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High-Resolution Optical Imaging of Zebrafish Larval Ribbon Synapse Protein RIBEYE, RIM2, and CaV 1.4 by Stimulation Emission Depletion Microscopy

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Abstract

The synaptic ribbon is a unique presynaptic structure with an intricate morphology in photoreceptors. Because of the resolution limit in conventional fluorescence microscopy, investigating ribbon protein locations has been challenging, especially in the early development stages of model animals. Here, we used stimulated emission depletion microscopy, a super-resolution imaging technique, to look at retina sections in 4 days post-fertilization (dpf) zebrafish. We observed that in photoreceptor cells, RIBEYE and RIM2 are expressed along the synaptic ribbon, with RIM2 consistently located inside of the horseshoe-shaped synaptic ribbon structure with RIBEYE located on the outside. The L-type calcium channel subunit, CACNA1F, exhibited small spot-like staining beneath the RIM2 and RIBEYE structures. Using morpholino antisense oligonucleotides to knock down RIBEYE expression, we observed fewer and shorter ribbons in the photoreceptor outer plexiform layers of 4 dpf fish retina as well as a reduction in RIM2 expression. The clustering of CACNA1F in these blind fish was no longer observed, but instead showed a diffuse expression in the photoreceptor terminal.

Keywords

STED; RIBEYE; zebrafish; retina; synapse; synaptic ribbon; calcium; vesicle

INTRODUCTION

Nonspiking cells of the retina and inner ear that have high rates of tonic neurotransmitter release have evolved specialized proteinaceous structures, known as synaptic ribbons, which tether hundreds of synaptic vesicles near active zones of synaptic release. To date, several proteins have been localized to the synaptic ribbon: CtBP1/BARS, the kinesin subunit KIF3A, RIM1 and RIM2, Munc13s, CASTs/ERCs/ ELKS, Piccolo and Bassoon, L-type

calcium channels, BRAG1 and RIBEYE (Brandstatter et al., 1999; Muresan et al., 1999; Schmitz et al., 2000; Dick et al., 2001, 2003; Prescott & Zenisek, 2005; tom Dieck et al., 2005; Katsumata et al., 2009; Zanazzi & Matthews, 2009). Of these proteins, only RIBEYE has been found to be unique to the synaptic ribbon. Moreover, RIBEYE is not only a specific protein in the ribbon, but also its major component (Schmitz et al., 2000), with one estimate suggesting that RIBEYE occupies approximately 67% of the ribbon volume (Zenisek et al., 2004). Therefore, RIBEYE is an essential component of the synaptic ribbon and understanding RIBEYE function is likely to provide insight into synaptic ribbon function.

Synaptic ribbons were originally identified in electron microscopy as electron dense structures (Sjostrand, 1958). More recently, ribbon proteins have been classified into two molecular compartments: a ribbon-associated complex, which colocalizes more closely with the dense synaptic ribbon in immuno-EM and that moves from the plasma membrane in bassoon mutant animals; and the plasma membrane/arciform density-associated complex, such as RIM2 and L-type calcium channel (tom Dieck et al., 2005; Cui et al., 2007).

To complement these previous studies, we have employed stimulated emission depletion (STED) microscopy to investigate the localization of synaptic ribbon proteins in zebrafish retina at high spatial resolution. STED is a super-resolution microscopy technique that provides resolution well below the diffraction limit (Hell & Wichmann, 1994) and is becoming more widely used in neuroscience (Willig et al., 2006; Wilhelm et al., 2010). STED achieves its resolution by implementing a second laser source to de-excite fluorophores in the periphery of a diffraction-limited spot to limit excitation to a small subdiffraction limited region. Commercially available STED systems can improve lateral resolution to approximately 60–70 nm. When using STED to image hair cell ribbon synapses, the CaV1.3 channel was observed to form clusters in the plasma membrane near the synaptic ribbons (Frank et al., 2010). Here we describe the use of this emerging technology to observe the 4 days post-fertilization (dpf) zebrafish (*Danio rerio*) retina photoreceptor cell ribbon proteins, RIBEYE, RIM2, and the calcium channel subunit, CACNA1F.

As visualized by STED microscopy, RIM2 expresses in the inner part of horseshoe-shaped photoreceptor synaptic ribbons, while RIBEYE is found on the outside and also is longer. CACNA1F clusters to one side of the ribbon, likely on the plasma membrane. Unlike mammals, zebrafish have two genes for ribeye (Wan et al., 2005). By using morpholinos (MO) to knockdown expression of one of the two RIBEYE genes, *ribeye a*, in zebrafish retina, we found that in MO-injected 4 dpf fish, the reduction of RIBEYE expression caused a decrease of RIM2 expression. In the morphants, there are fewer RIBEYE-positive spots, and the remaining spots are shorter than in wild-type fish. However, RIM2 still colocalized with RIBEYE in the remaining shorter structures. CACNA1F no longer formed clusters in the blind fish and did not specifically colocalize with RIBEYE, but rather was expressed diffusely throughout the presynaptic terminal.

METHODS

Zebrafish were kept in accordance with the Yale University Animal Use and Care Committee guidelines.

Morpholino Injections

To knockdown *ribeye a* expression, 500 nL of 0.25 mM MO oligonucleotide (5'-ATACCTTACTCACAGGGAAGAAAAG-3', targets to 12-to-36) (GENE Tools, LLC, Philomath, OR, USA) were injected into 1–4 cell stage embryos. Under these conditions,

about 50% of animals exhibit a negative OKR at 4 dpf. Higher levels of MO injection results in an abundance of fish with a small eye phenotype with poor differentiation of the retina as well as cell death in the retina (as previously described by Wan et al., 2005). The effects at higher concentrations of MO could not be rescued by RIBEYE overexpression (data not shown), suggesting either off-target effects of the MO or a requirement for ribeye expression at specific times and locations during development.

Optokinetic Response

Optokinetic responses were measured in MO-injected fish at 90 h after injection, and for DNA-injected fish and offspring at 4 dpf after injection. To do so, larvae were placed in a 3 cm petri dish in a solution of 4% methylcellulose. The dish was placed on a dissecting microscope stage surrounded by a cylinder with black and white stripes on its inner surface that each occupied 22.5° of the visual field. The cylinder rotated at a speed of 40° per second clockwise and fish were observed for visually driven saccades using the microscope.

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4–6 dpf zebrafish larval were anaesthetized and fixed in 4% paraformaldehyde in phosphate buffered saline (PBS) at room temperature for 10 min and equilibrated with 10%, 20%, and 30% sucrose sequentially for a minimum of 2 h each. Next, the fish larvae were embedded in optimal cutting temperature compound (Sakura, Tokyo, Japan). Cryosections (14 µm thick) on polylysine-coated slides were permeabilized in blocking buffer consisting of 1% bovine serum albumin (v), 3% normal goat serum (v/v), and 0.1% Triton X-100 in PBS at room temperature for 30 min, followed by overnight incubation with primary antibodies diluted in 1% normal goat serum, 3% normal goat serum (v/v), and 0.1% Triton X-100 in PBS at 4°C. The second morning, the samples were washed three times with PBS, 15 min each time, and incubated in diluted secondary antibody for 2 h. The primary antibody used for ribbon protein CtBP [CtBP (H-440, Santa Cruz Biotechnology, Inc., Santa Cruz, CA, USA)], RIM2 (anti-Rim2 PDZ domain, Synaptic Systems, Goettingen, Germany) and CACNA1F [CACNA1F antibody—cytoplasmic domain (ab81098), Abcam, Cambridge, MA, USA] were diluted 1:200. The secondary antibodies [Alexa Fluor 488/568 goat antimouse/ rabbit IgG(H+L), Molecular Probes, Eugene, OR, USA] were diluted 1:100. The secondary antibody ATTO 647N (STED) goat anti-rabbit IgG (Active Motif, Carlsbad, CA, USA) was diluted 1:1,000.

STED Microscopy

STED images were obtained using a commercial Leica TCS STED microscope equipped with a pulsed 640 nm diode laser (PDL 800-B, PicoQuant, Berlin, Germany) for excitation (~10–80 µW) and a modelocked Ti:Sapphire laser (Mai Tai, Spectra Physics, Santa Clara, CA, USA) tuned to 770 nm for depletion (~180 mW). Laser powers were measured at the objective back aperture. Fluorescence signal was collected by a 100×/1.4 NA oil immersion objective lens (HCX PL APO STED, Leica Microsystems, Wetzlar, Germany), bandpass filtered (FF01-685/40, Semrock, Rochester, NY, USA), and detected by either an avalanche photodiode (in STED mode) or a photomultiplier tube (in confocal mode) through a 0.5 Airy unit pinhole. The lateral resolution of the system in STED mode was measured by STED imaging of 20 nm diameter fluorescent beads (F8782, Invitrogen Corporation, Carlsbad, CA, USA). Since the size of the beads is well below the expected resolution, measuring the full-width at half-maximum of STED images of single beads well approximates the resolution of the system. Our measurements confirmed 60–70 nm resolution in the focal plane as specified by the manufacturer. In accordance with the Nyquist criterion, the pixel size was set to 30.3 nm. Images of 1024 × 1024 pixels were acquired at a scan speed of 1,000 lines per second with 16 line averages. Confocal images were taken sequentially after the STED

images using the 488 nm line of an argon laser for excitation and a fluorescence detection range of 494–541 nm. Confocal images were acquired with 6 line averages.

RESULTS

Superresolution Imaging of RIM2 and CACNA1F by STED Microscopy

Photoreceptor cell ribbons are about 30 nm thick, 0.2–1 μm in length, and are separated from the plasma membrane by ~ 20 nm (Sterling & Matthews, 2005). RIBEYE is the most abundant protein in the synaptic ribbon and is found throughout the structure, while RIM2 and L-type calcium channel localize beneath the ribbon in mouse photoreceptor ribbons (Morgans et al., 2001; tom Dieck et al., 2005). However, due to the small size of the underlying structure, localization of these proteins is only poorly resolved using conventional confocal microscopy (Morgans, 2001; Sterling & Matthews, 2005; tom Dieck et al., 2005).

Increasingly, zebrafish is becoming a popular model system for study of the visual system. One major advantage to the zebrafish as a model system is the availability of morpholino antisense oligonucleotides (MO) to knockdown gene expression in the developing embryo (Nasevicius & Ekker, 2000). Despite the power of the technique, MOs exhibit reduced effectiveness early in development (Bill et al., 2009; Goll et al., 2009). Therefore, it is critical to conduct associated research within a short time after injection. Zebrafish develop functional retina within 72 h of fertilization, which is within the effective working time of MOs (Wan et al., 2005; Sheets et al., 2011). Therefore, we focused our studies here on 3–4-day-old fish.

To study the distribution of synaptic ribbon proteins, we first used an antibody raised to CtBP, which recognizes both RIBEYE and the closely-related CtBP. Staining with this antibody revealed intense labeling of both the outer and inner-plexiform layers of the retina, as expected for synaptic ribbon labeling. When using traditional confocal microscopy with a 100×1.35 NA objective to look at 4 dpf zebrafish retina sections, it is possible to visualize single ribbons in photoreceptor cells (Figs. 1, 2, green). RIM2 (Fig. 1A) partially co-localized with RIBEYE. Similar to RIM2, the L-type calcium channel CACNA1F (Fig. 2A) also partially co-localized with RIBEYE (Fig. 2, green). These results are similar to those reported in mouse (Morgans et al., 2001; tom Dieck et al., 2005).

To further resolve these structures, we used STED microscopy and were able to obtain a significant resolution improvement (e.g., compare Figs. 1 and 2, left panels to right panels). Using STED microscopy, RIM2 fluorescence is restricted to a smaller region and appears localized to one side of the RIBEYE/CTBP staining (Fig. 1C). With the higher resolution provided by STED, the CACNA1F staining is revealed to be a collection of spots rather than a continuous cluster (Fig. 2B).

Using STED Microscopy to Image Single Ribbons at High Resolution

Next, we used STED microscopy to focus on the distribution of proteins in individual photoreceptor ribbons in three dimensions and compared the results to those obtained using confocal microscopy. To do so, we took a $0.25 \mu\text{m}$ step z-series of images through sections of retina (Fig. 3). After visualizing the z-axis projection, horseshoe-like ribbon structures were observed in the outer plexiform layer (Fig. 3, left). To better visualize these structures, we focused on individual ribbons and generated a two-dimensional projection of serial z-stack images. Choosing only frames containing a single ribbon projection, we found RIM2 localized to the base of the ribbons along the length of the entire ribbon (Fig. 3, right). Different from RIM2, CACNA1F, while still expressed at the base of the ribbon, was more separated from RIBEYE as a series of spots rather than as a continuous structure (Fig. 4). Of

note, the Leica STED system employed in these studies only improves the lateral resolution and axial resolution is still diffraction limited.

The Knockdown of RIBEYE Reduces RIM2 Expression and Changes the CACNA1F Distribution

We next looked at how RIBEYE expression affects localization of other ribbon proteins. Previous results by Wan et al. (2005) demonstrated that MO knockdown of RIBEYE caused blindness in larval zebrafish. We used the same RIBEYE a MO to knock down ribeye in the developing retina, and we investigated RIM2 and CACNA1F localization after the RIBEYE knockdown. Four days after RIBEYE MO injection, zebrafish were screened for visual defects using a behavioral optokinetic screen (see the Methods section). Seventy-one out of 86 MO-injected zebrafish failed our optokinetic screen, whereas none of the 80 fish injected with the injection buffer failed the screen, indicating that *ribeye a* MO injection compromised the ability of zebrafish to respond to an optical stimulus. We sectioned OKR negative fish, to look at RIBEYE, RIM2, and CACNA1F staining in their retinas. To get more complete structural information, we also obtained z-stacks using STED. MO-injected fish exhibited fewer CtBP-positive spots in both the OPL and IPL (Fig. 5A), and the remaining structures lacked the horseshoe shape typical of the photoreceptor ribbon (Fig. 5A, right). Surprisingly, we found that RIM2 expression level was also reduced (Fig. 5A), and the remaining protein still co-localized with the remaining small RIBEYE spots (Fig. 5A).

Dramatically different from RIM2, the expression level of CACNA1F was still apparent in the morphants, but the expression changed from a compact clustering near the ribbon to a diffuse distribution throughout the whole presynaptic terminal (Fig. 5B). Even at the remaining RIBEYE and RIM2-positive spots, we did not observe obvious clusters of CACNA1F (Fig. 5B, right).

DISCUSSION

Confocal microscopy is widely used in cell and tissue imaging. Because of the diffraction limit, they have a standard resolution at more than 200 or 250 nm. STED microscopy uses saturated de-excitation of fluorescent dyes to overcome the resolution limit imposed by diffraction. STED microscopy has been used in fixed tissue or cells and living cells (Willig et al., 2006; Nagerl & Bonhoeffer, 2010). In this study, we show that STED microscopy allows resolution of substructure of the ribbon synapse not possible with conventional confocal microscopy. Specifically, STED microscopy allowed for the discrimination of the localization of RIM2 and CACNA1F staining on the ribbon and the resolution of CACNA1F staining as individual spots rather than a continuous structure. These results are in good agreement with previous results in mammalian preparations, suggesting a common evolutionary arrangement of proteins on the ribbon (tom Dieck et al., 2005).

RIBEYE is a major and unique protein in synaptic ribbons, yet its function remains unknown. Its abundance in the synaptic ribbon (Schmitz et al., 2000; Zenisek et al., 2004), binding partners (Alpadi et al., 2008; Venkatesan et al., 2010), and importance for normal synaptic function (Wan et al., 2005; Sheets et al., 2011) point toward an important role for RIBEYE in synaptic ribbon function. Our results here add to this earlier data, by demonstrating that RIBEYE expression levels can direct localization and control protein levels of RIM2 on the synaptic ribbon and effect distribution of calcium channels in the retina. We found that knockdown of RIBEYE caused a dramatic reduction in RIM2 expression levels, indicating that RIBEYE influences either the stability or expression level of RIM2. RIM2 localizes to the base of the ribbon in retinal ribbon synapses near RIBEYE (tom Dieck et al., 2005). In conventional synapses, RIM proteins play important roles in

vesicle priming (Rizo & Rosenmund, 2008). Our results here mirror those of Nicolson and colleagues in hair cells (Sheets et al., 2011), who demonstrated that knockdown of both *ribeye a* and *ribeye b* causes the loss of presynaptic CaV1.3a channel clusters from hair cells and that ectopic aggregates of overexpressed ribeye colocalized with ectopic clusters of CaV1.3a channel in hair cells. We speculate that RIBEYE, by stabilization and localization of RIM2, ensures that primed vesicles are specifically released near the ribbon and that calcium channels are brought into close proximity to ribbons. Recently our lab has shown that acute disruption of the ribbon disrupts a step downstream of vesicle attachment to the ribbon, but upstream of exocytosis, consistent with a role for the ribbon in vesicle priming (Snellman et al., 2011). RIM proteins have also been described to play a key role in localizing calcium channels to vesicle release sites in conventional synapses (Kaesler et al., 2011). It should be noted, however, that a recently described direct interaction between RIM and calcium channels is specific for N and P/Q type channels, but not the L-type channels found at release sites in ribbon synapses (Kaesler et al., 2011). However, an indirect interaction involving RIM-binding protein has been described that could link L-type channels to RIM (Hibino et al., 2002). In light of these other findings, it was somewhat surprising that we failed to find calcium channels colocalized to the RIBEYE and RIM-2 positive spots in morphant animals (Fig. 5B, right). Although we do not know the reason for these results at this time, we speculate that either (1) a threshold amount of RIBEYE and RIM is required for calcium channel recruitment, (2) the RIBEYE b, which we did not knockdown, cannot effectively recruit calcium channels without RIBEYE a, or (3) calcium channel clusters near RIBEYE sites may have been obscured by the diffuse staining for calcium channels no longer anchored to ribbons. Regardless of the reason for this mislocalization, our results are consistent with RIBEYE directing key presynaptic proteins to active zones either through direct or indirect interactions.

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Figure 1.

Comparison of conventional confocal microscopy and STED microscopy images of RIM2 staining in 4 dpf zebrafish retina section. **A:** Confocal image of RIM2 anti-RIM2 stained section of zebrafish retina. **B:** Same region as image **A**, imaged using STED. **C:** Image **A** merged with confocal image of anti-CtBP staining (green). **D:** Image **B** merged with confocal image of anti-CtBP staining (green). Note that STED images of RIM2 are higher resolution (**B**) than confocal images (**A**). When costaining with the CtBP, STED imaging (**D**) reveals that RIBEYE and RIM staining are slightly out of register with one another. By contrast, RIBEYE and RIM staining appear aligned in confocal images (**C**). Scale bar is 1 μm .

Figure 2.

Comparison of conventional confocal microscopy and STED microscopy images of CACNA1F staining in 4 dpf zebrafish retina section. Confocal images of CaV 1.4 show a single spot (**A**), which colocalizes with RIBEYE (**C**). The higher resolution of STED microscopy reveals a cluster of smaller CACNA1F spots (**B**), near RIBEYE stained ribbons (**D**). Scale bar is 1 μm .

Figure 3.

Comparison of confocal images and STED three-dimensional (3D) projection images of RIM2 and RIBEYE in 4 dpf zebrafish photoreceptor cells. **A:** Confocal images. **B:** STED images of anti-RIM2 staining aligned with confocal image of anti-CtBP staining. **Left:** The Z projection of z-stack images. **Right:** Examples of single ribbon 3D reconstruction. Red is RIM2 staining, and green is CtBP antibody staining. Scale bar is 1 μm . The stack thickness is 3.75 μm .

Figure 4. Comparison of confocal and STED 3D projection images of RIM2 and CACNA1F in 4 dpf zebrafish photoreceptor cells. **A:** Confocal images. **B:** STED images. **Left:** The Z projection of z-stack images. **Right:** Single ribbon 3D reconstruction. Red is RIM2, and green is CtBP. Scale bar is 1 μm .

Figure 5.

A: Effect of *ribeye* MO knockdown on RIM2 and CACNA1F expression in 4 dpf zebrafish retina. **A, left:** Projection of a z-series of STED images of RIM2 (red) and confocal CtBP (green) staining in 4 dpf zebrafish retina subjected to MO knockdown of *ribeye*. Note that there are fewer and shorter ribbons (green) than in the uninjected zebrafish retina (Fig. 4). **A, right:** Magnified images of projections of individual ribbons highlighted by the boxes in image **A, left**. Note that RIM2 colocalizes with remaining RIBEYE spots (**A**, red). **B, left:** Projection of a z-series of STED images of CACNA1F (red) and confocal CtBP (green)

staining in 4 dpf zebrafish retina subjected to MO knockdown of ribeye. Note that CACNA1F expresses throughout the presynaptic terminal (**B**, red). Scale bar is 1 μm .