Ultrastructural Changes of Photoreceptor Synaptic Ribbons in Relation to Time of Day and Illumination

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Purpose. Electron microscopic sections through rod and cone ribbon synapses reveal mainly rodlike synaptic ribbon profiles, but a few unusual spherical and club-shaped profiles also occur. To elucidate the meaning of the latter two forms, the authors have investigated these ribbon synapses at different times during the 24-hour cycle and under various lighting conditions.

Methods. The various types of ribbon profiles were counted, and their sizes were measured by means of transmission electron microscopy in retinas of male BALB/c mice exposed to 12 hours light (lights on at 6 AM) and 12 hours dark (LD 12:12), continuous light, or continuous darkness for 4 days.

Results. A 24-hour study of mice exposed to LD 12:12 showed that spherical and club-shaped profile numbers ranged from 0% to 29%, depending on the time of day. They reached a maximum at 3 hours after light onset, followed by a gradual decrease to approach zero at night and reappearing after light onset the next morning. After 4 days of continuous light, the spherical profiles were significantly decreased in number (examined at 9 AM). After continuous darkness, the spherical and club-shaped profiles were significantly reduced in number. Administration of 4 hours of light after 92 hours of continuous darkness restored the number of spherical and club-shaped profiles to normal values. The rodlike ribbon profiles were found to be longer in darkness than in light. In rod terminals containing spherical profiles, the rodlike ribbon profiles were shorter than in terminals without spherical profiles.

Conclusions. The club-shaped and the spherical profiles were related to the turnover of the synaptic ribbons. Soon after light exposure in the morning, the synaptic ribbons formed distal swellings, giving rise to club-shaped profiles and a decrease in length. The swellings appeared to bud off, thus forming spherical synaptic bodies. This article discusses whether these changes are signs of degradation of spent ribbons, or whether they play a physiological role related to the inactivation of the ribbon synapses after light exposure. (Invest Ophthalmol Vis Sci. 1999;40: 2165–2172)
In addition to spherical profiles, club-shaped profiles are often present in which a rodlike profile has a distinct swelling at its distal end. Their presence poses the question of whether the swelling is the result of a fusion process of spherical synaptic bodies with platelike ribbons or whether they are signs of budding. It has been hypothesized that club-shaped synaptic ribbon profiles are signs of synaptic ribbon degradation.

To shed more light on the significance of the spherical and the club-shaped synaptic bodies in the retina, we studied these forms over a period of 24 hours and under different lighting conditions. We used BALB/c mice, because a preliminary interspecies comparison in this laboratory had shown that these unusual synaptic body profiles were most abundant in this mouse strain.

**Materials and Methods**

**Animals**

All experimental procedures conformed with the ARVO Resolution for the Use of Animals in Ophthalmic and Vision Research. Male BALB/c mice (n = 87; 25 g body weight) were kept under constant laboratory conditions (12 hour light, 12 hour dark [LD 12:12]; lights on at 6 AM; fluorescent strip lights providing 100 lux at the bottom of the cages; room temperature 21 ± 2°C; 60% relative humidity; food and water ad libitum) for 2 weeks before the experiments. The animals were anesthetized with ether and killed by decapitation at the times indicated, during darkness under dim red light.

In experiment 1, two groups of mice (n = 3 each) were killed at 9 AM (3 hours after lights on) or at midnight (after 6 hours in darkness). In experiment 2, mice (n = 35) were killed over a 24-hour period (n = 5 at 4-hour intervals; the 9 AM time point was repeated), and in addition in the early light phase killed 1 or 2 hours after lights on (at 7 AM or 8 AM, n = 3 per time point). In experiment 3, mice (n = 5) were exposed for 4 days to continuous room light. In experiment 4 mice were killed after 4 days of continuous darkness (n = 5) or after 92 hours of continuous darkness followed by 4 hours of room light (n = 5). In experiments 3 and 4, the experimental and the control animals (n = 5) were killed at 9 AM, with room lights on or under dim red light as required. The continuous-darkness experiment was repeated to verify the effects of the preceding experiment. Because there were no significant differences between the results of the two continuous-darkness experiments, the data were pooled.

**Electron Microscopy**

The eyes were rapidly removed and incised, and the retinas were taken out and fixed in fresh fixative (2% paraformaldehyde, 2.5% glutaraldehyde in 0.1 M phosphate buffer) for 15 hours. After a rinse in 0.1 M phosphate buffer containing 6.8% sucrose, the tissues were postfixed in 2% osmium tetroxide for 90 minutes, washed three times in 0.1 M phosphate buffer, dehydrated in a graded series of acetones, and flat embedded in Epon in such a way that transverse sections could be obtained through the retina. Ultrathin sections (50 - 60 nm) were cut on an ultratome (Reichert Jung, Vienna, Austria). They were mounted on one-hole Formvar-coated copper grids (Serva, Heidelberg, Germany). The sections were stained with 8% uranyl acetate (10 minutes), followed by lead citrate (according to Reynolds, 5 minutes). They were analyzed at primary magnifications of ×20,000 under an electron microscope (Model 109; Carl Zeiss; Oberkochen, Germany) with a morphomat (IDMS, IMA, Dortmund, Germany) attached. To avoid bias, the investigator was unaware of the exact experimental background of the material investigated.

**Ultrastructural Analysis**

One retina was quantitatively evaluated from each animal; in pilot studies, both retinas were used. From one randomly selected retinal section, synaptic body profiles in 100 neighboring photoreceptor terminals were systematically examined according to the following criteria: type of synaptic body profile, location within the terminal, and size. The profiles were classified as “attached” when they bordered the presynaptic membrane, with the arciform density interposed. Profiles unrelated to the presynaptic membrane were referred to as “free.” Size measurements were performed for attached and free profiles. Because the latter probably represented cuts through the distal areas of the attached crescent-shaped synaptic ribbons and because their size varied greatly, depending on the cutting angle, only the measurements of the attached profiles are provided for the functional aspects of this study. The data obtained are expressed as means ± SEM.

**Statistical Analysis**

Statistical analysis was performed by Student’s t-test when the data were normally distributed and had equal variances. Otherwise, the Wilcoxon Mann-Whitney test was used. P ≤ 0.05 was regarded as significant.
RESULTS

General Observations

As described for other species, in both rod and cone terminals of BALB/c mice the most common forms of synaptic ribbon profiles were rodlike (Figs. 1, 2A). In rods, varying numbers of spherical (SS; Fig. 2C) and club-shaped profiles (CSR; Figs. 1, 2B) with a distally located swelling were found. In cones, spherical profiles but no club-shaped profiles were encountered. Rodlike and club-shaped profiles were mostly seen close to the presynaptic membrane, the spherical profiles usually lay distant from it. The size of the distal swelling of the club-shaped profiles corresponded to that of the spherical profiles (approximately 0.16 μm). Similar to the rodlike profiles, synaptic vesicles surrounded the spherical and the club-shaped profiles.

Frequency of Ribbon Types

Experiments 1 and 2 indicated that the number of spherical and club-shaped profiles seen within rods changed more than 20-fold in mice kept under standard lighting conditions of LD 12:12 (Figs. 3, 4). They were at their highest frequencies in the morning at 9 AM (Figs. 3, 4). At this time point, 32.4% of the rod terminals contained more than one synaptic profile. During the remainder of the light period, a steady decrease in spherical and club-shaped profile numbers was noted, reaching zero at night (Fig. 4). In the middle of the dark phase, as few as 2.8% of the rod terminals contained more than one synaptic profile. After light onset, the club-shaped profiles increased in number at 7 AM and the spherical profiles at 8 AM (Fig. 4). In cones, spherical profiles were relatively rare (between 1.0% and 6.9%) and seen only in the light phase at 9 AM and 5 PM. Club-shaped profiles were never encountered.

To study whether the differences described above depended on the time of day or on conditions of illumination, mice were exposed to various lighting regimes. Continuous illumination for 4 days (LL) or continuous darkness for 4 days (DD) strikingly reduced the amounts of spherical profiles in rods (Figs. 5, 6). The club-shaped profiles tended to increase in number after continuous illumination in rods but decreased significantly after continuous darkness (Figs. 5, 6). When continuous darkness for 92 hours was followed by 4 hours of light...
(DD+L), spherical and club-shaped profiles reappeared (Fig. 6). In cones, spherical profiles were seen only in the light-adapted control animals (1.8%), and in the DD+L animals (0.6%). Club-shaped profiles were not seen in cones in these experiments.

**Size of Synaptic Body Types**

Table 1 shows the mean lengths of rodlike profiles in rod and cone terminals at different times during the 24-hour cycle and under various lighting conditions. Rodlike profiles in rods of animals kept under LD 12:12 exhibited a significant increase in length (16%) in the dark phase. After continuous darkness for 4 days, they showed an increase compared with control animals, identical with that of animals killed in the dark phase of the 24-hour cycle. Continuous darkness followed by 4 hours of light significantly reduced the mean length to control values. After continuous light for 4 days, a slight decrease in length was noted, but the difference was not significant. In cones, the rodlike profiles were significantly reduced in size only in one experimental group, 5 AM.

Table 2 shows the lengths of rodlike profiles in rod terminals with no and with one or more spherical profiles. The comparison showed that at each of the three time points when spherical profiles were present in sufficient numbers to be evaluated, the length of the rodlike profiles was significantly reduced in terminals containing spherical profiles. Rodlike profile length was even shorter when two or more spherical profiles were present in the terminal.

The comparison of the diameters of the spherical profiles showed that they were always significantly larger in rods (mean ranging from 0.15 to 0.22 μm) than in cones (0.10–0.15 μm; P < 0.01). They revealed no significant differences in size under the various experimental conditions.

**DISCUSSION**

**Numerical Changes of Synaptic Body Types**

In ribbon synapses, the occurrence of spherical and club-shaped profiles has long been enigmatic, the main reason being that they have been so little studied. The present study showed that these unusual profiles are a regular feature in the retina of BALB/c mice, are present in relatively large numbers (up to 29% of all the synaptic profiles encountered), exhibit a prominent day–night rhythm, and can be experimentally manipulated. That these profile types exhibit differences in number during day and night in the rat has been mentioned before,27,31 but the picture emerging from previous data is not clear. In one study27 the synaptic bodies in question amounted to 37% at 4:30 PM and were absent at 1:30 AM. In the other study31 involving eight evenly spaced time points, spherical profiles were always present but were relatively low in number (10%–25%) at 12 AM, 6 PM, 9 PM, and 12 PM, suddenly interrupted by a strong peak (85%) at 3 PM, and intermediate in number (45%–60%) at 3 AM, 6 AM, and 9 AM. Thus, not only the number, but also the relationship to light and darkness differ in the two studies.
The day–night results obtained in the present study show a pattern of changes that supports the notion of a rhythm regulated by light. Thus, spherical and club-shaped profiles were absent, or low in number, throughout the dark phase and up to 1 hour after light onset, followed by a striking increase in number during the next 2 hours and a steady decline during the remainder of the light phase. Moreover, the profiles under consideration were very low in number after continuous darkness for 92 hours but increased strikingly when the dark period was followed by 4 hours of light. Additional evidence for an influence of light is that in *pearl* mice these profiles were present in light-adapted but absent in dark-adapted animals and that in rats they occurred after exposure to strong light. For turtle rods, it has been concluded that cyclic light–dark

**FIGURE 6.** Influence of continuous darkness (DD) for 4 days and DD followed by 4 hours of light (DD+L). Note the striking decrease of spherical (SS) and club-shaped (CSR) profiles under DD and the reversal to light–dark control (LD) levels after 4 hours of light. Each bar represents the mean from 10 animals. **P < 0.01.**

**TABLE 1.** Synaptic Ribbon Profile Length in Rods and Cones from Albino Mice (BALB/c) at Different Time Points of a 24-hour Period (Light/Dark 12:12 Hours; Experiments 1 and 2) and under Various Lighting Conditions (See Text; Experiments 3 and 4)

<table>
<thead>
<tr>
<th>Experiment (no.)</th>
<th>Time Point</th>
<th>Animals/Group</th>
<th>Rods (µm)</th>
<th>Cones (µm)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Day–night comparison (1)</td>
<td>9 AM</td>
<td>3</td>
<td>0.27 ± 0.02</td>
<td>0.28 ± 0.03</td>
</tr>
<tr>
<td></td>
<td>Midnight</td>
<td>3</td>
<td>0.28 ± 0.01</td>
<td>0.29 ± 0.03</td>
</tr>
<tr>
<td>24-Hour cycle (2)</td>
<td>9 AM</td>
<td>5</td>
<td>0.27 ± 0.01*</td>
<td>0.25 ± 0.03</td>
</tr>
<tr>
<td></td>
<td>1 PM</td>
<td>5</td>
<td>0.26 ± 0.01*</td>
<td>0.23 ± 0.03</td>
</tr>
<tr>
<td></td>
<td>5 PM</td>
<td>5</td>
<td>0.26 ± 0.02*</td>
<td>0.33 ± 0.04</td>
</tr>
<tr>
<td></td>
<td>9 PM</td>
<td>5</td>
<td>0.31 ± 0.02</td>
<td>0.28 ± 0.07</td>
</tr>
<tr>
<td></td>
<td>1 AM</td>
<td>5</td>
<td>0.31 ± 0.02</td>
<td>0.25 ± 0.04</td>
</tr>
<tr>
<td></td>
<td>5 AM</td>
<td>5</td>
<td>0.31 ± 0.01</td>
<td>0.21 ± 0.01†</td>
</tr>
<tr>
<td></td>
<td>7 AM</td>
<td>3</td>
<td>0.27 ± 0.01*</td>
<td>0.24 ± 0.01</td>
</tr>
<tr>
<td></td>
<td>8 AM</td>
<td>3</td>
<td>0.26 ± 0.01*</td>
<td>0.25 ± 0.01</td>
</tr>
<tr>
<td></td>
<td>9 AM</td>
<td>5</td>
<td>0.26 ± 0.02*</td>
<td>0.26 ± 0.05</td>
</tr>
<tr>
<td>Control (3)</td>
<td>9 AM</td>
<td>10</td>
<td>0.27 ± 0.01</td>
<td>0.25 ± 0.04</td>
</tr>
<tr>
<td>Continuous light</td>
<td>9 AM</td>
<td>5</td>
<td>0.24 ± 0.02</td>
<td>0.21 ± 0.01</td>
</tr>
<tr>
<td>Continuous darkness</td>
<td>9 AM</td>
<td>10</td>
<td>0.32 ± 0.01</td>
<td>0.29 ± 0.02</td>
</tr>
<tr>
<td>Continuous darkness + light</td>
<td>9 AM</td>
<td>10</td>
<td>0.27 ± 0.01‡</td>
<td>0.25 ± 0.01</td>
</tr>
</tbody>
</table>

For each animal approximately 100 photoreceptor synapses were analyzed. Values are given as mean ± SEM.

* Significantly different from the values in the dark phase (9 PM, 1 AM, and 5 AM; P < 0.01). Note that the synaptic ribbon profile lengths of the light time points were pooled and compared with the pooled dark-time values.

† Significantly different versus 5 PM (P < 0.05).

‡ Statistically different from the continuous darkness value (P < 0.01).
TABLE 2. Synaptic Ribbon Profile Length in Individual Rod Terminals Containing No (0) or Different Numbers (1–3) of Spherical Profiles at Different Time Points in the Light Phase

<table>
<thead>
<tr>
<th>Number of Spherical Profiles Present</th>
<th>Synaptic Ribbon Length [µm]</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>9 AM</td>
</tr>
<tr>
<td>0</td>
<td>0.31 ± 0.02*</td>
</tr>
<tr>
<td>1</td>
<td>0.27 ± 0.01†</td>
</tr>
<tr>
<td>2</td>
<td>0.23 ± 0.02</td>
</tr>
<tr>
<td>3</td>
<td>0.19 ± 0.01</td>
</tr>
</tbody>
</table>

Data are from experiment 2. Note that rodlike ribbon profiles were longest when no spherical profiles were present and that they become shorter with increasing numbers of spherical profiles. n.d., not detectable (at these time points terminals containing a rodlike synaptic ribbon profile and, in addition, three spherical profiles, were not observed).

* Significantly different versus 2 and 3 (P < 0.05).
† Significantly different compared with 2 (P < 0.05) and 3 (P < 0.01).
§ Significantly smaller versus 2 (P < 0.05).
‡ Significantly different compared with the value of 2 (P < 0.01).

illumination is necessary to form spherical synaptic bodies. In view of the endogenous circadian control of disc-shedding in the rat retina, a circadian rhythm of ribbon changes seems entirely possible, as well.

Spherical synaptic bodies are distinct organelles in some species and in some organs other than retina. In the retina, they appear to be degradation products of the platelike ribbons, with the club-shaped profiles being intermediate stages in this process (Fig. 7). Club-shaped profiles have been shown to result from in vitro administration of Li or the application of the neurotoxin quisqualic acid. In all cases club-shaped profiles disintegrate into spherical profiles and finally disappear. Similarly, we found that, in the morning, club-shaped profiles occurred first, followed later by spherical profiles.

What is the significance of these morphologic changes? There are two possibilities. First, the changes may represent a regulatory mechanism for synaptic activity. Ribbon synapses are known to be designed for high output. Because the platelike ribbons become smaller when they form spherical bodies and because the spherical bodies are still surrounded by synaptic vesicles when moving away from the synaptic site, fewer synaptic vesicles are available near the synapse to be exocytosed. Perhaps, this process is related to the diminished neurotransmitter release after light onset, resulting in hyperpolarization of the rod photoreceptors. The spherical profiles may either disappear completely or later reassemble to form new platelike ribbons. It is also feasible that, when required, the spherical profiles fuse with platelike ribbons to increase their size.

The second possibility is that the changes observed are signs of degradation of exhausted synaptic ribbons, occurring at the end of their life span. However, not all the synapses exhibit the club-shaped and spherical profiles. Perhaps, the ribbons disintegrate only in those photoreceptors in which disc-shedding occurs simultaneously.

Concerning the synaptic ribbons in cones our data suggest that, here, synaptic ribbon turnover is similar to that seen in rods, with minor differences. Because we have not seen club-shaped profiles in cones, perhaps the budding process of the synaptic ribbons is faster, or less frequent, than that in rods. This may become clearer in future investigations of a species with a larger percentage of cones.

Changes in Synaptic Ribbon Size
Changes in photoreceptor synaptic ribbon size have been little examined, and the results obtained have been variable, showing no differences between light and dark, larger ribbons during light, or vague findings of larger ribbons during darkness. The variability of the literature data may be related to interspecies differences, measuring methods, and particularly to differences in the sectioning angle of the organelles. Therefore, in the present study we have discarded all the size data of ribbon profiles not associated with the presynaptic membrane, because they represent tangential and therefore highly variable orientations through the crescent-shaped ribbons. We restrict the discussion to the ribbons of rods, because here we have a sufficiently large amount of data, compared with that collected for cones.

In all our experiments, the data obtained from 7 to 9 AM are highly consistent, showing a mean ribbon profile length of 0.27 µm. In two of the three experiments involving light and darkness, we found that ribbon size was significantly larger in darkness (0.31 µm) than in light (0.27 µm). Because the experiment that showed no difference involved
only two time points (9 AM versus midnight) and a small number of animals, whereas the 24-hour study was performed at nine time points, we attached more importance to the data of the latter, in particular because the results obtained during the light (dark) phases were consistent in themselves. Moreover, an influence of dark and light was clearly revealed by our constant dark–light experiments, in which ribbon profile lengths measured 0.52 μm/0.24 μm, compared with 0.27 μm in control samples.

The greater size of the synaptic ribbons in darkness compared with those in light is in agreement with the observation that neurotransmitter (glutamate) release occurs during darkness\(^{41,42}\) and the hypothesis that synaptic ribbons function as conveyor belts for the transport of synaptic vesicles to the presynaptic membrane\(^{18,43}\) where exocytosis takes place. Bearing in mind that crescent-shaped synaptic ribbons span the invaginated postsynaptic elements (two horizontal cell processes and one or more bipolar cell processes), the increase in size would enlarge the conveyor belt and therefore provide a larger area of interaction between the presynaptic and postsynaptic elements.

Moreover, because synaptic ribbons have been shown to be more densely occupied by synaptic vesicles after 48 hours of constant dark compared with 48 hours of light,\(^{44}\) we feel that the currently described relatively small change of synaptic ribbon size, together with the differences in the packing density of synaptic vesicles, may represent an important regulatory mechanism in ribbon synapses.

We conclude that the change in ribbon size is related to the formation of club-shaped and spherical synaptic profiles in rod terminals. The summary figure (Fig. 7) shows this relationship graphically. Club-shaped and spherical profiles are relatively abundant when the rodlike ribbon profiles are short and vice versa. The same is observed in individual rod terminals where rodlike and spherical profiles are both present. We assume that the increase in ribbon size is brought about by a reincorporation of the previously released spherical synaptic bodies shown in Figure 7. However, an incorporation of newly synthesized spherical profiles cannot be excluded.

**Acknowledgment**

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**References**


