retina, the rod-dominant ON bipolar cell (so-called Mb₁ subtype) has an unusually large and accessible synaptic terminal that has been the subject of intensive physiological investigation. The ON bipolar cell resembles the photoreceptor in some respects: binding of glutamate by receptors located on the dendrites initiates a metabotropic transduction cascade that controls the rate of cation flow across the plasma membrane. Like photoreceptors, it utilizes a ribbon synapse and a high voltage-activated calcium channel to release glutamate and thereby communicate with third-order retinal neurons. On the other hand, the fact that the synapse between photoreceptors and ON bipolar cells is sign-inverting, i.e., a photoreceptor hyperpolarization results in an ON bipolar cell depolarization, has important consequences for transmitter release. ON bipolar cells maximally release neurotransmitter in response to light, whereas photoreceptors release at their maximal rate during darkness. Under physiological conditions, the maximal rate of neurotransmitter release from rod photoreceptors is much slower than that of ON bipolar cells. Furthermore, the calcium sensitivity of exocytosis appears to be very different in these two neurons (Heidelberg et al., 1994; Rieke and Schwartz, 1996; Thoreson et al., 2004).

Our review examines the process of neurotransmitter release by photoreceptors and compares it with the much more extensively studied ON bipolar cell. By examining the morphology, physiology and molecular biology of ribbon synapses in photoreceptor and bipolar cells, we delineate our current understanding of transmitter release at these synapses and identify some unanswered but fundamental questions. For example: does rapid, phasic release of neurotransmitter utilize a different calcium sensor than slow tonic release? How do vesicles move from the ribbon to the release sites? How many functional pools of synaptic vesicles exist for ribbon synapses? What are the mechanisms by which exocytosis from photoreceptors is regulated?

2. Structure and function of ribbon synapses

2.1. Structural organization

The anatomical organization of the ribbon synapses found in photoreceptor and bipolar cells has been reviewed many times (e.g., Dowling, 1987; Sterling and Matthews, 2005) and it is not our purpose to repeat that description. Instead we revisit this theme with two points in mind. One is to review the molecular organization of ribbon synapses. Now that many proteins involved in the process of synaptic function have been identified, which of them are shown to be present in ribbon synapses and where are they located? Are there any ribbon-specific proteins and, if so, what are their contributions to neurotransmitter release? The second point is to try to infer as best we may with the available data how ribbon synapses function, i.e., what are the mechanisms whereby vesicles are induced to fuse with the subsynaptic membrane and release their neurotransmitter (glutamate) content? As a corollary point we integrate the information about Ca²⁺ channels and currents reviewed above with the data concerning rates of transmitter release inferred from capacitance or fluorescence measures, to see whether the data sets are coherent.

At the ultrastructural level, the ribbon synapse of a photoreceptor or a bipolar cell differs from that of a conventional central synapse. At conventional synapses, 10–100 synaptic vesicles are clustered near the plasma membrane at the active zone. In contrast, at rod, cone and bipolar cell synapses, a large number of small clear-core synaptic vesicles are tethered by protein filaments to an electron dense, pentalaminar structure, the synaptic ribbon, which is located between two active zones situated on either side of the ribbon and just adjacent to it (Dowling

and Werblin, 1969; Lasansky, 1973; Raviola and Gilula, 1975; Townes-Anderson et al., 1985; Lenzi and von Gersdorff, 2001).

In three dimensions, the ribbons of photoreceptors are shaped like a slender bar, about 35 nm thick, up to 1.0 μm high and 1–2 μm in length. Although the ribbon is typically curved, it maintains a vertical orientation over a troughlike structure called the arciform density. This ribbon-arciform density complex is itself positioned over a specialized depression in the photoreceptor membrane called the synaptic ridge (Dowling and Werblin, 1969; Lasansky, 1973; Raviola and Gilula, 1975; Pierantoni and McCann, 1981; Matsumura et al., 1981; Schaeffer et al., 1982; Townes-Anderson et al., 1985; Rao-Mirotznik et al., 1995). The approximate spatial relationships of these synaptic structures is diagrammed in Fig. 1. As reviewed elsewhere (Vollrath and Spiwoks-Becker, 1996), photoreceptor ribbons exhibit diurnal changes in shape and size. However, in turtle cones where the ribbon surface area decreases almost two-fold in darkness, there is a compensatory two-fold increase in synaptic vesicle density on the ribbon face suggesting that the number of tethered vesicles remains nearly constant despite considerable changes in ribbon size (Pierantoni and McCann, 1981).

Although goldfish ON bipolar terminals are large, their ribbons are much smaller than those in photoreceptors, having a height of 0.15–0.25 μm , and a length of about 0.35 μm (von Gersdorff et al., 1996; Holt et al., 2004). In contrast to photoreceptors, bipolar cell ribbons lack an arciform density, permitting the ribbon to approach the surface membrane more closely. Vesicles at the base of the ribbon are in contact with both the plasma membrane and the ribbon.

In addition to their presence in vertebrate retinas, synaptic ribbons are found in other regions of the central nervous system. They demarcate the active zones of hair cells of the cochlear and vestibular systems and are present in pineal gland cells and fish electroreceptors (Parsons and Sterling, 2003).

2.2. Ribbon synapse-associated proteins

Four proteins, of which three are presumed to be structural proteins, are associated with synaptic ribbons. The first is KIF3A, a member of the kinesin superfamily and a component of the kinesin II motor protein holoenzyme (Kondo et al., 1994; Hirokawa and Takemura, 2004; Muresan et al., 1999). The concept of the ribbon as a molecular motor shuttling vesicles to the active zone was originally proposed by Bunt (1971), and the immunocytochemical localization of KIF3A to the ribbon is compatible with this long-standing hypothesis. However, KIF3A is not exclusively localized to synaptic ribbons: it is also found on some synaptic vesicles (Muresan et al., 1998, 1999) as well as on many cargo vesicles in the brain, where it mediates anterograde fast axonal transport (Kondo et al., 1994; Yamazaki et al., 1995; Hirokawa and Takemura, 2004). Thus, a possible function of KIF3A may be to transport active zone structures from the soma to the active zone (e.g., Garner et al., 2000; Kondo et al., 1994; Muresan et al., 1998, 1999). To date there is no functional evidence to support the view that molecular motors are involved in vesicle movements in the vicinity of the active zone (reviewed in Lenzi and von Gersdorff, 2001; Heidelberg et al., 2002). Finally, in the absence of evidence other than antibody recognition, one cannot rule out the possibility that a ribbon protein and KIF3A share a conserved epitope (Parsons and Sterling, 2003; Sterling and Matthews, 2005).

RIBEYE is the only known protein that localizes exclusively to the synaptic ribbon (Schmitz et al., 2000; tom Dieck et al., 2005). This 120 kDa protein is a splice variant of the transcriptional repressor C-terminal binding protein 2 (CtBP-2) possessing one domain nearly identical to CtBP-2 and a second novel domain. Antibodies raised against either domain label ribbon-style synapses, but not conventional synapses. In addition, the use of double-labeling techniques and immunogold-conjugated antibodies confirm that it is the ribbon-structure, not

synaptic vesicles or other presynaptic constituents, that contains RIBEYE (Schmitz et al., 2000). RIBEYE is thought to constitute a large fraction of the ribbon substance, possibly its central scaffold (Schmitz et al., 2000; Zenisek et al., 2004). Live-cell optical imaging of a fluorescent peptide that binds to RIBEYE was used to estimate that there are approximately 4000 RIBEYE molecules in a single Mb₁ bipolar cell synaptic ribbon (Zenisek et al., 2004). Similar estimates are not yet available for photoreceptors, but if RIBEYE is the main structural protein, one would expect that the larger ribbons of these neurons should contain correspondingly more molecules of RIBEYE.

Two other proteins present at synaptic ribbons, bassoon and piccolo (Brandstatter et al., 1999) are cytomatrix proteins that were first identified at conventional synapses in brain (Garner et al., 2000). Piccolo is found in the more distal portion of the ribbon whereas bassoon is located at the base of photoreceptor ribbons and in the subjacent cytoplasm (Dick et al., 2001). Inexplicably, bipolar cells do not immunolabel for bassoon (Dick et al., 2001). Thus, either they do not express this protein or the epitope recognized by the antibody is uniquely modified or somehow unavailable in the bipolar cell. Bassoon appears to be crucial for maintaining photoreceptor ribbon orientation in relation to other synaptic structures, judging from the finding that in a bassoon knockout (Dick et al., 2003), both cone and rod ribbons become free-floating. Moreover, tom Dieck et al. (2005) showed that bassoon is linked both to the ribbon and to the presynaptic plasma membrane/arciform density compartment, consistent with its proposed role as an anchoring protein for the ribbon. The effect of a bassoon knockout on bipolar cell ribbons was not reported. The presence of bassoon and the correct positioning of ribbons at active zones also are necessary for normal synaptic transmission in photoreceptors, as evidenced by altered b-waves in ERG's of bassoon mutants (Dick et al., 2003). Similarly, bassoon is necessary for the placement of synaptic ribbons at cochlear hair cell active zones and fast, synchronous neurotransmitter release (Khimich et al., 2005). Little is known about the specific role of piccolo in ribbon synapses, but this multidomain protein may be generally important as a cytomatrix scaffolding protein that binds calcium (Gerber et al., 2001) and functions in endocytosis (Fenster et al., 2003).

Of related interest, the nrc zebrafish mutant is characterized by the absence of an optokinetic response and disordered cone synapses in which synaptic ribbons are free-floating (Allwardt et al., 2001). Van Epps et al. (2004) showed that the nrc mutant had a loss of synaptojanin1, a protein implicated in cytoskeletal organization and endocytosis. In the mouse retina, Ball et al. (2002) found that when the $\beta 2$ subunit of the rod Ca²⁺ channel was not expressed so that the rod lacked a functional Ca²⁺ channel, the photoreceptor ribbon synaptic structure also failed to develop. Collectively, these studies indicate that the synaptic ribbon is closely linked to adjacent structures. It not only tethers a large population of synaptic vesicles close to their release sites but is probably also linked to the arciform density through protein–protein interactions. Detachment of the ribbon from these associated structures leads to loss of synaptic function and in some cases loss of the ribbons themselves.

2.3. Fusion machinery

The SNARE (SNAP receptor) complex is a universal mediator of fusion events in eukaryotes. In neurons, this complex typically consists of an integral vesicle protein, synaptobrevin (also known as vesicle-associated membrane protein [VAMP]), and two plasma membrane associated proteins, syntaxin 1 and SNAP-25 (synaptosomal-associated protein—25 kDa). These proteins bind to each other and together constitute the minimal core complex necessary for membrane fusion (reviewed by Bruns and Jahn, 2002; Sudhof, 2004). Syntaxin 1 also interacts at the molecular level with N-type calcium channels (Sheng et al., 1994). The latter is thought to assist in localizing these calcium channels to the sites of vesicle fusion (Sheng et al., 1998) and to modulate calcium channel gating (Stanley and Mirotznik, 1997; Stanley,

2003). Interestingly, syntaxin 1, the isoform present at conventional synapses, appears to be absent from rodent retinal ribbon synapses (Brandstatter et al., 1996b). However, it is replaced by syntaxin 3, which forms a complex with SNAP-25, synaptobrevin, and a cytoplasmic synapse-associated protein, complexin (Morgans et al., 1996). This isoform substitution may be related to the L-type presynaptic calcium channel used by photoreceptors and bipolar cells. Syntaxin 3 is also present in insulin-secreting β cells where it inhibits L-type Ca^{2+} channels and insulin secretion (Kang et al., 2002). The other components of the minimal core fusion complex, SNAP-25 and synaptobrevin, are now believed to be present in retinal ribbon synapses, despite earlier controversies (Von Kriegstein et al., 1999; Sherry et al., 2001, 2003b). In mouse retina, the synaptobrevin isoform VAMP2 is expressed at both conventional and ribbon synapses. VAMP1, however, is limited in distribution and is present in terminals of photoreceptors and a subset of ganglion cells, but not in bipolar cells (Sherry et al., 2003b). Thus, all retinal neurons, including those that utilize synaptic ribbons, possess the minimal components necessary for SNARE-mediated fusion. The major expressed isoform of a particular SNARE protein, however, may differ among synapses.

Additional proteins assist in holding the vesicles to the ribbon, transferring them to the docking sites and preparing them for release (reviewed in Morgans, 2000). Crucial to the release step is synaptotagmin, which has been identified as the Ca^{2+} sensor for neurotransmitter release (reviewed in Sudhof, 2004). Sudhof (2002) describes a large family of synaptotagmins having different Ca^{2+} sensitivities and locations within the presynaptic terminal. Synapses at which rapid, phasic release occurs utilize synaptotagmin I or II, proteins associated with the synaptic vesicle itself and having a low Ca^{2+} affinity (Sudhof, 2004). Other synaptotagmins are found on the plasma membrane, rather than the synaptic vesicle, and have a higher Ca^{2+} sensitivity, i.e., operate at lower $[\text{Ca}^{2+}]_i$. Given the unusual hyperpolarizing response of rods and cones, their apparently small Ca^{2+} currents (I_{Ca}) (Corey et al., 1984) and the relatively low $[\text{Ca}^{2+}]_i$ in their synaptic terminals (Rieke and Schwartz, 1996), one might expect to find a high-sensitivity synaptotagmin in photoreceptors.

Heidelberg et al. (2003) utilized a variety of anti-synaptotagmin I/II antibodies to probe this question. In the retinas of goldfish and salamander, two species where the physiology of exocytosis can be readily examined, the anti-synaptotagmin antibodies that gave the strongest labeling of conventional synapses did not immunostain photoreceptor terminals. On the other hand, both Heidelberg et al. (2003) and Berntson and Morgans (2003) reported anti-synaptotagmin I/II immunostaining in mouse photoreceptors, and Von Kriegstein et al. (1999) localized synaptotagmin I to the outer plexiform layer of the bovine retina. Synaptotagmin III, a plasma membrane synaptotagmin (Sudhof, 2002, 2004), was also present in the outer plexiform layers in both goldfish and mouse, although the immunolabeling was somewhat sparser in mouse (Berntson and Morgans, 2003). Although the role of synaptotagmin 3 in exocytosis is not yet fully understood, it is interesting to note that the *in vitro*, calcium-dependent phospholipid-binding properties of the C2A domain of synaptotagmin 3 mirror the calcium sensitivity of the highly calcium-sensitive pool of synaptic vesicles in salamander rods better than the C2A domain of synaptotagmin I does (Sugita et al., 2002; Thoreson et al., 2004; see also Section 4).

As with photoreceptors, there are differences in synaptotagmin isoform labeling between mammalian and non-mammalian bipolar cells. Whereas synaptotagmin III antibodies label PKC-positive, ON-type, rod-dominant bipolar cell terminals in the goldfish, they do not label rod bipolar terminals of the mouse. Furthermore, mouse bipolar cells are immunolabeled by synaptotagmin I/II, whereas their goldfish counterparts were not. The significance of these species differences is unclear because, on the one hand, the immunolabeling studies in non-mammalian vertebrates were performed using antibodies to mammalian isoforms and, on the other hand, little is known about the calcium dependence of release at mammalian retinal ribbon

synapses. A straightforward interpretation of isoform expression and the calcium sensitivity of release may be further complicated by the putative role of synaptotagmin in endocytosis and the different modes of endocytosis that a cell may preferentially utilize (Llinas et al., 2004; Holt et al., 2003; Paillart et al., 2003).

Many other synaptic vesicle-associated proteins including SV2B, rab3, SCAMP1, synaptogyrin, Munc18 and synaptophysin1 are present on synaptic vesicles of photoreceptors and bipolar cells (Von Kriegstein et al., 1999; Von Kriegstein and Schmitz, 2003; Sherry et al., 2001, 2003b; Morgans et al., 1996; Morgans, 2000; Brandstatter et al., 1996a, b; Wang et al., 1997; Ullrich and Sudhof, 1994). A small number of vesicle proteins appear to be absent from some or all ribbon synapses. For example, while the synaptic vesicle protein SV2B appears to be present at all ribbon synapses in the retina, SV2A is present in terminals of cones but not rods (Wang et al., 2003). SV2A is also found in some cone OFF bipolar cell terminals, as well as many conventional synapses of the retina. The functional consequences of this rod/cone difference are not yet fully understood but it is worth noting that SV2 proteins interact with synaptotagmin (Lazzell et al., 2004). The vesicle protein, synapsin 1, appears to be absent from all ribbon synapses (Mandell et al., 1990, 1992; Von Kriegstein et al., 1999). Synapsin 1 has been proposed to promote clustering of vesicles (Greengard et al., 1994; but see Sudhof, 2004) but at the ribbon, this function may be replaced by proteins tethering vesicles to the ribbon. As discussed later, the absence of synapsin may also facilitate greater vesicle mobility in ribbon synapses. Rab3 is identified as a neuronal GTP-binding protein that negatively regulates fusion of synaptic vesicles (Wang et al., 1997). Rabphilin, which associates with Rab3 at many central synapses, is also absent from ribbon synapses (Von Kriegstein et al., 1999) perhaps replaced by the cytoplasmic protein RIM (rab3 interacting molecule) which is shown to cluster around the ribbon (Wang et al., 1997). Mutations in RIM have been implicated in autosomal dominant rod-cone dystrophy (Johnson et al., 2003b). Yet another protein, RIM binding protein (RBP) has been studied in chicken retina by Hibino et al. (2002). Although it has a widespread distribution among the retinal layers, it co-localizes with SV2 and with the Ca^{2+} channels in photoreceptor terminals.

2.4. Synaptic vesicles

Synaptic vesicles in both photoreceptor and bipolar cell terminals contain the neurotransmitter glutamate, which is packed into the vesicle by the vesicular glutamate transporter type I (Sherry et al., 2003b; Johnson et al., 2003a). The concentration of glutamate in the vesicle is estimated to be on the order of 100 mM (Burger et al., 1989). Vesicle diameter has been measured by EM to be 29–36 nm in goldfish bipolar cell terminals (von Gersdorff et al., 1996; Lagnado et al., 1996) but 45–50 nm in diameter in salamander photoreceptor terminals (Lasansky, 1973; Thoreson et al., 2004). Synaptic vesicle membrane is derived from the surface membrane by endocytosis (Royle and Lagnado, 2003) but is modified by the addition of many proteins implicated in vesicle docking, priming and fusion, as described above.

The large synaptic terminals of Mb bipolar cells contain 400,000–1,000,000 vesicles scattered throughout the terminal (Lenzi and von Gersdorff, 2001). Cone terminals of lizard and turtle retinas (~6 μm diameter) contain approximately 250,000 synaptic vesicles (Pierantoni and McCann, 1981; Rea et al., 2004). Assuming a similar cytoplasmic concentration of vesicles, a mammalian rod with a terminal 2–3 μm in diameter would have 8000–27,000 vesicles within the terminal cytoplasm. Of this total pool, a much smaller number, estimated at ~700 vesicles in mammalian and amphibian rods (Rao-Mirotznik et al., 1995; Thoreson et al., 2004) as well as turtle cones (Pierantoni and McCann, 1981), is tethered to each synaptic ribbon. Bipolar cell ribbons tether a smaller number of vesicles, arranged as five rows of 11 vesicles on each ribbon face (von Gersdorff et al., 1996). The tethers are slender proteinaceous strands, up to 50 nm long and 8–10 nm in diameter in photoreceptors (Usukura and Yamada, 1987); those

in bipolar cell terminals are of similar dimensions (cf. Fig. 3, Sterling and Matthews, 2005). Vesicles appear to be arranged in a hexagonal array along the face of the ribbon (Pierantoni and McCann, 1981; Thoreson et al., 2004). The orderly array of ribbon-associated vesicles contrasts with the clustering of synaptic vesicles observed at conventional synapses. The absence of clusters is partly due to the physical separation of neighboring vesicles created by the tethers, but another contributing factor may be that photoreceptors and bipolar cells lack the protein, synapsin 1 (Mandell et al., 1990, 1992), that has been proposed to promote clustering (Greengard et al., 1994; but see Sudhof, 2004).

In addition to their ribbon synapses, cone photoreceptors also make so-called flat or basal contacts onto post-synaptic cells (Kolb, 1970; Lasansky, 1973). Unlike conventional synapses, flat contacts do not exhibit a concentration of vesicles, although these junctions do show prominent paramembranous densities on both pre- and post-synaptic sides (Lasansky, 1973). In mammalian retina, OFF bipolar cell dendrites are not directly associated with a ribbon synapse but instead make contact with cone bases only through flat junctions (Kolb, 1970). The absence of direct ribbon contacts in mammalian OFF bipolar cells prompted the suggestion that they must receive synaptic input from flat contacts (Lasansky, 1972; Dowling, 1987). Evanescent wave imaging studies of bipolar cell terminals show that a fraction of the total exocytosis occurs at sites away from the ribbon (Zenisek et al., 2000, 2003). Whether a similar phenomenon occurs in photoreceptors and whether such sites are associated with basal contacts are unanswered questions. It should be noted that in the salamander retina, in contrast to mammalian retina, OFF bipolar cells receive ~80% of their contacts from ribbons and ~20% from flat contacts whereas ~80% of the contacts onto ON bipolar cells are from flat contacts and ~20% are from ribbons (Lasansky, 1978). Because the status of flat contacts as either synaptic release sites or non-synaptic contact points is still undecided, in this review we confine our remarks to ribbon synapses.

2.5. Synaptic vesicle pools

Both anatomical and physiological data indicate that at least three vesicle pools exist in ribbon-synapse bearing terminals: cytoplasmic, ribbon related and docked. Vesicles from the cytoplasmic pool reach the ribbon either by diffusion or some transport process (see below). It is presumed that these vesicles are at least partially filled with glutamate and bear the appropriate vesicle proteins to initiate docking at the active zone and become fusion competent. Vesicles are docked through protein–protein interactions between proteins integral to the vesicle, proteins concentrated in the surface membrane at the active zone, and cytosolic proteins (cf. Sections 2.2 and 2.3).

The vesicles that fuse as a result of Ca^{2+} entry are distinguished by being positioned at specialized docking sites in the photoreceptor surface membrane—the active zone—where a set of proteins primes them for release. In freeze-fracture studies (Raviola and Gilula, 1975), the docking sites are recognized by circular profiles, which in photoreceptors are arranged in a hexagonal array, occupying 2–4 lines along the surface membrane, on either side of, and parallel to the ribbons (Raviola and Gilula, 1975; Fig. 1). A rough estimate based on freeze-fracture and transmission EM pictures is 100–130 docking sites/ribbon in mammalian photoreceptors (Raviola and Gilula, 1975; Rao-Mirotznik et al., 1995). In many species, it appears that only the lowest row of 20–25 vesicles at the base of the ribbon is docked in contact with the plasma membrane (Sterling and Matthews, 2005).

At conventional synapses, exocytosis is a sequential process, in which vesicles arrive at the active zone, are docked through protein–protein interactions, and are primed for release; the arrival of calcium triggers the fusion process with minimal delay. At ribbon synapses, circumstantial evidence suggests that vesicles tethered to the ribbon already are primed for rapid, calcium-dependent fusion, regardless of their proximity to the plasma membrane

(Heidelberg, 1998; Heidelberg et al., 2002). This raises the possibility that at ribbon synapses priming may occur prior to docking, although how this might work at a molecular level is unclear (reviewed by Heidelberg, 2001; Sterling and Matthews, 2005).

Following the elevation of internal Ca^{2+} , a vesicle's membrane fuses with the plasma membrane and releases its contents into the synaptic cleft. Release can occur through a small fusion pore sometimes called "kiss-and-run" exocytosis, or via the rapid dilatation of the pore and collapse of the vesicle into the plasma membrane, a process known as "full fusion" (reviewed by An and Zenisek, 2004). Whole-cell or whole-terminal membrane capacitance measurements commonly employed to study neurotransmitter release cannot readily distinguish between these modes (An and Zenisek, 2004). The evidence so far available, however, indicates that photoreceptors and bipolar cells do not employ kiss-and-run. For example, recent work using optical methods to observe the destaining time course of fluorescently labeled vesicles during exocytosis suggests that, in bipolar cells, full fusion is evoked by membrane depolarization (Zenisek et al., 2003; Llobet et al., 2003a). Although not all the details of vesicle movement in the exocytotic cycle are fully clarified, the physiological data described here and in subsequent sections indicate that it is the ribbon-associated vesicles which represent the releasable pool underlying fast neurotransmitter release. Typically, upon closer examination, a second, smaller pool may be observed with even faster fusion kinetics. This pool may represent that subset of releasable vesicles situated nearest to the presynaptic calcium channels and hence the ones which experience the highest calcium concentration.

2.6. Vesicle pools and versatility of ribbon synapses

In bipolar cells, the releasable pool of synaptic vesicles has been defined with remarkable consistency by different laboratories (reviewed in Heidelberg, 2001). This population of vesicles has already passed through all the ATP-dependent priming steps required for fusion (Heidelberg, 1998; Heidelberg et al., 2002) and is fully fusion competent. Other names used in the literature to describe this pool in bipolar cells include the release-ready pool and, confusingly, the reserve pool. From the average size of the capacitance jump (150 fF) and the average size of a bipolar cell vesicle (29 nm diameter), the calculated size of the release-ready pool from the physiological measurements is ≈ 5700 vesicles, in close agreement with the total number of ribbon-tethered vesicles in a bipolar cell terminal (5500–6000) determined by serial EM reconstructions (von Gersdorff et al., 1996). This excellent correspondence, in addition to the observations that smaller terminals have fewer synaptic ribbons and exhibit smaller capacitance changes whereas larger terminals have more ribbons and exhibit larger capacitance changes, provides strong circumstantial support for the belief that ribbon-associated vesicles constitute the releasable pool (von Gersdorff et al., 1996). Similarly, in salamander rod photoreceptors, a releasable pool of synaptic vesicles has been physiologically defined whose size agrees well with the total number of vesicles estimated to be tethered to the synaptic ribbons, based upon ribbon size, number and vesicle packing (Thoreson et al., 2004). Furthermore, in both bipolar cells and hair cells, which also contain synaptic ribbons, vesicle-attachment sites on a synaptic ribbon located nearest the plasma membrane become devoid of vesicles following stimulation, consistent with the supposition that ribbon-associated vesicles contribute to the exocytotic response (Holt et al., 2004; Lenzi et al., 1999).

The average rate of vesicle fusion for vesicles in the releasable pool in response to a depolarizing voltage step can be used to estimate an average maximal fusion rate of the releasable pool in response to calcium influx. In bipolar cells, this rate is about 500 vesicles/s/synaptic ribbon (Matthews, 1996). If one assumes that each of the 22 vesicles at the bottom of the ribbon contacting the plasma membrane is located at a site of vesicle fusion, then this rate is equivalent to ≈ 20 vesicles/s/release site, which is virtually identical to the estimated fusion rates for the conventional fast synapses of amacrine cells and hippocampal neurons

(Matthews, 1996). In other words, the secretory machinery of bipolar cell ribbon synapses is not intrinsically slow. More modest rates of release (900 vesicles/s or ≈ 15 vesicles/s/ribbon or ≈ 0.7 vesicles/s/release site) have been measured in response to long, mild depolarizations more typical of physiological stimulation (Rouze and Schwartz, 1998; see also Lagnado et al., 1996), suggesting that bipolar cell ribbon synapses are extremely versatile with respect to the speed and duration of their neurotransmitter release.

The fusion rate of the releasable pool of vesicles in the salamander rod photoreceptor can be similarly approximated. The maximal rate of vesicle fusion for the typical vesicle in the releasable pool evoked by membrane depolarization is estimated to be 2300 vesicles/s/synaptic ribbon, assuming a releasable pool of ≈ 3500 vesicles and five functional synaptic ribbons per terminal (Thoreson et al., 2004). To make a direct comparison with hippocampal neurons, one needs to know the number of vesicle docking or release sites. Estimates in the salamander rod photoreceptor, determined by counting the number of ribbon-associated vesicles in physical contact with the plasma membrane (Ellen Townes-Anderson, pers. comm.), suggest a total of 115–235 docked vesicles per synaptic terminal (≈ 25 /ribbon). If each docked vesicle represents a vesicle poised at a release site, then the average maximal rate of release in response to calcium channel activation is on the order of 50–90 vesicles/s/release site. This result suggests that, as for the bipolar cell, the secretory machinery of the photoreceptor is unlikely to be intrinsically slower than that of a conventional synapse.

Of course, a large depolarizing voltage step is not a physiological stimulus for a photoreceptor, so such high rates of fusion may never be achieved in vivo. Rieke and Schwartz (1996) have estimated a release rate of 400 vesicles/s at the dark resting potential. Again, assuming 125–235 docked vesicles, this value collapses to 2–3 vesicles/s/release site. Release from cone terminals in the turtle retina at the dark resting potential has been estimated from fluctuation analysis to be 20–80 vesicle/ribbon/s (Ashmore and Copenhagen, 1983). Turtle cone ribbons have similar dimensions to salamander rod ribbons and tether similar number of vesicles (Pierantoni and McCann, 1981). If we therefore assume turtle cones, like salamander rods, exhibit ≈ 25 docked vesicles/ribbon, then we once again obtain release rates of 2–3 vesicles/s/release site.

Sustained rates of release from mammalian photoreceptors appear to be at least as slow, if not slower. In mammalian rods, there appear to be ≈ 130 docking sites per ribbon (Sterling and Matthews, 2005) and computer simulations suggest rods release continuously at rates of 80–100 vesicle/ribbon/s (Rao-Mirotznik et al., 1998; van Rossum and Smith, 1998). Very low sustained rates of release from mammalian cones (≈ 18 vesicles/ribbon/s) have been estimated using noise/variance analysis techniques to analyze post-synaptic light responses in cone-driven bipolar cells (Berntson and Taylor, 2003). These different approaches suggest rates of release from mammalian photoreceptors of < 1 vesicle/s/release site. Thus, as for the bipolar cell, the available data indicate that the secretory machinery of photoreceptor ribbon synapses also is extremely versatile, accommodating both a burst of rapid neurotransmitter release, and sustained, but slower, rates of release.

In addition to the releasable pool, a smaller, faster pool of synaptic vesicles has been identified in bipolar cells (Mennerick and Matthews, 1996; Sakaba et al., 1997; Neves and Lagnado, 1999). This pool, referred to as the ultrafast or rapidly releasable pool, was discovered by examining the exocytotic response to a relatively brief (< 50 ms) activation of voltage-gated calcium channels (Mennerick and Matthews, 1996). Vesicles in this pool fuse with rates similar to the maximal rates achieved in flash-photolysis experiments, in which calcium was rapidly and globally elevated by uncaging calcium (Heidelberg et al., 1994). In the flash-photolysis experiments, fusion rates comparable to those of the ultrafast pool were achieved only when local intraterminal calcium was $> 200 \mu\text{M}$ (Heidelberg, 2001). Under physiological

conditions, such high values are expected very near the mouth of an open calcium channel, suggesting that this pool of vesicles might represent vesicles docked at ribbon-associated active zones, where fusion sites and calcium channels co-localize (Zenisek et al., 2004). Several additional pieces of evidence support this supposition. First, the fusion of this physiologically defined vesicle pool is not prevented by the addition of millimolar exogenous calcium buffer EGTA to the presynaptic cytosol, consistent with a vesicle location very near to the sites of calcium entry (Mennerick and Matthews, 1996; Sakaba et al., 1997). Secondly, there is an excellent correlation between the total number of vesicles docked at the base of a bipolar cell's synaptic ribbons and in physical contact with the plasma membrane (≈ 1000 – 1400 , for a small and large terminal respectively) and the number of vesicles that comprise the rapidly releasing pool of vesicles as determined by capacitance measurements (≈ 1100) (von Gersdorff et al., 1996; see also Neves and Lagnado, 1999). It is also noteworthy that the rate of fusion of vesicles in this pool is so rapid, that it is hindered by the activation kinetics of the presynaptic calcium channels (Mennerick and Matthews, 1996). When the calcium channels are bypassed, as in a flash photolysis of caged-calcium experiment, the brief delays that are observed between the rise in calcium and the onset of exocytosis (<1 ms for $\text{Ca}^{2+} > 200 \mu\text{M}$) can be attributed solely to the calcium-binding properties of the calcium sensor for exocytosis (Heidelberg et al., 1994). Thus, vesicles in this pool must be primed and situated at their fusion sites prior to calcium elevation.

In rod photoreceptors as well, pulse-duration studies have revealed two kinetic components of release, and it may be that the faster component ($\tau < 10$ ms) represents a rapidly releasable pool (Thoreson et al., 2004). From the calcium dependence of the rate of exocytosis derived from flash experiments on photoreceptors, an average vesicle in this putative pool might experience a calcium concentration in the 20 – $40 \mu\text{M}$ range (Thoreson et al., 2004). Although vesicles in this pool must be quite close to the calcium channels to experience such calcium concentrations, these vesicles still may not be as closely situated to the presynaptic calcium channels as their bipolar cell counterparts, which release with a time constant of 1.6 ms (Mennerick and Matthews, 1996) and are thought to sample $\approx 325 \mu\text{M}$ calcium (Heidelberg, 2001). This greater distance and the consequently lower calcium requirements in the rod could be related to the arciform density, which we suggest acts as a diffusion barrier to entering calcium and/or to prevent vesicles from docking beneath the synaptic ribbon.

A putative ultrafast pool is not easily distinguished in rod photoreceptors due to the high noise intrinsic to photoreceptor capacitance measurements and the potentially small size of the pool. Thus, the possibility that the kinetic component discussed here includes some contributions from the slower releasable pool cannot be excluded. Additional experimental evidence, such as the sensitivity of exocytosis to EGTA or a rigorous pool size determination, is needed to distinguish between these possibilities. Nonetheless, it is worth noting that paired recordings in retinal slices of photoreceptors and second-order neurons indicate that the post-synaptic current is detected within a few milliseconds of photoreceptor depolarization. As discussed below, this time frame is probably too short to allow significant vesicle movement. Furthermore, delays of a few milliseconds are plausibly accounted for by the calcium-binding properties of the rod's calcium sensor for exocytosis (Thoreson et al., 2004). Therefore, it is reasonable to postulate that a rapidly releasing pool of synaptic vesicles, located near fusion sites, is also present in rod photoreceptors.

2.7. Fusion scenarios and ribbon function

Exactly how a vesicle in the releasable pool arrives at the active zone is not known. In bipolar cells, the most distant vesicle in the releasable pool is located $\approx 0.15 \mu\text{m}$ from the plasma membrane (von Gersdorff et al., 1996). This distance is even greater in photoreceptors, with some ribbon-associated vesicles as much as $1.0 \mu\text{m}$ away from the plasma membrane

(Pierantoni and McCann, 1981). How do these more distant vesicles make their way to their fusion sites? One possibility is that vesicles are somehow escorted to the active zone, perhaps by an active transport mechanism or via protein–protein interactions. This putative mechanism would include the popular hypothesis that the ribbon somehow acts to convey vesicles along its length to the active zone, in addition to vesicle detachment, followed by escorted movement. A second possibility is that a vesicle detaches from its tether, perhaps in a calcium-dependent manner, and diffuses to the plasma membrane. A third possibility is that there is some form of compound fusion at play, in which only the bottom row of vesicles fuses directly with the plasma membrane (Edmonds et al., 2004; Parsons and Sterling, 2003) and subsequent vesicles fuse with the first. Fig. 2 illustrates some of these possibilities in schematic form. Distinguishing between these hypotheses is not possible at this time, but recent data raise some interesting points that are worth discussing.

In bipolar cells, several studies have permitted inferences about vesicle movement near the release sites. For example, in Mb₁ terminals, the actin cytoskeletal network was found to have no effect on exocytosis or on general vesicle mobility (Job and Lagnado, 1998; Holt et al., 2004). Vesicles in cone photoreceptors are also more mobile than those of conventional synapses (Rea et al., 2004). At conventional synapses, vesicles are anchored to the actin cytoskeleton via vesicular synapsin, and vesicle mobility is far more restricted. Accordingly, these mobility differences between ribbon and conventional synapses may reflect the absence or presence of vesicular synapsin. Even within 50–120 nm of fusion sites on the plasma membrane, fluorescently labeled synaptic vesicles in bipolar terminals observed using total internal fluorescence microscopy exhibited relatively random movements (Holt et al., 2004; Zenisek et al., 2003). Thus, evidence from optical measurements of vesicle movement in living terminals does not support highly directed movements, such as might occur along a defined track. In addition, microtubules or actin filaments needed for directed movements typically are not found near ribbon-style active zones (Usukura and Yamada, 1987; but see Gray, 1976).

In both bipolar cells (Holt et al., 2004) and cone photoreceptors (Rea et al., 2004), diffusion times for cytoplasmic vesicles have been calculated from fluorescence data. The estimated diffusion coefficients (*D*) (ca. 2×10^{-10} cm²/s for ON bipolar terminal and 1.1×10^{-9} cm²/s for the cone) are 1–2 orders of magnitude slower than expected for a vesicle-sized sphere diffusing in an unhindered medium. The cytoplasm, however, is filled with obstacles (e.g., endosomes and other vesicles) that contribute to the tortuosity (λ) of the medium, which reduces *D* by λ^2 . If free diffusion is hindered entirely by the tortuosity, then this suggests a value for λ of 3.3–10, in accordance with estimates of intracellular tortuosity obtained from studies on the diffusion of ficoll and dextran particles (reviewed in Luby-Phelps, 2000). However, such estimates cannot distinguish between Brownian diffusion and protein–protein interactions; the latter plausibly acts upon vesicles given the large number of membrane proteins they contain and the finding that vesicle-bound proteins associate with cytoplasmic proteins such as complexin (Morgans et al., 1996).

Assuming an array of vesicles stacked like cannonballs above the active zone, and given the estimated diffusion coefficients (Zenisek et al., 2000; Holt et al., 2004), a minimum delay for replacing an exocytotic vesicle with its nearest neighbor in the ON bipolar cell is about 20–40 ms. This corresponds to a rate of travel for a vesicle of approximately ≈ 0.75 – 1.5 μ m/s, in excellent agreement with the rate of vesicle movement observed using optical techniques near release sites (Zenisek et al., 2000). A vesicle may require an additional 250 ms after it arrives at the active zone before it is able to fuse (Zenisek et al., 2000). Under conditions of tonic release, vesicle fusion occurs no more frequently than once every 350ms in bipolar cells and photoreceptors. This rate is sufficiently slow to permit 20–40ms for replacement of an active zone vesicle and another 250 ms for the restoration of a release site to functionality. However, these rates are far too slow to explain the fusion of vesicles already in the ultrafast or rapidly

releasing pool, consistent with the belief that these vesicles are already primed and docked near fusion sites prior to stimulus arrival.

Neither prior priming, random diffusion, nor motor proteins can fully explain how the bipolar cell releasable pool, thought to comprise the entire cohort of ribbon-associated vesicles, can fuse in less than 1 ms in response to a rapid, global elevation of calcium (Heidelberg et al., 1994; Heidelberg, 1998). To explain these findings with a translocation model, a vesicle situated at the top of a bipolar cell ribbon would have to traverse approximately 120 nm in less than ≈ 350 μ s, and it must do this in the absence of ATP hydrolysis (Heidelberg, 1998). Both the speed and the ATP requirement argue against a role for kinesin and myosin motor proteins, including kinesin II (Howard, 1997; Zhang and Hancock, 2004). This movement is also too fast to be explained by simple diffusion, based on the published diffusion coefficients for vesicles in bipolar (Holt et al., 2004) and photoreceptor (Rea et al., 2004). One possible explanation is that vesicles fuse with each other to form compound vesicles prior to fusion with the plasma membrane (Fig. 2D). Compound vesicles have been observed in non-neuronal secretory cells (e.g., Alvarez de Toledo and Fernandez, 1990) and could potentially underlie the multivesicular release reported in bipolar cells (Singer et al., 2004) and cochlear hair cells (Glowatzki and Fuchs, 2002; Edmonds et al., 2004). On the other hand, it is difficult to reconcile the fusion of compound vesicles with the rise in membrane capacitance evoked by the rapid, global elevation of calcium, which is best described by a smooth, single exponential function indicative of the fusion of a small, homogeneous class of vesicle (Heidelberg et al., 1994). An alternative explanation is that in the presence of high, global calcium, a different form of compound fusion takes place, in which docked vesicles fuse with the plasma membrane and the next row of vesicles fuses with the docked vesicles, and so on up the ribbon. High calcium concentrations are known to facilitate vesicle-vesicle fusion in other systems and conceivably could do so in neurons (Gratzl et al., 1977). With this scenario (Fig. 2), as the line of fused vesicles grows in height, one might predict the ribbon to appear in cross section as though it were evaginating into the plasma membrane with the next available vesicle sitting at the crest of the membrane folding formed by the fused vesicles. Indeed, both evagination with increasing stimulation intensity and the placement of the next vesicle at the crest have been reported in lateral line ribbon synapses (Fields and Ellisman, 1985). Moreover, the relatively large, tubular endocytic figures observed after strong depolarization could represent the retrieval of several vesicles' worth of membrane (Paillart et al., 2003; Royle and Lagnado, 2003), such as might occur from retrieving several vesicles following compound fusion (although other interpretations are possible). Alternatively, but not exclusively, the global calcium stimulus provided by calcium uncaging might trigger the fusion of numerous vesicles outside the active zone. However, in both photoreceptors and bipolar cells, cross-depletion studies, in which a depolarization is given to deplete the releasable pool followed by rapid calcium uncaging to trigger exocytosis by the remainder of fusion-competent vesicles in the cell, have indicated that if there is a contribution of non-active zone vesicles, it is probably small and cannot account for the majority of the response (Heidelberg, 1998; Thoreson et al., 2004).

Under normal physiological conditions many of the ribbon-tethered vesicles are thought not to be exposed to the high concentrations of calcium normally found in restricted areas under the plasma membrane, near open calcium channels. If these upper row vesicles are used to refill the ultrafast pool, do they stay attached to their tethers and move along the ribbon or do they detach from their tethers and then move? The answer to this question is unknown. However, it is possible that the tethers which attach vesicles to the ribbon may act like synapsin proteins at conventional synapses in which the elevation of calcium stimulates an intracellular cascade that ultimately results in the release of synapsin from the synaptic vesicles, freeing them from the actin cytoskeleton (Fig. 2C; Sudhof, 2004). Consistent with such an idea, in the bipolar cell, elevated calcium is reported to accelerate the refilling of the rapidly releasable pool (von Gersdorff and Matthews, 1994; Gomis et al., 1999). In addition, an increase in

intraterminal calcium can cause the two temporal components of glutamate release reported with a bioassay to merge into one, fast component, again consistent with calcium-accelerated vesicle mobilization (von Gersdorff et al., 1998). At present, the molecular target(s) mediating this action are unknown.

3. Calcium channels

3.1. Number and location

As in other cells, calcium entry through voltage-gated calcium channels in bipolar and photoreceptor cell terminals triggers fusion of vesicles with the surface membrane and release of their contents into the synaptic cleft (reviewed in Thoreson and Witkovsky, 1999). In bipolar cells, a number of physiological studies provided indirect evidence that sites of Ca^{2+} entry are concentrated near the ribbons (Heidelberg et al., 1994; Mennerick and Matthews, 1996; Llobet et al., 2003b; Zenisek et al., 2003). Recently, however, Zenisek et al. (2004) used a fluorescent protein marker for the ribbon to show directly that sites of calcium influx were spatially coincident with the synaptic ribbons. Although comparable studies have not yet been carried out on photoreceptors, immunocytochemical studies (Nachman-Clewner et al., 1999; Morgans, 2001) indicate a clustering of Ca^{2+} channels near the ribbons. In addition, tom Dieck et al. (2005) demonstrated that the $\alpha 1$ subunit of the Ca channel co-localized with RIBEYE, the main structural protein of the synaptic ribbon. This association was lost in the freely floating ribbons of bassoon mutant photoreceptors. Depolarizing steps, moreover, stimulate localized “hot spots” of calcium influx into photoreceptor terminals (Fig. 3).

Freeze-fracture data show an aggregation of transmembrane particles in the synaptic ridge underlying the arciform density (Raviola and Gilula, 1975; Nagy and Witkovsky, 1981). Raviola and Gilula (1975) postulated that these particles, which are “polyhedral in shape and contain a central dimple”, represent the calcium channels. They estimated there were 500 such putative channels/ridge. The dimensions of the ridge derived from freeze-fracture images indicate a surface area of $1.1 \mu\text{m}^2$ which yields a Ca^{2+} channel density of $460/\mu\text{m}^2$. This value is relevant to possible overlap of intracellular Ca^{2+} ion domains when more than one Ca^{2+} channel is open simultaneously.

Physiological measurements of whole-cell and single-channel Ca^{2+} currents yield similar estimates of Ca^{2+} channel number. Cell-attached patch recordings from isolated salamander rod photoreceptor indicate that their Ca^{2+} channels exhibit a single-channel conductance of 22 pS (with 82 mM Ba^{2+} as the charge carrier) and peak mean open probability of ~ 0.1 (Thoreson et al., 2000; Fig. 4A), similar to L-type Ca^{2+} channels in other preparations (Fenwick et al., 1982; Fox et al., 1987; O'Dell and Alger, 1991; Slessinger and Lansman, 1991; Wang et al., 1993). At physiological Ca^{2+} levels, this conductance drops to about 2 pS (Church and Stanley, 1996; Rodriguez-Contreras et al., 2002). The amplitude of I_{Ca} in salamander rods averages 55 pA at its peak near -25 mV in 1.8 mM Ca^{2+} (Thoreson et al., 2003a, b). If one assumes a reversal potential for Ca^{2+} of $+50$ mV (Hille, 1993), then the resulting driving force of 75 mV yields an average whole-cell conductance of 733 pS. (A similar value is obtained from slope conductance measurements of I_{Ca} current/voltage relationships.) Assuming a peak mean open probability of 0.1 and a single-channel conductance of 2 pS, the calcium current is generated by a population of 3700 calcium channels, of which 370 are open at the peak of the current. $\sim 95\%$ of the calcium channels are clustered in the synaptic terminal (M. Slaughter, pers. comm.) and there are ~ 7 ribbons/rod terminal (Townes-Anderson et al., 1985) suggesting ~ 500 channels/ribbon. At a dark resting membrane potential of -45 mV, I_{Ca} attains $\sim 16\%$ of its peak amplitude (Thoreson et al., 2003a, b) so that at any given moment only a total of 60 channels or 8 channels/ribbon are open. If the rod is maintained near its dark potential for an extended period of time, some of these channels are likely to close as a result of Ca^{2+} -dependent inactivation (Rabl and Thoreson, 2002) resulting in an even smaller number of open channels.

Even though only a small number of channels are open at any given moment in darkness, we estimate that the diffusion of Ca^{2+} into the cell from the extracellular space is nevertheless rate limiting for photoreceptors in vivo. This conclusion is based on the following considerations. If we assume that the baseline current at -40 mV is 10 pA, then 5×10^{-17} moles of divalent charge carrier can enter every second, equivalent to $1.2 \times 10^{+6}$ Ca^{2+} ions/s. Calcium ions are constrained to enter the terminal close to the ribbons where Ca^{2+} channels are clustered (Nachman-Clewner et al., 1999). In the salamander rod, ribbons associate with post-synaptic processes at small invaginations about 0.6 μm in the vertical axis and 1.0 μm in the horizontal axis (Lasansky, 1973). More than one ribbon is typically associated with each invagination, so we estimate four such invaginations/rod. Assuming that the invagination has a spherical shape filled with post-synaptic processes, except for the 20 nm extracellular space surrounding each process, the total extracellular volume within the four invaginations is 2.4×10^{-16} L, which for a $[\text{Ca}^{2+}]_o$ of 1.8 mM, contains $2.6 \times 10^{+5}$ Ca^{2+} ions. In other words, the total number of Ca^{2+} ions in the immediate vicinity of the ribbons are insufficient to generate the baseline current, meaning that Ca^{2+} must diffuse in from the surrounding extracellular space. Based on the dimensions of the mammalian rod and estimates of the extracellular volume of its invagination (Rao-Mirotznik et al., 1995, 1998), the same considerations apply. Because the amount of Ca^{2+} entering the rod terminal exceeds the amount available in the extracellular space, sustained depolarization at the dark potential leads to a depletion of extracellular calcium that is sufficient to limit calcium influx significantly, as shown by Rabl and Thoreson (2002).

3.2. Molecular organization

Calcium channels are ubiquitous in animal cells, both nervous and non-nervous. The complete Ca^{2+} channel is a complex of multiple proteins, of which the $\alpha 1$ subunit forms the channel pore and contains the voltage-sensing sequence. In mammals, 10 genes coding for $\alpha 1$ subunits have been identified, and they form the basis for a standard nomenclature (Catterall et al., 2003). The 10 genes are grouped into three families (1, 2, 3) with multiple subtypes. In this review, we concentrate upon the L-type Ca^{2+} channels responsible for exocytosis from photoreceptor and bipolar cells (Corey et al., 1984; Wilkinson and Barnes, 1996; Thoreson et al., 1997; Schmitz and Witkovsky, 1997; Heidelberg and Matthews, 1992; Berntson et al., 2003; but see also Pan et al., 2001). Although formerly designated variously as L-type, high voltage-activated or dihydropyridine-sensitive, in the latest naming scheme, all are part of the Ca_v1 subfamily (Catterall et al., 2003). Ca_v1 channels encompass subtypes $\text{Ca}_v1.1$ – $\text{Ca}_v1.4$, which correspond to α_{1S} , α_{1C} , α_{1D} and α_{1F} , respectively; all of the naming terms for calcium channels continue to be widely used in the literature. Each calcium channel subtype is distinguished by its molecular organization, functional properties, pharmacological profile and modulation by ions and neuroactive substances, some of which work through second messenger-mediated pathways. The question of which subtypes are represented in vertebrate photoreceptors and bipolar cells is considered below. (N.B. The individual proteins of a given calcium channel are referred to as α , β , etc. To avoid confusion with the different subtypes of calcium channel, we spell out the protein subunit and use the Greek symbol for the channel subtype.)

Although the $\alpha 1$ subunit alone forms a functional Ca^{2+} channel in expression systems, Ca^{2+} channels in vivo are found in association with a set of additional proteins, termed $\alpha 2$, β , γ and δ . The β proteins are intracellular, while the $\alpha 2$ is complexed through disulfide bonds to the δ protein and is extracellular. α and β proteins have phosphorylation sites, whereas the $\alpha 2 \delta$ complex has multiple glycosylation sites. The γ protein is transmembrane but may not be present in every Ca^{2+} channel. Knowledge of the molecular structure of the Ca_v1 channels is critical for understanding how they interact with dihydropyridines, calmodulin and second-messenger-mediated systems. The α subunit is 190 – 250 kDa (Catterall, 2000) and has four repeated domains, each containing six

transmembrane segments, S1–S6. The pore is thought to be created in part by a hairpin turn between S5 and S6, similar to what is found in K channels (Rudy, 1988; Catterall, 1988).

Beta units are found in multiple subtypes, but in the mammalian retina, Ball et al. (2002) showed by elegant cloning techniques that only the beta2 subunit is able to combine with the alpha1 subunit to create a functional channel permitting rod to rod bipolar cell synaptic transfer. Correspondingly, in expression systems, cloned alpha1/beta2 subunits, in conjunction with a disulfide-linked alpha2/delta dimer, provide functional L-type Ca^{2+} channels with the properties associated with retinal photoreceptor and bipolar cells (Koschak et al., 2003; McRory et al., 2004; Baumann et al., 2004).

3.3. Which subtypes are present in photoreceptor cells?

When the entire rat retina was screened for Ca^{2+} channel mRNA (Kamphuis and Hendriksen, 1998), transcripts were found primarily for Ca_v2 family members α_{1A} and α_{1B} and Ca_v1 family α_{1D} subtype. Using antibodies against these subtypes reveals the presence of α_{1D} in most, but not all, tree shrew cones (Morgans, 1999), and in some types of mouse bipolar cells (Berntson et al., 2003). The possibility of different Ca^{2+} channel subtypes in different cone subtypes is supported by differences in the effects of cAMP on I_{Ca} in different types of cones in the salamander retina (Thoreson and Stella, 2000). Rodent rods express a newly described subtype, α_{1F} (Morgans, 2001), and the same subtype was found in rod bipolar cells of the mouse retina (Berntson et al., 2003). Parenthetically, molecular defects in the α_{1F} subtype have been shown to underlie an incomplete form of congenital stationary night blindness (Bech-Hansen et al., 1998). In non-mammalian vertebrates, α_{1D} immunoreactivity is reported for salamander (Henderson et al., 2001) and chicken photoreceptors (Hibino et al., 2002). The immunostaining data are in accord with the conclusion that salamander photoreceptors are of the α_{1D} subtype based on pharmacological and functional characteristics (reviewed in Barnes and Kelly, 2002).

Certain characteristics of I_{Ca} are extremely important. Studies of the cloned α_{1F} channel (McRory et al., 2004) reveal a complete absence of Ca^{2+} -dependent inactivation. However, long depolarizing steps can produce a slowly developing, but substantial, inactivation of I_{Ca} in salamander rods (Corey et al., 1984; Rabl and Thoreson, 2002); this inactivation is abolished by strong intracellular Ca^{2+} buffering (Corey et al., 1984) or after steps to very positive potentials to minimize Ca^{2+} influx (Rabl and Thoreson, 2002) suggesting that it is Ca^{2+} dependent. Calcium channels in bipolar cell terminals exhibit a similar, slowly developing Ca^{2+} -dependent inactivation (von Gersdorff and Matthews, 1996). The presence of Ca^{2+} -dependent inactivation is consistent with immunohistochemical data described above that the Ca^{2+} channels in salamander rods and goldfish bipolar cells are not likely to be of the α_{1F} subtype.

$\text{Ca}_v1.4$ channels show a slowly developing voltage-dependent inactivation (Baumann et al., 2004; Koschak et al., 2003; McRory et al., 2004). There is no demonstration of voltage-dependent inactivation in native channels but most studies were done using relatively short depolarizing test steps, whereas the voltage-dependent inactivation of $\text{Ca}_v1.4$ requires many seconds to develop. Steps to -40 mV produce only a small amount of voltage-dependent inactivation but it becomes much more pronounced with strong depolarizing steps. Since the physiological behavior of rods and cones is to hyperpolarize, voltage-dependent inactivation probably plays a small role in their normal function, but in depolarizing bipolar cells voltage-dependent inactivation may contribute to phasic release of transmitter.

In expression systems, $\text{Ca}_v1.4$ channels produce currents that activate at potentials more positive than native I_{Ca} (Yagi and MacLeish, 1994; Taylor and Morgans, 1998; De Vries and Schwartz, 1999; Thoreson et al., 2003a, b). However, co-expression of $\text{Ca}_v1.4$ channels with

a Ca^{2+} -binding protein that is specifically found in photoreceptors (CaBP4) shifts the activation function to a range of membrane potentials similar to those seen in native channels (Haeseleer et al., 2004). This important finding is a cautionary note for comparisons with studies of Ca^{2+} channels in expression systems in which the full complement of modulatory proteins may be lacking.

3.4. Impact of experimental conditions

Typically, studies of calcium channels and currents in retinal neurons are carried out either on isolated cells or cells in a slice preparation, using the whole-cell patch recording technique. Every technique has its limitations. The synapse of the isolated cell lacks the limited extracellular space, synaptic inputs and metabolic influences of the intact preparation; these conditions are partially mitigated by the slice preparation, but slices also have an altered metabolic state and expanded extracellular space relative to an intact preparation. By dialyzing the intracellular contents, the whole-cell patch clamp technique alters the I_{Ca} being recorded; such currents are well known to experience 'rundown', which may be slowed by the addition of ATP, GTP and other factors to the pipette solution. The perforated patch method is thus better in that intracellular constituents are maintained and rundown is less severe.

A further important consideration is the choice of charge carrying divalent cations. Many studies use Ba^{2+} to measure Ca^{2+} currents because Ca_v1 channels flux Ba^{2+} more than Ca^{2+} . Passage of divalent cations through the Ca^{2+} channel involves interactions of the ion with charged groups within the pore (Josephson et al., 2002). The different ion-channel interaction of Ba^{2+} compared to Ca^{2+} results in a shift of the Ba^{2+} -generated current-voltage relation to the left, a shift amounting to up to 10 mV (McDonald et al., 1994). Moreover, single-channel recordings (Rodriguez-Contreras and Yamoah, 2003) of L-type Ca^{2+} channels in hair cells reveal clearly that at any test voltage Ba^{2+} results in much greater activation of the channel than Ca^{2+} does, i.e., the channel has a much higher open probability in Ba^{2+} . Barium also fails to activate significantly calcium-dependent channels, including a Ca^{2+} -dependent potassium current (Bader et al., 1982; Moriondo et al., 2001) and a Ca^{2+} -dependent chloride current (ICl_{Ca}) (Maricq and Korenbrot, 1988; Thoreson et al., 2000). Thus, the use of Ba is beneficial because it allows better isolation of I_{Ca} , but it also obscures functionally important interactions between calcium and calcium-dependent channels (see Section 5.1).

The concentration of divalent cations has a potent influence on membrane surface charge. Increased screening of membrane surface charge by higher concentrations of divalent cations reduces the apparent transmembrane voltage, causing the peak of the current-voltage relation to shift to the right (Piccolino et al., 1999). Protons also screen membrane surface charge; a more acidic pH shifts the activation curve of the calcium current to the right along the voltage axis (Barnes et al., 1993; De Vries, 2001). In addition to surface charge screening effects, protons inhibit Ca^{2+} influx through the channels (Chen et al., 1996), reducing current amplitude.

4. Calcium-dependent exocytosis at the photoreceptor synapse

4.1. Relationship between membrane potential and calcium influx

The diverse factors described in the previous section contribute to the very substantial variability noted in the literature in the magnitude of recorded current and the placement of the current-voltage relation along the voltage axis. Such considerations are particularly crucial for photoreceptor function, because the membrane potential of rod and cone photoreceptors in darkness is close to -40 mV. Light moves the membrane potential in a graded fashion to more hyperpolarized levels, creating an operating range for the photoreceptors of -40 to -65 mV. The Ca^{2+} channel(s) of rods and cones must smoothly regulate Ca^{2+} entry in this voltage range

and it has long been a puzzle to investigators that -40 mV is just at the foot of the I_{Ca} activation function measured in rods and cones of lower vertebrates (Corey et al., 1984; Lasater and Witkovsky, 1991; Wilkinson and Barnes, 1996). In contrast, three studies of I_{Ca} in mammalian cones revealed a relatively large current at -40 mV (Yagi and MacLeish, 1994; Taylor and Morgans, 1998; De Vries and Schwartz, 1999). However, the early experiments on I_{Ca} in photoreceptors of lower vertebrates were typically done using high divalent cation concentrations. Much of the apparent difference between mammalian and non-mammalian photoreceptors disappears after appropriate adjustments are made for the surface charge effects of divalent cations and pH levels. When currents are measured with physiological levels of calcium ($1\text{--}2$ mM), L-type I_{Ca} in amphibian photoreceptors and mammalian cones show a similar current/voltage relationship in which they activate positive to -60 mV and attain half-maximal activation between -35 and -40 mV (Taylor and Morgans, 1998; Thoreson et al., 2003a, b). An additional variable is the membrane potential of the photoreceptor in darkness. In salamander retina, the dark resting potential of rods averages -44 mV whereas the cone resting potential is slightly more depolarized (-39 mV) (Thoreson et al., 2003b). In primate retina, rod dark membrane potentials were found to be more positive (-37 mV) than cone membrane potentials (-46 mV) (Schneeweis and Schnapf, 1995).

Whatever the value of the membrane potential and the precise position of the calcium current activation function on the voltage axis, changes in I_{Ca} accompanying a light response are accurately predicted from the Boltzmann function fit to I_{Ca} , as illustrated in Fig. 4 which shows the change in I_{Ca} from rod photoreceptors recorded when using a rod voltage response to light as the command waveform. At a dark potential of -44 mV in salamander rods, I_{Ca} attains $<20\%$ of its peak activity (Thoreson et al., 2003a). The probability that calcium channels remain open diminishes as the membrane hyperpolarizes in response to light and I_{Ca} becomes vanishingly small as the membrane potential passes below -55 mV. Inactivation of I_{Ca} combined with depletion of Ca^{2+} ions from the synaptic cleft reduces I_{Ca} by $\sim 50\%$ in darkness (Rabl and Thoreson, 2002). This reduction in I_{Ca} proceeds slowly and thus does little to shape the response over short time periods (<1 s), but can explain the result shown in Fig. 4 that the predicted change in I_{Ca} is about two-fold larger than the actual change (Fig. 4D).

One potential reason for the minimal activation of I_{Ca} in darkness is to reduce the prodigious Ca^{2+} load that photoreceptors must buffer or extrude in order to stay alive. A second possible reason is to avoid the generation of inverted light responses in second-order retinal neurons. A sufficiently large negative shift in I_{Ca} activation (e.g., accompanying lowered Ca^{2+} levels) moves the peak of I_{Ca} below the membrane potential, producing a situation in which hyperpolarizing light responses of photoreceptors increase, rather than decrease, I_{Ca} which in turn leads to a reversal of glutamate output and a reversal in the light-evoked responses of second-order neurons (Piccolino et al., 1999; Cadetti et al., 2004). Thus, the fact that the membrane potential is near the foot of the I_{Ca} activation curve in photoreceptors can be considered an adaptation that decreases the risk of visually confusing or potentially damaging increases in I_{Ca} activation.

4.2. Linearity between Ca^{2+} influx and exocytosis: mechanistic implications

Many well-studied CNS synapses exhibit a roughly third-order relationship between Ca^{2+} influx and release (Mintz et al., 1995; Borst and Sakmann, 1999b; Wu et al., 1999). In contrast, at rod photoreceptor terminals, there is a linear relationship between the magnitude of I_{Ca} and exocytosis (Thoreson et al., 2004). Evidence for linear or nearly linear relationships between I_{Ca} and release has also emerged from studies of insect photoreceptors, olfactory nerve terminals, mature hair cells and neuroendocrine cells (Engisch and Nowycky, 1996; Kurtz et al., 2001; Murphy et al., 2004; Johnson et al., 2004a, b).

Linearity in the transmission of small signals from rod photoreceptors to their post-synaptic targets has long been known (Tranchina et al., 1981; Sakai and Naka, 1987; Naka et al., 1987; Wu, 1985); it allows a rod to translate a small light-evoked hyperpolarization from the dark potential into a proportional small change in the magnitude of I_{Ca} and exocytosis. These studies of synaptic gain, however, do not establish an underlying linear mechanism because the predictions using models based either on linear or higher-order relations between Ca^{2+} influx and transmitter release do not differ greatly for small signals. With larger light-evoked voltage excursions, the sigmoidal relationship between I_{Ca} and rod membrane potential becomes increasingly evident (Attwell et al., 1987; Wu, 1988; Belgum and Copenhagen, 1988; Witkovsky et al., 1997). Fig. 4 illustrates that the light-evoked reduction in I_{Ca} shows a less pronounced initial transient (Fig. 4D) than the corresponding light-evoked hyperpolarization of the photoreceptor membrane (Fig. 4C), as predicted by the Boltzmann function. The finding that light-evoked reduction in glutamate release parallels the reduction in I_{Ca} is consistent with an underlying linearity (Witkovsky et al., 1997; Thoreson et al., 2003b).

One unusual property of exocytosis from photoreceptors is that it is triggered by submicromolar levels of Ca^{2+} (Rieke and Schwartz, 1996; Thoreson et al., 2004), in contrast to other neurons, including bipolar cells, in which calcium levels must exceed 10 μM to evoke release (Heidelberg et al., 1994; Beutner et al., 2001). The ability of submicromolar calcium levels to trigger release indicates that transmitter release from rods does not require the high calcium levels found only immediately adjacent to Ca^{2+} channels, a finding underlying the hypothesis that exocytosis from rods may be regulated by changes in spatially averaged calcium levels in the synaptic terminal (Rieke and Schwartz, 1996). A corollary consideration is that Ca^{2+} from other sources, e.g., intracellular stores, might also gate exocytosis (Krizaj et al., 1999). Plausibly, a dependence on spatially averaged calcium levels benefits vision by reducing the synaptic noise introduced by the stochastic properties of individual Ca^{2+} channels. Furthermore, use of a high-affinity release mechanism minimizes the need for large Ca^{2+} loads and thereby reduces the potential for Ca-dependent cell death. Over the physiological range of spatially averaged Ca^{2+} levels thought to be attained in rod terminals by voltages up to -40 mV (Rieke and Schwartz, 1996), exocytotic increases in membrane capacitance evoked by the instantaneous flash photolysis of caged calcium were approximately linearly related to $[Ca^{2+}]_i$ (Thoreson et al., 2004).

What are the implications of linearity for the underlying Ca^{2+} sensor? The data relating calcium current and release just cited notwithstanding, it is important to note that they do not necessarily imply a 1:1 binding of calcium to the calcium sensor. The data also are consistent with a model that assumes that the binding of up to three Ca^{2+} ions is needed to trigger membrane fusion. Over the limited range of calcium levels, the differences in the slope between the linear and nonlinear models are modest. The steeper slope with a nonlinear relationship becomes evident only with larger changes in calcium that occur only outside the physiological range of photoreceptor voltages (or in local microdomains surrounding individual calcium channels). Below we consider mechanisms by which linearity can be reconciled with multiple calciums binding to the calcium sensor.

The structure of Ca^{2+} microdomains can contribute to a linear relationship between Ca^{2+} influx and release. For example, the squid giant synapse exhibits a high cooperativity between Ca^{2+} and release, but a linear relationship between transmitter release and I_{Ca} is obtained when only a very small number of Ca^{2+} channels are open, due to the absence of overlap among adjacent Ca^{2+} microdomains at the release site (Augustine et al., 1991). Linearity results when the number of active Ca^{2+} microdomains, corresponding to active release sites, increase proportionally to the amplitude of I_{Ca} . This mechanism appears to account for the linearity between presynaptic Ca^{2+} and post-synaptic currents in cultured amacrine cells (Gleason et

al., 1994). In order for Ca^{2+} microdomains not to overlap at release sites, Ca^{2+} channels must be sparsely distributed, yet close to their associated release site (Neher, 1998; Kits and Mansvelder, 2000). Computer simulations of the kinetics of horizontal and bipolar cell light-evoked currents suggest essentially instantaneous Ca^{2+} kinetics in photoreceptor synaptic terminals, indicating that open channels are close to the release site (Thoreson et al., 2003b). And although calcium channels appear to be clustered closely together at the apex of synaptic ridge in photoreceptors, only a few channels are likely to be open at any given moment when the cell is at its dark resting potential, given the voltage dependence of Ca^{2+} channel opening described in Section 4.1.

Another way that intracellular Ca^{2+} dynamics might help to promote linearity between influx and release is indicated by studies on neuroendocrine cells. Melanotropes exhibit a linear relationship between Ca^{2+} influx and exocytosis despite an underlying third or fourth power relationship between internal Ca^{2+} and exocytosis (Mansvelder and Kits, 1998, 2000; Kits and Mansvelder, 2000). However, in these cells, Ca^{2+} channels are relatively distant (100–200 nm) from the release site, ruling out the possibility that the linear relationship arises from the presence of non-overlapping Ca^{2+} microdomains (Kits and Mansvelder, 2000). Computer simulations suggest instead that linearity stems from diffusion barriers that prevent intracellular Ca^{2+} from diffusing freely in all directions, resulting in local buffer saturation (Kits et al., 1999; Kits and Mansvelder, 2000). In photoreceptors, the arciform density overlying calcium channels on the synaptic ridge might act as a diffusion barrier, allowing Ca^{2+} to spill over onto the ribbon face only after saturating local buffers. This would minimize the contribution of microdomains and promote linearity between I_{Ca} and release.

A recent report raises the possibility that a second component of membrane addition with a high threshold Ca^{2+} sensor may co-exist with the low threshold pool in the rod photoreceptor (Kreft et al., 2003). This high threshold pool was extremely large, constituting more than 10% of the total cell capacitance or approximately 10 times the number of ribbon-associated vesicles (Kreft et al., 2003; Thoreson et al., 2004) raising concerns about ectopic release from non-synaptic sites. The functional significance of this pool remains unclear since it was not associated directly with neurotransmitter release or shown to be cross-depleted by a physiological stimulus. But it is conceivable that, as has been suggested for bipolar cells (Lagnado et al., 1996) and hippocampal neurons (Geppert et al., 1994), two different Ca^{2+} sensors for exocytosis may co-exist in the rod.

4.3. Rod– cone differences in calcium dynamics

Amphibian cones show a smaller gain reduction with strong hyperpolarization than do rods and thus cones communicate efficiently with second-order neurons over a larger range of light levels than rods (Attwell et al., 1987; Wu, 1988; Belgum and Copenhagen, 1988; Witkovsky et al., 1997). Like rods, however, synaptic output from cones appears to be linearly related to I_{Ca} (Witkovsky et al., 2001). One often ignored factor contributing to cone efficiency is its resting potential, which, in the amphibian retina, is positive to that of rods by about 5 mV. This difference means that the range of potentials over which release can be evoked from rods is more restricted than that of cones, because their resting potential lies closer to the foot of the I_{Ca} activation curve. In mammals, some cone classes utilize an $\alpha_{1\text{D}}$ channel whereas rods possess an $\alpha_{1\text{F}}$ channel, although possible behavioral differences stemming from channel subtype remain to be defined. Another possible mechanism for the less pronounced decrement with release from cones to strong hyperpolarization is a contribution from cGMP-gated cation channels. Calcium influx through cGMP-gated channels found in cone photoreceptor terminals, but not rods, stimulates release of glutamate (Rieke and Schwartz, 1994; Savchenko et al., 1997). Additionally, guanylate cyclase and guanylate cyclase activating protein (GCAP) are both present in cone terminals (Venkataraman et al., 2003). It has been suggested that the

ability of cGMP-gated channels to open at potentials below -55 mV may extend the useful operating range for cones to more negative potentials than reliance on calcium channels alone (Rieke and Schwartz, 1994). One difficulty with this hypothesis is that the increased driving force provided by membrane hyperpolarization should increase, not decrease, calcium influx. This anticipated increase might be offset by a divalent cation block of the cGMP channel which provides a modest inward rectification to the current. The appropriate voltage dependence might also be influenced by other mechanisms such as a voltage-dependent increase in cone terminal glutamate clearance (Gaal et al., 1998) or, more speculatively, voltage-dependent changes in cGMP concentration (e.g., by disinhibiting the calcium-sensitive GCAP, Venkataraman et al., 2003). At present the precise role for CNG channels in release from cones is unclear.

4.4. Is there calcium-independent transmission at ribbon synapses?

Based on the finding that light responses can persist in second-order horizontal cells even in the presence of solutions containing reduced $[Ca^{2+}]_o$ and divalent cations that block Ca^{2+} channels, it was proposed that photoreceptors are capable of Ca^{2+} independent exocytosis (Schwartz, 1986, 1987; Umino and Watanabe, 1987). However, subsequent studies showed that by altering surface charge, a lowered $[Ca^{2+}]_o$ causes a leftward (negative) shift in I_{Ca} activation that paradoxically promotes Ca^{2+} influx into the synaptic terminals of photoreceptor cells, even in the presence of divalent cations used to block I_{Ca} (Piccolino et al., 1996, 1999; Cadetti et al., 2004). Lowering $[Ca^{2+}]_o$ also enhances the amplitude of photoreceptor light responses (Bertrand et al., 1978; Cervetto et al., 1988). The combined effects of low divalent cation solutions on photoreceptor light responses and membrane surface charge are sufficient to account for the persistence of horizontal and bipolar cell light responses (Cadetti et al., 2004). Furthermore, Ca^{2+} influx is necessary to maintain synaptic transmission since light responses of horizontal cells in the retinal slice preparation are blocked completely when low Ca^{2+} solutions and/or divalent cations are superfused for long periods (Cadetti et al., 2004). Thus, we conclude that there is no Ca^{2+} -independent neurotransmission at the photoreceptor synapse.

4.5. Release of calcium from intracellular stores

An important structural component of the photoreceptor terminal is the smooth endoplasmic reticulum (SER; reviewed in Berridge, 1998; Krizaj and Copenhagen, 2002). Besides its involvement in endocytosis (a topic we do not consider but cf. Royle and Lagnado, 2003; Matthews, 2004), the SER forms cisternae which serve as calcium stores. Ca^{2+} is pumped into the SER by an ATP-requiring pump, the sarcoplasmic–endoplasmic reticulum calcium ATPase (SERCA) which in photoreceptors appears to be the SERCA2a splice variant (Krizaj et al., 2004). Release from stores occurs via activation of specialized receptors. The ryanodine receptor (RyR) senses cytoplasmic $[Ca^{2+}]$ and opens at some threshold concentration. The inositol trisphosphate receptor (IP3R) depends on a local increase of IP3, which is generated through G-protein-coupled pathways. Neuromodulators such as acetylcholine and dopamine can be coupled to IP3 generation. It would be interesting to know the precise spatial distribution of SER in rod and cone terminals because of the fact that Ca^{2+} channels are clustered near the ribbon. From this fact and the knowledge that free Ca^{2+} has a very restricted diffusion due to the abundance of mobile buffers such as calmodulin and immobile buffers such as mitochondria and SER, the release of Ca^{2+} from the SER triggered by influx of Ca^{2+} through voltage-gated Ca^{2+} channels [a process known as calcium-induced calcium release (CICR)] might be very local. These considerations take on significance because there is evidence that Ca^{2+} from stores supplements the Ca^{2+} entering the synaptic terminal through VGCCs in gating transmitter release (Krizaj et al., 1999). The possible functional role of IP3Rs is not well characterized and the evidence for the distribution of IP3Rs in photoreceptors is still controversial. One study (Peng et al., 1991) reported them to be in high concentration in photoreceptor inner segments,

whereas another study (Wang et al., 1999) found them in outer segments. More than one isoform of IP3R exists, however, so these reports are not necessarily contradictory. Moreover, in a mouse with genetically deleted phospholipase C, the enzyme responsible for IP3 generation, synaptic transmission from photoreceptors to ON bipolar cells was severely impaired (Jiang et al., 1996), indicating a role for the IP3R in photoreceptor signal transfer to second-order retinal neurons. CICR has been studied in different nerve cells, including photoreceptors, taking advantage of the fact that caffeine evokes release from stores by acting on the RyR. In investigations of cerebellar Purkinje cells (Kano et al., 1995) and salamander rods (Krizaj et al., 1999), it was found that the Ca^{2+} transient evoked by a constant caffeine puff increased as $[\text{Ca}^{2+}]_i$ increased. These data indicate that there is a threshold $[\text{Ca}^{2+}]_i$ for RyR-mediated Ca^{2+} release from stores. If CICR operated near the rod's membrane potential in darkness, but were diminished or cut off at stronger hyperpolarizations, it might contribute to increasing synaptic gain for small signals.

4.6. Calcium buffers and transporters

The cation current flowing through the cyclic nucleotide-gated channels of the photoreceptor outer segments is carried by Na^+ , Ca^{2+} and K^+ , with Ca^{2+} accounting for about 15% of the total current flow. A Na/Ca, K exchanger (Schnetkamp et al., 1989) maintains outer segment Ca^{2+} in a steady state by exchanging four Na^+ for one Ca^{2+} and one K^+ . When $[\text{Ca}^{2+}]_i$ falls below about 50 nM the exchanger is inactivated. Other mobile buffers help to maintain $[\text{Ca}^{2+}]_i$ in the outer segment (Palczewski et al., 2000). However, outer and inner segment mechanisms for Ca^{2+} homeostasis are different and substantially independent (reviewed in Krizaj and Copenhagen, 2002).

In the inner segment, including the synaptic base, control of Ca^{2+} buffering is complex. That aspect of control which concerns regulation of the VGCCs is reviewed below in Section 5. Other membrane currents, including I_h , a delayed K rectifier and the Ca-dependent K and Cl currents are important in limiting photoreceptor depolarization, thereby preventing Ca^{2+} spikes. Additional mechanisms include: Ca^{2+} sequestration in SER stores and in mitochondria, Ca^{2+} extrusion, and a variety of mobile buffers that bind Ca^{2+} . Extrusion of Ca^{2+} from the inner segment is effected by plasma membrane Ca-ATPases (PMCA) of which isoforms 1, 2 and 4 are found in photoreceptors (Krizaj et al., 2004). Morgans et al. (1998) showed that the Ca-ATPase is concentrated in both rod and cone terminals, but is distributed along the sides of the terminals, and is absent from the proximal terminal faces where the ribbon synapses are located.

Calcium handling mechanisms differ in rods and cones. Caffeine-evoked increases in inner segment calcium are observed consistently in rods but are seen only rarely in cones (Krizaj et al., 2003). However, caffeine-evoked calcium increases were revealed in cones after blocking plasma membrane calcium exchanger activity, indicating that the calcium released from intracellular stores is more efficiently extruded by PMCA in cones than in rods (Krizaj et al., 2003).

Although we still have much to learn about the function and regulation of buffering and extrusion mechanisms, it is clear that Ca^{2+} buffering within the photoreceptor terminal must be very powerful. If one considers the salamander rod terminal as a 6 μm sphere and assumes a baseline I_{Ca} of 10 pA at -40 mV in darkness, then the steady Ca^{2+} influx/s would raise $[\text{Ca}]_i$ to 500 μM in the absence of buffering, transport and sequestration, i.e., about three orders of magnitude higher than that measured by Rieke and Schwartz (1996). These considerations reinforce the suggestion that photoreceptors' dependence on I_{Ca} , which is only slightly active in the voltage operating range of the cell, may have evolved to avoid enormous Ca^{2+} loads.

4.7. Do post-synaptic responses conform to the quantal hypothesis?

Release at most spiking CNS synapses (Redman, 1990; Sakaba et al., 2002) appears to adhere to the quantal hypothesis of exocytosis developed by del Castillo and Katz (1954) from studies on the neuromuscular junction. According to this hypothesis, the excitatory post-synaptic current (EPSC) is a product of nPQ where n is the number of releasable vesicles, P is the probability that a vesicle will be released and Q is the PSC resulting from release of a single vesicle. Synaptic transmission at photoreceptor synapses exhibits some fundamental differences from transmission at conventional CNS synapses. These are exemplified by the graded response properties of photoreceptors, their ability to release glutamate for an indefinite period of time, the prominence of a high-affinity calcium sensor, and the more shallow relationship between local calcium and the rate of glutamate release. In addition, there are structural differences such as the presence of a presynaptic ribbon and the finding that pre- and post-synaptic processes are not as closely apposed as at other CNS synapses. In this context, it has been suggested that the difference between retinal neurons and other CNS neurons is that the post-synaptic responses of second-order retinal neurons reflect changes in the spatially integrated level of glutamate in the cleft determined by a balance between the release and re-uptake of glutamate (Gaal et al., 1998; Roska et al., 1998), an idea referred to as “cleft integration” (Rao-Mirotznik et al., 1998).

Rao-Mirotznik et al. (1998) calculated that the rate of release needed to sustain the mean level of glutamate in the cleft required by the cleft-integration model was 4000 vesicles/ribbon/s. In contrast, the rate needed to sustain responses using a quantal release model was determined to be only 100 v/ribbon/s. Although bipolar cells and photoreceptors are capable of releasing vesicles at the prodigious rates predicted by the cleft-integration model (von Gersdorff et al., 1996; Kreft et al., 2003), evidence discussed earlier suggests that tonic release in darkness involves much more modest rates of 18–400 vesicles/ribbon/s (Rieke and Schwartz, 1996; Ashmore and Copenhagen, 1983; Berntson and Taylor, 2003), consistent with a quantal release model.

The cleft-integration model predicts that glutamate is homogeneously distributed in the cleft at a concentration sufficient to activate a large fraction of the post-synaptic glutamate receptors when glutamate release is maximal in darkness. In contrast, the quantal release hypothesis predicts that the glutamate concentration in the cleft is generally low throughout the cleft except for brief instances in the spatially restricted regions of the cleft where vesicles have been recently released. Glutamate levels in the cleft have been estimated in a few different ways. Gaal et al. (1998) determined the glutamate concentrations necessary to produce different membrane potentials in horizontal cells. To attain a membrane potential of -30 mV, near the dark resting potential of horizontal cells, it was necessary to apply $20\text{--}30$ μM glutamate. If this reflects the mean glutamate level in the cleft, then glutamate receptors should be slightly more than 50% activated in darkness (Gaal et al., 1998).

The low-affinity GluR antagonist, kynurenic acid, has been used as tool for estimating the peak concentration of glutamate attained at various CNS synapses (Clements, 1996). This method takes advantage of the fact that the ratio between the binding rates of the transmitter and the more rapidly dissociating antagonist is similar to the ratio between their concentrations (Clements, 1996). Kim and Miller (1991, 1993) found that concentrations of $0.5\text{--}1$ mM kynurenic acid were required to block EPSCs in horizontal and OFF bipolar cells evoked by depolarizing steps applied to presynaptic cones and >5 mM kynurenic acid was required to block EPSCs in rod-driven horizontal and OFF bipolar cells. If one interprets these results in terms of the ratio of binding rates, the high concentrations of kynurenic acid needed to block EPSCs imply that when photoreceptor release is maximally stimulated by strong depolarization, post-synaptic GluRs adjacent to cones are exposed to glutamate concentrations in excess of 0.5 mM and those adjacent to rods are exposed to concentrations exceeding 5 mM.

This is consistent with other CNS synapses where it appears that the release of a vesicle causes a spatially restricted elevation of glutamate levels to high concentrations (1–5 mM; Clements, 1996). Anatomically detailed simulations of glutamate release from the synaptic ribbon of mammalian rod photoreceptors also suggest that glutamate can attain a concentration as high as 0.7 mM at post-synaptic bipolar cell dendrites (Rao-Mirotnik et al., 1998). Thus, the evidence that glutamate may attain saturating concentrations at post-synaptic GluRs favors the hypothesis that quantal release dictates the post-synaptic response in second-order retinal neurons.

A requirement of the quantal hypothesis is the presence of miniature EPSCs arising from single vesicle fusion events. In fact, quantal miniature EPSCs have been detected in OFF bipolar cells and uncoupled horizontal cells (Hirasawa et al., 2001a; Kawai, 1999; Maple et al., 1994). Noise analysis also suggests an underlying quantal structure to ON bipolar cell responses (Berntson and Taylor, 2003), although discrete miniature EPSCs in this cell type are obscured by the slow kinetics of the mGluR6 transduction cascade (Nawy and Jahr, 1990).

The detection of individual miniature EPSCs in second-order retinal neurons indicates that individual vesicles are capable of exerting discrete post-synaptic effects under certain experimental conditions. However, the quantal hypothesis rests on the additional assumption that each vesicle exerts an equal and independent post-synaptic effect. This assumption of statistical independence does not hold if glutamate spills over from one active zone onto another or if glutamate persists at a suprathreshold concentration in the synaptic cleft between subsequent release events (DiGregorio et al., 2002; Otis et al., 1996; Mennerick and Zorumski, 1995; Barbour et al., 1994). Spillover of glutamate between neighboring photoreceptors is minimized by the presence of glutamate transporters in Müller cell processes that ensheath the photoreceptor terminals (Sarantis and Mobbs, 1992; Burris et al., 2002). Additional glutamate transporters present on photoreceptor terminals (Eliasof and Werblin, 1993; Grant and Werblin, 1996) also limit spillover of glutamate (Pow et al., 2000) onto GluRs at neighboring active zones. However, it remains an open question whether these mechanisms are adequate to prevent spillover or glutamate persistence in the cleft during darkness when photoreceptors release transmitter at their maximal rate. Thus, while it is clear that glutamate is released from vesicles in quantal packets, the degree to which this quantal structure of release is retained at the post-synaptic membrane needs further study.

5. Modulation of calcium channels and exocytosis at photoreceptor terminals

5.1. Intrinsic mechanisms that regulate I_{Ca}

Exocytosis from photoreceptor terminals is continuously regulated by a large number of extrinsic and intrinsic mechanisms. Most of the research to date on the modulation of exocytosis in photoreceptor terminals has focused on the regulation of calcium channels. Thus, although we also consider some other possible sites of modulation (e.g., direct regulation of the exocytotic apparatus), we focus our discussion of this topic accordingly.

The relatively depolarized membrane potentials of photoreceptors and bipolar cells are a crucial component of their excitability. What mechanisms maintain a membrane potential near -40 mV? The dark current (Owen, 1987) provides the necessary depolarizing input, since when the light-sensitive channels close, photoreceptors undergo a strong hyperpolarization. Inwardly rectifying currents in rods also assist in maintaining a relatively depolarized potential. But what prevents the cell from depolarizing beyond -40 mV in darkness? As discussed previously, I_{Ca} is partially active at -40 mV and when stimulated by extrinsic depolarization or by a strong depolarizing input from the receptive field surround, I_{Ca} can become regenerative active allowing photoreceptors to produce large, and often long-lasting, calcium-dependent transients (Burkhardt et al., 1988; Gerschenfeld and Piccolino, 1980; Akopian et al., 1997). One way that

photoreceptors minimize these depolarizing overshoots is by the activation, above -30 mV, of large outward currents (I_{Kx} , I_{KCa} , $I_{Cl(Ca)}$; Owen, 1987; Moriondo et al., 2001). In addition, there are a number of intrinsic feedback mechanisms that help to put the brakes on I_{Ca} and prevent its regenerative activation. By intrinsic, we refer to mechanisms that are obligatorily engaged whenever calcium channels are open and synaptic release is stimulated. We discuss some possible intrinsic mechanisms including Ca^{2+} -dependent inactivation of I_{Ca} , depletion of synaptic cleft Ca^{2+} , and activities of glutamate transporters, vesicular protons and vesicular zinc.

The amplitude and voltage dependence of photoreceptor I_{Ca} also are affected by a large number of extrinsic neuromodulators, including dopamine (Stella and Thoreson, 2000), somatostatin (Akopian et al., 2000), cannabinoids (Straiker and Sullivan, 2003; Fan and Yazulla, 2003), adenosine (Stella et al., 2002), nitric oxide (Kurenniy et al., 1994), insulin (Stella et al., 2001), polyunsaturated fats (Vellani et al., 2000) and various ions. The list of substances that can modulate photoreceptor I_{Ca} is probably not yet complete. These neuromodulators exert their effects through a number of signal transduction pathways. I_{Ca} in rods and cones can be regulated by cAMP-dependent protein kinase (Stella and Thoreson, 2000). Tyrosine kinase inhibitors also alter rod I_{Ca} , suggesting that tyrosine phosphorylation might regulate channel activity (Stella et al., 2001). The ineffectiveness of cGMP analogs (Kurenniy et al., 1994) argues against modulation by a cGMP-dependent protein kinase. The question of whether other enzymes such as PKC might regulate photoreceptor I_{Ca} remains unanswered.

As discussed earlier (Section 4.1) the tonic activity of I_{Ca} in darkness means that small changes in I_{Ca} voltage dependence and amplitude will have large effects on synaptic output. The many neuromodulators capable of regulating I_{Ca} leads one to ask how photoreceptors maintain a stable activation level of I_{Ca} necessary for stable synaptic output. It appears that in addition to assisting in the maintenance of a stable membrane potential, the various intrinsic feedback mechanisms considered below are also important for stabilizing the amplitude and voltage dependence of I_{Ca} in the face of waxing and waning levels of multiple neuromodulators.

5.1.1. Calcium-dependent inactivation and depletion of synaptic cleft $[Ca^{2+}]$ —

Corey et al. (1984) showed that I_{Ca} in salamander rod photoreceptors exhibited a substantial degree of Ca^{2+} -dependent inactivation. Using the perforated patch technique to maintain endogenous Ca^{2+} buffering and extrusion mechanisms, Rabl and Thoreson (2002) found that the Ca^{2+} -dependent inactivation of I_{Ca} produced by long depolarizing steps ranged from 15% at -40 mV to $>80\%$ at -10 mV (Rabl and Thoreson, 2002). Interestingly, when the same experiment was repeated using synaptically connected rods in a retinal slice, a more profound (up to 50%) depression of I_{Ca} was observed following steps to -40 mV. Thus, consistent with calculations presented earlier that diffusion of calcium may be rate limiting, it appears that the sustained influx of Ca^{2+} at the dark resting potential reduces the amplitude of I_{Ca} by depleting Ca^{2+} from the synaptic cleft (Rabl and Thoreson, 2002). Depletion of synaptic cleft Ca^{2+} has also been shown to reduce presynaptic I_{Ca} at calyceal synapses (Borst and Sakmann, 1999a; Stanley, 2000). The combined effects of Ca^{2+} -dependent inactivation and synaptic cleft depletion at the photoreceptor synapse can produce a slow reduction in I_{Ca} that exceeds 50% at the dark potential; recovery from these effects also requires many seconds (Rabl and Thoreson, 2002). While such slow changes in I_{Ca} are unlikely to shape synaptic output to brief light flashes, they may contribute to post-receptor light adaptation. Although hyperpolarization of the rod membrane during a light flash initially reduces I_{Ca} , the resulting decrease in Ca^{2+} influx will, over the long term (>1 s), attenuate the degree of Ca^{2+} -dependent inactivation and depletion of synaptic cleft Ca^{2+} . These changes promote a slow, partial recovery of I_{Ca} which in turn allows for a partial recovery of synaptic transmission and post-synaptic light responses.

5.1.2. Ionic modulation of calcium channels: chloride—Reducing the concentration of Cl^- in the bathing medium suppresses light-evoked currents of bipolar and horizontal cells by reducing glutamate release from photoreceptors (Thoreson and Miller, 1996). The inhibition of glutamate release by low Cl^- solutions was found to result from an inhibition of I_{Ca} in rods and cones (Thoreson et al., 1997). In addition to reducing the amplitude of the current, replacing Cl^- with various anions produces a hyperpolarizing activation shift in I_{Ca} (Thoreson et al., 1997; Stella and Thoreson, 2000). This inhibition of I_{Ca} is not a consequence of the increase in Ca^{2+} -dependent inactivation that could accompany such a hyperpolarizing activation shift (Thoreson et al., 1997). Nor is the inhibition of I_{Ca} due to antagonism by the substituting anion or anion-induced changes in extracellular or intracellular pH (Thoreson et al., 1997). Instead, the evidence summarized below suggests that there is a low-affinity chloride binding site on the interior face of the channel that facilitates calcium channel activity.

The inhibition of I_{Ca} by anions follows the Hofmeister sequence: $\text{Cl}^- \sim \text{Br}^- < \text{NO}_3^- < \text{I}^- < \text{ClO}_4^-$ where the anion with lowest charge density, ClO_4^- , produces the greatest suppression (Stella and Thoreson, 2000). Anion-induced hyperpolarizing activation shifts also follow the Hofmeister sequence. Correlation with the Hofmeister sequence indicates that anion effects on amplitude and voltage dependence derive from their ability to approach a surface, suggesting that anions influence I_{Ca} by interactions at the membrane surface.

Perfusion with replacement anions initially produces a large leftward activation shift, presumably due to an increase in negative surface charge as anions approach the extracellular membrane. With continued perfusion of replacement anions, the initial leftward shift is subsequently reduced by a secondary shift back toward more positive values which may reflect an accumulation of replacement anions at the intracellular membrane surface. A reduction in I_{Ca} amplitude accompanies this secondary right shift, providing the first clue that the inhibition of I_{Ca} may be mediated by effects at the intracellular membrane surface (Stella and Thoreson, 2000).

The hypothesis that replacement anions inhibit I_{Ca} by actions at the intracellular surface was shown more directly in single-channel studies (Thoreson et al., 2000). Intracellular $[\text{Cl}^-]$ was reduced by superfusing cells with nearly Cl^- -free solutions while recording in the cell-attached patch configuration to maintain the extracellular surface of a single Ca^{2+} channel in a high Cl^- pipette solution. The single-channel conductance was unchanged by reducing intracellular $[\text{Cl}^-]$, but the peak mean open probability fell from 0.1 to 0.03. This 70% reduction in open probability accounts for the 2/3 reduction in whole-cell I_{Ca} produced by the same low Cl^- solution. The reduction in open probability is due to increases in the dwell times of both closed states of the channel. Thus, it appears that reducing extracellular Cl^- inhibits whole-cell I_{Ca} by depleting intracellular Cl^- and thereby increasing the time that Ca^{2+} channels spend in the two closed states.

Chloride-dependent modulation of I_{Ca} appears to have physiological significance. I_{Ca} is significantly inhibited by replacing relatively small amounts of Cl^- with various anions, including physiological anions like sulfate and phosphate. Although normal changes in extracellular $[\text{Cl}^-]$ may be limited to a few millimolar (Dietzel and Heinemann, 1986), intracellular changes can be much larger. For example, illumination can cause a 20 mM reduction in intracellular $[\text{Cl}^-]$ of horizontal cells (Djamgoz and Laming, 1987). Reducing $[\text{Cl}^-]$ by a similar amount in photoreceptor terminals would inhibit I_{Ca} and synaptic transmission by >40% (Thoreson et al., 1997; Stella and Thoreson, 2000). As presented below, there is evidence that anion flux through Ca -activated Cl^- channels (Barnes and Hille, 1989) and Cl^- channels coupled to glutamate uptake (Eliasof and Werblin, 1993) regulate I_{Ca} by altering intraterminal Cl^- concentrations.

5.1.3. Calcium-dependent chloride channels—One feature of photoreceptor terminals is the presence of a large Ca^{2+} -activated Cl^- current [$I_{\text{Cl}(\text{Ca})}$] activated by influx through I_{Ca} (Corey et al., 1984; Maricq and Korenbrot, 1988; Barnes and Hille, 1989; Yagi and MacLeish, 1994; Taylor and Morgans, 1998). Cl^- flux through these channels can be substantial. For example, during a 1.4 s depolarizing step, the charge movement accompanying activation of $I_{\text{Cl}(\text{Ca})}$ has been estimated to be 8.5 times that produced by activation of I_{Ca} alone (Barnes and Hille, 1989).

The intraterminal Cl^- changes described above alter I_{Ca} amplitude in photoreceptors leading to the hypothesis that Cl^- flux through $I_{\text{Cl}(\text{Ca})}$ might regulate I_{Ca} amplitude. Cl^- flux can also alter membrane potential and a balance between these two effects may help to stabilize the tonic activation level of I_{Ca} (Thoreson et al., 2003a). To better understand these simultaneous feedback interactions, we consider the case of rods where E_{Cl} is positive to the dark membrane potential (Bader et al., 1982; Somlyo and Walz, 1985; Burkhardt et al., 1991; Thoreson et al., 2000, 2003a). In this scenario, activation of I_{Ca} stimulates $I_{\text{Cl}(\text{Ca})}$, producing a depolarization that leads to further activation of I_{Ca} . In contrast, the Cl^- efflux which accompanies activation of $I_{\text{Cl}(\text{Ca})}$ depletes intracellular Cl^- and thus inhibits I_{Ca} through direct effects of Cl^- on Ca^{2+} channel open probability (Thoreson et al., 2000). In support of such a feedback relationship between I_{Ca} and $I_{\text{Cl}(\text{Ca})}$ in rods, calcium and chloride imaging experiments revealed that increases in $[\text{Ca}^{2+}]_i$ evoked by modest depolarization produced appreciable reductions in $[\text{Cl}^-]_i$ and, conversely, reductions in $[\text{Cl}^-]_i$ inhibited depolarization-evoked increases in $[\text{Ca}^{2+}]_i$ (Thoreson et al., 2003a). In cones, E_{Cl} appears to be near or slightly below the dark resting potential (Kaneko and Tachibana, 1986; Thoreson and Burkhardt, 1991; Kraaij et al., 2000; Thoreson and Bryson, 2005), whereas in rods it is 25 mV positive to the dark resting potential (Thoreson et al., 2002). When E_{Cl} is negative, activation of $I_{\text{Cl}(\text{Ca})}$ is expected to enhance I_{Ca} via direct actions of Cl^- but to inhibit I_{Ca} via membrane hyperpolarization. Thus, whether E_{Cl} is above or below the resting dark potential, an equilibrium between these two processes may help to stabilize the tonic level of I_{Ca} activation and thereby synaptic output. As discussed below in the section on dopamine, this feedback interaction appears to play a role in reducing the enhancement of I_{Ca} by D_2 dopamine receptor activation (Thoreson et al., 2002).

5.1.4. Calcium-dependent K channels—Large conductance calcium-dependent K^+ (BK) channels are concentrated in photoreceptor terminals (Barnes and Hille, 1989; Xu and Slaughter, 2004) and play an important role in maintaining the dark potential near -40 mV (Moriondo et al., 2001). A recent report suggested that K^+ efflux accompanying activation of Ca^{2+} -dependent K^+ channels in photoreceptor terminals alters the voltage dependence of activation, inhibiting I_{Ca} by a direct action analogous to the effects of Cl^- on calcium channels (Xu and Slaughter, 2004).

5.1.5. Vesicular zinc— Zn^{2+} is concentrated in photoreceptor terminals and is proposed to be co-released with glutamate in synaptic vesicles (Akagi et al., 2001; Wu et al., 1993). The Zn^{2+} chelator, histidine, enhances the ERG b-wave in fish retina (Redenti and Chappell, 2003; Rosenstein and Chappell, 2003). Zn^{2+} released from photoreceptors might act at a number of pre- and post-synaptic sites. For example, Zn^{2+} inhibits ionotropic and metabotropic glutamate receptors (Zhang et al., 2002; Rosenstein and Chappell, 2003), as well as GABA_A (Feigenspan and Weiler, 2004) and GABA_C (Qian et al., 1997) receptors in horizontal cells. At low concentrations (<100 μM), Zn^{2+} also stimulates GABA_A receptors in fish horizontal cells (Qian et al., 1997) and currents through connexin 35 hemichannels (Chappell et al., 2003) which are thought to be present in photoreceptor terminals and may contribute to ephaptic feedback regulation of I_{Ca} (Kamermans et al., 2001). Like vesicular protons, Zn^{2+} blocks Ca^{2+} channels in photoreceptors and exerts potent surface charge actions that produce a positive

shift in activation (Cadetti et al., 2004). Thus, Zn^{2+} may be yet another negative feedback modulator of I_{Ca} .

5.1.6. Vesicular protons—Glutamatergic synaptic vesicles are acidified by an ATP-dependent proton pump that helps to establish the electrochemical gradient needed to load vesicles with glutamate. Both the amplitude and voltage dependence of I_{Ca} are altered by changes in pH (Barnes and Bui, 1991; Barnes et al., 1993). De Vries (2001) found that during depolarizing steps applied to cones there is a transient reduction in I_{Ca} that matches the time course for exocytosis, is blocked by pH buffers, but is unaffected by glutamate transport inhibitors. He concluded that the release of glutamate from cones was accompanied by a transient reduction in extracellular pH that inhibits presynaptic I_{Ca} in cone terminals. The reduction in I_{Ca} results from both an inhibition of the peak amplitude of I_{Ca} and a positive shift in I_{Ca} activation. The former effect results from proton block of the channel, the latter results from neutralization of membrane surface charge (Krafte and Kass, 1988; Barnes and Bui, 1991; Klockner and Isenberg, 1994). Thus, the release of protons that accompanies release of L-glutamate acts as a negative feedback mechanism to inhibit I_{Ca} and reduce further exocytosis.

5.1.7. Glutamate—Glutamate transporters located on rod and cone terminals are associated with a significant anion conductance (Picaud et al., 1995; Grant and Werblin, 1996; Larsson et al., 1996; Eliasof and Werblin, 1993; Rabl et al., 2003). By activating these transporters, glutamate inhibits I_{Ca} in rods (Rabl et al., 2003). Thus, besides removing glutamate from the synaptic cleft, glutamate transporters inhibit I_{Ca} , providing a negative feedback signal that further inhibits L-Glu release.

Because E_{Cl} is positive to the resting potential in rods, activation of glutamate transporters stimulates a Cl^- efflux (Rabl et al., 2003). Analogous to the feedback interaction postulated between I_{Ca} and $I_{\text{Cl}(\text{Ca})}$, the resulting depletion of intraterminal chloride appears to be responsible for the transporter-mediated inhibition of I_{Ca} . Accordingly, inhibition of I_{Ca} is occluded by prior reduction of intraterminal Cl^- with low Cl^- solutions introduced through the patch pipette (Rabl et al., 2003). By facilitating a Cl^- efflux, the chloride conductance associated with glutamate transporter activation appears to play a key role in the inhibition of I_{Ca} in rod terminals.

The group III mGluR, mGluR8a, is found presynaptically in both rod and cone terminals of the mammalian retina (Koulen et al., 1999, 2005; Koulen and Brandstatter, 2002). Activation of group III mGluRs by L-AP4 reduces intracellular calcium levels in isolated mammalian photoreceptors (Koulen et al., 1999) and, consistent with an inhibition of presynaptic release, reduces the rate of spontaneously occurring miniature EPSCs in OFF bipolar cells of the fish retina (Hirasawa et al., 2002). However, group III mGluR agonists do not inhibit rod or cone I_{Ca} (Hirasawa et al., 2002; Rabl et al., 2003) suggesting that group III mGluRs may inhibit Ca^{2+} influx by stimulating K^+ channels to induce membrane hyperpolarization (Hirasawa et al., 2002). Studies on responses of bipolar and horizontal cells suggest that group II metabotropic receptors may also inhibit glutamate release from photoreceptors (Higgs and Lukasiewicz, 2002).

5.2. Extrinsic neuromodulators

5.2.1. Dopamine—Dopamine is an important circadian neuromodulator in the retina (Witkovsky, 2004). Although circadian mechanisms increase dopamine release modestly during prolonged darkness at night (Weiler et al., 1997), dopamine release is substantially enhanced (up to 3–4-fold) by increasing illumination (Godley and Wurtman, 1988; Boatright et al., 1989; Witkovsky et al., 1993). Increasing dopamine levels in daylight illumination diminish the strength of rod signals and enhance cone signals in second-order neurons

(Witkovsky et al., 1988, 1989). The effects of dopamine on photoreceptor output are mediated in part by D₂-like (D₂ or D₄) dopamine receptors on rods and cones (Muresan and Besharse, 1993). Stella and Thoreson (2000) examined the effects of dopamine on I_{Ca} in rods and cones of the salamander retina. Surprisingly, activation of D₂-like receptors by quinpirole enhanced I_{Ca} in rods and inhibited I_{Ca} in red-sensitive, large single cones which form the largest population of cones in the salamander retina. As found in rods, I_{Ca} in red, blue and UV-sensitive small single cones was also enhanced by quinpirole. Additional experiments indicated that D₂ receptors in both rods and cones modulate I_{Ca} by activating a pertussis toxin-sensitive G protein that inhibits production of cAMP.

How does activation of D₂-like receptors enhance rod I_{Ca} yet suppress rod inputs to its post-synaptic targets? Thoreson et al. (2002) tested the hypothesis that D₂ receptor enhancement of I_{Ca} might increase $I_{Cl(Ca)}$, thereby stimulating an efflux of Cl⁻ from rods that in turn provided a negative feedback inhibition of I_{Ca} . For measurements of I_{Ca} , $I_{Cl(Ca)}$ was blocked with niflumic acid and by the use of Ba²⁺ as the charge carrier. Consistent with the enhancement of I_{Ca} observed electrophysiologically, depolarization-evoked increases in [Ca²⁺]_i measured with Fura-2 were enhanced by quinpirole when $I_{Cl(Ca)}$ was blocked by niflumic acid. Furthermore, in the presence of niflumic acid, quinpirole no longer inhibited rod inputs into horizontal and bipolar cells. When niflumic acid was omitted from the bathing medium and Ca²⁺ was used as the charge carrier, quinpirole increased $I_{Cl(Ca)}$, resulting in an efflux of Cl⁻ from the rod. Furthermore, when $I_{Cl(Ca)}$ was not blocked, quinpirole inhibited depolarization-evoked increases in [Ca²⁺]_i in rods. A similar effect was obtained by directly lowering intracellular Cl⁻ using a low [Cl⁻] solution. Collectively, these results suggest that the quinpirole-induced inhibition of Ca²⁺ influx was a result of the Cl⁻ efflux accompanying enhanced activation of $I_{Cl(Ca)}$. These experiments are consistent with the hypothesis that feedback interactions between I_{Ca} and $I_{Cl(Ca)}$ help to shape synaptic output and responses to neuromodulation.

5.2.2. Adenosine—Adenosine acts as a neuromodulator at many sites in the nervous system. It is generated primarily by the catabolism of ATP through an ectonucleotidase cascade (Cunha et al., 1992, 1998; Dunwiddie et al., 1997). Adenosine acts at G-protein-coupled cell surface receptors classified as A₁, A_{2A}, A_{2B} and A₃ (Ralevic and Burnstock, 1998). In the retina, adenosine is accumulated selectively by rod-driven horizontal cells (Studholme and Yazulla, 1997); receptors for adenosine have been localized to the outer nuclear layer and photoreceptors (Paes de Carvalho et al., 1990, 1992; Kvant et al., 1997). Adenosine is released from retina in the dark (Blazynski and Perez, 1991), when photoreceptors are depolarized and L-glutamate release from photoreceptors is at its greatest (Schmitz and Witkovsky, 1997).

Adenosine elicited a dose-dependent inhibition of I_{Ca} in rod photoreceptors by stimulating A_{2A}-like adenosine receptors that in turn stimulate adenylyl cyclase activity (Stella et al., 2002, 2003). A₂ receptors have been shown to inhibit I_{Ca} at other synapses, but the other examples occur at inhibitory synapses and thus activation of A₂ receptors is generally thought to increase excitability (Edwards and Robertson, 1999). The inhibition of I_{Ca} at the photoreceptor synapse is the first example of A₂ receptors acting to inhibit glutamate release.

Studies on the light responses of rods and second-order neurons revealed that inhibition of I_{Ca} by A₂ agonists inhibited synaptic transmission from rods. Furthermore, an A_{2A} antagonist, ZM-241385, enhanced light-evoked currents in second-order neurons, suggesting the presence of endogenous levels of adenosine sufficient to tonically inhibit transmitter release from rods.

Activation of D₂ dopamine receptors stimulates I_{Ca} but nonetheless inhibits glutamate release from rods, at least partly as the result of a feedback loop involving the activation of $I_{Cl(Ca)}$ (Thoreson et al., 2002, 2003a). Invoking the same sort of feedback loop, one would predict

that inhibition of I_{Ca} by A_2 receptors should stimulate glutamate release, but instead A_2 receptor activation inhibits synaptic transmission. To reconcile these results we hypothesize that the feedback loop involving $I_{Cl(Ca)}$ may be engaged only when Ca^{2+} reaches high levels following stimulation of I_{Ca} (e.g., with dopamine) but not when I_{Ca} is inhibited. (e.g., with adenosine). A rectification in the feedback loop would result if high levels of Ca^{2+} were needed to activate $I_{Cl(Ca)}$. Consistent with this postulate, Ca^{2+} -activated Cl^- channels in salamander olfactory neurons do not attain half-maximal activation until Ca^{2+} levels reach 5 μM (Kleene and Gesteland, 1991).

These studies suggest that endogenously released adenosine in the outer retina may inhibit glutamate release from rod terminals by inhibiting Ca^{2+} influx. Adenosine levels in the retina are likely to be elevated by high metabolic activity in the dark as well as by the circadian release of adenosine (Ribelayga and Mangel, 2005). Although ATP did not replicate the effects of adenosine, it is possible that ATP metabolites (including adenosine) may be co-released in vesicles as is found at other synapses (Fredholm, 1995). Adenosine might thus play a role in controlling synaptic transmission at the first synapse in the retina in response to changing circadian conditions or levels of illumination.

5.2.3. Cannabinoids—Cannabinoids, the active components of marijuana, can induce a number of visual effects, including increased sensitivity to light (Dawson et al., 1977). Cannabinoids act on G-protein-coupled receptors (CB1 and CB2). RT-PCR and in situ hybridization indicate the presence of both CB1 and CB2 receptors throughout the retina (Lu et al., 2000; Buckley et al., 1998; Porcella et al., 2000). CB1 receptor antibodies label many neurons in the retina, including rod and cone terminals (Straiker et al., 1999; Yazulla et al., 1999; Straiker and Sullivan, 2003). Although the endogenous cannabinoid, anandamide, has not been detected, the CB1 agonist 2-arachidonylglycerol and CB2 agonist palmitoylethanolamide were found to be present in high levels in retina (Straiker et al., 1999). Fatty acid amide hydrolase, which hydrolyzes anandamide and 2-arachidonylglycerol, is also widespread in retina and prominently expressed in horizontal cells (Yazulla et al., 1999).

The CB1 agonist WIN 55212-2 (1 μM) enhances I_{Ca} in rods but inhibits I_{Ca} in red-sensitive large single cones of the salamander retina (Straiker and Sullivan, 2003). Another red-sensitive cone subtype in salamander retina, the accessory member of double cones, was not affected by WIN55212-2. These effects were blocked by a cannabinoid antagonist (SR141716A). In zebrafish retina, WIN55212-2 inhibited I_{Ca} at concentrations above 1 μM but enhanced I_{Ca} at concentrations below 1 μM (Fan and Yazulla, 2003), a biphasic effect not seen in salamander rods (Straiker and Sullivan, 2003). Cannabinoid receptors also act on potassium and chloride channels in photoreceptors that influence synaptic transmission through their effects on membrane potential and excitability (Straiker and Sullivan, 2003; Fan and Yazulla, 2003). Activation of CB1 receptors by higher concentrations of WIN55212-2 stimulates pertussis toxin-sensitive G proteins ($G_{i/o}$) that in turn inhibit adenylyl cyclase, similar to the signaling cascade engaged by D_2 -like dopamine receptors (Straiker and Sullivan, 2003; Fan and Yazulla, 2003). The stimulatory effects of low concentrations of WIN55212-2 on zebrafish cones were unaffected by pertussis toxin, but blocked by a PKA inhibitor and cholera toxin, suggesting that low concentrations of the CB1 agonist activate cholera toxin-sensitive Gs proteins which act to stimulate adenylyl cyclase (Fan and Yazulla, 2003).

The observation that D_2 dopamine and CB1 cannabinoid receptors both converge on a signaling pathway involving pertussis toxin-sensitive G proteins and inhibition of adenylyl cyclase prompted Fan and Yazulla (2004) to examine interactions between the two types of receptors. They found that activation of D_2 receptors with quinpirole blocked both the stimulatory effects of low concentrations of Win55212-2 and the inhibitory effects of high concentrations of

Win55212-2, even when quinpirole exerted no detectable effect by itself. The sites and pathways for these interactions are not yet clear, but this finding raises the possibility of interactions among other signaling pathways regulating voltage-dependent currents in photoreceptors.

5.2.4. Somatostatin—Somatostatin, also called somatotropin release-inhibiting factor (SRIF), is found throughout the nervous system, including the vertebrate retina (Tornqvist et al., 1982). SRIF immunoreactivity is found in primarily in a subtype of amacrine cell. Its actions are mediated by several classes of G-protein-coupled receptors, labeled sst_{1-5} (Moller et al., 2003). Although there is evidence that all classes of SRIF receptor are present in the vertebrate retina (Cristiani et al., 2002; Thermos, 2003), it is a splice variant of the sst_2 receptor, sst_{2a} which has been the focus of most physiological investigations of SRIF function. In rabbit (Johnson et al., 1998), rat (Johnson et al., 1999) and salamander (Akopian et al., 2000) retinas, sst_{2a} receptors are found on rod and cone terminals. Akopian et al. (2000) studied the actions of SRIF on isolated salamander rod and cone cells, using whole-cell patch clamp techniques. SRIF increased a delayed rectifier K current in both rods and cones, an action blocked by pertussis toxin or GDP β S, indicating a G-protein mediated pathway. SRIF had a differential action on I_{Ca} , increasing that of cones, but decreasing the rod I_{Ca} . SRIF actions on rod/cone I_{Ca} are opposite to those induced by dopamine (Stella and Thoreson, 2000) or CB1 cannabinoid receptor activation (Straiker and Sullivan, 2003). Ca^{2+} imaging experiments showed that SRIF reduced the Ca^{2+} fluorescent signal induced by elevated $[K]_o$ in rods and increased that in cones. The effect of SRIF on I_{Ca} was rapid (<1 min) suggesting an underlying membrane-delimited pathway. In the rat retina, Johnson et al. (2001) found that SRIF reduced Ca^{2+} influx into rod bipolar terminals.

5.2.5. Insulin—Insulin inhibits both the ERG b-wave (Gosbell et al., 1996) and L-type Ca^{2+} channels in rod photoreceptors (Stella et al., 2001). The potency of insulin's actions ($IC_{50} = 2$ nM) and the effectiveness of an insulin receptor-specific tyrosine kinase inhibitor suggest that receptors for insulin, and not insulin-like growth factor, are responsible for these effects (Stella et al., 2001). Insulin in the retina is derived from both the pancreas and the retina itself. Circulating insulin derived from the pancreas is transported across the blood–retinal barrier by a mechanism involving the binding to insulin receptors that allows about 80% of the transported insulin to remain intact (Reiter and Gardner, 2003). The retina also is capable of synthesizing insulin (Tesoriere et al., 1994), in situ hybridization and RT-PCR show that Müller cells contain mRNA for the preproform of insulin (Das et al., 1987; Budd et al., 1993), and insulin and insulin-like immunoreactivity have been demonstrated throughout the retina, particularly in Müller cells (Das et al., 1987). By influencing calcium influx, alterations in retinal insulin levels or sensitivity to insulin (e.g., during diabetes) may affect photoreceptor cell survival and neurotransmission.

5.2.6. Nitric oxide—Nitric oxide synthase is present throughout the retina, including horizontal and bipolar cell processes in the OPL and photoreceptor inner segments (e.g., Kurenni et al., 1995; Djamgoz et al., 1996; Blute et al., 1997; Cao and Eldred, 2001; Haverkamp and Eldred, 1998). In addition to stimulating cGMP synthesis and thus CNG channels, application of NO donors inhibit I_{Ca} in salamander cones and produce a negative activation shift in the I_{Ca} of salamander rods (Kurenni et al., 1994; Kurenni et al., 2004). Application of an nNOS inhibitor evokes a relative reduction in rod vs. cone inputs to horizontal cells (Kurenni et al., 2004), consistent with effects of NO donors on I_{Ca} , suggesting that endogenously released NO inhibit cone inputs and stimulate rod inputs into horizontal cells. Although the patterns of production of NO under different conditions of illumination are not fully understood, it is reasonable to speculate that NO production by photoreceptors might increase in darkness when they are relatively depolarized and calcium levels are accordingly

elevated. If so, then the reciprocal regulation of rod and cone calcium channels by NO might contribute to a shift in the retina to more rod-dominated conditions (Kurenniy et al., 2004).

5.2.7. Retinoids and polyunsaturated fats—Retinoids, including all-trans retinal, and long chain polyunsaturated fatty acids, including arachidonic acid and docosahexanoic acid, can potently inhibit I_{Ca} in photoreceptors (Vellani et al., 2000). Inhibitory effects of polyunsaturated fats were potentiated by membrane depolarization but manipulations of various signaling molecules including cyclo-oxygenase, lipoxygenase, G proteins, protein kinases A and C, and protein phosphatases failed to block these effects. From these data, Vellani et al. (2000) proposed that polyunsaturated fats interfere with calcium entry into the channel by binding near its extracellular face. Light activates phospholipase A2 (Jelsema and Axelrod, 1987) leading to a light-induced increase of arachidonic acid and docosahexanoic acid release (Jung and Reme, 1994; Reinboth et al., 1996). Retinoid levels also are increased by light (McCaffery et al., 1996). It is postulated that light-induced increases in these substances may diffuse to rod terminals to reduce synaptic output and thus contribute to post-receptor light adaptation (Vellani et al., 2000).

5.3. Feedback from horizontal cells to cones

In the context of the present review, this topic takes on importance in view of recent findings that feedback from horizontal cells affects the I_{Ca} of cones. There is no credible evidence for horizontal cell feedback to rods. The possibility of feedback arose from findings by Baylor et al. (1971) that cones had a receptive field with a small excitatory center (but still larger than the diameter of a single cone, indicating cone–cone coupling) and a concentric surrounding area, which when illuminated reduced the central response. The crucial finding was that hyperpolarizing current injected into the horizontal cell resulted, after a short delay, in a depolarizing response in the cone. The delay and the polarity reversal seemed to indicate a chemical junction. In the lower vertebrates which were the main object of study in the 1970s and 1980s, horizontal cells are GABAergic (Marc et al., 1978) and much energy was expended in trying to prove that feedback to cones operated through a GABA-dependent mechanism. In spite of many claims and counterclaims, however (reviewed in Burkhardt, 1993; Kamermans and Spekrijse, 1999), a GABAergic basis for feedback could not be established firmly.

Other evidence pointed to an effect of feedback on photoreceptor I_{Ca} . O'Bryan (1973), Gerschenfeld and Piccolino (1980) and Burkhardt et al. (1988) described Ca^{2+} -dependent depolarizations which sometimes led to spikes in turtle cones, responses that were elicited by flashes of annular illumination in the presence of steady central illumination or by current injection into cones. Thoreson and Burkhardt (1990) found that the depolarizing responses in cones elicited by current injection were blocked by submicromolar concentrations of cobalt, suggesting that they depended on calcium. The same low concentrations of cobalt are sufficient to block the surround responses of bipolar cells without attenuating the response to central illumination (Vigh and Witkovsky, 1999).

Kamermans and his co-workers (Verweij et al., 1996; Kraaij et al., 2000; Kamermans et al., 2001; reviewed in Kamermans and Fahrenfort, 2004) have elaborated a hypothesis whereby feedback shifts the operating range of the I_{Ca} in cones. In the goldfish retina, illuminating the cone with a surround displaced the Ca-activation curve to the left by about -7.5 mV. Feedback-induced shifts in I_{Ca} were blocked by AMPA receptor antagonists that disrupt cone to horizontal cell communication. GABA-related drugs, on the other hand, were without effect.

Calcium-dependent chloride currents contribute to depolarizing surround responses when the cone membrane potential is below E_{Cl} (Thoreson and Burkhardt, 1990). E_{Cl} in cones appears to be near or slightly negative to the dark resting potential (Kaneko and Tachibana, 1986; Thoreson and Burkhardt, 1991; Kraaij et al., 2000; Thoreson and Bryson, 2005) but in the

presence of background illumination, the cone membrane potential often passes below E_{Cl} . Blocking $I_{Cl(Ca)}$ with niflumic acid blocks feedback depolarization in goldfish cones, but fails to block depolarizing responses in biphasic horizontal cells that are thought to arise from horizontal cell to cone feedback (Kraaij et al., 2000). These data are interpreted as suggesting that post-receptor consequences of feedback are potentially influenced by feedback-induced shifts in I_{Ca} but only weakly influenced by feedback depolarization per se.

The precise mechanism whereby the feedback response shifts I_{Ca} remains controversial. New evidence on this point (Hirasawa and Kaneko, 2003) suggests that pH may play a crucial role. The susceptibility of I_{Ca} to changes in pH was established earlier by Barnes and Bui (1991) and Barnes et al. (1993). In the newt retina, Hirasawa and Kaneko (2003) found that strong pH buffering largely eliminated the surround-induced shift in the cone I_{Ca} . They postulate that in an intact preparation, the pH of the synaptic cleft is more acidic (by about 0.2 pH unit) than the extracellular pool, and that surround illumination, by hyperpolarizing the horizontal cell, reduces the proton level in the cleft. Increased alkalinization of the bathing medium shifts the Ca^{2+} activation function to the left. It is important to point out that these changes occur in a bicarbonate-buffered system (which resembles the situation in vivo), but not in a HEPES-buffered medium. The mechanism of pH change is still not established, but may involve a depolarization-dependent Na/HCO_3 co-transport in glial cells resulting in uptake of HCO_3^- and release of protons into the extracellular space. Neuronal hyperpolarization would remove K^+ from the extracellular space, causing a hyperpolarization of the glia and a reversal of the effects of the transporter. This hypothesis still has to be tested rigorously in the retina.

An alternative hypothesis was put forward by Kamermans et al. (2001). It depends on the demonstration of connexin 26 immunoreactivity on horizontal cell dendrites in the cone invagination (Janssen-Bienhold et al., 2001). The connexins are postulated to form hemichannels near the glutamate release sites, which when open permit positive current flow into the horizontal cell. The efflux of cations from the extracellular space of the synaptic cleft makes it more negative, reducing the transmembrane potential of the cone, and thereby shifting the Ca -activation function to the right. The crucial experimental test of the Kamermans hypothesis was to block the hemichannel conductance with carbenoxolone, resulting in a loss of the surround response. Carbenoxolone, however, is not a clean drug and ultimately leads to rundown of all responses. In particular, Vessey et al. (2004) find that carbenoxolone directly inhibits the I_{Ca} of cone photoreceptors.

To summarize, the evidence that horizontal cell to cone feedback acts primarily on the cone I_{Ca} seems clear, even if the mechanism by which this occurs is not yet fully clarified. The effect of large surround area illumination, a particularly effective stimulus for horizontal cells, is to shift the cone Ca^{2+} activation function to the left, thus increasing Ca^{2+} influx, resulting in increased glutamate release by the cone.

5.4. Vesicular glutamate transporters

Synaptic transmission from photoreceptors relies on the release of glutamate packaged in presynaptic vesicles (reviewed by Thoreson and Witkovsky, 1999). The packaging of glutamate into vesicles is performed by members of a family of vesicular glutamate transporters: VGlut1, VGlut2 and VGlut3. VGlut1 was previously named the brain, Na-dependent Pi (BNPI) transporter because of its homology to inorganic phosphate transporters in the kidney. However, subsequent experimentation established that VGlut1, 2 and 3 are present on vesicular membranes and function as glutamate transporters (Bellocchio et al., 2000; Takamori et al., 2000). By contrast with the plasma membrane glutamate transporters (Eliasof et al., 1998), vesicular glutamate transporters are sodium-independent and exhibit a more than 10-fold lower K_m (2 mM: Bellocchio et al., 2000). In place of sodium-dependence,

glutamate uptake into vesicles relies primarily on the electrical component ($\Delta\Psi$) of the electrochemical gradient across the vesicle membrane.

Different VGlut subtypes show complementary expression patterns in different regions of the CNS. VGlut2 appears to be largely absent from rodent retina, whereas VGlut1 is present in photoreceptor and bipolar cell terminals (Sherry et al., 2003a; Johnson et al., 2003a). VGlut3 is limited to a subset of amacrine cells in many species (Johnson et al., 2004a, b; Haverkamp and Wässle, 2004). In cat retina, there is immunohistochemical evidence suggesting that VGlut2 may co-localize with VGlut3 in some ganglion cells and VGlut2 may co-localize with VGlut1 in S cone photoreceptors (Fyk-Kolodziej et al., 2004).

Changes in the glutamate content of vesicles can alter post-synaptic responses when post-synaptic receptors are not fully saturated by the concentration of glutamate in the synaptic cleft. It remains to be determined whether the concentration of glutamate in the photoreceptor synaptic cleft is sufficient to saturate post-synaptic glutamate receptors, as found at many (Frerking and Wilson, 1996), but not all (McAllister and Stevens, 2000), CNS synapses. If the receptors are not saturated, modulation of VGlut activity would provide a means of regulating post-synaptic response amplitude.

An interesting property of vesicular glutamate uptake is the presence of a bell-shaped chloride dependence with optimal uptake achieved with low millimolar chloride (Naito and Ueda, 1985; Bellocchio et al., 2000). In rod terminals, E_{Cl} is well above the resting potential and thus the opening of calcium-activated chloride channels or the chloride channels associated with plasma membrane glutamate transporters produces a chloride efflux (Thoreson et al., 2002, 2003a; Rabl et al., 2003). By altering the rate of glutamate uptake, changes in intraterminal chloride levels might therefore alter the strength of synaptic output from photoreceptors.

Another interesting property of vesicular glutamate transporters is that, like plasma membrane glutamate transporters, they are associated with an anion channel. As discussed previously, intracellular chloride levels can influence calcium channel open probability (Thoreson et al., 2000). Intravesicular chloride levels are not known, but significant chloride flux across the vesicular membrane would be expected to alter calcium channel activity in the photoreceptor terminal. Such a mechanism would allow the activity of VGlut to influence the calcium channels responsible for controlling vesicle release.

5.5. Modulation of exocytotic proteins

Various second messengers and kinases act directly on exocytotic proteins to modulate synaptic exocytosis (reviewed by Takahashi et al., 2003). Examples include cAMP-dependent protein kinase (reviewed by Evans and Morgan, 2003), PKC (Minami et al., 1998), CaMKinase, MAP kinase (Chi et al., 2003) and src family kinases (Ohnishi et al., 2001). Modulation of release from bipolar cell terminals by PKC has been described (Minami et al., 1998), but the direct modulation of exocytosis by these and other mechanisms has not yet been investigated in photoreceptors.

6. Conclusions

6.1. Relation of synaptic release to the underlying light-evoked responses of ON bipolar and photoreceptor cells

The great majority of studies we cite in this review utilized preparations reduced from their *in vivo* state, a logical choice whose goal was to reduce the complexities of experimentation and interpretation inherent in studying the intact retina. In this concluding section, we return to the whole retina to try and relate what is known about ribbon synapses to the behavior of the retinal neurons that utilize them.

Two subtypes of ON bipolar cell have been described in the much studied salamander retina: transient and sustained (Awatramani and Slaughter, 2000). In response to a bright test flash, the transient cell responds with an initial depolarizing transient that peaks in ca. 100 ms, then decays with $\tau = 400$ ms to a maintained plateau that is only 13% of peak depolarization. Ichinose et al. (2005) showed that a TTX-sensitive Na current contributes to the initial peak. Sustained ON bipolar cells also show rolloff from peak depolarization, but the plateau voltage is ca. 40% peak. The light-evoked response of the goldfish Mb₁ ON bipolar, upon which virtually the whole data set of transmitter release mechanisms for bipolar cells is based, is of the transient subtype (Toyoda, 1973). Given the fast rates of exocytosis elicited by depolarizing steps described in Section 2.6, a light-evoked depolarizing transient in the ON bipolar cell should result in a phasic release of glutamate whose magnitude will vary with light intensity. For a step of light, when the bipolar cell voltage relaxes to its plateau, exocytosis will diminish sharply, but does it fall to zero?

The answer may depend upon the intensity and duration of the light stimulus. Using a bioassay for glutamate release, von Gersdorff et al. (1998) have shown that if a depolarization is strong enough to deplete an entire vesicle pool, then glutamate release from the Mb₁ bipolar cell will be relatively transient. Conversely, if the stimulus is weaker, then the transient component is reduced in amplitude and a second, more sustained component of release is revealed (von Gersdorff et al., 1998; see also Sakaba et al., 1997). Similarly, paired recordings in mammalian retina between rod bipolar cells and AII amacrine cells reveal an initial transient burst of glutamate release that is followed by a smaller, sustained component of release (Singer and Diamond, 2003). As with the Mb₁ bipolar cell, the intensity of the stimulus determines the relative ratios between the transient and sustained components. These two components share similar sensitivities to exogenous calcium buffers and may represent the fusion of vesicles located near calcium channels (Singer et al., 2004). Thus, there could potentially be release during the plateau phase, depending upon stimulation intensity. In addition, other factors in the intact retina, such as negative feedback via reciprocal inhibitory synapses, inactivation of the presynaptic calcium current by released protons, and depletion of cleft calcium may further refine the pattern of glutamate release. Therefore, to firmly address the magnitude of exocytosis during the plateau phase, additional experiments using physiological stimuli and intact preparations will be required.

Based on capacitance measures, exocytosis from ON bipolar cell terminals occurs only when $[Ca]_i$ rises beyond 10 μ M (Heidelberg et al., 1994; von Gersdorff and Matthews, 1994; Heidelberg, 1998). On the other hand, using an optical technique based on activity-dependent dyes, Rouze and Schwartz (1998) reported that exocytosis still occurs, albeit at a relatively slow rate, when intracellular calcium was increased to between 0.3 and 3.0 μ M. This raises a central, and still unresolved question: do ON bipolar cells communicate synaptically using one calcium sensor or two? And, as a related question: if two sensors exist, are they coupled to the same vesicle pool?

One candidate for the high-affinity component of release is the asynchronous release that occurs following closure of calcium channels after a stimulus that evokes a very large influx of calcium (von Gersdorff et al., 1998; Singer and Diamond, 2003). The available evidence indicates that asynchronous release is triggered by residual calcium, rather than the high calcium levels found near open calcium channels (Singer and Diamond, 2003), making it a prime candidate for a high-affinity receptor. In the Mb₁ bipolar cell, asynchronous release may occur over hundreds of milliseconds and yet represent only a small fraction ($\approx 10\%$) of the total stimulus-evoked release (von Gersdorff et al., 1998). Thus, this form of release may be missed in capacitance measurements, particularly under recording conditions that mimic the endogenous calcium buffering capacity (Pan et al., 2001; Singer and Diamond, 2003). That said, the role of the high-affinity calcium receptor and asynchronous release in synaptic

signaling is presently unclear. Further experiments are warranted to identify which vesicles are released by a high-affinity receptor, to determine if this form of exocytosis occurs in the intact circuit when inhibitory feedback is active and to assess what type of information it conveys.

In regard to the second question, cross-depletion experiments (using a depolarizing voltage step to deplete the releasable pool of vesicles prior to flash photolysis of caged calcium) indicate clearly that a low-affinity calcium sensor underlies the dominant portion of ON bipolar cell exocytosis. This same pool may underlie the initial burst of release recorded in response to a light stimulus (e.g., Trexler et al., 2005). It is worth noting that if an experiment shows that perceptible exocytosis occurs when the spatially averaged calcium is, say, 1 μM or less, this result does not per se implicate a high-affinity calcium sensor. That is, even when only a few calcium channels are open, they still create a local region of high $[\text{Ca}]$. In fact, as the photoreceptor or bipolar cell hyperpolarizes, e.g., when the ON bipolar cell relaxes from its peak depolarization to a maintained plateau, the driving force for a calcium current increases, even as the number of open calcium channels decreases. Thus, the spatially averaged $[\text{Ca}]$ will fall, whereas the local $[\text{Ca}]$ at the mouth of the calcium channel will rise.

In contrast to the ON bipolar cell, for the rod photoreceptor the preponderance of data indicate that a high-affinity calcium sensor controls exocytosis, particularly when considered from the standpoint of the in vivo operating range of photoreceptor voltages. Thus, between the dark membrane potential of -40 to -45 mV and light-induced hyperpolarizations to -55 mV, below which calcium currents become undetectably small, $[\text{Ca}]_i$ varies from 0.3 to 2.0 μM . In this range, exocytosis increases linearly with $[\text{Ca}]$ and can be measured as an increase in membrane capacitance (Rieke and Schwartz, 1996; Thoreson et al., 2004). These data notwithstanding, there is evidence for a component of exocytosis in photoreceptors that occurs only when Ca levels exceed 10 μM (Kreft et al., 2003). As for bipolar cells, locally high levels of Ca might be attained even with the modest depolarizations anticipated to occur under physiological conditions, but cross-depletion studies (Thoreson et al., 2004), of the sort just described, indicate that light-evoked control of exocytosis in photoreceptors depends primarily on a high-affinity calcium sensor. Based on the prevailing view that the calcium sensor is synaptotagmin (Sudhof, 2004), but noting that many isoforms of synaptotagmin exist, a definitive answer to the questions about calcium sensors will be greatly aided by conditional knockouts of synaptotagmins I–III, the forms so far identified at ribbon synapses.

The apparent differences in primary calcium sensor between ON bipolar cell and photoreceptors are reflected in the organization of their respective ribbon synapses. Bipolar cells lack an arciform density at the base of the ribbon, and thus the lowest row of ribbon-related vesicles is docked at active zones, which are very close to the calcium channels. In photoreceptors, the location of the arciform density appears to create a greater distance between calcium channels and active zones. Although this conclusion retains an element of uncertainty, because the precise locations and distribution of active zones in photoreceptors are not fully resolved, it appears to reflect the difference in spatial separation of calcium channels and active zones characterized by Augustine (2001) as nanodomain vs. microdomain. Given the very steep falloff of calcium as distance from the mouth of the calcium channel increases, the bipolar cell active zone (nanodomain) can experience calcium concentrations of hundreds of micromolar, whereas in photoreceptors, the calcium concentration at the active zone (microdomain) is predicted to be tens of micromolar.

These data still beg the question of exactly what calcium concentrations are needed at the rod photoreceptor active zone to evoke fusion. Granted that the data of Rieke and Schwartz (1996) suggest that submicromolar calcium is sufficient. We note, however, that their experiments utilized release of caged calcium, whereas in vivo the primary source of calcium

is entry through voltage-gated channels. Release of caged-calcium results in a spatially uniform increase in $[Ca]_i$ whereas entry of Ca^{2+} through membrane channels creates local microzones of elevated $[Ca]_i$. Moreover, there is evidence in rod photoreceptors that calcium released from intracellular stores results in transmitter release (Krızaj et al., 1999). Whether release by stores acts as entry through channels does in creating very local elevations of $[Ca]$ is presently unknown. That is, the store receptors (IP3R and/or RyR) might be situated very close to active zones, possibly giving rise to calcium 'sparks' (Rizzuto et al., 1993). The general point is to determine the effective calcium concentration at the active zones where the calcium sensors are located.

Whatever the final resolution of these questions, it is noteworthy that exocytosis from rods is triggered by only a very small number of open calcium channels: <10 /ribbon even at -45 mV, and a still smaller number when the rod is hyperpolarized from this level (see Section 3.1 for the calculations). How does the rod avoid a high level of synaptic noise that inevitably results from the random openings of a few calcium channels? Three types of post-synaptic cells are excited by rods: horizontal, ON bipolar and OFF bipolar cells. Horizontal cells minimize noise through extensive coupling to neighboring horizontal cells, whereas ON bipolar cells reduce noise through the slowness of their own metabotropic cascade. On the other hand, OFF bipolars are uncoupled and have fast responding ionotropic glutamate receptors. Recordings from OFF bipolar cells (Pang et al., 2004) indicate that the photoreceptor–OFF bipolar cell synapse is, in fact, noisy. In the cone circuit, noise is reduced by utilizing desensitized AMPA/KA receptors (De Vries and Schwartz, 1999). Voltage noise in amphibian, but not mammalian rod is reduced by rod–rod coupling (Krızaj et al., 1998; Schneeweis and Schnapf, 1995), but this does not greatly diminish the noisy Ca^{2+} signals arising from stochastic openings of single calcium channels. Pooling of noisy signals through integration within the OFF bipolar cell dendrites, however, does occur in all vertebrate retinas. Another possibility is that exocytosis from rods truly is triggered by a calcium sensor with a very high affinity. To the extent that this turns out to be true, a greater portion of the calcium microdomain is above the threshold of the sensor. Accordingly, calcium ions released from the open channel (or from calcium stores) and which diffused to multiple nearby active zones, might be sufficient to trigger release. Under this scenario, moreover, CICR assumes importance in the control of exocytosis.

There is general consensus now that ribbon synapses are organized to promote fusion and exocytosis of a large number of vesicles with a single effective stimulus, e.g., a brief but bright light flash (Sterling and Matthews, 2005). Nevertheless, fundamental questions about the function of the ribbon itself are unresolved, of which the main one is just what role does it play in the stimulus-related release of vesicles? The original notion of a conveyor belt formulated by Bunt (1971) seems to be ruled out on kinetic grounds and the finding that ATP hydrolysis is not required for the release of vesicles already in the releasable pool. But even if one accepts that diffusion is sufficient to convey vesicles from the ribbon to the active zone, is there still a signal (or multiple signals) that governs attachment and release of vesicles to their slender tethers? What sorts of proteins make up the tethers? Calcium enhanced refilling of rapidly releasable pool (Mennerick and Matthews, 1996) raises the possibility that tethers are sensitive to calcium, a topic that calls for further study, perhaps along the lines of testing whether elevations in intracellular $[Ca]$ release vesicles from ribbons.

As a final note on the importance of the ribbon synapse to vision, gene defects in proteins found at the ribbon synapse of rods and cones result in photoreceptor dystrophies. To take just a few examples, gene defects in the Rab3A-interacting molecule (RIM1) found at ribbon synapses lead to an autosomal dominant rod–cone dystrophy (Johnson et al., 2003b). A late onset cone–rod dystrophy is produced by mutations in HRG4 which is highly enriched at photoreceptor ribbon synapses and which appears to be associated with synaptic vesicles (Kobayashi et al., 2000). Although the function of this protein at ribbon synapses is unclear, its *C. elegans*

orthologue, UNC119, has been shown to activate SRC tyrosine kinases (Cen et al., 2003). X-linked congenital stationary night blindness arises from mutations in the $Ca_v1.4$ calcium channel (CACNA1F) found in synaptic terminals of rods and rod bipolar cells (Bech-Hansen et al., 1998). Given the intense investigative focus now being applied to the molecular biology of ribbon synapses, we expect that further photoreceptor dystrophies resulting from mutations in ribbon synapse-associated genes will come to light in the near future.

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Fig. 1.

(A) Schematic of a photoreceptor ribbon synapse visualized by freeze fracture. The fracture plane has split the plasma membrane, exposing its external face (below) and protoplasmic face (curving upward). Ribbon synaptic structures described in the text are labeled. Presumed (hence the question mark) calcium channels are concentrated in the synaptic ridge, which is that portion of the plasma membrane underlying the arciform density. After Raviola and Gilula (1975) with permission of the authors and the Rockefeller Press. (B) A photoreceptor ribbon synapse viewed by transmission electron microscopy. The electron dense synaptic ribbon is surrounded by a halo of synaptic vesicles connected to the ribbon by slender tethers. The arciform density is positioned between the base of the ribbon and the plasma membrane. Three post-synaptic processes (1, 2, 3) are closely apposed to the photoreceptor near the ribbon. Adapted from Schaeffer et al. (1982).

Fig. 2.

Possible fusion scenarios at a bipolar cell ribbon synapse. (A) A front-on schematic of a bipolar cell ribbon synapse at rest with five rows of vesicles tethered by fine filaments to each face of a synaptic ribbon. The bottom row of vesicles is in physical contact with the plasma membrane and may be considered “docked”. (B) Following the brief opening of voltage-gated calcium channels, the bottom row of vesicles fuses with the plasma membrane. (C) In response to a longer depolarization, entering calcium may build up at the active zone, releasing vesicles from their tethers. These vesicles may then move downward to their release sites. (D) In response to a high and sustained rise in intraterminal calcium, the bottom row of vesicles rapidly fuses with the plasma membrane. Vesicles at higher positions also sense the elevated calcium and fuse with their neighbors, participating in a wave of compound fusion.

Fig. 3. Depolarizing pulse stimulated a localized influx of calcium (arrow) into a rod synaptic terminal. Single confocal slice (55 ms frame) of a rod filled with the calcium-sensitive dye, Oregon Green 6F ($K_{Ca} = 20 \mu M$). (A) Control image and (B) image obtained during a 50 ms depolarizing pulse (-70 to -10 mV). Scale bar = $10 \mu M$.

Fig. 4.

(A) Opening of a single calcium channel recorded in cell-attached patch mode from a rod terminal using 82 mM Ba^{2+} as the charge carrier. (B) Voltage dependence of I_{Ca} in salamander rod photoreceptors. I_{Ca} was measured using a ramp voltage protocol, averaged from seven different rods, and fit with a Boltzmann function corrected for driving force ($V_{50} = -30$ mV, slope factor = -6.3). (C) Smoothed rod light response used as a voltage command waveform. (D) A comparison of the measured change in I_{Ca} (noisy trace) and the predicted I_{Ca} (solid line) when rods at -40 mV were modulated by the voltage command shown in (C). Passive membrane properties were subtracted using a P/8 protocol. Data in (B) and (D) were obtained from the same seven rods. Figure modified with permission from Thoreson et al. (2003b).