# EVIDENCE FOR SYNERGISTIC AND COMPLEMENTARY ROLES OF BASSOON AND DARKNESS IN ORGANIZING THE RIBBON SYNAPSE

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**Abstract—Ribbon synapses are tonically active high-throughput synapses. The performance of the ribbon synapse is accomplished by a specialization of the cytomatrix at the active zone (CAZ) referred to as the synaptic ribbon (SR). Progress in our understanding of the structure–function relationship at the ribbon synapse has come from observations that, in photoreceptors lacking a full-size scaffolding protein Bassoon ($Bsn^{\Delta Ex4/5}$), dissociation of SRs coincides with perturbed signal transfer. The aim of the present study has been to elaborate the role of Bassoon as a structural organizer of the ribbon synapse and to differentiate it with regard to the ambient lighting conditions. The ultrastructure of retinal ribbon synapses has been compared between wild-type (Wt) and $Bsn^{\Delta Ex4/5}$ mice adapted to light (low activity) and darkness (high activity). The results obtained suggest that Bassoon and environmental illumination synergistically and complementarily act as organizers of the ribbon synapse. Thus, light-dependent and Bassoon-independent regulation involves initial SR tethering to the membrane and a basic shape transition of ribbon material from spherical to rod-like, since darkness induces these features in $Bsn^{\Delta Ex4/5}$ rod spherules. However, the tight anchorage of the SR via an arciform density and the proper assembly of SRs to the full-sized horseshoe-shaped complex depend on Bassoon, as these steps fail in $Bsn^{\Delta Ex4/5}$ rod spherules. © 2012 IBRO. Published by Elsevier Ltd. All rights reserved.**

Key words: bassoon, retina, ribbon synapse, synaptic ribbon, light/dark-cycle.

## INTRODUCTION

Sensory neurons of the retina and inner ear face the challenge of transmitting sensory signals over a broad range of intensity magnitude. For this reason, they need dynamic and adjustable synaptic machinery, which is provided by a unique type of chemical synapse, the ribbon synapse (tom Dieck and Brandstätter, 2006). Their most prominent organelle is the so-called synaptic ribbon (SR), which is thought to tune the synaptic vesicle cycle in a dynamic manner for speed, precision, and endurance (Zanazzi and Matthews, 2009). SRs constitute electron-dense structures of various forms and considerable size (Vollrath and Spiwoks-Becker, 1996). They are capable of tethering hundreds of synaptic vesicles (Rao-Mirotznik et al., 1995), which are positioned by the ribbon in close proximity to the presynaptic neurotransmitter release site, namely the active zone.

Most of the ribbon synapses in the outer plexiform layer of the mouse retina form part of the rod terminals, whereas the cone terminals account for less than 2% of the photoreceptor terminals. In the rod photoreceptor ribbon synapse, the prototype of a ribbon synapse, SRs mostly appear as rod-like structures in ultrathin sections, whereas three-dimensionally, they are thin plates with a horseshoe-like shape that is easily detected by immunofluorescence light microscopy. They are 30–50 nm in thickness, protrude upward with an average of 300 nm into the presynaptic cytoplasm, and can extend up to 2 µm in length parallel to the presynaptic plasma membrane. The concave inner base of the ribbon is anchored to the presynaptic membrane by means of the arciform density, a second electron-dense structure belonging to the ribbon complex. The morphology of photoreceptor SRs is dynamic (Vollrath et al., 2001; Regus-Leidig et al., 2010a) and undergoes daily changes in BALB/c mice (Adly et al., 1999; Spiwoks-Becker et al., 2004). Although they are large and smooth during the dark phase, after light exposure in the morning, they form distal swellings that bud off, thus forming spherical synaptic bodies (synaptic spheres, SSs), whereas at the onset of darkness, the reverse process occurs (Spiwoks-Becker et al., 2004). Accordingly, SSs can be considered as being the disintegrated building block pool of ribbon material.

A major component of the SRs is the protein RIBEYE (Schmitz et al., 2000; Magupalli et al., 2008; Venkatesan et al., 2010). Other molecular components found to be enriched at the photoreceptor SRs are a variety of proteins of the cytomatrix at the active zone (CAZ) as found in conventional active zones, including the

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*Abbreviations:* Bc, bipolar cells; CAZ, cytomatrix at the active zone; hc, horizontal cells; PBS, phosphate-buffered saline; SS, spherical synaptic bodies; SR, synaptic ribbon; wt, wild-type.

scaffolding protein Bassoon (tom Dieck et al., 2005). These findings have led to the concept that SRs are a specialization of the CAZ present at conventional synapses (Zhai and Bellen, 2004). The ribbon complex of photoreceptor cells can be segregated into a ribbon-associated compartment and an active zone compartment. Bassoon is enriched at the proximal/concave site of the ribbon and appears to contribute essentially to the link between the two compartments (tom Dieck et al., 2005). At the ribbon site, it physically interacts with RIBEYE (tom Dieck et al., 2005), whereas the interacting protein at the active zone compartment has not yet been identified.

In $Bsn^{\Delta Ex4/5}$ mutant mice, the region of Bassoon interacting with RIBEYE is deleted (Altrock et al., 2003; Dick et al., 2003; tom Dieck et al., 2005; Frank et al., 2010). These mice produce a 180-kDa Bassoon mutant protein, which is not tightly anchored to the CAZ and is easier to extract (Altrock et al., 2003; Dick et al., 2003). Consistent with the loss of the Bassoon-RIBEYE interaction, SRs in light-adapted $Bsn^{\Delta Ex4/5}$ rod spherules are not anchored to the presynaptic active zone but float freely in the cytoplasm (Dick et al., 2003). Since the function of SRs is to tether synaptic vesicles close to the release sites, this seems to explain why retina signal transfer from photoreceptors to the second-order neuron is disturbed in the $Bsn^{\Delta Ex4/5}$ mutant (Dick et al., 2003). The observation that, in $Bsn^{\Delta Ex4/5}$ rod spherules, the shape of anti-RIBEYE-stained SRs changes from horseshoe-like to punctate (tom Dieck et al., 2005) hints at the possibility that Bassoon also influences the morphology of SRs, and that perturbed signal transmission in $Bsn^{\Delta Ex4/5}$ mutant photoreceptor terminals might therefore be attributable to several structural deficiencies (tom Dieck et al., 2005; Specht et al., 2009).

The aim of the present study was to dissect the role of Bassoon as a structural organizer of the ribbon synapse in dependence of the ambient lighting conditions. To this end, the ultrastructure of ribbon synapses was systematically compared between $Bsn^{\Delta Ex4/5}$ and wild-type (wt) mice during the light phase when synaptic transmission is low and the dark phase when synaptic transmission is high.

## EXPERIMENTAL PROCEDURES

### Animals

All animal experiments were performed in accordance with the guidelines issued by the Max Planck Society. In total, 17 wt and 11 Bassoon mutant mice ($Bsn^{\Delta Ex4/5}$; Altrock et al., 2003; Dick et al., 2003) were examined under light- (4 h after light onset) or dark-adapted (4 h after dark onset) conditions from two independent experiments with an age of 8–15 weeks. Homozygous $Bsn^{\Delta Ex4/5}$ mice and wt controls (mixed background: 129/Sv × 129/SvJ from R1 ES-cells, backcrossed to C57Bl/6) were both obtained as littermate offspring from intercrosses of heterozygous $Bsn^{\Delta Ex4/5}$ mice. The mice were kept under constant laboratory conditions (12-h light, 12-h dark; lights on at 6 a.m. and off at 6 p.m.; less than 100–200 lux at the bottom of the cages; food and water *ad libitum*) for a minimum of one week prior to the experiments. They were anesthetized with isoflurane and decapitated prior to removal of the eyes during darkness under dim red light. Usually, both eyes/retinae per animal were removed by using the Winkler method (Winkler, 1972).

### Electron microscopy and morphometry

For the electron-microscopical evaluation, the retinae were dissected from rapidly removed eye balls and fixed in freshly prepared fixative, viz., 2% paraformaldehyde, 2.5% glutaraldehyde in phosphate-buffered saline (PBS) for 15 h. Subsequently, tissue was rinsed in PBS containing 6.8% (w/v) sucrose, postfixed in osmium tetroxide (2% (w/v) in PBS) for 90 min, washed three times in PBS, and dehydrated in a graded series of acetone. Tissue was flat-embedded in Epon (Serva, Heidelberg, Germany). Transverse sections (50–60 nm thick) were mounted onto one-hole Formvar-coated copper grids (Serva), stained with 8% (w/v) uranyl acetate (10 min), and contrasted with lead citrate for 5 min. Sections were viewed in an LEO 906 transmission electron microscope (Zeiss, Oberkochen, Germany), and all synapses were photographed. Morphometry was carried out by using Analysis 3.2. Software (Soft Imaging Systems, Münster, Germany).

### Quantitative analysis

From one randomly selected retinal section, synaptic body profiles in approximately 50 neighboring photoreceptor terminals were systematically examined according to the following criteria: type of SR profile (e.g., rodlike, spherical, or polymorphic) and size and location of the SR within the terminal. The SRs were classified as "tethered" when they bordered on the presynaptic membrane, in wt photoreceptors, typically via the arciform density and, in bipolar cells (BC), via small dense plaques. SR profiles not bordering on the presynaptic membrane were referred to as being "free".

Because rod-like SRs of rod photoreceptor are sections through horseshoe-shaped structures, and because the structure might be cut at various angles, transverse cuts yield shorter profiles with a smaller variability than horizontal cuts, which represent the SR length, and therefore sections were taken through the distal SR area (see also Fig. 3A). However, transversely cut profiles can be easily recognized, since, unlike the horizontally cut profiles, the underlying arciform density is seen together with the ribbon.

In general, within a given synapse, the number of SRs and SR fields were also estimated. Synapses without SR profiles were classified as "empty" endings. Furthermore, measurements of the area of the postsynaptic horizontal and bipolar processes were performed. Tissue and data analyses were performed blind, i.e., without knowledge of the genotype and by using coded specimens.

### Serial section analysis

To draw conclusions on the three-dimensional shape, the location of the SRs, and their arrangement at the synaptic complex, serial sections of randomly chosen profiles of light- and dark-adapted mutant photoreceptor terminals were photographed. The synaptic areas of adjacent sections were superimposed by using the Adobe Photoshop (Adobe Systems Inc.).

### Statistical analysis

The data obtained were expressed as means ± standard error of the mean. For statistical analysis, Student's *t*-test or the Wilcoxon-Mann–Whitney *U*-test was used. A *p*-value of smaller than or equal to 0.05 was regarded as significant. The values are based on pooled data from two independent experiments.

## RESULTS

### Light-dependent ultrastructural changes in rod ribbon synapses lacking functional Bassoon

In adult wt mice (8–15 weeks), presynaptic electron-dense SRs typically anchored to the so-called arciform density