Ribeye is required for presynaptic CaV1.3a channel localization and afferent innervation of sensory hair cells

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SUMMARY
Ribbon synapses of the ear, eye and pineal gland contain a unique protein component: Ribeye. Ribeye consists of a novel aggregation domain spliced to the transcription factor CtBP2 and is one of the most abundant proteins in synaptic ribbon bodies. Although the importance of Ribeye for the function and physical integrity of ribbon synapses has been shown, a specific role in synaptogenesis has not been described. Here, we have modulated Ribeye expression in zebrafish hair cells and have examined the role of Ribeye in synapse development. Knockdown of ribeye resulted in fewer stimulus-evoked action potentials from afferent neurons and loss of presynaptic CaV1.3a calcium channel clusters in hair cells. Additionally, afferent innervation of hair cells was reduced in ribeye morphants, and the reduction was correlated with depletion of Ribeye punctae. By contrast, transgenic overexpression of Ribeye resulted in CaV1.3a channels colocalized with ectopic aggregates of Ribeye protein. Overexpression of Ribeye, however, was not sufficient to create ectopic synapses. These findings reveal two distinct functions of Ribeye in ribbon synapse formation – clustering CaV1.3a channels at the presynapse and stabilizing contacts with afferent cells during synaptogenesis (Hermes et al., 1992). These aggregates of Piccolo, Bassoon and Rim1 have been reported in photoreceptor synapses, and it consists of an N-terminal, proline rich A-domain, and a C-terminal B-domain that is identical to the transcriptional co-repressor C-terminal protein binding protein 2 (CtBP2) (Schmitz et al., 2000). A recent study revealed that exogenous expression of Ribeye in transfected R28 cells formed protein aggregates that were often surrounded by vesicles, suggesting that Ribeye alone can form ribbon-like structures (Magupalli et al., 2008). Furthermore, electron-dense structures containing Ribeye, Piccolo, Bassoon and Rim1 have been reported in photoreceptor cells during synaptogenesis (Hermes et al., 1992). These aggregates decrease in number during early postnatal stages when ribbon bodies take on a mature form (Regus-Leidig et al., 2009). Altogether, these studies predict that Ribeye plays an essential role in the formation of ribbon synapses. Despite the implicated role for Ribeye in ribbon formation, its specific function in synaptogenesis has not yet been examined.

One effective strategy to characterize the role of Ribeye in the development of ribbon synapses would be targeted deletion of the gene. However, ribeye knockout mice have not been reported, perhaps because this protein is the product of alternative splicing of the gene encoding CtBP2, and mice harboring deletions of Ctbp2 are not viable (Hildebrand and Soriano, 2002). An alternative approach to depleting Ribeye, by blocking mRNA translation with morpholino antisense oligos (MOs), was previously shown to be an effective way to examine its function in zebrafish (Wan et al., 2005). Moreover, through transgenic expression of Ribeye in a tissue-specific manner, one can directly compare the effects of Ribeye overexpression with that of Ribeye depletion. We therefore took advantage of these techniques in order to characterize the role of Ribeye in ribbon synaptogenesis in hair cells.

Here, we characterized localization of pre- and postsynaptic proteins in ribbon synapses of zebrafish hair cells during development. We then examined the effect of Ribeye knockdown or overexpression on synapse formation. Our results show that Ribeye is crucial for clustering of CaV1.3a channels in the basolateral membrane and is required for afferent innervation of hair cells.

INTRODUCTION
Hair cells of the auditory and vestibular system transmit a dynamic range of sensory information and do so both continuously and with exquisite temporal precision (Beutner et al., 2001; Brandt et al., 2005; Moser et al., 2006). These properties require specialized synapses containing structures called synaptic ribbons. Synaptic ribbons are electron-dense presynaptic specializations that tether synaptic vesicles and are crucial for fast synaptic transmission at sensory cell synapses (Glowatzki and Fuchs, 2002; Hull et al., 2006; Khimich et al., 2005; Lenzi et al., 2002; Li et al., 2009; Schmitz, 2009). Although it has been observed that the molecular organization of ribbon synapses is functionally important (Brandt et al., 2005; Li et al., 2009), very little is known about ribbon synapse biogenesis.

Several studies indicate that the ribbon synapse component Ribeye may play an essential role in ribbon synapse formation (Hermes et al., 1992; Magupalli et al., 2008; Regus-Leidig et al., 2009). Ribeye is the only known protein that is unique to ribbon synapses, and it consists of an N-terminal, proline rich A-domain, and a C-terminal B-domain that is identical to the transcriptional co-repressor C-terminal protein binding protein 2 (CtBP2) (Schmitz et al., 2000). A recent study revealed that exogenous expression of Ribeye in transfected R28 cells formed protein aggregates that were often surrounded by vesicles, suggesting that Ribeye alone can form ribbon-like structures (Magupalli et al., 2008). Furthermore, electron-dense structures containing Ribeye, Piccolo, Bassoon and Rim1 have been reported in photoreceptor cells during synaptogenesis (Hermes et al., 1992). These aggregates decrease in number during early postnatal stages when ribbon bodies take on a mature form (Regus-Leidig et al., 2009). Altogether, these studies predict that Ribeye plays an essential role in the formation of ribbon synapses. Despite the implicated role for Ribeye in ribbon formation, its specific function in synaptogenesis has not yet been examined.

One effective strategy to characterize the role of Ribeye in the development of ribbon synapses would be targeted deletion of the gene. However, ribeye knockout mice have not been reported, perhaps because this protein is the product of alternative splicing of the gene encoding CtBP2, and mice harboring deletions of Ctbp2 are not viable (Hildebrand and Soriano, 2002). An alternative approach to depleting Ribeye, by blocking mRNA translation with morpholino antisense oligos (MOs), was previously shown to be an effective way to examine its function in zebrafish (Wan et al., 2005). Moreover, through transgenic expression of Ribeye in a tissue-specific manner, one can directly compare the effects of Ribeye overexpression with that of Ribeye depletion. We therefore took advantage of these techniques in order to characterize the role of Ribeye in ribbon synaptogenesis in hair cells.

Here, we characterized localization of pre- and postsynaptic proteins in ribbon synapses of zebrafish hair cells during development. We then examined the effect of Ribeye knockdown or overexpression on synapse formation. Our results show that Ribeye is crucial for clustering of CaV1.3a channels in the basolateral membrane and is required for afferent innervation of hair cells.

MATERIALS AND METHODS

Fish strains
Transgenic lines and mutant alleles were maintained in Tübingen, Top Long Fin and WIK wild-type backgrounds. The transgenic line Tg(neurod:GFP) and cavl3a alleles have been previously described (Obholzer et al., 2008; Sidi et al., 2004).
Generation of Tg(−6mysin 6b: ribeye b-GFP) transgenic line

A plasmid was made by inserting full-length ribeye b cDNA (NCBI Accession Number NM_001015064) into the EcoRI/KpnI sites of a modified pEGFP-N1 vector (Clontech, Mountain View, CA, USA) containing −6.5 kb of the mysin 6b promoter (Obholzer et al., 2008) and two ISceI sites flanking myo6b: ribeye b-GFP. A solution containing 50 ng/ml plasmid, 6% ISceI enzyme and 3% Phenol Red was injected directly into the single cell of a one-cell stage embryo. Transiently expressing larvae were raised and their offspring screened for stable expression of ribeye b-GFP. Larvae with ectopic accumulations of Ribeye b were generated by incressing Tg(−6mysin 6b: ribeye b-GFP) fish.

In situ hybridization

Digoxigenin-labeled antisense probes to ribeye a (nucleotides 10-973, NCBI Accession Number AY878349) or ribeye b (nucleotides 116-1096) were synthesized from linearized modified pEGFP-N1 vectors containing full-length cDNAs. Whole-mount in situ hybridization was performed as previously described (Stiller et al., 2004). Larvae were imaged on a Zeiss Axio bright-field microscope (Carl Zeiss Microimaging, Oberkochen, Germany) using a 10×0.3 N.A. dry lens objective. Images were acquired via an AxioCam MRC5 color digital camera using Axiovision software and processed with ImageJ (US National Institutes of Health, Bethesda, MD, USA) and Adobe Photoshop (San Jose, CA, USA).

Morpholino injection

MOs against the start codon of ribeye a 5′-CTGGAGACTCAACATGAG-GAAGAAGAT-3′ (Wan et al., 2005)), the ribeye b start codon 5′-AACCCTGACTCCAGTCCAGATG-3′ and the intron 1 splice donor site of ribeye b 5′-CAGTTGACATCATTCATGTTCCCGG-3′, as well as inverse controls, were obtained from GeneTools (Philomath, OR, USA). Approximately 2 nl of 50-100 μM ribeye a MO, 0.75 mM-1.0 mM of ribeye b MO or both MOs diluted in RNAse-free double distilled water with 3% Phenol Red were pressure injected into one-cell stage wild-type and Tg(neurod:GFP) outcross embryos.

Larvae were screened for impaired acoustic startle response at 4 dpf (Nicolson et al., 1998).

RT-PCR

Groups of 10 larvae were collected at various time points after fertilization and their total RNA extracted using the RNeasy fibrous tissue mini kit (Qiagen, Germantown, MD, USA). Reverse transcription (RT) PCR was performed using the Sprint RT Complete Oligo(dT) kit (Clontech, Mountain View, CA, USA). Primers used for each transcript are as follows: ribeye a, 5′-CTATACGGCTAATGCAATG-3′ and 5′-GCA-TTCACACATGCTTCC-3′; ribeye b, 5′-CAGGAGGGCTGCC-GAGAACGTTCGCCG-3′ and 5′-ACAAGAAGCGAGGTGCGA-3′; cav1.3a, 5′-ACAAGAAGCGAGGTGCGA-3′ and 5′-CATTTCGTAATGCTTCC-3′; pcd15cd3, 5′-AACTGCGCTGAT-3′ and 5′-CCGTTTTCAGGTGTTGTT-3′; pcd15cd3, 5′-CGTATCCAGATGTCCATCCTCC-3′ and 5′-CCGAGAGGCTCT-CACAGC-3′; gapdh, 5′-GATACAGGAGACCAGGTGTT-3′ and 5′-GCCATACGGTCATACACGG-3′.

Antibodies

The following affinity-purified primary antibodies were generated against Danio rerio: mouse monoclonal against Ribeye b (amino acids 12-33; Open Biosystems, Huntsville, AL) and rabbit polyclonals against Ribeye a (amino acids 1-466; Proteintech, Chicago, IL, USA), Ribeye b (amino acids 4-483; Proteintech) and Cav1.3a (amino acids 42-56; Open Biosystems). We used the K28/86 (NeuroMab, Davis, CA, USA) to label MAGUKs.

Whole-mount immunohistochemistry and fluorescent imaging

Zebrafish larvae were fixed with 4% paraformaldehyde in phosphate buffer for 4 hours at 4°C, permeabilized with ice-cold acetone at 4-6 dpf, or 1% Tween in phosphate-buffered saline (PBS) at 20-50 hpf and blocked with PBS buffer containing 2% goat serum, 1% bovine serum albumin (BSA) and 1% dimethyl sulfoxide (DMSO). They were then incubated with primary antibodies diluted in PBS containing 1% BSA and 1% DMSO overnight followed by diluted secondary antibodies coupled to Alexa 488, Alexa 647 ( Molecular Probes, Invitrogen, Carlsbad, CA, USA) or DyLight 549 (Jackson ImmunoResearch, West Grove, PA, USA), and labeled with DAPI (Molecular Probes, Invitrogen, Carlsbad, CA, USA). Z-stack images of neuromasts (spaced by 0.5 μm over 5-10 μm) were acquired with an Olympus FV1000 confocal microscope using a 60×0.5 N.A. oil immersion lens. Excitation for DAPI (405 nm), Alexa 488/ GFP (488 nm) DyLight 549 and Alexa 647 was provided by blue-violet diode, Argon-ion, Green HeNe and Red HeNe lasers, respectively, using the lowest laser power possible to minimize photobleaching. For each experiment, the microscope parameters were adjusted using the brightest specimen so that the darkest pixels had a brightness value of around zero and the brightest pixels had a brightness value of 4095. Digital images were processed using MetaMorph ( Molecular Devices, Sunnyvale, CA, USA) and ImageJ software.

Image analysis

Maximal projections of z-stack confocal images were created and analyzed using MetaMorph software. Individual neuromasts were delineated using the region tool, and an extended threshold was applied to isolate the pixels occupied by immunolabeled punctae. Immunolabeled punctae were defined as regions where the pixel intensity was at least threefold above the average intensity measured in the whole neuromast. The integrated morphometry analysis function was used to quantify the number of punctae, as well as the area occupied by fluorescent pixels and the intensity of fluorescent pixels within individual punctae. Innervating afferent processes were analyzed using the Metamorph Neurite Outgrowth Module. The module-generated neuronal trace for each neuromast was visually inspected, and the settings individually adjusted until the resulting trace accurately represented that of the afferent branches. From each resulting trace, the number of branch points was quantified and the total length of neurite outgrowth was measured and corrected for diagonal lengths. Statistical analysis was performed using Prism 5 (GraphPad Software, San Diego, CA, USA).

Electron microscopy

Electron microscopy was performed on 4 dpf wild-type, inverse control and Ribeye b MO-injected larvae as well as high expressing Ribeye b-GFP transgenic larvae as previously described (Obholzer et al., 2008).

Vestibular induced eye movement

Larvae were tested at 3 dpf as previously described (Mo et al., 2010).

Action current recordings

Action current recordings were obtained from 4 dpf larvae as previously described (Trapani and Nicolson, 2010). Briefly, larvae were anesthetized, mounted and microinjected in the heart with 125 μM c-bungarotoxin to suppress muscle activity. Larvae were then rinsed and maintained in normal extracellular solution [in mM: 130 NaCl, 2 KCl, 2 CaCl2, 1 MgCl2 and 10 HEPES (pH 7.8)]. Borosilicate glass pipettes were pulled (P-97, Sutter Instruments, Novato, CA, USA) to tip diameters smaller than 1 μm and had resistances between 5 and 15 MΩ. Signals were collected with an EPC 10 amplifier and Patchmaster software (Heka Electronic, Bellmore, NY, USA). Extracellular currents were acquired in voltage-clamp mode from an individual afferent neuron in loose-patch configuration. Recordings were sampled at 100 μS/pt and filtered at 1 kHz. Neuromast hair-cell stimulation was performed with a pressure clamp (HSPC-1, ALA Scientific, NY) attached to a glass micropipette (tip diameter ~30 μM) filled with normal extracellular solution. The waterjet pipette was positioned (MP-265, Sutter Instruments) ~100 μm from an innervated neuromast (determined by sequential stimulation of multiple neuromasts) and cupula displacement was verified by eye. The pressure clamp received a sinusoidal analog voltage command driven concurrently by the EPC 10. Waterjet pressure was monitored with a feedback sensor located on the HSPC-1 headstage and was collected in Patchmaster alongside the current recording. Data were analyzed using custom software written in Igor Pro (Wavemetrics, Lake Oswego, OR, USA) and were plotted with Prism 5 (Graphpad Software).
RESULTS
Ribeye expression and localization in developing and mature hair cells
As zebrafish have two copies of ribeye (Wan et al., 2005), we determined the temporal expression pattern of both genes using whole-mount in situ hybridization (Fig. 1A). Both ribeye a and ribeye b genes (ctbp2 and ctbp2l – Zebrafish Information Network) were expressed in the developing zebrafish ear by 28 hours post fertilization (hpf; Fig. 1A). Expression was also apparent in the retina (ribeye a) and in clusters of superficial hair cells called neuromasts (Fig. 1A; 52 hpf).

To address whether Ribeye a and Ribeye b colocalize during synapse development, we examined their localization in the developing ear. At 22 hpf, nascent hair cells contained small, colocalized Ribeye a and Ribeye b punctae throughout the cell body (Fig. 1B). By 26 hpf, these punctae formed larger aggregates at the basal end of hair cells where synaptic contacts are formed (Fig. 1C) and by 28 hpf, Ribeye immunolabel was predominantly at the basal end of the cell (Fig. 1D,E). This pattern of immunolabel is consistent with the idea that Ribeye is a component of transport units that are actively transported to presynaptic active zones and ultimately form a mature ribbon synapse (Magupalli et al., 2008; Regus-Leidig et al., 2009).

Previous studies of neuromasts in developing zebrafish reported that innervating afferent neurons form both thin, motile neurites that make short lived contacts with hair cells as well as thick, bulging neurites that probably correspond to stable synaptic connections (Faucherre et al., 2010; Faucherre et al., 2009; Nagiel et al., 2008). Therefore, we examined localization of Ribeye with respect to innervating afferent neurons at 2 days post fertilization (dpf). By visualizing afferent neurons with stable GFP expression (Obholzer et al., 2008), we detected fine neuronal processes in proximity to neuromast hair cells (see Fig. S1A in the supplementary material, white arrows) and thick dendritic varicosities adjacent to Ribeye immunolabeled punctae (see Fig. S1A in the supplementary material, white arrowheads). This data supports the idea that the presence of Ribeye punctae corresponds with stable synaptic innervation during hair-cell development. By 4 dpf, the fibers had a complex branching pattern that overlapped with several Ribeye punctae per hair cell and included a few thin neurites that did not localize with Ribeye (see Fig. S1B in the supplementary material).

To further characterize synaptogenesis in zebrafish hair cells, we compared Ribeye staining with a postsynaptic marker. The PSD-95 family of membrane associated guanylate kinases (MAGUKs) are scaffolding proteins that are thought to regulate the assembly of postsynaptic densities in excitatory synapses (Boeckers, 2006; Meyer et al., 2005). Although MAGUK immunolabel initially appeared between 26 and 30 hpf, within proximity of the basal end of hair cells, it did not juxtapose Ribeye (see Fig. S1C,D in the supplementary material). Later, we observed that larger aggregates of MAGUK had formed and were now adjacent to hair cells (see Fig. S1E in the supplementary material). In mature hair cells, MAGUK immunolabel appeared to partially overlap with Ribeye (see Fig. S1F in the supplementary material) in a similar manner as Ribeye and GLUR2/3 label in mouse auditory hair cells (Nemzou et al., 2006).

Knockdown of ribeye a and ribeye b in hair cells
To examine the function of Ribeye in hair-cell development, we employed ribeye MOs. We used a previously published MO to inhibit ribeye a (Wan et al., 2005) and designed two MOs that specifically targeted either the translation start region or a splice site in ribeye b, but not ribeye a or CtBP2b. To further confirm the specificity of the ribeye b splice site MO, we performed RT-PCR on control and ribeye b splice MO injected morphants. We found that the MO specifically blocked the correct splicing of ribeye b exon 1 and did not disrupt splicing of ribeye a exon 1 (see Fig. 1, ribeye a and ribeye b expression and localization during zebrafish hair-cell development. (A) Expression of ribeye a and ribeye b mRNA in the developing ear and neuromasts. ribeye b is expressed strongly in the otic vesicle at 20 hpf, while ribeye a is expressed after 20 hpf, and at lower levels (black arrowheads). At 52 hpf, ribeye a is detectable in the retina and ear (black arrowhead), whereas ribeye b is expressed in both the ear (black arrowhead) and lateral line neuromasts (black and white arrowheads). Scale bar: 200 μm. (B-E) Representative maximum confocal z-projections of hair cells in wild-type larvae. Scale bars: 3 μm. (B-D) Ribeye immunofluorescent labeling in nascent hair cells in the developing ear at 22 hpf (B), 26 hpf (C) and 28 hpf (D). Ribeye a and Ribeye b proteins form aggregates that, by 26 hpf, are accumulated at the basal end of hair cells (white arrowheads). (E) Ribeye immunofluorescent labeling in a cross-section of a neuromast at 5 dpf. Merged images include DAPI labeling of cell nuclei (blue).
Fig. 2). Because we observed comparable phenotypes in ribeye b morphant larvae injected with either the translation or the splice blocking MO (Fig. 4B, data not shown), we used the ribeye b splice blocking MO for the majority of the experiments in this study.

After injection of larvae with either control or antisense MOs, we examined immunolabeling of Ribeye in neuromast hair cells. Imaging neuromast hair cells is advantageous because they are located near the surface of the skin and are therefore highly accessible. Typically each neuromast on the trunk contains eight to 12 hair cells at 120 hpf, and each hair cell contains approximately four ribbon bodies (Obholzer et al., 2008). Thus, neuromast hair cells are more amenable to quantification of immunolabel than hair cells of other vertebrates, which contain far more ribbon bodies (Moser et al., 2006). Embryos injected with the MO that inhibited ribeye a translation (75-100 μM) initially showed knockdown of ribeye a, whereas the intensity of Ribeye b immunolabel was equivalent to 2 dpf stage-matched control animals (Fig. 2A,B; Fig. 3F). The number of Ribeye b-labeled punctae per neuromast was also comparable with control (Fig. 2E).

A different result was obtained with ribeye b MO (0.75 mM; Fig. 2C,E,F). Ribeye b immunolabel was effectively eliminated, whereas Ribeye a immunolabel intensity was significantly higher than control larvae (Fig. 2C,F; Mann-Whitney U-test, P<0.0001). When ribeye a and b MOs were injected together, immunolabel of both Ribeye a and b punctae was severely reduced (Fig. 2D). We were unable to quantify Ribeye labeling in double morphants because we could not detect Ribeye a or b immunolabel above background levels.

With injections of either ribeye a or b MO, knockdown of the target gene was still apparent at 4 dpf (Fig. 3A-E). In contrast to what we observed at 2 dpf, we detected reduced labeling of the untargeted isoforms of Ribeye at this later stage: Ribeye b immunolabel was less intense in ribeye a (100 μM) morphants compared with control injected larvae (Fig. 3B,E,F), and a similar reduction in Ribeye a immunolabel was observed in larvae injected with the splice site MO against ribeye b (0.75-1.0 mM; Fig. 3C,D,F). Overall, depleting either Ribeye protein led to a less severe, but significant, reduction of the other, i.e. hair cells in larvae injected with ribeye b MO had fewer Ribeye a punctae with significantly less total intensity and vice versa (Fig. 3D-F; Mann-Whitney U-test: P<0.0001 and P<0.003, respectively).

We also examined knockdown of both ribeye isoforms in 4-day-old larvae. In general, we found that the ribeye a MO was more toxic than the ribeye b MO. Therefore, we injected lower doses of both morpholinos and restricted our analysis to morphologically normal larvae. When we injected low concentrations of both morpholinos (50 μM ribeye a and 0.75 mM ribeye b MO) approximately one-third of 4-day-old larvae were excluded from our experiments because of edema and abnormal morphology (short trunk, small head, etc.). The remaining double morphants presented a wide range of phenotypes (Fig. 3G; Fig. 7C-E). Moreover, the average total pixel intensity of Ribeye a immunolabeled punctae was not significantly different from control (Fig. 3H; Mann-Whitney U-test: P=0.2). This may be due to less ribeye a knockdown with a lower dose of MO in the double

![Fig. 2. Morpholino-mediated knockdown of ribeye a and ribeye b in 2-day-old zebrafish larvae. (A-D) Representative confocal z-projections of Ribeye a (red) and Ribeye b (green) immunolabel in neuromast hair cells of wild-type (A), ribeye a morphant (B), ribeye b morphant (C), and ribeye a and ribeye b morphant larvae (D). Merged images include DAPI (blue). Scale bar: 3 μm. (E) The average number of Ribeye a punctae per neuromast (white circles) in control (11±2) and ribeye b MO-injected (10±2) larvae, and the average number of Ribeye b punctae (black circles) in control (7±1) and ribeye a MO-injected (9±2) larvae. Each circle represents neuromast 1 in the trunk of an individual larva. Error bars indicate s.e.m. (F) Total pixel intensity (A.U.) of Ribeye a immunolabel in control and ribeye b morphants (Mann-Whitney U-test: P<0.0001), and Ribeye b in control and ribeye a morphants (Mann-Whitney U-test: P=0.80). Whiskers indicate the 10th and 90th percentiles. (G) RT-PCR analysis of control (C) and antisense ribeye b splice site MO-injected whole larvae. cav1.3a was used as a control.](image-url)
morphants combined with the initial compensatory increase in ribeye a expression in response to knockdown of ribeye b (see Fig. 2C,F).

Depletion of Ribeye causes hearing and balance defects and reduced postsynaptic activity

Because both ribeye genes are expressed in hair cells, it is likely both isoforms are needed for hearing and balance. To address this possibility, we injected either ribeye a or ribeye b MO alone and tested the acoustic startle reflex of 4-day-old morphants (Fig. 4A). A significant percentage of ribeye a and ribeye b MO injected larvae either did not respond to acoustic stimuli, indicating deafness (Nicolson et al., 1998), or were unable to remain upright while swimming, indicative of a balance defect. Consistent with a requirement of both isoforms, the percentage of larvae lacking an acoustic startle reflex was greater with increasing doses of either MO, or when both MOs were combined (Fig. 4A). Because of the toxicity issues with the ribeye a MO mentioned above, combined with the finding that high doses of ribeye b MO also depleted Ribeye a and led to a comparable percentage of larvae with a defective acoustic startle reflex, we performed the following functional experiments with ribeye b MO alone.

A direct measure of vestibular function can be obtained by testing compensatory eye movements in response to rotation of larvae in the dark with infrared illumination to eliminate visual cues (Mo et al., 2010). In this manner, we quantified vestibular defects in ribeye b morphants. Vestibular-induced eye movements in response to rotation of the head were significantly reduced in morphant larvae compared with control MO-injected larvae (Fig. 4B; Mann-Whitney U-test; P<0.0002). In summary, neither ribeye isoform fully compensated for the other, as knockdown of either gene led to hearing and/or balance defects in zebrafish.
Our results, showing that Ribeye b depletion led to fewer and smaller synaptic ribbons (Fig. 3D,F and see Fig. S2 in the supplementary material) and consequent defects in hearing and balance, suggested that synaptic transmission was disrupted in hair cells. To test this hypothesis, we recorded evoked action potentials (spikes) from afferent neurons in larvae with variable degrees of ribeye b knockdown. Previously, we have shown that in the lateral-line organ, synaptic transmission from hair cells is required for postsynaptic spiking (Obholzer et al., 2008). Thus, spiking recorded in response to direct mechanical stimulation of innervated hair cells provides an indirect measure of hair-cell transmission (Trapani et al., 2009). We found that compared with control larvae, a morphant injected with 0.75 mM ribeye b MO showed a slight reduction in the number of spikes recorded in response to a 20 Hz stimulus (Fig. 5A, gray circle). Furthermore, morphants injected with a higher concentration of ribeye b MO (1 mM) did not respond to acoustic stimuli and displayed a greater reduction in afferent spikes (Fig. 5A, blue and red circles). The timing of the spikes did not appear to be drastically altered as the degree of phase locking to the stimulus was unaffected (Fig. 5B). To correlate the apparent reduction in synaptic transmission with the severity of Ribeye depletion, we then examined Ribeye immunolabel from the three morphant larvae recorded in Fig. 5A. The number of Ribeye labeled punctae was correlated to the degree of reduction in spiking (Fig. 5C,D; Pearson’s r = 0.79, P<0.005; n=11). This correlation supports the notion that the behavioral phenotype observed in the ribeye morphants is due to reduced synaptic transmission.

Afferent innervation is reduced when Ribeye is depleted

The reduction in number of evoked spikes prompted us to examine the degree of afferent innervation in morphants. In wild-type zebrafish, each neuromast is innervated on average by two neurons that branch at several points to make contact with multiple hair cells. Our results, showing that Ribeye b depletion led to fewer and smaller synaptic ribbons (Fig. 3D,F and see Fig. S2 in the supplementary material) and consequent defects in hearing and balance, suggested that synaptic transmission was disrupted in hair cells. To test this hypothesis, we recorded evoked action potentials (spikes) from afferent neurons in larvae with variable degrees of ribeye b knockdown. Previously, we have shown that in the lateral-line organ, synaptic transmission from hair cells is required for postsynaptic spiking (Obholzer et al., 2008). Thus, spiking recorded in response to direct mechanical stimulation of innervated hair cells provides an indirect measure of hair-cell transmission (Trapani et al., 2009). We found that compared with control larvae, a morphant injected with 0.75 mM ribeye b MO showed a slight reduction in the number of spikes recorded in response to a 20 Hz stimulus (Fig. 5A, gray circle). Furthermore, morphants injected with a higher concentration of ribeye b MO (1 mM) did not respond to acoustic stimuli and displayed a greater reduction in afferent spikes (Fig. 5A, blue and red circles). The timing of the spikes did not appear to be drastically altered as the degree of phase locking to the stimulus was unaffected (Fig. 5B). To correlate the apparent reduction in synaptic transmission with the severity of Ribeye depletion, we then examined Ribeye immunolabel from the three morphant larvae recorded in Fig. 5A. The number of Ribeye labeled punctae was correlated to the degree of reduction in spiking (Fig. 5C,D; Pearson’s r = 0.79, P<0.005; n=11). This correlation supports the notion that the behavioral phenotype observed in the ribeye morphants is due to reduced synaptic transmission.

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The reduction in number of evoked spikes prompted us to examine the degree of afferent innervation in morphants. In wild-type zebrafish, each neuromast is innervated on average by two neurons that branch at several points to make contact with multiple hair cells. To test this hypothesis, we recorded evoked action potentials (spikes) from afferent neurons in larvae with variable degrees of ribeye b knockdown. Previously, we have shown that in the lateral-line organ, synaptic transmission from hair cells is required for postsynaptic spiking (Obholzer et al., 2008). Thus, spiking recorded in response to direct mechanical stimulation of innervated hair cells provides an indirect measure of hair-cell transmission (Trapani et al., 2009). We found that compared with control larvae, a morphant injected with 0.75 mM ribeye b MO showed a slight reduction in the number of spikes recorded in response to a 20 Hz stimulus (Fig. 5A, gray circle). Furthermore, morphants injected with a higher concentration of ribeye b MO (1 mM) did not respond to acoustic stimuli and displayed a greater reduction in afferent spikes (Fig. 5A, blue and red circles). The timing of the spikes did not appear to be drastically altered as the degree of phase locking to the stimulus was unaffected (Fig. 5B). To correlate the apparent reduction in synaptic transmission with the severity of Ribeye depletion, we then examined Ribeye immunolabel from the three morphant larvae recorded in Fig. 5A. The number of Ribeye labeled punctae was correlated to the degree of reduction in spiking (Fig. 5C,D; Pearson’s r = 0.79, P<0.005; n=11). This correlation supports the notion that the behavioral phenotype observed in the ribeye morphants is due to reduced synaptic transmission.
cells (Faucherre et al., 2009; Nagiel et al., 2008; Obholzer et al., 2008). Unexpectedly, we observed reduced afferent innervation in larvae with fewer Ribeye punctae. Innervating nerve fibers appeared shorter and less complex with greater Ribeye depletion (Fig. 6A). In 4-day-old controls and larvae injected with both ribeye a and ribeye b MOs, we quantified the number of Ribeye a punctae in primary neuromast 2 of the posterior lateral line and compared it with the total length of innervating afferent terminal fibers, as well as the complexity of arborization. Both the total process length and the number of innervating branches were correlated to the number of Ribeye punctae in ribeye morphants (Fig. 6B,C; Pearson’s r = 0.92, P < 0.0001 and r = 0.78; P < 0.003, respectively). Because we also observed fewer hair cells in morphant neuromasts compared with wild type (8±2 hair cells in ribeye a morphants. Each representative confocal z-projections showing GFP-labeled innervating afferent neurons (green), Ribeye a immunolabel (red) and DAPI label (blue) in lateral line neuromast 2 from three individual 4 dpf larva. Scale bars: 3 μm. (B,C) Plots of the relationship between either the total length of innervating afferent processes (B) or the number of afferent branch junctions (C) and the number of Ribeye a immunolabeled punctae in ribeye a and ribeye b morphants. Each point represents neuromast 2 in an individual larva. (D,E) Plot of the relationships between the total length of innervating afferent processes (D) or the number of afferent branch junctions (E) and the number of Ribeye a immunolabeled punctae in wild-type and control MO-injected larvae. There was no significant correlation between dendritic length (Pearson’s n=0.4525; P=0.08) or the number of afferent branches (Pearson’s n=0.0129; P=0.97) and the number of Ribeye a punctae. The linear regressions of the plots in either B or C are included for comparison (broken red line).

Fig. 6. Depletion of Ribeye results in reduced afferent innervation. (A) Representative confocal z-projections showing GFP-labeled innervating afferent neurons (green), Ribeye a immunolabel (red) and DAPI label (blue) in lateral line neuromast 2 from three individual 4 dpf larva. Scale bars: 3 μm. (B,C) Plots of the relationship between either the total length of innervating afferent processes (B) or the number of afferent branch junctions (C) and the number of Ribeye a punctae in primary neuromast 2 of the posterior lateral line and compared it with the total length of innervating afferent terminal fibers, as well as the complexity of arborization. Both the total process length and the number of innervating branches were correlated to the number of Ribeye punctae in ribeye morphants. Each point represents neuromast 2 in an individual larva. (D,E) Plot of the relationships between the total length of innervating afferent processes (D) or the number of afferent branch junctions (E) and the number of Ribeye a punctae. The linear regressions of the plots in either B or C are included for comparison (broken red line).

The number of afferent branch junctions (C) and the number of Ribeye a punctae. The linear regressions of the plots in either B or C are included for comparison (broken red line).

Fig. 7. Localization of the postsynaptic density in ribeye morphants. (A-E) Representative confocal z-projections of pan-MAGUK (green) and Ribeye a (red) immunolabel in posterior lateral line neuromast 2 of 4 dpf larvae. Scale bars: 3 μm (main panels), 1 μm (far right panels). (A,B) MAGUK immunolabel juxtaposes Ribeye-labeled punctae in wild-type (A; n=24) and ribeye b MO-injected larvae (B; n=15). Yellow pixels present in the far right panels indicate the close proximity of Ribeye to MAGUK immunolabel. The brightness of Ribeye a immunolabel in B was increased in the merged panel to better visualize its localization. (C) Ribeye immunolabel was absent in 18% (n=33) of ribeye a and ribeye b MO-injected larvae. (D) MAGUK immunolabel appeared to overlap Ribeye in 49% (n=33) of ribeye a and ribeye b MO-injected larvae. (E) MAGUK immunolabel did not overlap Ribeye in 33% (n=33) of ribeye a and ribeye b MO-injected larvae. Yellow pixels are reduced in the far right panel.
background (Fig. 7C). Notably, even in the absence of Ribeye immunolabel, we saw accumulations of MAGUK immunolabel, presumably at the tips of the remaining afferent fibers. The majority of double morphants analyzed showed Ribeye immunolabel: in 49% of the larvae examined (16/33) MAGUK immunolabel was still tightly juxtaposed to Ribeye (Fig. 7D), but in 33% of the larvae (11/33) there was loss of juxtaposition between Ribeye and MAGUK immunolabel (Fig. 7E). These results indicate that levels of Ribeye influence the accumulation of postsynaptic components.

Modulation of Ribeye protein levels affects calcium channel localization
CaV1.3 channels preferentially localize to ribbon active zones in hair cells (Brandt et al., 2005; Sidi et al., 2004). Therefore, we examined the effect of modulation of ribeye expression on CaV1.3a localization. In control larvae, CaV1.3a channel clusters were observed at the basal end of hair cells, and were colocalized with Ribeye punctae (Fig. 8A). However, CaV1.3a clusters were absent in larvae with knockdown of both ribeye isoforms (Fig. 8B) or severe knockdown of ribeye b alone (Fig. 8C). Additionally, in neuromasts where Ribeye b levels were weakly depleted, there were fewer CaV1.3a-postive clusters, and these clusters colocalized with intensely labeled Ribeye b positive aggregates (Fig. 8D). Altogether, these findings are consistent with a requirement for Ribeye protein for proper localization of CaV1.3a channels.

To determine whether the absence of CaV1.3a channels in ribeye morphants was due to an effect on cav1.3a transcript levels, we performed RT-PCR using the tails of uninjected or MO injected larvae. Tail tissue was used to specifically isolate cav1.3a expressed in hair cells of the posterior lateral line and exclude

Fig. 8. Ribeye depletion disrupts clustering of presynaptic CaV1.3a channels in hair cells. (A-D) Ribeye b (green) and CaV1.3a (red) immunolabel in cross-sections of anterior lateral line neuromasts at 4 dpf. Merged images include DAPI (blue). Scale bar: 3 μm. (A) CaV1.3a clusters are colocalized with Ribeye b-labeled punctae in control MO-injected larvae. (B) CaV1.3a clusters are absent in ribeye a & b double morphants (50 μM and 750 μM, respectively). (C) CaV1.3a clusters are absent in ribeye b MO-injected (1 mM) larvae. (D) CaV1.3a clusters in ribeye b MO-injected D (500 μM) larvae. CaV1.3a colocalized with the larger Ribeye b punctae in a neuromast with weak Ribeye b knockdown. (E) RT-PCR analysis of tails from 4 dpf larvae injected as in A and B. gapdh was used as a loading control and pcdh15 was used as a hair-cell specific transcript control.
cav1.3a expressed in the brain (Sidi et al., 2004). We found that cav1.3a mRNA levels were similar between control and ribeye morphants (Fig. 8E).

Given our result that ribeye knockdown abolished CaV1.3a immunolabel, we examined whether overexpression of Ribeye would also affect the localization of Cav1.3a channels. We elicited high expression of exogenous Ribeye by generating transgenic fish carrying multiple copies of GFP-tagged Ribeye b, which resulted ectopic ribbon bodies at the apical end of hair cells (Fig. 9D,E; Fig. 10A',C'). Interestingly, in these transgenic larvae we observed intensely labeled Cav1.3a clusters that colocalized with ectopic Ribeye b-GFP punctae (Fig. 10A'), whereas cav1.3a transcript levels appeared similar to wild type (Fig. 10B). This finding illustrates a crucial role for Ribeye in localizing Ca²⁺ channels near synaptic ribbons. To determine whether the ectopic ribbon bodies formed synapses, we immunolabeled transgenic hair-cell postsynaptic densities. We were unable to detect MAGUK immunolabel near ectopic Ribeye punctae (Fig. 10C,C'). In addition, we did not observe afferent fibers that projected beyond the basal half of the hair cells (data not shown). We did, however, observe MAGUK immunolabel juxtaposed to Ribeye punctae near the basal end of hair cells. Despite the presence of larger ribbon bodies, the MAGUK immunolabel did not appear to be enlarged compared with wild type (Fig. 10C,C'). We counted the number of synapses and found that the number of MAGUK-positive punctae per hair cell did not increase in larvae expressing high levels of exogenous Ribeye b-GFP (Fig. 10D).

**DISCUSSION**

In the current study, we examined how Ribeye contributes to the formation of ribbon synapses in hair cells. We observed that (1) depletion of Ribeye was correlated to reduced synaptic transmission and afferent innervation, (2) ribeye knockdown led to a loss of CaV1.3a channel clustering, and (3) ectopic accumulations of Ribeye in transgenic Ribeye b-GFP larvae colocalized with ectopic Cav1.3a channel clusters. Combined, these results indicate that Ribeye is necessary for both the stabilization of afferent innervation and Cav1.3a channel clustering.

During development, afferent neurons of the lateral line organ in zebrafish feature both fine dynamic processes and stable bulging termini that form contacts with hair cells and correspond with ribbon synapses (Faucherre et al., 2009; Nagiel et al., 2008). Our observations that Ribeye localizes across from afferent bulges during development, and that depletion of Ribeye correlates with fewer afferent synaptic contacts, support a necessary role for Ribeye in stabilizing synaptic contacts with innervating neurons. We postulate that this function of Ribeye is not dependant on synaptic transmission. A recent study examined afferent innervation of lateral line neuromasts in zebrafish mutants lacking vesicular glutamate transporter 3 (Vglut3), the transporter responsible for filling hair cell synaptic vesicles with glutamate (Nagiel et al., 2009; Obholzer et al., 2008). They reported no significant difference in the mean fraction of hair cells innervated by a single afferent neuron in vglut3 mutants versus wild type, and concluded that synaptic transmission did not appear to be essential for synaptic maintenance (Nagiel et al., 2009). These results suggest that Ribeye is probably playing a crucial structural role, perhaps by recruiting additional molecules to stabilize synaptic connections. It is notable that in transgenic larvae with high levels of Ribeye b-GFP, MAGUK does not juxtapose ectopic accumulations of Ribeye and Cav1.3a. Therefore, while Ribeye appears necessary for stabilizing synaptic contacts, additional localized molecular cues may be required for afferent neurons to form synapses with hair cells.

**Fig. 10.** Ectopic aggregates of overexpressed Ribeye colocalize with ectopic clusters of Cav1.3a channel in hair cells. Merged images include DAPI (blue). Scale bars: 3 μm. (A,A') Ribeye b (green) and Cav1.3a (red) immunolabeling in cross-sections of anterior lateral line neuromasts in 6 dpf larvae. (B) RT-PCR analysis of cav1.3a transcripts in wild-type and transgenic larvae. Gapdh was used as a loading control. (C,C') Ribeye b (green) and MAGUK (red) immunofluorescent labeling in cross-sections of neuromasts. (D) The average number of MAGUK-positive punctae in wild-type and larvae stably expressing high levels of GFP-tagged Ribeye b. Error bars indicate s.e.m.
Why zebrafish hair cells express both ribeye a and ribeye b is not clear. Both isoforms are expressed early in hair-cell development, show considerable colocalization before synapse formation, and tightly colocalize at mature synapses. In 4-day-old morphants with knockdown of one isoform, the protein levels of the untargeted Ribeye isoform were reduced by approximately half, suggesting that both Ribeye a and b are required to form a stable complex. Accordingly, the remaining MAGUK label, although properly localized, also appeared reduced at later stages, indicating that a stable Ribeye complex is important for maintenance of localized postsynaptic components. By contrast, depletion of both Ribeye isoforms appeared to disrupt juxtaposition of pre- and postsynaptic components in approximately one-third of the morphants. This result suggests that the presence of either one of the Ribeye proteins during the initial stages of synaptogenesis, or possibly the ratio of the two proteins later in development, may contribute to the alignment of pre- and postsynaptic components.

How might Ribeye orchestrate the clustering of Cav1.3a channels? One possibility is that Cav1.3a is trafficked with Ribeye during development. Several studies indicate that this scenario is unlikely. Presynaptic spheres in retinal bipolar cells contain the proteins Piccolo, Bassoon and Ribeye, but do not contain L-type Ca\(^{2+}\) channel \(\alpha 1\) subunit (Regus-Leidig et al., 2009). Furthermore, in inner hair cells of developing mouse cochlea only about 50% of synaptic ribbons colocalize with Cav1.3 (Zampini et al., 2010). Alternatively, the Ribeye aggregates at the plasma membrane may direct the clustering of Cav1.3a. This hypothesis is supported by observations made from mice deficient for the protein Bassoon, the absence of which prevents anchoring of the synaptic ribbon in photoreceptors (Dick et al., 2003) and hair cells (Khimich et al., 2005). In bassoon\(^{-/-}\) photoreceptor terminals, Ribeye immunolabeled punctae do not colocalize with Ca\(^{2+}\) channel \(\alpha 1\) subunit, and the Ca\(^{2+}\) channel labeling appears more diffuse than wild type (Tom Dieck et al., 2005). Additionally, studies examining Drosophila neuromuscular junctions report that Bruchpilot, a structural protein that is an integral part of Drosophila presynaptic densities, is responsible for clustering Ca\(^{2+}\) channels at the active zone, but not for Ca\(^{2+}\) channel trafficking to the presynapse (Fouquet et al., 2009; Kittle et al., 2006). Collectively, these studies combined with our results support a model in which Ribeye assembles synaptic ribbons by forming presynaptic spheres containing Bassoon, accumulating into aggregates at the presynaptic terminal, and recruiting additional proteins, e.g., Cav1.3, once attached to the plasma membrane.

Taken together, our results reveal two important functions for Ribeye in the formation of ribbon synapses in zebrafish hair cells and support a role for Ribeye as a synaptic organizer. Future studies exploring the molecular mechanisms underlying both Ribeye-mediated Cav1.3a channel clustering and postsynaptic stabilization will not only shed light on how the ribbon synapse matures during development, but may also give insight into functional ribbon-synapse plasticity (Emran et al., 2010; Henry and Mulroy, 1995).

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**Compelling interests statement**

The authors declare no competing financial interests.


