Published in final edited form as:

J Neurophysiol. 2006 October ; 96(4): 2025–2033. doi:10.1152/jn.00364.2006.

Diurnal Changes in Exocytosis and the Number of Synaptic Ribbons at Active Zones of an ON-Type Bipolar Cell Terminal

Court Hull¹, Keith Studholme², Stephen Yazulla², and Henrique von Gersdorff¹

¹The Vollum Institute, Oregon Health and Science University, Portland, Oregon

²Department of Neurobiology and Behavior, Stony Brook University, Stony Brook, New York

Abstract

The number and morphology of synaptic ribbons at photoreceptor and bipolar cell terminals has been reported to change on a circadian cycle. Here we sought to determine whether this phenomenon exists at goldfish Mb-type bipolar cell terminals with the aim of exploring the role of ribbons in transmitter release. We examined the physiology and ultrastructure of this terminal around two time points: midday and midnight. Nystatin perforated-patch recordings of membrane capacitance (C_m) revealed that synaptic vesicle exocytosis evoked by short depolarizations was reduced at night, even though Ca^{2+} currents were larger. The efficiency of exocytosis (measured as the ΔC_m jump per total Ca^{2+} charge influx) was thus significantly lower at night. The paired-pulse ratio remained unchanged, however, suggesting that release probability was not altered. Hence the decreased exocytosis likely reflects a smaller readily releasable vesicle pool at night. Electron microscopy of single sections from intact retinas averaged 65% fewer ribbons at night. Interestingly, the number of active zones did not change from day to night, only the probability of finding a ribbon at an active zone. Additionally, synaptic vesicle halos surrounding the ribbons were more completely filled at night when these ON-type bipolar cells are more hyperpolarized. There was no change, however, in the physical dimensions of synaptic ribbons from day to night. These results suggest that the size of the readily releasable vesicle pool and the efficiency of exocytosis are reduced at night when fewer ribbons are present at bipolar cell terminal active zones.

INTRODUCTION

Sensory synapses that contain active zone specializations called synaptic ribbons typically operate using continuous, graded membrane potential changes. Synaptic ribbons are osmiophilic, proteinaceous sheets or spheres directly adjacent to the active zone and typically have a closely associated halo of clear-core synaptic vesicles (guinea pig photoreceptors, Sjöstrand 1958; rat bipolar cells, Ladman 1958; Lenzi and von Gersdorff 2001). It has long been suspected that synaptic ribbons play a direct role in exocytosis, and their proximal association with sites of calcium influx and vesicle release has provided good evidence for this hypothesis (Issa and Hudspeth 1994; Zenisek et al. 2004). Despite this correlation, however, it has remained unclear how synaptic ribbons participate in exocytosis or whether plastic changes in these structures can alter synaptic function. In particular, it has been reported that exocytosis can occur at sites that are not associated with synaptic ribbons (frog hair cells: Lenzi et al. 2002).

Photoreceptor synaptic ribbons are highly dynamic structures that can change in number and shape in response to certain visual stimuli and/or during the diurnal/circadian cycle (reviewed by Vollrath and Spiwoks-Becker 1996). Recently, changes in mouse photoreceptor ribbon size with illumination have been observed (Spiwoks-Becker et al. 2004), and the highest visual threshold was observed at a time during the diurnal cycle when ribbons are the shortest (Balkema et al. 2001). In the fish retina, the number of synaptic ribbons in photoreceptor terminals changes on a circadian cycle (at night ribbons tend to disassemble) (Allwardt et al. 2001; Wagner 1975). By comparison, diurnal changes in bipolar cell ribbons have been little studied. If ribbons do play a role in synaptic vesicle exocytosis, perhaps by anchoring a readily releasable pool of vesicles (Khimich et al. 2005; Thoreson et al. 2004), it is likely that the release properties of a synapse undergoing dynamic ribbon changes also would be affected. Because the goldfish Mb-type bipolar cell terminal is large enough for direct patch clamp recording and can be acutely dissociated from its soma, it is ideal for measurements of synaptic vesicle exocytosis using time-resolved membrane capacitance measurements. Thus we chose to investigate whether ribbons change on a diurnal cycle at Mb bipolar cells, and how such changes might alter the properties of synaptic vesicle exocytosis. We used capacitance measurements to assay vesicle exocytosis at two time points in the diurnal cycle. To relate exocytosis to ribbon dynamics, we also used electron microscopy (EM) to track whether synaptic ribbons change on a diurnal cycle. Using perforated-patch recordings that do not dialyze the terminals, we find that the efficiency of exocytosis triggered by short depolarizing pulses (2 or 20 ms) is reduced at night, while EM studies of intact goldfish retinas revealed a significant decrease in bipolar cell synaptic ribbon numbers at night. These results suggest that a reduced number of synaptic ribbons at active zones may decrease the efficiency of exocytosis at bipolar cell terminals during nighttime.

METHODS

Goldfish (*Carassius auratus*) ~12–15 cm standard body length, with eyes 6–7 mm in diameter, were obtained from a commercial supplier. The goldfish were maintained at 22°C in aerated tanks filled with tap water circulating through a polyester fiber/charcoal filter system. They were kept on a 12-h on/ 12-h off light cycle. Animals were treated according to the guidelines of the National Institutes of Health and Association for Research in Vision and Ophthalmology.

“Day” fish were removed from their tanks at about noon, and all dissections and processing took place under room-light conditions. “Night” fish were maintained in the dark from 6 p.m. until midnight. For electron microscopy, all dissections took place under indirect darkroom-red safety light. Subsequent processing through fixation took place in the dark. The dark-adaptive state of the retina was confirmed histologically by the fully extended position of the cones and retracted position of rods that are observed during subjective night (Burnside and Nagel 1983; Malchow and Yazulla 1986). For electro-physiological experiments, retinas were exposed briefly to room light (<5 min) before dissociation of the bipolar cells and their presynaptic terminals.

Electron microscopy

Goldfish were cervically transected, and the eyes were removed and hemisected. Eyes from four fish were used for each condition (day and night). Eyecups were cut in half along the superior-inferior axis and placed vitreous-side down on Millipore filter paper that was positioned on a Swinnex-filter holder. Gentle suction was applied to remove adhering vitreous humor. This procedure improves the penetration of fixative into the inner retina from the vitreal margin. Retinas were fixed in mixed aldehydes (2.4% paraformaldehyde, 1% glutaraldehyde) for 12 h, rinsed, fixed in 1% OsO₄ for 1 h, dehydrated and embedded in

LX-112 epoxy resin. Silver/gold sections were collected on formvar-coated 1×2 -mm slot grids, counterstained with 0.1% lead citrate and 2% uranyl acetate in 70% ethanol, and viewed on a JEOL 1200EX electron microscope. Micrographs were photographed at a magnification of 12,000–15,000. Electron micrographs were printed on 8×10 -in paper from negatives. Prints that were selected for publication were scanned on an HP 5470C flatbed scanner and acquired as TIFF files at 600 dpi. Photoshop (v.6.0) was used to optimize all TIFF files for brightness and contrast and both Photoshop and Corel Draw (v.9.0) were used for the composition and labeling of figures.

Data acquisition

Synaptic terminals of Mb bipolar cells are in the most proximal inner plexiform layer and are 8–10 μm in diameter (Ishida and Stell 1980; Sherry and Yazulla 1993). Smaller profiles in a single section could represent a section through the peripheral portion of a Mb bipolar cell terminal or a full section through a cone bipolar cell terminal. As these cannot be differentiated without serial reconstruction, analysis was restricted to the large ($>8 \mu\text{m}$) terminals. All Mb bipolar cell terminals in a section were analyzed regarding properties of synaptic ribbons. The density of synaptic vesicles in the region of synaptic ribbons was determined using an acetate sheet with a grid of $1 \mu\text{m}^2$ placed over a synaptic ribbon in electron micrograph prints. The apex of the ribbon was positioned in one corner of a grid and vesicles only within the $1\text{-}\mu\text{m}^2$ grid were counted. Synaptic vesicles within the grid were marked with ink, tabulated and expressed as vesicles/ μm^2 . Counts were obtained for 21 Mb terminals for each of the day and night conditions. A two-tail *t*-test was used to determine statistical significance.

Electrophysiology

Bipolar cell terminals from the goldfish retina were acutely dissociated according to Heidelberger and Matthews (1992). All recordings were performed within 2–4 h of plating. Recordings at “day” were from 12:00 to 4:00 pm, and at “night” from 12:00 to 4:00 am. Retinal slice preparation and optics follow the methods of Palmer et al. (2003b). Dissociated terminals were plated on a glass cover slip and visualized under a Zeiss Axioscope 2FS upright microscope (Zeiss, Germany) using standard DIC optics and a CCD camera (Hamamatsu, Japan). In the retinal slice, bipolar cell terminals with severed axons were identified in the inner plexiform layer based on the resting membrane time constant (single exponential for isolated terminals), the presence of an L-type calcium current and C_m jump, and Mb shaped terminal morphology (Palmer et al. 2003b). Picrotoxin (50 μM , Sigma) was included in all slice recordings to block GABAergic input from amacrine cells (Hull and von Gersdorff 2004).

The external recording solution contained (in mM) 120 NaCl, 2.5 KCl, 2.5 CaCl_2 , 1.0 MgCl_2 , 10 HEPES, and 12 glucose (pH 7.2; osmolarity: 260–265). Patch pipettes were pulled from leaded capillary glass with a PP-830 Narishige vertical puller and coated with dental wax to reduce pipette capacitance. Internal pipette solutions contained: 110 Cs-Gluconate, 15 TEA-Cl, 25 HEPES, 3 Mg-ATP, 0.5 Na-GTP, and 0.5 EGTA. Cesium gluconate internals were prepared from purified cesium gluconate salt extracted from CsOH and glu-conic acid according to a protocol provided by Dr. J.G.G. Borst (Erasmus University, Rotterdam). All internal solutions were set to pH 7.2 with CsOH and an osmolarity of 260 (checked with a vapor pressure osmometer, Wescor, Vapro 5520). Perforated-patch recordings were made with nystatin (250 $\mu\text{g/ml}$, 0.4% DMSO). All recordings were performed with a digital manometer (WPI) attached to the pipette holder tubing for recording and controlling pipette pressure. Pipette pressure was kept slightly negative (–0.01 psi) to achieve the most reproducible endocytosis (Heidelberger et al. 2002).

Isolated bipolar cell terminals were voltage-clamped in the whole cell or perforated patch mode using a HEKA EPC-9 double patch-clamp amplifier in conjunction with Pulse software running the X-chart extension (Pulse v. 8.53). The Sine+DC technique was used for real-time measurements of membrane capacitance (C_m). Briefly, a 30-mV peak-to-peak 1-kHz sine wave was superimposed on the cells' holding potential (-60 mV) and used by on-line analysis software to calculate time-resolved membrane capacitance. Off-line data analysis was performed using "IgorPro" software (v. 4.07, Wavemetrics, Lake Oswego, OR). After a C_m jump, slow (>0.5 s) changes in membrane capacitance due to endocytosis were fit with exponentials according to the criteria of Hull and von Gersdorff (2004). Series resistance (R_s) averaged 14.4 ± 1.4 M Ω ($n = 10$) in the whole cell mode and 28.6 ± 2.0 M Ω ($n = 10$) under nystatin perforated patch (averaged over the entire course of the recording period). Statistics were calculated using Prism (v. 3, GraphPad Software) with two tailed t -test to compare two data sets and one-way ANOVAs for comparison of three or more data sets. Data are reported as means $\pm$ SE.

RESULTS

Membrane capacitance measurements

To determine whether exocytosis or endocytosis changed from day to night, we performed membrane capacitance (C_m) measurements from Mb bipolar cell terminals during both time periods. It is important to note that day retinas were briefly dark adapted (20 min) to allow removal of the retina without pigment epithelium from the eyecup, though they were then subjected to continuous light during dissection and preparation of the dissociated terminals. Night retinas were exposed to dim light for ~ 5 min during the dissection. Therefore this study was intended to explore only the effect of any diurnal/circadian changes in synaptic ribbon number independent of brief changes in ambient light. Because the number of synaptic ribbons in zebrafish and carp photoreceptors change on a circadian cycle regardless of dark adaptation or light exposure (Allwardt et al. 2001; Vollrath and Spiwoks-Becker 1996), we felt this was an adequate paradigm for our experiments. Therefore the possibility of short-term ribbon modulation due to brief light/dark exposure is not addressed here.

We first tested the exocytosis responses of acutely dissociated terminals (axon severed) at night under whole cell mode. When terminals were depolarized to elicit calcium entry via the L-type calcium current ($I_{Ca^{2+}}$) and exocytosis (membrane capacitance jumps, ΔC_m), there was no significant difference in the amplitude of $I_{Ca^{2+}}$ or ΔC_m at night from what we have previously found during the day [day: $I_{Ca^{2+}} = 270 \pm 22$ pA, $\Delta C_m = 167 \pm 14$ fF, $n = 23$ (see also Hull and von Gersdorff 2004); Night: $I_{Ca^{2+}} = 235 \pm 36$ pA, $\Delta C_m = 183 \pm 24$ fF, $n = 6$; $I_{Ca^{2+}}$: $P = 0.09$, ΔC_m : $P = 0.85$; depolarizing pulses from -60 to 0 mV for 200 ms]. Similarly, we recorded from isolated bipolar cell terminals in the retinal slice during the night in whole cell mode. Even though these experiments were performed in the intact retinal slice and therefore received constant illumination under the recording microscope, only isolated terminals that had their axon cut during the slicing procedure were studied. Thus these terminals did not receive light input from their normal direct pathways over the course of recording. They could, however, have been affected by indirect synaptic pathways or diffuse modulators activated by light. In the retinal slice under whole cell mode recording conditions, there was no difference in the amplitude of calcium currents or membrane-capacitance jumps at night after 200-ms depolarizations from what we observed during the day (day: $I_{Ca^{2+}} = 296 \pm 20$ pA, $\Delta C_m = 205 \pm 14$ fF, $n = 35$; night: $I_{Ca^{2+}} = 298 \pm 80$ pA, $\Delta C_m = 206 \pm 44$ fF, $n = 7$; $I_{Ca^{2+}}$: $P = 0.93$, ΔC_m : $P = 0.42$).

Although we did not find any differences from day to night in the amplitude of $I_{Ca^{2+}}$ or exocytosis in whole cell mode recordings from acutely dissociated terminals or isolated terminals in slices, it is possible that whole cell dialysis masked exocytosis differences by

washing out either the endogenous calcium buffer or another mobile factor that affects exocytosis. Because the endogenous calcium buffer is thought to lie in the range of 2 mM EGTA (Burrone et al. 2002; Hull and von Gersdorff 2004), our standard use of 0.5 mM EGTA in whole cell patch pipettes may have allowed an elevated exocytosis response that minimized the importance of any changes in ribbon number. Thus to maintain physiological Ca^{2+} buffers and other intracellular constituents intact, exocytosis was next tested by eliciting voltage-clamp depolarizations under the nystatin perforated-patch mode of recording.

Terminals were depolarized for 2, 20, or 200 ms from -60 to 0 mV to elicit I_{Ca}^{2+} and exocytosis (ΔC_m ; see Table 1). For all depolarization lengths, the average peak calcium current was significantly larger at night than during the day ($*P < 0.05$). The integrated calcium charge (Q_{Ca}^{2+}), however, was statistically larger only for 200-ms depolarizations ($P = 0.03$). Despite having consistently larger peak calcium currents, terminals at night did not have a corresponding increase in exocytosis. For 2- and 20-ms depolarizations, the amount of exocytosis was actually reduced at night when the calcium currents were larger (though the difference in ΔC_m was only significant for 20-ms depolarizations, $P = 0.003$). For 200-ms depolarizations, there was no difference in exocytosis from day to night. The ΔC_m jumps (both night and day) after 200-ms depolarizations are similar to the ΔC_m jumps reported under nystatin-perforated patch from a previous study (Hull and von Gersdorff 2004).

Because the larger calcium currents at night produced smaller capacitance jumps for 2- and 20-ms depolarizations, the amount of exocytosis per calcium influx was reduced at night. We therefore divided each capacitance jump (ΔC_m) by its corresponding calcium charge (Q_{Ca}^{2+}) for every cell and averaged these values to obtain a measurement of exocytosis efficiency in the day versus night. Importantly, there was no difference in the size of acutely dissociated terminals from day to night (day: 2.9 ± 0.1 pF, night: 2.8 ± 0.2 pF). Based on this measurement, the efficiency of exocytosis was significantly reduced at night for 2- and 20-ms depolarizations (day, 2 ms: 96.9 ± 11.8 fF/pC, night, 2 ms: 47.6 ± 12.1 fF/pC, $P = 0.0076$; day, 20 ms: 13.2 ± 1.4 fF/pC; night, 20 ms: 5.2 ± 0.7 fF/pC, $P < 0.0001$; Fig. 1). The efficiency of exocytosis for 200-ms depolarizations was also reduced at night; however, this did not reach statistical significance (day: 2.1 ± 0.3 fF/pC; night: 1.6 ± 0.3 fF/pC, $P = 0.25$). Because 2- and 20-ms depolarizations are more selective for releasing vesicles docked near the plasma membrane in close association with both Ca^{2+} channels and synaptic ribbons, it is reasonable to expect these stimuli to be most sensitive to a reduction in ribbon number. Longer depolarizations of 200 ms, however, lead to increased calcium spread within the terminal, and could also stimulate ectopic release (Zenisek et al. 2002), or allow time for additional vesicles to reload the ribbons and thus make changes in ribbon-associated efficiency difficult to detect.

Apart from the change in exocytosis efficiency, we did not find any other difference in the release properties of this terminal measured under perforated patch (Fig. 2). Endocytosis was well fit by double exponentials with similar decay kinetics in both day- and nighttime recordings (Fig. 2, *A* and *B*). Similarly, neither paired-pulse depression nor recovery from paired-pulse depression was altered from night to day (Fig. 2*C*). This suggests that although the absolute efficiency of exocytosis was reduced under perforated patch recordings (less vesicles were released per calcium influx for each stimuli), the mechanism of vesicle replenishment at the membrane remained unchanged. Additionally, the averaged I - V relationships for L-type calcium currents (I_{Ca}^{2+}) did not change from night to day (Fig. 2*D*) nor did the activation kinetics of I_{Ca}^{2+} (day: I_{Ca}^{2+} $\tau = 789 \pm 7$ ms, $n = 10$; night: I_{Ca}^{2+} $\tau = 774 \pm 11$ ms, $n = 10$; $P = 0.92$).

Finally, because the expression of retinal calcium-binding proteins has been reported to change on a circadian cycle (Gabriel et al. 2004) and a change in calcium buffering could also explain our changes in exocytosis efficiency, we checked whether there was a difference in the calcium-activated, chloride tail current ($I_{Cl(Ca)}$) under nystatin-perforated patch from night to day. Comparing 10 terminals from each time with average peak calcium current amplitudes that were not statistically different (day: $I_{Ca} = 207 \pm 18$ pA, $n = 10$; night: $I_{Ca} = 249 \pm 20$ pA, $n = 10$; $P = 0.13$), we found no difference in the amplitude of $I_{Cl(Ca)}$ after 200-ms depolarizations (day: $I_{Cl(Ca)} = 31.1 \pm 2.5$ pA, $n = 10$; night: $I_{Cl(Ca)} = 34.0 \pm 3.4$ pA, $n = 10$; $P = 0.39$). The dependence of $I_{Cl(Ca)}$ on I_{Ca} was also the same from night to day for 20-ms depolarizations (data not shown) and could not be measured for 2-ms depolarizations due to the small size of $I_{Cl(Ca)}$ after these short pulses. The constant relationship between $I_{Cl(Ca)}$ and I_{Ca} from night to day suggests that calcium buffering does not change on a circadian time scale. However, although the amplitude of $I_{Cl(Ca)}$ has been shown to change after manipulations that significantly alter the mobile calcium-buffering capacity in bipolar terminals (Burrone et al. 2002; Hull and von Gersdorff 2004), perhaps this current is not an adequate measure of small changes in Ca^{2+} -buffer capacity. In addition, it is possible that the number of $I_{Cl(Ca)}$ channels also changes diurnally, offsetting a change in calcium buffering and thus making them unsuitable detectors. Because the identity of the endogenous calcium buffer(s) is not known at this terminal, we did not attempt to histologically stain for changes from day to night, though this approach would likely be necessary for a conclusive assessment of any role for diurnal calcium buffer changes.

Ultrastructure of synaptic ribbons

Using electron microscopy (EM), synaptic ribbon contacts of fish bipolar cells were defined as per Witkovsky and Dowling (1969). Ribbons appear as a flattened disk, ~50 nm thick, 170 nm high (extending into the cytoplasm), and 250 nm along the presynaptic ridge. In cross-section, synaptic ribbons appear as electron dense bars and/or pentalaminar structures associated with a halo of vesicles that are in immediate proximity to a presynaptic active zone. Two or three postsynaptic profiles are also clearly present with asymmetric postsynaptic densities. Single-electron micrographs through a giant Mb bipolar cell terminal of goldfish or carp rarely show more than two synaptic ribbons at a time (see for example, Marc et al. 1978; von Gersdorff et al. 1996; Witkovsky and Dowling 1969; Yazulla and Studholme 1992). Serial reconstruction of two isolated Mb bipolar cell terminals from goldfish yielded 45 and 65 synaptic ribbons (von Gersdorff et al. 1996). Our results are based on samples of 84 separate synaptic terminals from day fish and 117 separate synaptic terminals from night fish. Occasionally, three-section series were followed for individual synaptic ribbons.

With one exception, the general appearance of synaptic ribbons from day and night fish looked very similar (Figs. 3 and 4). In well-oriented sections, synaptic ribbons appeared as pentalaminar structures that extended into the cytoplasm, directly opposed to a presynaptic active zone. Other planes of section showed ribbons laid over to one side or en face. The major difference in ribbon appearance was that night ribbons were often less electron dense (Fig. 4, *A* and *D*). The halo of vesicles was still present, but the ribbon was less prominent. This was a characteristic of an individual ribbon that remained consistent through several sections. Not all night ribbons had this semi-translucent appearance; typical electron density was observed in other ribbons of night Mb terminals (Fig. 4*B*).

Quantitative analysis showed that the height of synaptic ribbons (extension into the cytoplasm from the active zone) was the same in day and night fish [$n = 25$; 169 ± 8.2 vs. 183 ± 12.2 (SE) nm, respectively; $t = 0.97$, $P > 0.33$]. The equivalence of ribbon size in day and night fish simplified the ribbon-frequency analysis per Mb terminal because the probability of encountering a ribbon in a single section of an Mb terminal was the same for

both conditions. Far fewer synaptic ribbons were observed in a single section of an Mb terminal in the night condition compared with the day. A frequency histogram (Fig. 5) shows that 75% (63/84) of day terminals had at least one ribbon while only 27% (32/117) of night terminals had at least one ribbon. There were 88 ribbons in the 63 day terminals with ribbons and 38 ribbons in the 32 night terminals with ribbons. A χ^2 test that showed the two distributions in Fig. 5 to be significantly different ($P < 0.001$). There was thus about a 65% reduction in the observed frequency of synaptic ribbons/Mb terminal at night compared with daytime. Given that there were fewer ribbons in night terminals, the question arose as to whether there were also fewer active zones. In serial sections, a synaptic ribbon rarely appears in more than three 80-nm-thick sections; usually, in a subsequent section an active zone will be visible in the absence of the ribbon. As such, there is a lower probability of encountering a ribbon at a ribbon synapse than the active zone that is associated with the ribbon. Thus in a single section of a “ribbon synapse,” the ribbon plus active zone would be expected to be visible in 75% of the cases and just the active zone in the remaining 25%. An active zone was identified by the presence of two postsynaptic densities in a typical dyad arrangement, perhaps with synaptic vesicles, except that there was no ribbon as illustrated in Fig. 4D (▲). All of the Mb terminals were analyzed to determine the percentage of active zones that were accompanied by ribbons in day terminals compared with night terminals. During the day, 74% of the active zones were associated with a synaptic ribbon, whereas at night only 43% of the active zones were associated with a synaptic ribbon. The 74% value observed in day terminals is consistent with what would be expected from the serial section samples as indicated in the preceding text. However, the 43% value observed in night terminals is far below this expectation and is consistent with a decrease in the number of synaptic ribbons at night. Recall that the size of the ribbons was the same in day and night and thus the probability of encountering a synaptic ribbon in a single section was the same for each condition. If we combine the active zones with synaptic ribbons and the active zones without ribbons, 86% of day terminal sections had at least one active zone, whereas 77% of night terminals had at least one active zone. The frequency histograms of active zones were not significantly different as determined by a χ^2 test ($P > 0.25$), indicating that day and night terminals differed with respect to the number of anchored synaptic ribbons but not the number of active zones.

The halo of vesicles surrounding ribbons also appeared more organized in night terminals. Vesicle halos were rated as disorganized, moderate, and very organized on a scale of 0, 1, 2 as illustrated in Fig. 6. Quantification of halo organization (Fig. 6) shows that halos were far more regular in night terminals than day terminals; the distributions are near mirror images of each other. For example, night terminals had very few (5%) disorganized terminals, whereas day terminals had few (11%) organized terminals. These distributions differed significantly as determined by a χ^2 test ($P < 0.001$).

In addition to a more orderly array of vesicles around the synaptic ribbon, there was a higher density of vesicles around ribbons in Mb terminals from night retinas. The density of synaptic vesicles within $1 \mu\text{m}^2$ of a synaptic ribbon was significantly greater ($P < 0.01$; $n = 21$) for the night retinas [62 ± 2.9 (SE) vesicles/ μm^2] compared with day retinas (50 ± 3.6 vesicles/ μm^2). This result, and the finding that day terminals had fewer organized terminals, may reflect the tendency of ON-type bipolar cells to be more depolarized during the daytime and thus more depleted of synaptic vesicles near their ribbons.

DISCUSSION

Given the mounting evidence that retinal synaptic ribbons are dynamic structures controlled by diurnal/circadian rhythms (reviewed by Vollrath and Spiwoks-Becker 1996; Wagner 1997), we attempted to correlate the physiology of ribbon-containing Mb bipolar cell

terminals with the ultrastructure of their ribbons during the day and at night. ΔC_m measurements showed that these terminals are capable of producing robust exocytosis and endocytosis during both time periods, and long depolarizing pulses of 200 ms did not reveal any differences in exocytosis or endocytosis from day to night. Exocytosis, however, was significantly less efficient at night when measured using shorter depolarizations. The decrease in exocytosis efficiency was thus apparent only for short depolarizations. This difference may result from the selectivity of shorter depolarizations for releasing ribbon-associated vesicles docked close to the plasma membrane (Mennerick and Matthews 1996; von Gersdorff et al. 1998), whereas longer depolarizations may release nonribbon associated docked vesicles (“outliers” that fuse at nonpreferred sites of the plasma membrane) (Zenisek et al. 2002).

Past studies at the goldfish Mb bipolar cell terminal have shown (and/or estimated) widely varying total numbers of ribbons per synaptic terminal depending on the technique used (45 and 65 ribbons for 8- and 10- μ m diameter terminals, respectively, von Gersdorff et al. 1996; 90 ribbons, Marc and Liu 2000; 30 ribbons, Llobet et al. 2003; 30 ribbons, TIRF microscopy with whole cell dialysis of fluorescent peptides, Zenisek et al. 2004). Some of these estimates are derived from intact, fixed retinas (EM studies) and some from acutely dissociated whole cell terminal recordings (TIRF studies). Because whole cell recordings can disrupt endogenous Ca^{2+} buffers and are subject sometimes to rapid rundown of exocytosis and endocytosis (e.g., see Fig. 8A of Hull and von Gersdorff 2004), it is perhaps not surprising that there is some disparity between EM and TIRF microscopy estimates. This is why we have chosen to concentrate the results of this study almost exclusively on perforated-patch recordings and EM studies of intact, fixed retinas, because these methods may be less disruptive to synaptic ribbons.

Diurnal synaptic ribbon plasticity

The first evidence of circadian ribbon plasticity came from guinea pig pinealocytes (Vollrath 1973), and later reports have showed the same phenomena in goldfish pinealocytes (McNulty 1981). In these cells, however, synaptic ribbons consistently disappear during the day rather than at night. Interestingly, ribbons detach from squirrel photoreceptor terminals during hibernation and then reattach after hibernation ends (Reme and Young 1977). Studies from zebrafish retina have shown a circadian regulation of photoreceptor synaptic ribbon number, with large decreases in ribbons at night (disassembly) and reassembly during the day (Allwardt et al. 2001). In the teleost fish, *Nannacara anomala* (Wagner 1973), the number of synaptic ribbons in cone pedicles decreased by 80% after dark-adaptation, a reduction later shown to follow a circadian rhythm (Wagner 1975). Also, in a photomontage of the inner plexiform layer, the number of synaptic ribbons in bipolar cell terminals as a group decreased by ~66% in daytime dark-adapted *Nannacara* (Wagner 1973). This early report is virtually identical to the 65% reduction in synaptic ribbons we observed in a larger sample of identified goldfish Mb terminals at nighttime in EM single sections. We suggest that a reduction in cone ribbon number and function during the night (or during hibernation) may be evolutionarily advantageous for the conservation of metabolic energy because the continuous exocytosis of glutamate at night (when an animal is sleeping) may be devoid of useful visual information.

The study of Allwardt et al. (2001) found that the cone pedicles of wild-type zebrafish larva lost their synaptic ribbons at night and that a few hours of light or dark adaptation was not enough to change this circadian ribbon plasticity. Similarly, 24 h of constant light or constant darkness did not reverse the circadian pattern of ribbon formation during the day and degradation at night. In our EM results, we did not find a complete loss of synaptic ribbons at night. Moreover, we emphasize that Mb bipolar cells are mixed bipolar cells that receive both rod and cone input. Unlike rods and cones, they thus need to operate during the

day and the night. Thus it is perhaps unsurprising that bipolar cell ribbons do not completely disappear at night. In summary, the diurnal ribbon loss at Mb terminals we observed is less dramatic than what has been reported for zebrafish photoreceptors (Allwardt et al. 2001), and it may thus require a very sensitive and less disruptive detection method such as perforated-patch recordings and EM to be observed.

Synaptic ribbons and Ca^{2+} channels

Synaptic ribbons are known to be located near clusters of Ca^{2+} channels (Issa and Hudspeth 1994). Evanescent wave microscopy has shown that the sites with synaptic ribbons and “hot-spots” of calcium influx are also the preferred sites of vesicle exocytosis at the Mb bipolar cell terminal (Zenisek et al. 2003). For this reason, it has remained an attractive idea that the synaptic ribbons serve to couple release-competent vesicles with highly localized Ca^{2+} influx. Accordingly, photoreceptor ribbons become unanchored in mice lacking the $\beta 2$ subunit of the L-type Ca^{2+} channel (Ball et al. 2002). In addition, it has recently been shown that reserve synaptic vesicles move freely throughout ribbon-type terminals (Holt et al. 2004; Rea et al. 2004), a finding that may suggest a need for a presynaptic specialization that catches vesicles and traps them near release sites in preparation for fusion with the plasma membrane. At this synapse, as at most ribbon-type synapses, there is an extremely large reserve pool of vesicles. Sequestering a uniform number of these vesicles near release sites could therefore serve to normalize the amount of release (and/or allow for a transient component of release) at a terminal that would otherwise experience a much broader profile of vesicle fusion events. Our EM and nystatin results support the idea that ribbons aid in the coupling of release machinery with release-competent vesicles because exocytosis efficiency is decreased when fewer ribbons are present at night.

Despite the changes in exocytosis efficiency, we did not observe any difference in paired pulse depression (or ratio) or recovery from paired pulse depression from day to night (Fig. 2, *C* and *D*). These results thus suggest that there was no change in release probability and/or the rate of vesicle delivery to the membrane; rather, only the size of the readily releasable pool and the efficiency with which vesicles were released was changed at night when there were fewer synaptic ribbons. Indeed, if we assume that a 20-ms pulse to 0 mV depletes the readily releasable pool of vesicles (Mennerick and Matthews 1996) perhaps corresponding to the bottom row of vesicles in the ribbon (von Gersdorff et al. 1996), we observed a significantly smaller capacitance jump (46%) at night for 20-ms depolarizing pulses, while our EM analysis indicated on average 65% fewer ribbons at night.

We also observed significantly larger Ca^{2+} current amplitudes during nighttime as compared with daytime recordings (1.5-fold larger at night; Table 1). Interestingly, a diurnal modulation of Ca^{2+} current amplitudes was also observed for neurons in the mammalian suprachiasmatic nucleus (1.7-fold larger Ca^{2+} currents during the daytime) (Pennartz et al. 2002), and a majority of GABAergic synapses in the suprachiasmatic nucleus exhibit paired-pulse depression during the day but not during the night (Gompf and Allen 2004). However, the shape of the Ca^{2+} current I - V curve did not change from night to daytime in the Mb bipolar cell terminal (Fig. 2*D*). Changes in Ca^{2+} currents can also occur because of external pH changes (Palmer et al. 2003a), which do occur during the circadian cycle of the goldfish retina (Dmitriev and Mangel 2000), but these should not be present in our acutely dissociated bipolar cell terminals in HEPES-based external solutions.

The EM results also showed qualitative differences in the structure of ribbons from day to night with more ribbons appearing electron translucent at night. This change in ribbon structure may reflect an intermediate stage of ribbon disassembly, consistent with the reduced number of ribbons at night. It is also noteworthy that the number of active zones did not change at night, but the probability of finding a ribbon at an active zone was reduced.

Therefore we do not suggest a change in the number of synapses per se, only that there is a loss of ribbons at some active zones. Additionally, vesicle halos were more organized and complete at night, though this difference likely reflects the tendency of *ON*-type bipolar cell terminals to be more depolarized during the day (or under lighted conditions) and undergoing continuous, high levels of exocytosis and vesicle recycling. Thus ribbons partially denuded of vesicles appear to be more prominent during daytime (compare Fig. 3 with Fig. 4). Likewise, denuded ribbons are also found at fish electroreceptors after intense stimulation that depresses the synapse (Fields and Ellisman 1985), and a depletion of docked vesicles and tethered vesicles has also been observed at hair cell dense bodies after strong and prolonged depolarizations (Lenzi et al. 2002).

Synaptic ribbons and the efficiency of exocytosis

Recent studies of mutant zebrafish indicate that ribbons anchored to active zones are necessary for normal photoreceptor function and morphology (Van Epps et al. 2004). Indeed, zebrafish with a severe deficiency in the ribbon specific protein RIBEYE display dramatic physiological deficits in optokinetic responses, increased retinal apoptosis, and a loss of large synaptic terminals (Wan et al. 2005). Similarly, mice that have the scaffolding protein Bassoon knocked-out lack active-zone anchored photoreceptor ribbons and exhibit a greatly reduced b-wave ERG (Dick et al. 2003). Somewhat in contrast, the Bassoon KO-mouse cochlear inner hair cell still produces robust exocytosis in response of long depolarizations (Khimich et al. 2005). Likewise, for 200-ms depolarizations, we observed robust C_m jumps at night and day times (Fig. 1A). Interestingly, however, the hair cell Bassoon mutants have a reduced capacity for fast, synchronous exocytosis and show a reduction in the efficiency of exocytosis following short depolarizations of the same type we report here (Fig. 1B). Furthermore, inner hair cells undergo dramatic changes in the number and shape of synaptic ribbons during postnatal development, and this is paralleled by an increase in the efficiency of exocytosis with age (Beutner and Moser 2001; Johnson et al. 2005). Therefore we suggest that ribbons do play an important role in sensory physiology, and larger-scale ribbon changes may reveal a more significant change in the efficiency of synaptic vesicle release. Although we were limited by the requirements of patch-clamp techniques to use the giant Mb terminals from goldfish retina, a future study at zebrafish cone photoreceptors, where stronger circadian rhythms lead to a more complete ribbon loss (Allwardt et al. 2001), would perhaps be useful in further elucidating the relationship between synaptic ribbons and exocytosis.

Acknowledgments

We thank D. Zenisek (Yale University) and K. Matsui (National Institute for Physiological Sciences, Okasaki) for helpful discussions.

GRANTS C. Hull was supported by National Institutes of Health (NIH) Grant NRSA NS-42506, S. Yazulla was supported by NIH Grant EY-001682, and H. von Gersdorff was supported by a Human Frontier Science Program grant and NIH Grant EY-14043.

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FIG. 1.

The efficiency of exocytosis is reduced in nighttime recordings. *A*: typical nystatin perforated patch recordings from acutely dissociated bipolar cell terminals during the day (1:45 p.m., resting $C_m = 3.3$ pF) and night (3:25 a.m., resting $C_m = 2.4$ pF). L-type calcium currents were elicited by stepping the membrane voltage from -60 to 0 mV for 2, 20, or 200 ms. *B*: dividing the exocytosis (ΔC_m) by the calcium charge ($Q_{Ca^{2+}}$) yields a measure of release efficiency (fF of exocytosis per pC of calcium entry). This measurement shows a significantly higher efficiency of exocytosis during the daytime for the short 2 and 20 ms depolarizations (*, $P < 0.05$). There is no statistical difference for the efficiency of exocytosis between day and nighttime recordings for depolarizations of 200 ms. Error bars represent SE.

FIG. 2.

Endocytosis, recovery from paired-pulse depression, and the L-type calcium current I - V relationship are the same during nighttime and daytime recordings in nystatin perforated-patch. **A:** endocytosis recorded from 1 nighttime (black, 1:47 AM, resting $C_m = 3.02$ pF) and 1 daytime (red, 1:18 PM, resting $C_m = 3.75$ pF) terminal after successive 200-ms depolarizations from -60 to 0 mV. **B:** average kinetics of endocytosis after 200-ms depolarizations were the same during the day- and nighttime. Endocytosis traces were fit with double exponentials (day: $\tau_{\text{fast}} = 2.0 \pm 0.3$ s, 52% of ΔC_m , $\tau_{\text{slow}} = 18.6 \pm 2.8$ s, $n = 17$; night: $\tau_{\text{fast}} = 1.4 \pm 0.1$ s, 51% of ΔC_m , $\tau_{\text{slow}} = 15.7 \pm 2.1$ s, $n = 9$). The kinetics of endocytosis were also the same during the day- and nighttime for 2- and 20-ms depolarizations (not shown, 2-ms endocytosis: day: $\tau_{\text{fast}} = 1.1 \pm 0.4$ s, 32% of ΔC_m , $\tau_{\text{slow}} = 15.3 \pm 2.5$ s, $n = 4$; night: $\tau_{\text{fast}} = 1.3 \pm 0.3$ s, 26% of ΔC_m , $\tau_{\text{slow}} = 15.1 \pm 4.8$ s, $n = 5$; 20-ms endocytosis: day: $\tau_{\text{fast}} = 1.5 \pm 0.4$ s, 34% of ΔC_m , $\tau_{\text{slow}} = 21.6 \pm 4.2$ s, $n = 5$; night: $\tau_{\text{fast}} = 1.8 \pm 0.3$ s, 38% of ΔC_m , $\tau_{\text{slow}} = 26.6 \pm 5.9$ s, $n = 5$). Error bars represent SE. **C:** recovery from paired-pulse depression was measured for several interpulse intervals (interpulse interval, number of cells day/night: 50 ms, 6/5; 100 ms, 8/7; 250 ms, 7/6; 500 ms, 9/7; 1 s, 9/7; 3 s, 9/4; 5 s, 9/6; 7 s, 8/2; 10 s, 8/5; 15 s, 7/6). Although the curves diverge at the 15-s interpulse interval, there is no statistical difference between those night and day points ($P = 0.06$). Both curves are double exponential (day: $\tau_{\text{fast}} = 359$ ms, 67% of recovery, $\tau_{\text{slow}} = 2.9$ s; night: $\tau_{\text{fast}} = 421$ ms, 64% of recovery, $\tau_{\text{slow}} = 7.0$ s). **D:** typical example of paired-pulse depression from this synapse at night (2:10 a.m.) and the averaged L-type calcium current I - V relationships for night- and daytime recordings. Note that both the daytime and nighttime averaged I - V curves peak near 0 mV.

FIG. 3.

Electron micrographs illustrate the variety of appearances of synaptic ribbons ($\rightarrow$) in Mb bipolar cells terminals from “daytime” retinas. Classically oriented ribbons at a dyad (A) were relatively rare. More often ribbons appeared either laid over at the dyad (B), as an electron dense “smear” (C), or even without associated synaptic vesicles (D). A regular array of vesicles usually was not associated with the ribbons. Calibration bar = 0.25 μm .

FIG. 4.

Electron micrographs illustrate the variety of appearances of synaptic ribbons (→) in Mb bipolar cells terminals from “midnight” retinas. They had the same overall appearance and size of daytime ribbons. However, the electron density of ribbons at a dyad varied from quite dense (*B*) to very light (*A* and *D*) and intermediate (*C*). A regular array of vesicles usually was associated with the ribbon. This halo of synaptic vesicles frequently appeared to be tethered to the ribbon by thin filaments. ▲, putative active zone without a presynaptic ribbon. Note the uniform space of the synaptic cleft, the postsynaptic thickening, and fuzz between the BC terminal and what is likely a ganglion cell dendrite. Calibration bar = 0.25 μm .

FIG. 5.

This histogram illustrates the relative frequency of synaptic ribbons in Mb bipolar cells obtained from midnight and daytime retinas. What is represented on the abscissa is the number of synaptic ribbons that appeared in a single electron micrograph of the full extent of an Mb terminal. A higher percentage of daytime terminals had at least one synaptic ribbon compared with midnight terminals, most of which had no synaptic ribbons. These distributions were significantly different as determined by a χ^2 test ($P < 0.001$).

A Distribution of the relative organization of vesicular halo

B

Vesicular halo classes from least (0) to most (2) organized.

FIG. 6.

There were differences in the relative organization of the vesicular halo around synaptic ribbons between midnight and daytime terminals. *A*: histogram showing that daytime terminals have very few regular halos and a larger percentage of disorganized halos. Midnight terminals, however, had very few disorganized halos and a larger percentage of organized halos. These distributions were significantly different as determined by a χ^2 test ($P < 0.001$). *B*: electron micrographs illustrate the rating of a vesicular halo from least organized (0) to most organized (2).

TABLE 1

Day- and nighttime recordings in nystatin perforated patch mode

Depolarization	ΔC_m , fF	Peak $I_{Ca^{2+}}$, pA	$Q_{Ca^{2+}}$, pC	<i>n</i>
2 ms				
Day	30.2 ± 3.2	172.9 ± 10.4	0.32 ± 0.02	13
Night	22.7 ± 4.1	265.8 ± 34.8*	0.41 ± 0.05	12
<i>P</i> value	0.1593	0.0144	0.0986	
20 ms				
Day	50.1 ± 4.8	189.9 ± 8.1	3.81 ± 0.87	21
Night	27.3 ± 3.1**	276.4 ± 18.4*	5.42 ± 0.37	15
<i>P</i> value	0.0003	<0.0001	0.1450	
200 ms				
Day	80.2 ± 10.0	218.8 ± 16.8	41.61 ± 3.09	23
Night	87.1 ± 13.0	304.8 ± 34.1*	55.19 ± 6.08*	13
<i>p</i> value	0.6786	0.0159	0.0334	

Averaged exocytosis (ΔC_m) peak calcium current ($I_{Ca^{2+}}$) calcium charge ($Q_{Ca^{2+}}$ integral of the calcium current), and the number of terminals (*n*) recorded with nystatin perforated-patch mode during day- and nighttime. Voltage-clamp depolarizations were elicited from a holding potential of −60 to 0 mV for 2, 20, or 200 ms. For each depolarization, the peak calcium current was significantly larger for the set of terminals recorded at night (**P* < 0.05). For 2- and 20-ms depolarizations, the average ΔC_m was larger during the day than at night although this difference was only significant at 20 ms (***P* = 0.003). Averages have been rounded to the nearest significant digit.