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Enlargement of ribbons in zebrafish hair cells increases calcium currents, but disrupts afferent spontaneous activity and timing of stimulus onset

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Title: Enlargement of ribbons in zebrafish hair cells increases calcium currents, but disrupts afferent spontaneous activity and timing of stimulus onset

Abbreviated title: Relating hair-cell ribbon size to function

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ABSTRACT

In sensory hair cells of auditory and vestibular organs, the ribbon synapse is required for the precise encoding of a wide range of complex stimuli. Hair cells have a unique presynaptic structure—the synaptic ribbon—that organizes both synaptic vesicles and calcium channels at the active zone. Previous work has shown that hair-cell ribbon size is correlated with differences in postsynaptic activity. However, additional variability in postsynapse size presents a challenge to determining the specific role of ribbon size in sensory encoding. To selectively assess the impact of ribbon size on synapse function, we examined hair cells in transgenic zebrafish that have enlarged ribbons, without postsynaptic alterations. Morphologically, we found that enlarged ribbons had more associated vesicles and reduced presynaptic calcium-channel clustering. Functionally, hair cells with enlarged ribbons had larger global and ribbon-localized calcium currents. Afferent neuron recordings revealed that hair cells with enlarged ribbons resulted in reduced spontaneous spike rates. Additionally, despite larger presynaptic calcium signals, we observed fewer evoked spikes with longer latencies from stimulus onset. Taken together, our work indicates that hair-cell ribbon size influences the spontaneous spiking and the precise encoding of stimulus onset in afferent neurons.

Significance statement

Numerous studies support that hair-cell ribbon size corresponds with functional sensitivity differences in afferent neurons and, in the case of inner hair cells of the cochlea, vulnerability to damage from noise trauma. Yet it is unclear whether ribbon size directly influences sensory encoding. Our study reveals that ribbon enlargement results in increased ribbon-localized calcium signals, yet reduces afferent spontaneous activity and disrupts the timing of stimulus onset—a distinct aspect of auditory and vestibular encoding. These observations suggest that varying ribbon size alone can influence sensory encoding, and give further insight into how hair cells transduce signals that cover a wide dynamic range of stimuli.

INTRODUCTION

Hair cells, the sensory receptors of auditory, vestibular, and lateral-line organs, utilize specialized ribbon synapses to encode the timing and intensity of sensory information. Hair-cell ribbon synapses are capable of rapid neurotransmitter release within milliseconds of stimulus onset, and sustained neurotransmitter release over many seconds and longer (Parsons et al., 1994; Moser and Beutner, 2000; Matthews and Fuchs, 2010). What features enable the hair-cell ribbon synapse to perform such fine tasks is not well understood.

Hair-cell ribbon synapses are defined by a unique presynaptic structure known as the synaptic ribbon, which is a dense specialization that tethers glutamate-filled vesicles adjacent to clusters of the presynaptic calcium channel $Ca_V1.3$ (Usukura and Yamada, 1987; Brandt et al., 2005; Obholzer et al., 2008; Seal et al., 2008; Schmitz, 2009; Frank et al., 2010). The major component of ribbons is Ribeye, a unique protein that is vital for the physical integrity and function of ribbon synapses (Schmitz et al., 2000; Zenisek et al., 2004; Frank et al., 2010; Sheets et al., 2011; Lv et al., 2016). The size and shape of ribbons vary depending on species and hair-cell type, but it is unclear how these differences impact synapse function (Moser et al., 2006). Ribbons have been shown to tether and stabilize vesicles at the presynaptic active zone (Smith and Sjostrand, 1961; Khimich et al., 2005; Buran et al., 2010). The number of tethered vesicles increases with larger ribbons (reviewed in Nouvian et al., 2006), although the functional implications of the additional vesicles is not clear. Previous studies have also shown that hair-cell ribbons are able to recruit $Ca_V1.3$ channels, indicating an intimate relationship between these structures (Frank et al., 2010; Sheets et al., 2011, 2012; Wong et al., 2014; Lv et al., 2016).

In the mammalian auditory system, substantial work has been done to determine how ribbon size, $Ca_V1.3$ channels, and vesicle populations ultimately impact sensory encoding. Studies have shown that, in auditory inner hair cells (IHCs) are innervated by multiple afferent-nerve fibers (Pfeiffer and Kiang, 1965; Liberman, 1982; Taberner and Liberman, 2005; Johnson et al., 2008). There is evidence that synapses with larger ribbons and smaller postsynapses have a low rate of spontaneous release and correspond to high-threshold nerve fibers, while synapses with smaller ribbons and larger postsynapses show a higher rate of spontaneous release and correspond to low-threshold nerve fibers (Liberman et al., 2011). Whether differences in afferent activity are due to morphological differences at the postsynapse or at the ribbon is not well understood. In auditory IHCs, larger ribbons have also been shown to localize more $Ca_V1.3$ channels and have larger synaptic calcium signals compared to smaller ribbons (Meyer et al., 2009; Ohn et al., 2016). Despite larger calcium signals, larger ribbons were not correlated with more afferent activity, which has been attributed to a depolarizing shift in calcium channel activation present at larger ribbons compared to smaller ribbons. (Ohn et al., 2016). Overall, additional differences in $Ca_V1.3$ channel numbers and variability in the size of the postsynapse across ribbons in IHCs makes it is difficult to isolate the impact of ribbon size on auditory encoding.

To address how ribbon size influences hair-cell ribbon-synapse function, we used a transgenic zebrafish line that overexpresses Ribeye and enlarges ribbons (Sheets et al., 2011). Although hair-cell ribbons are enlarged in this transgenic line, there was no significant effect on postsynaptic size. Using a multipronged approach, we used this transgenic line as a model to understand how hair-cell ribbon size alters ribbon-synapse morphology and function. We

found, at the ultrastructural level, that enlarged ribbons have more associated synaptic vesicles yet a similar number of docked vesicles. Functionally, both global and ribbon-localized calcium signals are increased in hair cells with enlarged ribbons. Despite increased calcium signaling, presynaptic $Ca_V1.3$ channel density does not appear to scale up with ribbon enlargement, and channel density is reduced. Despite increases in calcium current and more associated vesicles, ribbon enlargement resulted in a reduction in spontaneous action potentials, and a longer latency to fire following stimulus onset.

MATERIALS AND METHODS

Fish Strains and Reagents. Adult zebrafish (*Danio rerio)* were maintained with a 14-hour light, 10-hour dark cycle using standard methods. Zebrafish work performed at the NIH was approved by the Animal Use Committee at the NIH under animal study protocol #1362-13. At Oregon Health and Sciences University, zebrafish work was overseen by the Institutional Animal Care and Use Committee. At Massachusetts Eye and Ear, zebrafish work was performed with the approval of the Massachusetts Eye and Ear Animal Care Committee and in accordance with NIH guidelines for use of zebrafish, protocol #13-001A. All zebrafish work at the University of Sheffield was licensed by the UK Home Office under the Animals (Scientific Procedures) Act 1986 and approved by the University of Sheffield Ethical Review Committee. Larvae were examined at 3-7 day post fertilization (dpf) unless stated otherwise. At these ages sex cannot be predicted or determined, and therefore sex of the animal was not considered in our studies. Zebrafish larvae were raised in E3 embryo media in mM: 5 NaCl, 0.17 KCl, 0.33 $CaCl_2$ and 0.33 $MgSO_4$, buffered in HEPES, at 30°C. All wild-type controls were non-transgenic siblings unless

stated otherwise. Previously described transgenic zebrafish strains used in this study include: *Tg(-6myo6b:ribeye b-EGFP)*^{vo67Tg}, *Tg(-6myo6b:RGECO)*^{vo10Tg} and *Tg(-6myo6b:GCaMP6s-CAAX)*^{idc1Tg} (Sheets et al., 2011; Maeda et al., 2014; Jiang et al., 2017).

Vector Construction and Transgenic Lines. To create additional Ribeye transgenic fish, plasmid construction was based on the tol2/Gateway zebrafish kit developed by the lab of Chi-Bin Chien at the University of Utah (Kwan et al., 2007). *Ribeye a* (NCBI Accession Number NM_001195491.1) and *Ribeye b* (NM_001015064.1) were cloned into the middle entry vector pDONR221 to create pME-*ribeye a* or pME-*ribeye b*. From the tol2 kit, vectors p3E-*mCherry* (388), pDestTol2 (395, 394) and p3E-polyA (302) were recombined with p5E -*6myosin6b* (Kindt et al., 2012), and our engineered plasmids to create the following constructs: -*6myosin6b:ribeye a-mCherry*, and -*6myosin6b:ribeye b-mCherry.*

To generate transgenic fish from these constructs, plasmid DNA (25-50 ng/µl) along with *tol2* transposase mRNA (25-50 ng/µl) was injected into zebrafish embryos at the single-cell stage. Transgenic lines were screened in the F1 and F2 generation for single copy integrations and expression level. The *Tg(-6myo6b:ribeye b-mCherry)*^{idc3Tg} transgenic strain was selected because using immunolabel (see methods below), it had normal number and size of ribbons compared to wild-type (ribbon area normalized to the wild-type median area, wild-type: 0.924 ± 0.073 a.u., n = 245 ribbons; *ribeye b-mCherry*: 0.909 ± 0.051 a.u., n = 264 ribbons, p = 0.867; synapses per hair cell via immunolabel: wild-type: 3.06 ± 0.13, n = 8 neuromasts; *ribeye b-mCherry*: 2.97 ± 0.14, n = 6 neuromasts, p = 0.601). *Tg(-6myo6b:ribeye a-mCherry)*^{idc2Tg} was chosen because similar to the *Tg(-6myo6b:ribeye b-EGFP)* ^{vo67Tg} transgenic strain, two copies of *Tg(-6myo6b:ribeye a-mCherry)*^{idc2Tg} resulted in ribbons that were significantly enlarged

compared to wild-type (ribbon area normalized to the wild-type median area, wild-type: 0.924 ± 0.073 a.u., n = 245 ribbons; *ribeye a-mCherry X 2*: 1.90 ± 0.190 a.u., n = 377 ribbons, p = 0.0006; synapses per hair cell via immunolabel: wild-type: 3.06 ± 0.13 n = 8 neuromasts; *ribeye a-mCherry X 2*: 2.86 ± 0.14, n = 8 neuromasts, p = 0.304). This analysis was performed on z-stack images acquired on a Zeiss LSM780 microscope (see methods below). All Ribeye transgenic fish used in this study had a similar number of hair cells, and a normal startle reflex and balance, indicating that our transgenes do not overtly alter auditory or vestibular function.

For electron microscopy, immunohistochemistry, whole-cell recordings and afferent recordings, an incross of *Tg(-6myo6b:ribeye b-EGFP)*^{vo67Tg} was used to compare larvae with 2 copies of Ribeye b-EGFP to wild-type, nontransgenic siblings. For cytosolic calcium measurements, *Tg(-6myo6b:RGECO1)*^{vo10Tg}*; Tg(-6myo6b:ribeye b-EGFP)*^{vo67Tg} X 2 triple transgenic hair cells were compared to *Tg(-6myo6b:RGECO1)*^{vo10Tg} single transgenic hair cells. For ribbon-localized calcium responses, *Tg(-6myo6b:GCaMP6s-CAAX)*^{idc1Tg}*; Tg(-6myo6b:ribeye a-mCherry)*^{idc2Tg} X 2 triple transgenic hair cells with enlarged ribbons were compared to *Tg(-6myo6b: GCaMP6s-CAAX)*^{idc1Tg}; *Tg(-6myo6b:ribeye b-mCherry)*^{idc3Tg} double transgenic hair cells with wild-type sized ribbons.

Zebrafish Immobilization and Hair Cell Mechanical Stimulation. To suppress muscle activity, larvae were anesthetized with 0.03% 3-amino benzoic acid ethyl ester (MS-222, Western Chemical, WA, USA), mounted with tungsten pins, and microinjected in the heart with 125 µM α-bungarotoxin (Tocris, Bristol, UK) to suppress muscle activity. Larvae were then rinsed and maintained in normal extracellular solution in mM: 130 NaCl, 2 KCl, 2 $CaCl_2$, 1 $MgCl_2$ and 10 HEPES, pH 7.3, 290 mOsm. Stimulation of neuromast hair cells was performed as

described previously (Trapani and Nicolson, 2010). Briefly, we used a pressure clamp (HSPC-1, ALA Scientific, New York) attached to a glass micropipette (inner tip diameter ~ 30 µm) filled with normal extracellular solution to mechanically stimulate hair cells. The waterjet pipette was positioned (MP-265, Sutter Instruments) approximately 100 µm from a given neuromast and displacement (3-5 µm) of the kinocilial tips was verified by eye. For recordings of lateral-line afferents, the pressure clamp was driven by a voltage command delivered by the recording amplifier and pressure was monitored from a feedback sensor located on the HSPC-1 headstage and collected concurrently. For calcium imaging experiments, the pressure clamp was driven by a voltage step command. An outgoing voltage signal from the imaging software was used to coordinate imaging with the pressure clamp stimulus.

Electrophysiology, Lateral-line Afferent Recordings. Our recording setup for action currents has been described in detail (Trapani and Nicolson, 2010; Olt et al., 2016b). For all experiments, recordings were performed in normal extracellular solution (see above) on afferent neurons innervating zebrafish primary neuromasts (L1–L4). For extracellular recordings, borosilicate glass pipettes were pulled (P-97, Sutter Instruments, Novato, CA) with a long taper and had resistances between 5 and 15 MΩ in extracellular solution. Signals were collected with a Multiclamp 700B, a Digidata 1550 data acquisition board, along with pClamp10 software (Molecular Devices, LLC, Sunnyvale, CA). Extracellular currents were acquired from an individual lateral-line afferent neuron in the loose-patch configuration (seal resistances ranged from 20 to 80 MΩ in extracellular solution). Recordings were done in voltage-clamp mode, sampled at 50 µs/pt, and filtered at 1 kHz. Spontaneous spike rate was quantified from measurements of 500 spontaneous events per neuron. The innervated neuromast for a

recorded neuron was identified by progressively stimulating primary neuromasts of the posterior lateral line until phase-locked spiking was detected.

Electrophysiology, Lateral-line Hair-cell Recordings. Whole-cell patch clamp experiments were performed from hair cells of the zebrafish primary neuromasts (L1–L4) as previously described (Olt et al., 2014, 2016b). The zebrafish were placed in a microscope chamber, with continuous perfusion via a peristaltic pump in the following extracellular solution in mM: 135 NaCl, 1.3 $CaCl_2$, 5.8 KCl, 0.9 $MgCl_2$, 0.7 NaH_2PO_4, 5.6 D-glucose, 10 Hepes-NaOH. Sodium pyruvate (2 mM), MEM amino acids solution (50X, without L-Glutamine) and MEM vitamins solution (100X) were added from concentrates (Fisher Scientific, UK). The pH was 7.5. For calcium current recordings, the extracellular solution was as the above but with 2.8 mM $CaCl_2$ instead of 1.3 mM (NaCl was reduced to 133 mM to keep the osmolality of the solution constant).

Calcium current and changes in membrane capacitance recordings were conducted at zebrafish body temperature (28.5°C). All other experiments (examination of K^+ currents and voltage responses) were performed at room temperature (21–24°C). Patch pipettes were made from soda glass capillaries (Harvard Apparatus Ltd, Edenbridge, UK) and had a typical resistance in the extracellular solution of 3-5 MΩ. To reduce the fast electrode capacitive transient, the shank of each capillary was coated with surfboard wax (Mr. Zog's SexWax, Sexwax Inc., Carpinteria, CA, USA). Current and voltage recordings were performed using the following intracellular solution in mM: 131 KCl, 3 $MgCl_2$, 1 EGTA-KOH, 5 Na_2ATP, 5 Hepes-KOH, and 10 sodium phosphocreatine, pH 7.3. For calcium current recordings and capacitance measurements, the intracellular solution contained in mM: 85 Cs-glutamate, 20 CsCl, 3 $MgCl_2$, 1

232 EGTA-CsOH, 5 Na_2ATP, 5 Hepes-CsOH, 10 Na_2-phosphocreatine, 0.3 Na_2GTP, 15 4-
233 aminopyridine (4-AP), and 20 TEA, pH 7.3. Recordings were made with an Optopatch amplifier
234 (Cairn Research Ltd, Faversham, UK). Data acquisition was performed using pClamp software
235 with a Digidata 1322A data acquisition board (Molecular Devices, LLC, Sunnyvale, CA).
236 Recordings were sampled at 5 kHz or 100 kHz, low pass filtered at 2.5 kHz or 10 kHz (8-pole
237 Bessel) and stored on computer for offline analysis using Origin 2016 (OriginLab Corp.,
238 Northampton, MA, USA) and pClamp 10 (Molecular Devices, LLC, Sunnyvale, CA). Membrane
239 potentials in voltage clamp were corrected for the voltage drop across the uncompensated
240 residual series resistance (*R*s: 5.3 ± 0.5 MΩ, *n* = 56) and for a liquid junction potential,
241 measured between electrode and bath solutions, of −4 mV for the KCl-based and −9 mV for Cs-
242 glutamate-based intracellular solution. Current responses are referred to a holding potential of
243 −84 mV or −79 mV, and are set to 0-current for easy comparison between recordings from
244 different hair cells.

245 *Calcium Imaging.* Optical measurements were made as previously described (Kindt et
246 al., 2012; Zhang et al., 2016). Briefly, calcium imaging experiments were performed in normal
247 extracellular solution (see above). Recordings were made at 10 Hz for cytosolic RGECO1
248 measurements and 20 Hz for ribbon-localized GCaMP6s-CAAX measurements. For cytosolic
249 calcium measurements using RGECO1*,* a Nikon Eclipse NiE widefield system with a 60× 1.0 NA
250 CFI Fluor water-immersion objective was used with excitation: 540/25 565LP and emission:
251 620/60 filters. The microscope was equipped with an Orca D2 camera (Hamamatsu,
252 Hamamatsu City, Japan), controlled using Elements software (Nikon Instruments Inc., Melville,
253 NY). For cytosolic RGECO1 measurements, a central imaging plane at the level of hair cell

nucleus was used. Calcium measurements at presynaptic ribbons made using GCaMP6s-CAAX were acquired on a Swept-field confocal system built on a Nikon FN1 upright microscope (Bruker Corporation, Billerica, MA) with a 60× 1.0 NA CFI Fluor water-immersion objective. The microscope was equipped with a Rolera EM-C2 EMCCD camera (QImaging, Surrey, Canada), controlled using Prairie view (Bruker Corporation, Billerica, MA). GCaMP6s-CAAX and Ribeye-mCherry were excited using 488 and 561 nm solid state lasers. The microscope was equipped with a Dual-View beam splitter (Photometrics, Tucson, AZ) using the following filters: dichroic 565; GCaMP6 emission 520/30; mCherry emission 630/50 (Chroma, Bellows Falls, VT) to enable dual imaging of GCaMP6s-CAAX calcium signals and Ribeye-mCherry to detect ribbon location. The Ribeye-mCherry signal was used to select a GCaMP6s-CAAX imaging plane containing ribbons in multiple hair cells. For comparisons, calcium imaging experiments were done using a minimum of 4 animals and 8 neuromasts per group.

The L-type calcium channel antagonist isradipine (Sigma-Aldrich, St. Louis, MO) was prepared in normal extracellular solution with 0.1 % dimethyl sulfoxide (DMSO) and used at 10 µM. Larvae were incubated in drugs for 10 min prior to calcium imaging.

Transmission Electron Microscopy. For electron microscopy, 4 dpf wild-type siblings and *ribeye b-EGFP* transgenic larvae were fixed in freshly prepared 2% paraformaldehyde and 4% glutaraldehyde (Electron Microscopy Sciences (EMS), Hatfield, PA) in 0.1 M phosphate buffer pH 7.4 for 30 minutes at room temperature, followed by a 2 hour incubation at 4°C. Larvae were washed with 0.1 M cacodylate buffer 3x5 min, and then fixed in 2% glutaraldehyde for 15 min, and washed again with 0.1 M cacodylate buffer 3x5 min. Larvae were then placed in 0.1 M osmium tetroxide buffer for 30 min and then washed with 0.1 M cacodylate buffer 3x10 min.

Larvae were then dehydrated in ethanol: 3x5 min 50% ethanol, 15 min 50% ethanol with 1% uranyl acetate, then 2x5 min 75% ethanol, 1x10 min 95% ethanol, 3x10 min 100% ethanol. After dehydration, larvae were placed in propylene oxide (PO), and the incubated in Epon:PO :: 1:1 for 1 hour, Epon:PO :: 2:1 for 1 hour, and lastly pure Epon overnight. Epon embedded samples were then placed in an oven at 64°C for 24 hours. Transverse serial sections (60–80 nm thin sections were placed on a single-slot, formvar/carbon coated nickel grid, 2x1 mm-EMS, Hatfield, PA) were used to section through cranial neuromasts located between the eyes, or neuromasts located between the eye and the ear. Samples were imaged on a JEOL JEM-2100 electron microscope (JEOL Inc., Tokyo, Japan). Whenever possible, serial sections were used to restrict our analysis to central sections of ribbons that were directly adjacent to a plasma membrane and near a well-defined afferent postsynaptic density. For our analysis, we analyzed 6 *ribeye b-EGFP* and 9 wild-type neuromasts, examining up to 7 sections per neuromast. Micrographs containing ribbons were scored blinded. Vesicles with a diameter of 30–50 nm and adjacent (within 60 nm of the ribbon) to the filamentous "halo" surrounding the ribbon were counted as tethered vesicles. Readily releasable vesicles were defined as vesicles between the ribbon and the plasma membrane. The distance of tethered vesicles from the ribbon was defined as the linear distance between the edge of a vesicle and the point on the ribbon closest to it. Average distance was calculated from five independent measurements of tethered, but not readily releasable, vesicles around the perimeter of a ribbon. All distances and perimeters were measured in ImageJ (Schneider et al., 2012).

Whole-Mount Immunohistochemistry. Zebrafish larvae were fixed with 4% paraformaldehyde and 4% sucrose in phosphate buffer with 0.2 mM $CaCl_2$ for 4.5-6 hrs at 4°C.

Larvae were then permeabilized with ice cold acetone for 5 min, and blocked with PBS buffer containing 2% goat serum, 1% bovine serum albumin (BSA), and 1% DMSO. Primary antibodies were diluted in PBS buffer containing 1% BSA and 1% DMSO, and larvae were incubated in the solution overnight at 4°C. Custom made primary antibodies for Ribeye a (rabbit polyclonal, 1:500), Ribeye b (IgG2a, 1:2000), and $Ca_V1.3a$ (rabbit polyclonal, 1:1000), and a commercially available antibody for Membrane-associated Guanylate Kinases (MAGUK, IgG1, 1:500, NeuroMab AB_10698179) have been described and used previously (Sheets et al., 2011). After removal of primary antibodies, diluted secondary antibodies coupled to Alexa 488, Alexa 647 (Life Technologies, Carlsbad, CA), or DyLight 549 (Jackson ImmunoResearch, West Grove, PA) were added. Hair-cell nuclei were labeled with DAPI (Life Technologies, Carlsbad, CA).

Confocal Imaging. Confocal images of fixed samples were obtained as previously described (Sheets et al., 2011). Briefly, z-stack images of whole neuromasts (spaced by 0.3 µm over 5-10 µm) were acquired with an Olympus FV1000, a Leica SP8, or a Zeiss LSM780 confocal microscope using 60x 1.3 NA oil, 63x 1.3 NA glycerol, or 63x 1.4 NA oil immersion objectives respectively. For quantitative measurements, confocal imaging parameters, including gain, laser power, scan speed, dwell time, resolution and zoom were maintained between comparisons. For super resolution imaging (Figure 3F-G), a Zeiss LSM780 microscope with Airyscan was used with a 63x 1.4 NA oil objective, a digital zoom of 25x, acquired at 256 X 256 in 0.19 µm sections. Images were processed in Zen (Carl Zeiss, Jena, Germany) with an Airyscan processing factor of 6.0. For live images of samples (Figure 1A, 5A-B, 6A-B), a Nikon C2 (10x 0.3 NA air or 60× 1.0 NA water objective) confocal system was used to image and excite EGFP, GCaMP6s, RGECO or mCherry using the appropriate solid-state laser. For each experiment, the microscope

parameters were adjusted using the brightest control specimen.

Confocal Image Processing. Maximal projections of z-stack confocal images were created and analyzed using MetaMorph (Molecular Devices, LLC, Sunnyvale, CA) or ImageJ software. Images containing immunolabel were corrected for background; within maximum-intensity projections a 7 μm^2 region containing the highest level of background was selected, and the average-fluorescence intensity of that region was subtracted from each pixel within the image stacks.

To quantitatively measure fluorescent intensities and areas of immunolabeled puncta in Metamorph, individual neuromasts were delineated using the region tool, and between comparisons, the same inclusive threshold was applied to isolate the pixels occupied by immunolabeled puncta within the neuromast. A punctum was defined as a region of immunolabel where the pixel intensity was at least threefold (Ribeye) or fivefold ($Ca_V1.3a$ and MAGUK) above the average intensity measured in the whole neuromast. Once the appropriate threshold was applied, the Integrated Morphometry Analysis function was used to automatically quantify the number of puncta, the area of each immunolabeled punctum, and the integrated intensity of fluorescent pixels within each individual punctum.

$Ca_V1.3a$ immunolabeled puncta adjacent or juxtaposing MAGUK immunolabel, i.e. $Ca_V1.3a$ puncta that, due to the resolution limits of light microscopy, appeared to partially overlap with MAGUK immunolabel (Sheets et al., 2012), were defined as presynaptic. To determine if ribbons were adjacent to PSDs (postsynaptic densities), custom software written in C++ was used as previously described (Liberman et al., 2011) to produce, for each ribbon or PSD, a thumbnail image of an x-y max-projection of a voxel cube (1 μm^2) extracted from

confocal image stacks and centered on each ribbon synapse using independently derived x, y, and z coordinates of all ribbons and PSDs. The numbers of intact ribbon synapses i.e. ribbons adjacent to PSDs were determined by visual inspection of these thumbnail arrays (see Figure 2C,D). Subsequent image processing for display within figures was performed using Photoshop and Illustrator software (Adobe, San Jose, CA).

Signal Analysis and Statistics. Afferent electrophysiology data were analyzed using custom software written in Igor Pro (Wavemetrics, Lake Oswego, OR) and were plotted with Igor Pro and Prism 7 (Graphpad, La Jolla, CA). For calcium imaging, image registration and peak detection were performed using custom scripts written in Matlab (The Mathworks, Natick, MA) as described previously (Hilliard et al., 2005; Zhang et al., 2016). For RGECO1 calcium measurements, a circular region of interest (ROI) with a diameter of 3 µm (215 nm per pixel) was placed on each hair cell within a neuromast. For ribbon-localized GCaMP6s-CAAX measurements, a circular ROI with a 1 µm diameter (268 nm per pixel) was placed on the center of an individual ribbon. Ribbon location was determined by either simultaneous or subsequent image capture of Ribeye-mCherry labeled ribbons.

Values in the text and data on graphs are expressed as mean ± SEM. Whenever possible an effort was made to minimize Type II error with appropriate population numbers. In some of our more challenging electrophysiology experiments with low N values, we were not able to achieve a power of >0.8 to indicate no significant difference. In these cases, we state, “we were unable to detect differences” between the two populations. Where appropriate, datasets were confirmed for normality using a Kolmogorov-Smirnov normality test, and for equal variances using a F test to compare variances. Statistical significance between two conditions was

364 determined by either paired or unpaired, two-tailed Student's *t* tests, or a Mann-Whitney U
365 test, as appropriate. When multiple t-tests were performed on the same data, a Holm-Siadk
366 method was used to correct for multiple comparisons.
367
368 **Results**

369 To examine how ribbon enlargement altered ribbon-synapse morphology and synapse
370 activity in hair cells, we took advantage of a transgenic zebrafish line with enlarged ribbons. In a
371 previous study, we created a stable transgenic line *Tg[myosin6b:ribeye b-EGFP]* that
372 overexpresses exogenous Ribeye b fused to EGFP in zebrafish inner ear hair cells and in
373 neuromast hair cells in the lateral-line system. Upon incrossing this transgenic strain, we
374 observed high levels of Ribeye b expression in hair cells (Figure 1A; expressing 2 copies and
375 subsequently referred to as *ribeye b-EGFP*) and substantially enlarged ribbons (Sheets et al.,
376 2011).

377 **Ribeye b-EGFP expressing hair cells have larger ribbons and more tethered synaptic vesicles**

378 Prior to investigating synaptic activity, we characterized the synaptic architecture of hair
379 cells with enlarged synapses and compared these measurements to wild-type synapses. We
380 examined TEM sections that featured ribbon bodies adjacent to postsynaptic densities in
381 zebrafish neuromasts from wild-type (n = 18) and *ribeye b-EGFP* transgenic (n = 19) ribbons. We
382 used TEM to quantify ribbon-body areas and found that ribbon areas were significantly larger
383 (2X) in *ribeye b-EGFP* hair cells compared to wild-type hair cells (Figure 1B-D,H; ribbon area,
384 wild-type: 0.065 ± 0.009 μm^2*; ribeye b-EGFP*: 0.138 ± 0.023 μm^2, p = 0.008).

We predicted that enlargement of ribbons would increase the number and distribution of synaptic vesicles associated with the ribbon. Synaptic vesicles were defined as circular structures 30-50 nm in diameter that were directly apposed to the filamentous halo surrounding the ribbon body (Schnee et al., 2005; Obholzer et al., 2008). We observed a similar vesicle density along wild-type and *ribeye b-EGFP* ribbon perimeters (Figure 1B-D,I; vesicle density per 500 nm, wild-type: 12.07 ± 0.80 vesicles*; ribeye b-EGFP*: 11.79 ± 0.76 vesicles, p = 0.80). The average size of individual vesicles was also similar at wild-type and *ribeye b-EGFP* ribbons (vesicle area, wild-type: 2236 nm^2 ± 129.1*; ribeye b-EGFP*: 2345 ± 73.7 nm^2, p = 0.46). In contrast, we found that synaptic vesicles were slightly closer to the ribbon in *ribeye b-EGFP* hair cells compared to wild-type (Figure 1B-D,L; vesicle to ribbon distance, wild-type: 55.52 ± 3.65 nm*; ribeye b-EGFP*: 45.43 ± 2.99 nm, p = 0.039), which indicates that vesicle tethering might be slightly altered in the *ribeye b-EGFP* transgenic line. Importantly, our cross section measurements revealed a significantly greater number of synaptic vesicles (1.3X more) associated with *ribeye b-EGFP* ribbons compared to wild-type ribbons (Figure 1J; associated or tethered vesicles, wild-type: 17.67 ± 1.58 vesicles; *ribeye b-EGFP*: 22.53 ± 1.57 vesicles, p = 0.036). However, we did not observe a significantly greater number of vesicles docked at the active zones of enlarged ribbons, which we defined as vesicles beneath the ribbon and adjacent to the plasma membrane that were also opposed to the postsynaptic density (Figure 1B-D,K; docked vesicles, wild-type: 2.83 ± 0.62; *ribeye b-EGFP:* 2.74 ± 0.34, p = 0.890). These results indicate that enlarged ribbons in *ribeye b-EGFP* transgenic hair cells have significantly more tethered vesicles but a similar number of docked synaptic vesicles compared to wild-type ribbons.

In addition to larger ribbons associated with postsynaptic densities (synaptic ribbons), we also observed ectopic ribbons in *ribeye b-EGFP* transgenic hair cells that were not associated with postsynaptic densities (Figure 1E-G). Ectopic ribbons were rarely seen in wild-type TEM sections; we observed over 60 ectopic ribbons in *ribeye b-EGFP* transgenic sections, but only 1 ectopic ribbon in a similar number of wild-type sections (n = 33 wild-type and n = 25 *ribeye b-EGFP* sections). Overall, we determined that most ectopic ribbons were located above the nucleus and were associated with membranous organelles or endosomes (Figure 1E-G). Only a small subset of the ectopic ribbons were found along the membrane of hair cells (Figure 1E), and the majority were associated with filaments in the cytosol (Figure 1F), or near the apical cuticular plate of hair cells (Figure 1G). Together, our TEM studies revealed that overexpression of Ribeye results in larger synaptic ribbons that are associated with more vesicles, as well as additional ectopic ribbons that populate the hair-cell body.

Enlarged ribbons do not affect postsynapse morphology or synapse number

To assess whether ribbon enlargement also affected hair-cell postsynaptic morphology, we used immunohistochemistry to examine the afferent postsynaptic densities (PSDs) at ribbon synapses in lateral-line hair cells. We visualized afferent PSDs using an antibody against the PSD-95 family of membrane-associated guanylate kinases (MAGUKs) and ribbons using antibodies specific to both paralogs of zebrafish Ribeye—Ribeye a and Ribeye b (Sheets et al., 2011). Because we observed numerous ectopic aggregates of Ribeye in our transgenic line (Figure 1A, E-G; Figure 2B,D), we quantified only Ribeye and MAGUK immunolabel at 'complete' ribbon synapses, i.e. presynaptic Ribeye-labeled puncta juxtaposing MAGUK-labeled patches (Figure 2C,D).

Because ribbon synapse components are too small to resolve accurately using conventional confocal microscopy, we approximated the sizes of pre-and postsynaptic components by examining the relative areas of Ribeye and MAGUK puncta. Corresponding to our TEM results, the relative areas of Ribeye puncta were significantly larger within *ribeye b-EGFP* hair cells compared to wild-type hair cells (Figure 2A-E; ribbon area (normalized to wild-type median), wild-type: 1.082 ± 0.057 a.u., n = 192 ribbons; *ribeye b-EGFP*: 4.565 ± 0.252 a.u., n = 132 ribbons, $p < 0.0001$). By contrast, relative areas of MAGUK puncta were comparable to wild-type (Figure 2A-E; PSD area (normalized to wild-type median), wild-type: 1.147 ± 0.059 a.u., n = 192 PSDs; *ribeye b-EGFP*: 1.311 ± 0.064 a.u., n = 210 PSDs, $p = 0.052$). We also examined whether the amount of PSD protein was altered at *ribeye b-EGFP* postsynapses by examining the integrated intensity of MAGUK immunolabel fluorescence per puncta, and found that compared to *ribeye b-EGFP* puncta, amount of MAGUK within the PSDs was comparable to wild-type puncta (MAGUK integrated intensity, wild-type: 18200 ± 1250 a.u., n = 192 PSDs; *ribeye b-EGFP*: 20000 ± 1250 a.u., n = 210 PSDs, $p = 0.1884$). In addition, there was no significant difference in the number of intact ribbon synapses between *rib b-EGFP* and wild-type hair cells (Figure 2F; synapses per hair cell, wild-type: 2.44 ± 0.10, n = 11 neuromasts; *ribeye b-EGFP*: 2.32 ± 0.13, n = 12 neuromasts, $p = 0.468$), and the PSDs appeared to correctly localize adjacent to the ribbons (Figure 2B,D). These results indicate that enlarging the ribbon via overexpression of Ribeye b does not proportionally affect PSD size, nor does it change the number of ribbon synapses.

$Ca_V1.3a$ channels are less tightly clustered at enlarged ribbon synapses

Previously, we reported that the voltage-gated calcium channel $Ca_V1.3$—the presynaptic calcium channel in mammalian and zebrafish hair cells—is localized to synaptic ribbons (juxtaposing a PSD) and to ectopic aggregates of Ribeye b-EGFP (Figure 3B,B'; (Sheets et al., 2011)). First, we determined whether $Ca_V1.3a$ distribution was affected at enlarged synaptic ribbons. We reasoned that more tightly clustered Cav1.3a immunolabeled puncta would, on average, display higher average fluorescent intensities, and therefore examined the average immunolabel intensity of $Ca_V1.3a$ puncta adjacent to a postsynapse. We found, at synapses with enlarged synaptic ribbons, the average intensity of $Ca_V1.3a$ immunolabel was reduced compared to wild-type synapses (Figure 3A-C; $Ca_V1.3a$ average intensity, wild-type: 11900 ± 205 a.u., n = 282 synapses; *ribeye b-EGFP*: 8930 ± 334 a.u., n = 125 synapses, $p < 0.0001$). Interestingly, the integrated immunolabel intensity per puncta, which represents the total amount of $Ca_V1.3a$ per synapse, was comparable between *ribeye b-EGFP* and wild-type synapses (Figure 3D; $Ca_V1.3a$ integrated intensity, wild-type: $6.50 \times 10^5 \pm 0.38$ a.u., n = 282 synapses; *ribeye b-EGFP*: $1.08 \times 10^6 \pm 0.14$ a.u., n = 125 synapses, $p = 0.156$).

As our confocal imaging indicated $Ca_V1.3a$ labeling was less clustered at *ribeye b-EGFP* synapses, we subsequently examined $Ca_V1.3a$ channel labeling more closely at synapses using super resolution microscopy. We observed that $Ca_V1.3a$ channels appeared less clustered at enlarged ribbons compared to wild-type ribbons (Figure 3F,G). Profile plots of $Ca_V1.3a$ immunolabel showed multiple peaks at wild-type synapses (Figure 3F'), corresponding with clustered $Ca_V1.3a$ labeling at the ribbon (Figure 3F). In *ribeye b-EGFP* transgenic synapse, $Ca_V1.3a$ labeling appeared more diffuse and spread out over a larger area (Figure 3G,G').

Collectively, these data suggest that there are a similar number of $Ca_V1.3a$ channels at enlarged synaptic ribbons compared to wild-type, but the channels cluster at a lower density.

Similar to what we previously observed, in *ribeye b-EGFP* hair cells, there were many additional ectopic ribbons with no associated PSD; these ectopic ribbons were also able to recruit $Ca_V1.3a$ channels ((Sheets et al., 2011), Figure 3B,B'; example, see arrowhead). When we summed the intensities of both ectopically and synaptically-localized $Ca_V1.3a$ immunolabel, we observed significantly more overall $Ca_V1.3a$ present in *ribeye b-EGFP* transgenic hair cells compared to wild-type hair cells (Figure 3E; total $Ca_V1.3a$ intensity per neuromast, wild-type: $3.29 \times 10^5 \pm 0.47$ a.u., n = 13 neuromasts; *ribeye b-EGFP*: $5.03 \times 10^5 \pm 0.42$ a.u., n = 14 neuromasts, p = 0.010). These findings confirm that ectopic ribbons can recruit $Ca_V1.3a$ without a postsynapse and suggest that the amount of $Ca_V1.3a$ allocated to each synaptic ribbon is fixed. Perhaps, if the amount of $Ca_V1.3a$ at synaptic ribbons is fixed, then increases in Ribeye and ribbon size can influence $Ca_V1.3a$ channel distribution and consequently serve as a mechanism to adjust the overall density of $Ca_V1.3a$ channels at the active zone.

Whole-cell calcium currents, but not capacitance measurements, are increased in hair cells with enlarged ribbons

To investigate how enlarged ribbons altered hair-cell function, we performed whole-cell recordings from lateral-line hair cells of wild-type and of *ribeye b-EGFP* transgenic larvae. The resting membrane potential of Ribeye b-EGFP expressing hair cells was similar to that measured in wild-type hair cells (V_m, wild-type*:* −69.5 ± 4.8 mV, 3-5 dpf, n = 4; *ribeye b-EGFP:* −72.8 ± 3.8 mV, 3-5 dpf, n = 6, p = 0.602). In addition, at rest, we were unable to detect any difference in cell membrane capacitance between genotypes (wild-type: 2.8 ± 0.3 pF, n = 25; *ribeye b-EGFP*:

493 3.3 ± 0.6 pF, n = 31, p = 0.491), which suggests that the overall size of the hair cells is similar.
494 Furthermore, there were no differences in the complement of K^+ currents, which included $I_{K,Ca}$
495 and I_A as previously described, between wild-type and *ribeye b-EGFP* hair cells (data not shown,
496 (Olt et al., 2014, 2016b).

497 Our immunolabel results indicate that the distribution of $Ca_V1.3a$ channels at *ribeye b-EGFP*
498 synapses is disrupted compared to wild-type synapses (Figure 3). We therefore examined
499 whether the calcium currents (I_{Ca}) expressed in *ribeye b-EGFP* hair cells were altered relative to
500 wild-type hair cells (Figure 4A,B). I_{Ca} was recorded using 2.8 mM extracellular calcium at 28.5°C,
501 and in the presence of the K^+ currents blockers 4-AP and TEA in the Cs-based intracellular
502 solution (see Methods, Figure 4A; example I_{Ca}). *Ribeye b-EGFP* hair cells had larger calcium
503 currents compared to wild-type hair cells (Figure 4B; at −31 mV, the peak of I_{Ca}, wild-type: −7.0
504 ± 1.4 pA, n = 13; *ribeye b-EGFP:* −13.2 ± 1.8 pA, n = 10, p = 0.012).

505 In hair cells, voltage-gated calcium currents are tightly associated with vesicle fusion, i.e.
506 exocytosis. To investigate whether *ribeye b-EGFP* hair cells had altered exocytosis, we
507 measured changes in cell-membrane capacitance (ΔC_m) following a 1.0-s depolarizing voltage
508 step (Figure 4C; example ΔC_m, (Moser and Beutner, 2000; Johnson et al., 2009; Olt et al., 2014,
509 2016a)). Despite the larger I_{Ca} in *ribeye b-EGFP* hair cells, the ΔC_m was similar to that measured
510 in wild-type cells (Figure 4C,D; ΔC_m 1.0-s step, wild-type: 9.0 ± 3.0 fF, n = 6; *ribeye b-EGFP*: 6.8 ±
511 1.0 fF, n = 7, p = 0.47), and similar to that reported in wild-type hair cells using the same
512 recording conditions (Olt et al., 2014, 2016b). These data show that, despite more associated
513 vesicles at enlarged ribbons and larger calcium currents in *ribeye b-EGFP* hair cells, the overall
514 amount of exocytosis does not correspondingly increase in response to a 1.0-s stimulus.

Calcium imaging confirms larger cytosolic calcium responses in hair cells with enlarged ribbons

To further test calcium dynamics in hair cells with enlarged ribbons, we examined mechanically-evoked calcium responses in a transgenic line expressing RGECO1 in hair cells (Zhao et al., 2011; Maeda et al., 2014). RGECO1 is a red-shifted calcium indicator that enabled us to spectrally separate Ribeye b-EGFP and RGECO1 and examine calcium responses within the cytosol of hair cells with enlarged ribbons (Figure 5B; RGECO1 plus 2 copies of *ribeye b-EGFP)* and wild-type ribbons (Figure 5A; RGECO1 alone). After a 2.0-s step-wise deflection of the cupula of neuromasts, we measured and quantified mechanically-evoked calcium responses (fluorescence response, $\Delta F/F_0$), in both genotypes. On average, calcium responses were significantly larger in hair cells with enlarged ribbons compared to wild-type hair cells (Figure 5C,D; RGECO1 $\Delta F/F_0$, wild-type: 14.39 ± 1.03 %, n = 54 hair cells; *ribeye b-EGFP*: 21.89 ± 2.32 %, n = 55 hair cells, $p = 0.02$). This finding is consistent with what we observed in our whole-cell calcium current measurements, where hair cells with enlarged ribbons had significantly larger calcium currents. In addition to increased mechanically-evoked calcium responses, we also observed that baseline calcium levels, as measured by baseline RGECO1 intensity, were significantly elevated in *ribeye b-EGFP* transgenic hair cells compared to wild-type hair cells (Figure 5E; RGECO1 baseline, wild-type: 610.9 ± 18.01 a.u., n = 74 hair cells; *ribeye b-EGFP;* 916.0 ± 28.62 a.u., n = 96 hair cells, $p < 0.0001$). Overall, elevated baseline calcium and increased cytosolic calcium responses support the results from our whole-cell recordings, and indicate that hair cells with enlarged ribbons have increased calcium signaling.

Local calcium signals at ribbons are larger in hair cells with enlarged ribbons

Our immunohistochemistry data indicate there are the same number of $Ca_V1.3a$ channels at enlarged ribbons compared to wild-type ribbons, but the channels cluster at a lower density. Functionally, our whole-cell recordings and cytosolic calcium imaging results show larger global calcium responses in *ribeye b-EGFP* hair cells. To determine whether reduced $Ca_V1.3a$ channel clustering altered the local calcium responses at enlarged synaptic ribbons, we established a method to detect ribbon-localized calcium responses at ribbons. To do this, we used a transgenic zebrafish that expresses the calcium indicator GCaMP6s (Chen et al., 2013) in hair cells, localized to the plasma membrane with a CAAX motif (Figure 6A-C, (Jiang et al., 2017)). We predicted that this line could be used to detect presynaptic calcium signals adjacent to ribbons. To test this prediction, we created an additional transgenic line, *ribeye b-mCherry* that has wild-type sized ribbons (see Methods) to mark ribbon location in order to measure GCaMP6s-CAAX, ribbon-localized calcium signals. Using this double transgenic line (Figure 6A), we were able to measure robust, mechanically-evoked calcium responses (fluorescence response, $\Delta F/F_0$) basally in hair cells, in response to deflection of the cupula of neuromasts (Figure 6C,C'). The presynaptic calcium signals were localized within a focal hotspot located at ribbons (Figure 6C,C'). We detected ribbon-localized calcium signals by centering a region of interest (ROI) with a 1 µm diameter over an individual ribbon (Figure 6C). Compared to an adjacent ROI, calcium signals at the ribbon were greater (peak GCaMP6s calcium response for a 2.0-s stimulus, on ribbon: 158.10 ± 0.23 %; off ribbon: 32.08 ± 0.04 %, n = 12 hair cells, $p < 0.0001$, examples shown in Figure 6C,C'). All presynaptic signals were blocked below detection by application of the L-type calcium channel antagonist isradipine, with complete block observed in 12/12 cells in response to a saturating stimulus (see example dashed traces in

559 Figure 6C'). Together, our imaging and pharmacological results support the use of GCaMP6s-
560 CAAX for detecting local, $Ca_V1.3$a-dependent calcium signals at ribbons.

561 Due to spectral overlap between EGFP and GCaMP6s, the *ribeye b-EGFP* and *GCaMP6s-*
562 *CAAX* transgenic lines are not compatible for imaging. To overcome this obstacle, we created an
563 additional transgenic line with high levels of Ribeye expression. Instead of EGFP, we fused
564 Ribeye to mCherry. Similar to the *ribeye b-EGFP* transgenic line, when incrossed, *ribeye a-*
565 *mCherry* transgenic ribbons were enlarged compared to wild-type ribbons (see Methods and
566 Figure 6B). This enlargement is not observed in our control *ribeye b-mCherry* transgenic line,
567 where ribbon size is comparable to wild-type (see Methods and Figure 6A). We used these two
568 mCherry lines to mark ribbon position and determine if ribbon-localized calcium signals (using
569 *GCaMP6s-CAAX*) were altered at enlarged ribbons (*ribeye a-mCherry X 2*) compared to control
570 ribbons (*ribeye b-mCherry*). We found, during a 0.5-s step stimulus, that the magnitude of the
571 ribbon-localized calcium responses at enlarged ribbons was similar compared to control (Figure
572 6D). We reasoned that the GCaMP6s-CAAX indicator could become saturated during this
573 stimulus. Therefore, we also measured the magnitude of the ribbon-localized calcium response
574 during a shorter, 0.1-s step stimulus. During a 0.1-s step, we observed that calcium responses
575 were larger at enlarged ribbons when compared to control ribbons (Figure 6E,E'; peak
576 GCaMP6s ribbon calcium response, *ribeye b-mCherry* (WT): 36.94 ± 7.69 %, n = 27 ribbons;
577 *ribeye a-mCherry* (enlarged): 65.89 ± 11.27 %, n = 29 ribbons, p = 0.042). In addition, for the
578 0.1-s step stimulus, the slope (rate of change toward peak) of the response was increased at
579 enlarged ribbons compared to control (Figure 6E''; slope, *ribeye b-mCherry* (WT): 228.8 ± 45.04,
580 n = 27 ribbons; *ribeye a-mCherry* (enlarged): 407.2 ± 65.41, n = 29 ribbons, p = 0.041).

In addition to synaptic ribbons, we also examined GCaMP6s-CAAX responses at ectopic ribbons. We observed that despite associating with $Ca_V1.3a$ channels (Figure 3B,B'), at ectopic ribbons, we were not able to measure a GCaMP6s-CAAX signal that was substantially different from the surrounding background (n = 15 ectopic ribbons). This suggests that ectopic ribbons may not contribute to the differences in global calcium signals that we measured using whole-cell recordings or the calcium indicator RGECO1.

Overall, our measurements indicate that for short stimuli, ribbon-localized calcium responses at enlarged synaptic ribbons are both faster and larger when compared to responses at wild-type-sized synaptic ribbons. An increase in calcium signals at enlarged ribbons is in line with our measurements of whole-cell calcium currents and our experiments using cytosolic RGECO1 where we observed increased calcium responses. Taken together, these results indicate that both local ribbon-localized calcium dynamics as well as global calcium dynamics are increased with ribbon enlargement.

Ribbon enlargement alters spontaneous and evoked afferent activity

Presynaptic measurements of capacitance changes to estimate exocytosis were unable to detect any differences in evoked neurotransmission between hair cells from wild-type or *ribeye b-EGFP* transgenic fish. Due to the limited number of ribbons and associated vesicles in zebrafish hair cells, it is likely that a large depolarizing stimulus may be required to detect changes in exocytosis using capacitance measurements. This could confound our ability to detect subtle differences in vesicle fusion between genotypes. Therefore, we examined whether postsynaptic activity was affected in *ribeye b-EGFP* transgenic fish by performing extracellular recordings of action currents from the cell bodies of the afferent neurons that

innervate lateral-line hair cells (Trapani and Nicolson, 2010). For these recordings, both spontaneous and evoked action currents (spikes) are generated by neurotransmitter release from hair cells that is dependent on $Ca_V1.3a$ activity (Trapani and Nicolson, 2011; Olt et al., 2016b). Spontaneous spikes result from a receptor potential that is within the activation range of $Ca_V1.3a$ channels when the hair cell is not stimulated. Evoked spikes occur when hair cells are stimulated and result from depolarizing mechanotransduction currents that lead to rapid activation of $Ca_V1.3a$ channels (Moser and Beutner, 2000; Jørgensen and Kroese, 2005; Trapani and Nicolson, 2011).

Using our whole-animal, *in vivo* approach, we examined spontaneous and evoked activity from afferent neurons of wild-type and *ribeye b-EGFP* transgenic fish. We first examined the spontaneous activity of single afferent neurons and found that hair cells with enlarged ribbons displayed significantly reduced rates of spontaneous activity with corresponding larger interspike intervals (ISI) compared to wild-type hair cells (Figure 7A,B; spontaneous ISI, wild-type: 0.147 ± 0.031 s, n = 19 cells; *ribeye b-EGFP*: 0.416 ± 0.085 s, n = 18 cells, p = 0.0006). This result suggests that, despite having more synaptic vesicles localized to larger ribbons compared to wild-type ribbons, *ribeye b-EGFP* transgenic hair cells have a lower probability of vesicle fusion at rest.

We next examined evoked afferent activity to determine whether ribbon size played a role in the encoding of mechanical stimuli. Stimuli were presented as two different types of neuromast cupula deflections: a 200-ms sine wave at 20 Hz and a 500-ms square-step stimulus. During a 20 Hz sine wave stimulus, we observed fewer spikes during two phases of the stimulus that activated hair cells in *ribeye b-EGFP* larvae compared to wild-type larvae (Figure 7C,D). In

contrast, during a 500-ms step stimulus, we were unable to detect a difference in the average number of spikes in *ribeye b-EGFP* afferents compared to wild-type afferents (Figure 7F,G; spikes per 500-ms step, wild-type: 8.84 ± 1.86 spikes, n = 11 cells; *ribeye b-EGFP*: 7.64 ± 1.19 spikes, n = 11 cells, p = 0.67). These results are consistent with our capacitance measurements where we observed no difference between *ribeye b-EGFP* transgenic and wild-type hair cells after 1.0-s step stimuli (Figure 4C,D). When reconciling why the number of spikes were reduced for 20 Hz, but not the 500-ms step stimulus, we noted that there were fewer spikes during the first 25 ms of the 500-ms step in *ribeye b-EGFP* transgenic fish compared to wild-type (Figure 7H; wild-type: 2.26 ± 0.26 spikes, n = 11 cells; *ribeye b-EGFP*: 1.35 ± 0.60 spikes, n = 11 cells, p = 0.022). This initial reduction of spikes suggests that fewer vesicles fuse at enlarged ribbons during the onset of a stimulus. For the 20 Hz sine-wave stimulus, the depolarizing phase of each 50-ms cycle (labeled A-D for the 200-ms stimulus duration in Figure 7C) is 25 ms, which may not be enough time for enlarged ribbons to achieve wild-type levels of exocytosis. Together, the decrease in afferent activity in response to short-phasic 20 Hz stimuli and at the onset of a longer-lasting saturating stimulus supports the hypothesis that there is reduced exocytosis at enlarged ribbons at stimulus onset.

Our examination of encoding of stimulus onset also revealed that the mean of the first spike latency (FSL) in response to a 500-ms step was longer in *ribeye b-EGFP* afferent neurons compared to wild-type neurons (Figure 7I; first spike latency for a 500-ms step, wild-type: 9.47 ± 1.59 ms, n = 11 cells; *ribeye b-EGFP*: 23.40 ± 6.27 ms, n = 11 cells, p = 0.007). In addition, FSLs appeared slightly longer for each phase of the 20 Hz sine-wave stimulus; however, these individual values were not statistically different from wild-type (Figure 7E). Overall, hair cells

with enlarged ribbons resulted in a significantly reduced rate of spontaneous activity in downstream afferent neurons, and an alteration in evoked activity with fewer postsynaptic spikes and longer first spike latencies at stimulus onset.

DISCUSSION

A specific role for the synaptic ribbon in sensory encoding has been remarkably challenging to define. Genetic studies aimed to perturb ribbons have yet to give a complete story of what features enable ribbon synapses to encode a particular auditory or vestibular stimulus. We used a transgenic zebrafish line that overexpresses Ribeye and enlarges hair-cell ribbons in order to examine how ribbon size alters the morphology and function of hair-cell synapses. Morphologically, hair cells with enlarged ribbons had more associated vesicles and failed to tightly cluster presynaptic $Ca_V1.3a$ channels, while PSDs appeared unaltered. Functionally, whole-cell calcium currents, cytosolic calcium responses, and ribbon-localized calcium responses were larger in hair cells with enlarged ribbons. Despite increased calcium signaling, enlarging ribbons resulted in reduced afferent spontaneous activity and disruptions in evoked release at the onset of stimuli. Overall, our results suggest that alterations to presynaptic ribbon size can influence postsynaptic activity and the encoding properties of ribbon synapses.

Ribeye and $Ca_V1.3$ channel clustering

Numerous reports have shown that $Ca_V1.3$ channels are clustered beneath hair-cell ribbons and are required for fast exocytosis at ribbon synapses (Frank et al., 2010; Moser and Beutner, 2000; Roberts, 1994; Sheets et al., 2012; Wong et al., 2014). Two recent studies

examining the genetic reduction of Ribeye in zebrafish hair cells and mouse retina have also indicated a close relationship between Ribeye levels and Ca_V1 localization. In these studies, disruption of Ribeye levels resulted in mislocalization of $Ca_V1.3a$ and $Ca_V1.4$ respectively (Lv et al., 2016; Maxeiner et al., 2016). We also observed a strong association between $Ca_V1.3a$ and Ribeye; $Ca_V1.3a$ was recruited to all ribbons, including ectopic ribbons generated by overexpression of Ribeye (Figure 3B,B'). Yet our observations in this study reveal that augmenting Ribeye at synapses is not sufficient to recruit additional $Ca_V1.3a$ channels to synapses. We observed that the number of $Ca_V1.3a$ at individual synaptic ribbons did not scale up with increased Ribeye levels and ribbon enlargement. Instead, the integrated intensity of $Ca_V1.3a$ channel immunolabel at enlarged synaptic ribbons was similar to wild-type (Figure 3D), but spread out over larger ribbon areas (Figure 3A-D,F-G). This result suggests that Ca_V1-channel density at the synapse may be regulated independently of Ribeye, perhaps through another ribbon-associated scaffolding protein such as Bassoon, or a RIM protein (Frank et al., 2010; Jung et al., 2015).

The ribbon's influence on coupling calcium influx and vesicle fusion

Previous work compiled from TEM studies has shown that larger ribbons have more ribbon-associated vesicles (Nouvian et al., 2006; Matthews and Fuchs, 2010; Graydon et al., 2011). Likewise, our TEM data also indicate that enlarged ribbons have an increased number of tethered vesicles compared to wild-type ribbons, which predicts the potential for an increase in vesicle fusion (Figure 1J). In addition, our measurements of whole-cell calcium currents and ribbon-localized calcium signals found that calcium responses were elevated in hair cells with enlarged ribbons (Figure 4A,B, Figure 6E,E'). Yet despite larger calcium currents and more

691 tethered vesicles at enlarged ribbons, we observed no increase in evoked activity and a
692 significant reduction in spontaneous vesicle fusion (Figure 7). These results are similar to what
693 has been observed in mammalian auditory IHCs, where larger ribbons are correlated with
694 elevated calcium currents and less spontaneous activity (Taberner and Liberman, 2005;
695 Liberman et al., 2011)

696 Our results suggest that other presynaptic factors may be important to couple calcium
697 influx and vesicle fusion. There are several scenarios wherein ribbon enlargement could impact
698 this coupling and ultimately the probability of vesicle fusion driving both spontaneous and
699 evoked activity. These mechanisms include changes in $Ca_V1.3$ channel activation properties, an
700 inhibitory effect on vesicle release by the ribbon itself, and/or a disruption of coupling between
701 presynaptic calcium signaling and vesicle fusion.

702 Alterations to $Ca_V1.3$ channel activation threshold could result in decreased
703 spontaneous release and set a higher threshold for fusion of the vesicles at stimulus onset
704 (Brandt et al., 2005; Magistretti et al., 2015). Recent work in mouse IHCs found that, compared
705 to smaller ribbons, $Ca_V1.3$ channels at larger ribbons within the same hair cell were activated at
706 more depolarized membrane potentials creating a higher threshold for activation (Ohn et al.,
707 2016). By contrast, we saw a similar activation of the calcium current in transgenic hair cells
708 compared to wild-type hair cells (Figure 4B). Additionally, we did not observe differences in the
709 resting potential in our whole-cell recordings (see Results). Overall, our data support that the
710 reduced afferent activity we observed in our transgenic is not likely due to altered $Ca_V1.3a$
711 channel activation.

712 Another possibility is the physical size or composition of the ribbon could impact vesicle
713 fusion. For example, previous studies in bipolar cells suggest one function of the ribbon may be
714 to stabilize a group of vesicles for evoked release while minimizing their spontaneous release
715 (Zenisek, 2008; Vaithianathan et al., 2013). We did observe that overexpression of Ribeye
716 decreased the distance between tethered vesicles and the ribbon (Figure 1L), which provides
717 some evidence that Ribeye may play a role in vesicle tethering, and perhaps could act to
718 stabilize vesicles at the ribbon. If enlarged ribbons restrain vesicles and inhibit their release, this
719 mechanism could explain why we observed larger calcium currents (Figure 4A,B) and more
720 tethered vesicles (Figure 1J) in hair cells with enlarged ribbons, but less spontaneous afferent
721 activity and disruptions in evoked activity (Figure 7).
722 In addition to the physical size of the ribbon and the amount of Ribeye influencing
723 vesicle fusion, reduced $Ca_V1.3a$ channel clustering observed at enlarged ribbons (Figure 3F-G)
724 could impact the coupling of presynaptic calcium influx to vesicle fusion. Coupling of
725 presynaptic calcium influx to vesicle release in hair cells has been previously described by two
726 models. The first is the nanodomain model, in which the opening of one or a few $Ca_V1.3$
727 channels is coupled to exocytotic calcium sensors on adjacent vesicles that trigger fusion
728 (Brandt et al., 2005; Goutman and Glowatzki, 2007; Wong et al., 2014). In the second, the
729 microdomain model, the opening of several, more distant $Ca_V1.3$ channels is necessary to
730 collectively raise calcium levels to overcome mobile calcium buffering and drive vesicle fusion
731 (Roberts, 1994; Beaumont et al., 2005). Our current study was not designed to study the spread
732 of calcium at the presynapse; therefore our results cannot be used to conclusively distinguish
733 between either of these models. Regardless of the mechanism, it is possible that reduced

734 $Ca_V1.3$ channel clustering impacts the coupling of presynaptic calcium influx with vesicle fusion.
735 In future studies, it will useful to design experiments using this transgenic line to directly
736 explore how enlarged ribbons affect the coupling of presynaptic calcium influx and vesicle
737 fusion in more detail.

738 **The relationship between ribbon size and afferent neuron sensitivity**

739 Ribbon size correlates with the sensitivity of auditory nerve fibers innervating IHCs of
740 the cochlea, with smaller ribbons associated with low-threshold/high-spontaneous rate fibers
741 and larger ribbons associated with high-threshold/low-spontaneous rate fibers (Taberner and
742 Liberman, 2005; Liberman et al., 2011). A recent study characterizing the firing properties of rat
743 auditory neurons supports that differences observed in afferent spike timing can be attributed
744 to the properties of presynaptic vesicle release mechanisms (Wu et al., 2016). When we
745 enlarged synaptic ribbons in the zebrafish lateral line, we observed a shift in afferent fiber
746 sensitivity; that is, enlarged ribbons resulted in a reduced rate of spontaneous firing in afferent
747 neurons (Figure 7A,B). This finding is consistent with the correlation described in IHCs,
748 suggesting that varying ribbon size may be a sufficient mechanism for tuning afferent
749 sensitivity.

750 In addition to reduced spontaneous spike rates in afferent neurons, enlarged ribbons
751 also resulted in an increase in first spike latency at stimulus onset. Moreover, we observed
752 decreases in evoked spikes in response to shorter-duration stimuli (20 Hz, 25-ms stimulus per
753 phase), and during the onset (first 25 ms) of longer, sustained stimuli. In larval zebrafish, the
754 fidelity and latency of the first spike is an important fast encoding mechanism utilized to rapidly
755 generate an escape reflex that can be critical for survival (Troconis et al., 2017). In mammals,

the first spike is important for perceptual encoding (Heil, 2004; Johansson and Birznieks, 2004; Chase and Young, 2007) as well as sound localization (Furukawa and Middlebrooks, 2001).

Our observations in the zebrafish lateral line reveal that enlargement of ribbons in hair cells is sufficient to alter afferent activity. This work provides insight into the mechanisms of hair-cell synapse heterogeneity and suggests that hair cells could use a simple strategy—varying ribbon size—to achieve sensitivity over a broad dynamic range of stimuli. It is possible that our study is relevant from a clinical perspective, as recent work has found that ribbon enlargement following moderate noise exposure is accompanied by coding deficits in auditory nerve fibers (Song et al., 2016). It will be useful in future studies to determine if morphological changes to hair-cell ribbons directly contribute to functional deficits associated with noise exposure.

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924 **FIGURE LEGENDS**

925

926 ***Figure 1.*** Overexpression of Ribeye b-EGFP increases ribbon size and number of tethered

927 vesicles.

928 ***A,*** Image of a live transgenic zebrafish expressing two copies of *ribeye b-EGFP (rib b-EGFP X 2)* in

929 hair cells at 4 dpf. Inset shows a top-down projection of a neuromast cluster of lateral-line hair

930 cells expressing Ribeye b-EGFP. ***B-D*** TEM images of neuromast ribbons in wild-type (WT)

931 siblings (B) and *ribeye b-EGFP* transgenic fish (C,D) at 4 dpf. ***E-G,*** Ectopic ribbons in hair cells

932 overexpressing of Ribeye b-EGFP. Shown are ectopic ribbons located above the nucleus. Ectopic

933 ribbons are located by the plasma membrane (E), associated with filaments (F), and near the

934 cuticular plate (G). The white arrow (E) indicates the plasma membrane of the hair cell. White

935 arrowheads (F) indicate filamentous structures in the cytosol. Black arrowheads (G) denote

936 stereocilia at the apex of the hair cell. ***H-L,*** Quantification of TEM images from n = 18 wild-type

937 and n = 19 *ribeye b-EGFP* ribbons from 9 and 6 neuromasts respectively. In *ribeye b-EGFP* hair

938 cells ribbon area (H) and number of tethered vesicles (J) was increased, while vesicle density (I)

939 and number of docked vesicles (K) was not altered. Compared to wild-type, vesicles were

940 slightly closer to the ribbon in *ribeye b-EGFP* hair cells (L). * $P < 0.05$, ** $P < 0.01$ using a t-test.

941 Scale bars: A = 500 µm, inset = 5 µm, B = 100 nm (same scale bar for B-G). The open circles or
942 squares (H-L) correspond to the example images shown in B-D.
943
944 ***Figure 2*** Overexpression of Ribeye b-EGFP increases the size of ribbons but does not increase
945 the size or number of PSDs.
946 ***A,B,*** Representative maximum intensity top-down (x-y) and side-view (x-z) projections of wild-
947 type (WT) (A) and transgenic *ribeye b-EGFP* (B) neuromasts with Ribeye a and Ribeye b labeling
948 ribbons (magenta) and MAGUK labeling PSDs (green) at 5 dpf. Ribeye a and b were labeled with
949 two spectrally separate fluorophores, but were both merged as magenta in the displayed
950 images. Cell nuclei labeled were with DAPI (blue). ***C,D,*** High-power confocal 1 μm^2 thumbnails
951 of synapses sorted by size in a representative WT (C) and a *ribeye b-EGFP* neuromast (D). A
952 number of ribbons in (D) do not have corresponding PSDs (green). ***E,*** Relative area (normalized
953 to wild-type median) of Ribeye puncta adjacent to PSDs, n = 192 WT and n = 132 *ribeye b-EGFP,*
954 and MAGUK puncta at ribbon-localized PSDs, n = 192 WT and n = 210 *ribeye b-EGFP* at 5 dpf. ***F,***
955 Number of complete synapses per hair cell (HC), estimated by taking the total number of
956 synapses per neuromast, divided by the number of hair cells per neuromast, n = 11 wild-type
957 and n = 12 *ribeye b-EGFP* neuromasts. Each circle represents a single neuromast in an individual
958 zebrafish. Scale bars: A and B = 5 µm. **** $P < 0.0001$ was determined using a Mann-Whitney U
959 test. Synapse counts were not significantly different (unpaired t-test).
960
961 ***Figure 3.*** $Ca_V1.3a$ channel immunolabel is less clustered at enlarged ribbons.

962 ***A,B,*** Immunolabel of $Ca_V1.3a$ in wild-type (WT) (A) and *ribeye b-EGFP* (B) hair cells at 5 dpf.
963 ***A',B'***, Overlay of $Ca_V1.3a$ (magenta) and the PSD label MAGUK (green) in wild-type (A') and
964 *ribeye b-EGFP* (blue) (B') hair cells. Inset on right highlights a single ribbon and corresponds to
965 boxed ROI in A' and B'. White arrowheads in B and B' indicate an enlarged ectopic ribbon with
966 no PSD, yet relatively strong $Ca_V1.3a$ immunolabel. $Ca_V1.3a$ immunolabeled puncta localized to
967 complete synapses, i.e. adjacent to PSDs, were examined in C and D. ***C,D,*** The average $Ca_V1.3a$
968 immunolabel intensity at sites adjacent to PSD label is greater at wild-type synapses compared
969 to *ribeye b-EGFP* synapses (C), while the total or integrated $Ca_V1.3a$ immunolabel intensity per
970 punctum is unchanged between these groups (D), n = 282 WT and n = 125 *ribeye b-EGFP*
971 $Ca_V1.3a$ puncta in C and D. ***E,*** The total amount of $Ca_V1.3a$ immunolabel per neuromast is
972 greater in *ribeye b-EGFP* transgenics compared to wild-type. This includes $Ca_V1.3a$ immunolabel
973 at both ectopic ribbons and at complete (PSD apposing) ribbon synapses, n = 13 WT and n = 14
974 *ribeye b-EGFP* neuromasts. ***F,G,*** Two example airyscan images of $Ca_V1.3a$ and Ribeye b
975 immunolabel at wild-type (F) and *ribeye b-EGFP* ribbons (G). Right image panel in F and G is
976 $Ca_V1.3a$ immunolabel alone, left image panel is $Ca_V1.3a$ (magenta) and Ribeye b (green)
977 immunolabel merged. ***F',G',*** Intensity profile plots represent the pixel intensity of the yellow
978 line drawn through the $Ca_V1.3a$ immunolabel in F and G. **** $P < 0.0001$ using a Mann-Whitney
979 U test in C; * $P = 0.01$ using an unpaired t-test in E. Scale bars: B' = 5 µm (same scale bar for A-
980 B'), B inset = 1 µm, F = 1 µm (same scale bar for F-G).
981
982 ***Figure 4.*** Calcium currents and capacitance measurements in hair cells with enlarged ribbons**.**

983 **A,** Calcium currents (I_{Ca}) recorded from wild-type (WT, 7 dpf, black) or *ribeye b-EGFP* hair cells
984 (5 dpf, gray). Currents were elicited by a series of depolarizing voltage steps in 10 mV
985 increments (200 ms in duration) from the holding potential of −79 mV. For clarity, only the
986 trace at the holding potential and at the peak of I_{Ca} are shown. ***B,*** I-V curves of whole-cell I_{Ca} in
987 wild-type (black) and *ribeye b-EGFP* (gray) hair cells (5-8 dpf), n = 13 wild-type and n = 10 *ribeye*
988 *b-EGFP.* At the peak of I_{Ca} (−31 mV), calcium currents were significantly larger in *ribeye b-EGFP*
989 hair cells, $p > 0.05$. ***C,*** Changes in membrane capacitance (ΔC_m) recorded from hair cells of wild-
990 type (5 dpf, black) and *ribeye b-EGFP* transgenics (6 dpf, gray). Recordings were obtained in
991 response to 1.0-s voltage steps from the holding potential of −79 mV to near the peak of I_{Ca}
992 (−31 mV). ***D,*** Average ΔC_m elicited following 1.0-s depolarization step to −31 mV from hair cells
993 of both wild-type (6-8 dpf, black) and *ribeye b-EGFP* (5-8 dpf, gray) hair cells was not
994 significantly different using a t-test, n = 6 wild-type and n = 7 *ribeye b-EGFP*.
995
996 ***Figure 5.*** Mechanically-evoked and baseline cytosolic calcium measurements are increased in
997 hair cells with enlarged ribbons.
998 ***A,B,*** Live, confocal image of hair cells expressing the calcium indicator RGECO1 (A) or RGECO1
999 and Ribeye b-EGFP (B). ***C,*** Average mechanically-evoked calcium response of hair cells
1000 expressing RGECO1 (WT, solid blue) or RGECO1 and Ribeye b-EGFP (*ribeye b-EGFP*, dashed
1001 green) at 5-6 dpf, n = 54 WT and n = 55 *ribeye b-EGFP* hair cells. The gray bar indicates the
1002 duration of the mechanical stimulus (2.0-s). ***D,*** Scatterplot of the magnitude of individual hair
1003 cell responses plotted in C. ***E,*** Baseline RGECO calcium signals in wild-type and *ribeye b-EGFP*
1004 transgenic hair cells, n = 74 wild-type and n = 96 *ribeye b-EGFP* hair cells. A Mann-Whitney U

1005 test was used to compare calcium responses and baseline calcium signals in D and E. * P < 0.05,
1006 **** P < 0.0001. Scale bar: A = 5 µm.
1007
1008 **Figure 6.** Ribbon-localized calcium signals are increased at enlarged ribbons.
1009 ***A,B,*** Live images of hair cells with wild-type (WT) sized ribbons, *ribeye b-mCherry;GCaMP6s-*
1010 *CAAX* (A) and enlarged ribbons, *ribeye a-mCherry X 2;GCaMP6s-CAAX* (B). ***C,C'*** Two ribbons in a
1011 single hair cell are depicted in C. In response to a saturating, 2.0-s 5 Hz stimulus, in control hair
1012 cells (*ribeye b-mCherry*) focal calcium hotspots are observed at individual ribbons (purple ROIs)
1013 compared to an adjacent region (orange ROIs). Isradipine completely blocks all presynaptic
1014 calcium signals (dashed traces in C'). ***D,E*** Average ribbon-localized calcium responses during a
1015 0.5-s (D) or 0.1-s (E) stimulus at wild-type ribbons and enlarged ribbons. ***E',E''*** Data from E
1016 quantified with respect to magnitude (E') and slope (E''). Measurements were performed at 4-5
1017 dpf. In D, traces represent the average response of n = 29 wild-type (*ribeye b-mCherry)* and n =
1018 37 enlarged (*ribeye a-mCherry*) ribbons. In E-E'', traces and data represent the average
1019 response of n = 27 wild-type (*ribeye b-mCherry)* and n = 29 enlarged (*ribeye a-mCherry*) ribbons.
1020 The gray bar indicates the duration of the mechanical stimulus in C', D and E. Dashed lines in D
1021 and E represent SEM. * P < 0.05, using a Mann-Whitney U test. Scale bar: A = 5 µm, C = 1 µm.
1022
1023 ***Figure 7.*** Enlarged ribbons disrupt evoked and spontaneous afferent activity.
1024 ***A,*** Representative example of a 60 s recording of spontaneous action currents from wild-type
1025 and *ribeye b-EGFP* afferent neurons. ***B,*** Quantification of mean spontaneous inter-spike
1026 intervals from wild-type (WT) and *ribeye b-EGFP*, n = 19 wild-type and n = 18 *ribeye b-EGFP*

neurons. ***C,*** Example trace from a wild-type and *ribeye b-EGFP* afferent neuron in response to a 200-ms 20 Hz sine wave stimulus. Each stimulus has four cycles with positive (stimulatory) phases A-D. ***D,*** Average spike number for each stimulus phase (A-D) is reduced in *ribeye b-EGFP* compared to wild-type, n = 8 wild-type and n = 10 *ribeye b-EGFP* neurons. ***E,*** Mean time to first spike for each stimulus phase in *ribeye b-EGFP* compared to wild-type. ***F,*** Example sweep from the same wild-type and *ribeye b-EGFP* afferent neuron as C, in response to a 500-ms step stimulus. ***G,*** Average spike number per 500-ms step is the same for *ribeye b-EGFP* compared to wild-type, n = 11 neurons for wild-type and *ribeye b-EGFP.* ***H,*** Spike number for the first 25 ms of the 500-ms step stimulus is significantly lower in in *ribeye b-EGFP* compared to wild-type. ***I,*** The mean first spike latency is longer in *ribeye b-EGFP* neurons compared to wild-type. * $P < 0.05$, ** $P < 0.01$, *** $P < 0.001$ using an unpaired t-test in D, E, G, H, I. Multiple t-tests in D and E were corrected for multiple comparisons using the Holm-Sidak method. A Mann-Whitney U test was used for B.