Bassoon and the Synaptic Ribbon Organize Ca\textsuperscript{2+} Channels and Vesicles to Add Release Sites and Promote Refilling

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SUMMARY

At the presynaptic active zone, Ca\textsuperscript{2+} influx triggers fusion of synaptic vesicles. It is not well understood how Ca\textsuperscript{2+} channel clustering and synaptic vesicle docking are organized. Here, we studied structure and function of hair cell ribbon synapses following genetic disruption of the presynaptic scaffold protein Bassoon. Mutant synapses—mostly lacking the ribbon—showed a reduction in membrane-proximal vesicles, with ribbonless synapses affected more than ribbon-occupied synapses. Ca\textsuperscript{2+} channels were also fewer at mutant synapses and appeared in abnormally shaped clusters. Ribbon absence reduced Ca\textsuperscript{2+} channel numbers at mutant and wild-type synapses. Fast and sustained exocytosis was reduced, notwithstanding normal coupling of the remaining Ca\textsuperscript{2+} channels to exocytosis. In vitro recordings revealed a slight impairment of vesicle replenishment. Mechanistic modeling of the in vivo data independently supported morphological and functional in vitro findings. We conclude that Bassoon and the ribbon (1) create a large number of release sites by organizing Ca\textsuperscript{2+} channels and vesicles, and (2) promote vesicle replenishment.

INTRODUCTION

Sensory encoding in the auditory and visual system of vertebrates relies on transformation of graded receptor potentials into rates of neurotransmitter release at ribbon synapses. The synaptic ribbon, an electron-dense structure anchored at the active zone, tethers a halo of synaptic vesicles (Glowatzki et al., 2008; Nouvian et al., 2006; Sterling and Matthews, 2005). Aside from its major component, RIBEYE/CtBP2 (Khimich et al., 2005; Schmitz et al., 2000; Zenisek et al., 2004), the ribbon also contains scaffold proteins such as Bassoon and Piccolo (Dick et al., 2001; Khimich et al., 2005; tom Dieck et al., 2005). Genetic disruption of Bassoon perturbs the anchoring of ribbons to the active zones (AZs) of photoreceptors (Dick et al., 2003) and cochlear inner hair cells (IHCs) (Khimich et al., 2005). At the IHC synapse, where the functional effects of Bassoon disruption and ribbon loss are best studied, fast exocytosis is reduced (Khimich et al., 2005), and sound encoding by the postsynaptic spiral ganglion neurons impaired (Buran et al., 2010). Moreover, IHCs of these Bassoon mouse mutants (Bsn\textsuperscript{-Ex4/5}) show smaller Ca\textsuperscript{2+} currents. However, matching Ca\textsuperscript{2+} currents by reducing the driving force for Ca\textsuperscript{2+} in wild-type IHCs does not equalize fast exocytosis between wild-type and mutant IHCs. This, together with an unaltered rate constant of fast exocytosis in mutant IHCs—indicating a normal vesicular release probability—led to the previous hypothesis that the defect primarily reflects a reduction of the readily releasable pool of vesicles (RRP) due to the loss of the ribbon (Khimich et al., 2005).

However, the exact structural and functional correlates of the RRP reduction remained unclear. For example, potential differences between mutant AZs that still have a ribbon (ribbon occupied) and their ribbonless counterparts have not yet been investigated. Moreover, it is not known to which degree and by which mechanism Ca\textsuperscript{2+} influx is affected at the level of individual synapses and how this might contribute to the exocytic deficit. Several mechanisms may explain the impairment of fast exocytosis in IHCs of Bsn\textsuperscript{-Ex4/5} mutants. First, mutant AZs may contain...
fewer vesicular docking sites and/or closely colocalized Ca\(^{2+}\) channels. Together, they have been suggested to constitute the numerous release sites of the IHC AZ at which vesicle fusion is controlled by the Ca\(^{2+}\) nanodomain of one or few nearby active Ca\(^{2+}\) channels (Brandt et al., 2005; Moser et al., 2006; Goutman and Glowatzki, 2007). Vesicles docked and primed in these “slots” probably constitute the RRP, of which the released fraction but not the release kinetics depends on the number of slots recruited by a given stimulus (Brandt et al., 2005; Furukawa and Matsuura, 1978; Wittig and Parsons, 2008). Therefore, fewer release sites, because of fewer Ca\(^{2+}\) channels (Neef et al., 2009) and/or fewer docking sites, could explain impaired fast exocytosis as a deficit of RRP size. Second, even if the number of release sites was unchanged, the standing RRP would be diminished if vesicle occupancy at each of these sites was reduced in Bsn\(^{-Ex4/5}\) IHCs, e.g., because of impaired replenishment or enhanced undocking of vesicles. Third, the coupling between Ca\(^{2+}\) influx and Ca\(^{2+}\) sensors of the exocytosis machinery could be altered, such that not all vesicles can contribute to fast exocytosis, even after proper docking and biochemical priming. This point subsumes changes in diffusion, buffering, or homeostasis of [Ca\(^{2+}\)]\(_i\), as well as an increased distance between channels and Ca\(^{2+}\) sensors, positional priming (Neher and Sakaba, 2008), as it was reported at the Drosophila neuromuscular junction after disruption of the presynaptic scaffold protein Bruchpilot (Kittel et al., 2006). Finally, the intrinsic Ca\(^{2+}\) sensitivity of exocytosis could be altered.

The availability of a number of techniques such as improved stimulated emission depletion (STED) microscopy, and fast confocal imaging of Ca\(^{2+}\) influx, as well as the generation of another Bassoon-deficient mouse line (Bsn\(^{-D}\)) now allowed us to address these questions. Here, we used in vitro and in vivo physiology in combination with light and electron microscopy and computational modeling to study in detail structural and functional effects of Bassoon disruption at both ribbon-occupied and ribbonless AZs. Our results indicate that both functional inactivation of Bassoon and ribbon loss reduce the number of synaptic Ca\(^{2+}\) channels. Membrane tethering of vesicles was improved but not fully normal at ribbon-occupied mutant AZs, suggesting a partial function of these ribbons. Mutant IHCs showed a reduction in the number of release sites while maintaining an intact coupling of Ca\(^{2+}\) influx to exocytosis. Vesicle replenishment was slightly impaired in in vitro experiments. We conclude that the multiprotein complex of the synaptic ribbon and Bassoon organize Ca\(^{2+}\) channels and synaptic vesicles at the AZ, thereby creating a large number of release sites.

RESULTS

The most prominent morphological phenotype of IHCs associated with the disruption of Bassoon function in mouse mutants with partial gene deletion (Bsn\(^{-Ex4/5}\)) is the loss of synaptic ribbons from their AZs (Buran et al., 2010; Khimich et al., 2005). In IHCs of immunolabeled whole-mounted organs of Corti from 3-week-old mice, we used confocal microscopy to count ribbon synapses as juxtaposed spots of presynaptic CtBP2/RIBEYE (labeling ribbons) and postsynaptic GluR2/3 (labeling glutamate receptor clusters). Per IHC in Bsn\(^{-Ex4/5}\), we found on average 2.5 ribbon-occupied synapses (22% of 1240 synapses, n = 112 IHCs) instead of 11.9 ribbon-occupied synapses in Bsn\(^{-wt}\) (97% of 1028 synapses, n = 84 IHCs). Consistent with observations at retinal photoreceptor ribbon synapses (Dick et al., 2003), we detected expression of the N-terminal Bassoon fragment in IHCs of Bsn\(^{-Ex4/5}\) mice (Figure S1A, available online) but found that it was not localized to afferent IHC synapses, arguing against a residual function at the AZ. This observation and the absence of an auditory deficit in 8-week-old heterozygous Bsn\(^{-Ex4/5}\) mice (data not shown) do not support the idea of a dominant negative effect of the N-terminal Bassoon fragment. We also observed fewer ribbon-occupied synapses in IHCs of the newly generated Bassoon-deficient mouse line Bsn\(^{-D}\) (4.8 versus 9.6 ribbon-occupied synapses per IHC in wild-type), which, like Bsn\(^{-Ex4/5}\) mice, showed a mild hearing impairment (threshold increase by 23 dB for click stimuli in four Bsn\(^{-D}\) mice compared to three wild-type littermates versus 37 dB increase in Bsn\(^{-Ex4/5}\); Pauli-Magnus et al., 2007). A weak Bassoon immunolabeling was observed at a small subset (approximately 10%) of synapses in Bsn\(^{-D}\) IHCs (Figure S1B), potentially explaining the higher number of ribbon-occupied AZs in Bsn\(^{-D}\) IHCs.

Reduction of Membrane-Proximal Vesicles at Hair Cell Synapses of Bassoon Mutants

We studied effects of Bassoon disruption and ribbon loss on synaptic ultrastructure in electron micrographs of 80 nm sections (Figures 1A and 1B). Membrane-proximal vesicles at apparently ribbonless Bsn\(^{-Ex4/5}\) AZs showed an altered distribution. When measuring their lateral position relative to the presynaptically projected center of the postsynaptic density, we observed a broad and seemingly random distribution of those vesicles at the AZ (Figure 1C, gray bars). In contrast, membrane-proximal vesicles at AZs of Bsn\(^{-wt}\) IHCs fell into two categories: ribbon-associated (red open bars) and non-ribbon-associated (black open bars). The latter population was indistinguishable from membrane-proximal vesicles at ribbonless Bsn\(^{-Ex4/5}\) AZs (p = 0.27, Kolmogorov-Smirnov test). We then counted the total number of those vesicles in single 80 nm sections and observed significantly fewer vesicles at apparently ribbonless (1.5 ± 0.2 vesicles, 53 AZs) and ribbon-occupied Bsn\(^{-Ex4/5}\) synapses (2.0 ± 0.4 vesicles/AZ section, 10 AZs) than at ribbon-occupied Bsn\(^{-wt}\) synapses (4.2 ± 0.4 vesicles/AZ section, 26 AZs, p < 0.01 for both comparisons).

Because the absence of a synaptic ribbon cannot unequivocally be concluded from not seeing a ribbon in a single 80 nm synaptic section, we used electron tomography to address potential differences between ribbon-occupied and ribbonless AZs in Bsn mutant mice (Figures 1E–1H). We used Bsn\(^{-D}\) mice for these experiments because of their larger fraction of ribbon-occupied AZs. In electron tomography, we counted vesicles that were tethered to the plasma membrane by filamentous linkers (see Figure 1D for examples; Fernández-Busnadiego et al., 2010). Indeed, we found a trend toward more membrane-tethered vesicles when a ribbon was present (6.4 ± 0.8, n = 10 versus 3.7 ± 1.1, n = 6 at ribbonless Bsn\(^{-D}\) AZs; p = 0.1), probably reflecting the addition of a ribbon-associated vesicle population. As in the analysis of 80 nm sections of Bsn\(^{-Ex4/5}\) AZs, vesicle numbers at ribbon-occupied Bsn\(^{-D}\) AZs did not reach Bsn\(^{-wt}\) numbers.
levels (10.6 ± 0.7, n = 5, p < 0.01). We also observed that, unlike at Bsn<sup>wt</sup> synapses, ribbons of Bsngt tended to be farther away from the plasma membrane (Figure 1I). In fact, we found a spectrum of ribbon-anchorage phenotypes: from wild-type-like proximity to loosely anchored ribbons (often accompanied by a second detached ribbon) to complete ribbon absence. It is tempting to speculate that loosely anchored ribbons may not fully promote membrane tethering of vesicles. We note that

Figure 1. Synaptic Ultrastructure and Vesicle Distribution in the Presence and Absence of the Synaptic Ribbon
(A and B) Electron micrographs of single thin sections of Bsn<sup>wt</sup> (A) and Bsn<sup>ΔEx4/5</sup> ribbon-occupied IHC ribbon synapses (B). The following abbreviations are used: r, ribbon; SV, synaptic vesicle; PSD, postsynaptic density; aff, afferent bouton.
(C) Distribution of membrane-proximal synaptic vesicles in Bsn<sup>wt</sup> and Bsn<sup>ΔEx4/5</sup> IHCs as a function of distance from the PSD center. The histogram was normalized to the number of sections analyzed in the respective genotype (Bsn<sup>wt</sup>, n = 58 SVs, 18 sections; Bsn<sup>ΔEx4/5</sup>, n = 74 SVs, 36 sections).
(D) Example slices from single-axis electron tomograms showing membrane-tethered synaptic vesicles. Tethers are marked by arrowheads SC denotes synaptic cleft. The scale bars represent 40 nm.
(E–H) Single slices from tomograms for Bsn<sup>wt</sup> (E1), Bsngt ribbon-occupied (F1 and G1), and Bsngt ribbonless synapses (H1). (E2–H2, upper) Tomogram-based model of Bsn<sup>wt</sup> (E2), Bsngt ribbon-occupied (F2 and G2), and Bsngt ribbonless synapses (H2). Vesicles distant from the ribbon and the plasma membrane are not shown. (Lower) Same models as in upper but only showing membrane-proximal SVs used for analysis (see Results).
(i) Bar plot showing mean minimal distance between ribbon and plasma membrane as measured in electron tomograms of Bsn<sup>wt</sup> AZs (black; n = 5 ribbons/5 AZs), of just the proximal ribbons at Bsngt AZs (red; n = 10 ribbons/5 AZs), and of all ribbons at Bsngt AZs (light red; n = 16 ribbons/10 AZs). The error bars represent standard error of the mean (SEM).
(j) Bar plot, showing average number of membrane-proximal SVs per thin section for wild-type (black; n = 46 AZs, pooled data from Bsn and CaV<sub>b</sub>2 wild-type littermates) and mutant synapses. Bsn<sup>ΔEx4/5</sup> (red; n = 67 AZs), but not CaV<sub>b</sub>2 knockout synapses (blue; n = 32 AZs), had approximately one-half the numbers of membrane-proximal SVs. The error bars represent SEM.
Figure 2. Decreased Immunofluorescence and Altered Shape of CaV1.3 Clusters

(A) Projections of confocal sections of IHCs of apical cochlear coils immunolabeled for synaptic ribbons (CtBP2, red) and Ca\(^{2+}\) channels (CaV1.3, green) as used for analysis in (B and C). (Left) Four Bsr\(^{wt}\) IHCs (n is an abbreviation for nuclei). (Middle) Enlargement of part of the synaptic layer (white box, left) showing colocalization of CtBP2 and CaV1.3. (Right) In the partial gene deletion mutant (Bsr\(^{delEx4/5}\)), Ca\(^{2+}\) channels still cluster but few ribbons remain (P28). Arrowheads point to ribbonless CaV1.3 clusters in wild-type and mutant. Arrow points to a ribbon-occupied CaV1.3 cluster. Asterisk labels a floating ribbon.

(B) CaV1.3 immunofluorescence intensity (mean ± SEM, a.u.) was less at Bsr\(^{delEx4/5}\) synapses when analyzing only CaV1.3 clusters that colocalized with GluR2 (gray; Bsr\(^{delEx4/5}\) versus Bsr\(^{wt}\), p < 0.0005) or when counting the ten brightest clusters per hair cell (black; Bsr\(^{delEx4/5}\) versus Bsr\(^{wt}\), p < 1e\(^{-20}\)). In both Bsr\(^{wt}\) and Bsr\(^{delEx4/5}\), the presence of a ribbon (CtBP2 colocalized: ribbon occupied, red) was associated with greater CaV1.3 intensity when compared to ribbonless synapses (blue). Bsr\(^{wt}\) ribbon occupied versus Bsr\(^{wt}\) ribbonless, p < 0.05; Bsr\(^{delEx4/5}\) ribbon occupied versus Bsr\(^{delEx4/5}\) ribbonless, p < 0.005.

(C) CaV1.3 cluster intensity histogram for Bsr\(^{wt}\) (solid line) and Bsr\(^{delEx4/5}\) (dotted line). Each distribution is decomposed into ribbon-occupied (red) and ribbonless (blue) clusters.
even in the 250 nm tissue sections that were used for tomography, the reported vesicle numbers represent underestimates of the full complement of membrane-proximal vesicles because synapses were not completely included along one dimension. However, this error equally affected each synapse type, and tomograms fully contained the synapse in the other two dimensions. Notably, we found that the electron-dense material lining the presynaptic plasma membrane (presynaptic density) was longer and thicker at ribbon-occupied BsnWT AZs than the spot-like presynaptic densities at BsnBt AZs (regardless of ribbon presence; Figures 1E2–1H2 and Table S1), which sometimes harbored more than one density (Figures 1F2 and 1H2).

Finally, we also studied AZs in IHCs of mouse mutants that contain fewer Ca\(^{2+}\) channels because of a lack of the \(\beta_2\) subunit (Ca\(\beta_2\); Neef et al., 2009). Ca\(\beta_2\)-deficient IHCs display a 70% reduction of both Ca\(^{2+}\) influx and RRP exocytosis despite the presence of synaptic ribbons. Number (Figure 1J, data from wild-type littermates of Bsn\(^{\Delta \text{Ex4/5}}\) and Ca\(\beta_2\) mutants were pooled) and distribution (data not shown) of membrane-proximal vesicles were unaltered in 80 nm sections, suggesting that proteins of the macromolecular ribbon complex, but not Ca\(^{2+}\) channels, are required for the formation of vesicle docking sites.

### Fewer Ca\(^{2+}\) Channels and Altered Shape of Ca\(^{2+}\) Channel Clusters

Voltage-gated Ca\(^{2+}\) influx is decreased in IHCs of Bsn mutants (Bsn\(^{\Delta \text{Ex4/5}}\); Khimich et al., 2005). Here, we explored changes of synaptic Ca\(^{2+}\) signaling by morphological and functional imaging. First, we studied synaptic Ca\(^{2+}\) channel clusters by confocal and STED microscopy following immunolabeling of Ca\(\text{v}_{1.3}\) channel proteins of Corti that had been processed for immunohistochemistry in parallel and following the same protocol were acquired with identical microscope settings and analyzed for intensity and parallel and following the same protocol were acquired with identical microscope settings and analyzed for intensity and function. In experiments colabeling for Ca\(\text{v}_{1.3}\) and the synaptic ribbon marker RIBEYE/CtBP2, we were able to separate ribbon-occupied AZs from ribbonless AZs in Bsn\(^{\Delta \text{Ex4/5}}\) and BsnWT mice. Because of the absence of an additional synaptic marker at ribbonless Bsn\(^{\Delta \text{Ex4/5}}\) AZs, and to exclude nonsynaptic Ca\(\text{v}_{1.3}\) immunofluorescent spots from analysis, we considered only the ten brightest spots in each cell for both genotypes.

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To resolve a potential substructure within Ca\(\text{v}_{1.3}\) clusters, we used a custom-built STED microscope (STED*; lateral point spread function [PSF] less than 100 nm at a tissue depth of 15 to 25 \(\mu\)m; yellow range in Figure 2E), Ca\(^{2+}\) channel clusters of Bsn\(^{\Delta \text{Ex4/5}}\) AZs typically displayed one to three stripes of Ca\(\text{v}_{1.3}\) immunofluorescence (Figure 2F). Parallel confocal observation of the associated CtBP2/RIBEYE immunofluorescence suggested that these synapses featured one ribbon regardless of the number of stripes, although two closely-spaced ribbons may fall within the confocal PSF and thus may not be resolved as individual ribbons. In contrast, Bsn\(^{\Delta \text{Ex4/5}}\) AZs showed Ca\(\text{v}_{1.3}\) immunofluorescence spots rather than stripes (Figure 2F; full width at half maximum of long and short axes: 120 ± 7.9 nm and 95 ± 5.5 nm, \(n = 13\)) with ribbon-occupied AZs typically harboring more spots than ribbonless AZs. Ca\(\text{v}_{1.3}\) immunofluorescent stripes and spots were reminiscent of the patterns of presynaptic density observed in electron tomography (Figures 1E2–1H2). In summary, the abundance of synaptic Ca\(^{2+}\) channels and the cluster shape are altered upon Bassoon disruption, which might reflect the loss of a direct Bassoon action on Ca\(^{2+}\) channel clustering or of the Bassoon-mediated ribbon anchorage. To test for a potential role of Bassoon in the direct synaptic anchoring of Ca\(^{2+}\) channels, we determined whether Bassoon and the Ca\(\text{v}_{1.3}\) channel interacted in a heterologous expression system. We did not find evidence that Bassoon coimmunoprecipitated or colocalized with Ca\(\text{v}_{1.3}\) in transfected HEK293T cells (Figure S4). Therefore, the role of Bassoon in
recruiting Ca\textsuperscript{2+} channels to the AZ may not involve a direct association of the two proteins.

**Reduced Synaptic Ca\textsuperscript{2+} Influx Results from Fewer Channels and Lower Open Probability**

To study synaptic Ca\textsuperscript{2+} influx in Bsn\textsuperscript{Ex4/5} IHCs, we performed whole-cell patch-clamp recordings of Ca\textsuperscript{2+} current (I\textsubscript{Ca}) and confocal imaging of presynaptic Ca\textsuperscript{2+} microdomains (Frank et al., 2009). We found a reduction of peak whole-cell I\textsubscript{Ca} amplitude in Bsn\textsuperscript{Ex4/5} IHCs of 3-week-old mice (Figure 3, Table 1, Figure S2, and Table S1). It ranged between 62% (ruptured-patch, 5 mM Ca\textsuperscript{2+}; Figure 3A and Table 1) and 69% (perforated-patch, 10 mM Ca\textsuperscript{2+}; Figure S2 and Table S1) of Bsn\textsuperscript{wt} amplitude. The difference from Bsn\textsuperscript{wt} was alleviated in the presence of the dihydropyridine agonist BayK8644 (77%, ruptured-patch, 10 mM Ca\textsuperscript{2+}; Figure 3B and Table 1), suggesting that Ca\textsuperscript{2+} channel open probability is reduced in Bsn\textsuperscript{Ex4/5} IHCs in the absence of BayK8644. Moreover, we found that Ca\textsuperscript{2+} current activation was slowed in Bsn\textsuperscript{Ex4/5} IHCs (Figure 3C and Figure S2), while it was indistinguishable from wild-type IHCs in the presence of BayK8644 (Figure 3D). Finally, the Ca\textsuperscript{2+} currents inactivated slightly more in Bsn\textsuperscript{Ex4/5} IHCs (Figure 3E and Table S1).

To test whether the observed reduction in I\textsubscript{Ca} was caused by changes in channel number (N\textsubscript{Ca}), unitary current (i\textsubscript{Ca}), or open

### Table 1. Summary of IHC Physiology in Bsn\textsuperscript{wt} Wild-Type and Mutant Mice

<table>
<thead>
<tr>
<th>Parameter</th>
<th>Bsn\textsuperscript{wt}</th>
<th>Bsn\textsuperscript{Ex4/5}</th>
<th>p value</th>
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<tbody>
<tr>
<td>Whole-Cell Ca\textsuperscript{2+} Current</td>
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<tr>
<td>Peak I\textsubscript{Ca} (pA; 5Ca\textsuperscript{2+}/−BayK)</td>
<td>−179.5 ± 9.6 (N = 31)</td>
<td>−111.1 ± 6.2 (N = 38)</td>
<td>p &lt; 0.001 (W)</td>
</tr>
<tr>
<td>Peak I\textsubscript{Ca} (pA; 10Ca\textsuperscript{2+}/+BayK)</td>
<td>−417.5 ± 29.0 (N = 29)</td>
<td>−321.0 ± 33.9 (N = 19)</td>
<td>p &lt; 0.05 (W)</td>
</tr>
<tr>
<td>N\textsubscript{Ca}; 10Ca\textsuperscript{2+}/+BayK</td>
<td>1574 ± 92 (N = 27)</td>
<td>1227 ± 111 (N = 22)</td>
<td>p &lt; 0.01 (W)</td>
</tr>
<tr>
<td>Synaptic Ca\textsuperscript{2+} Microdomains</td>
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<tr>
<td>ΔF\textsubscript{avg} (a.u.; 5 Ca\textsuperscript{2+}/−BayK)</td>
<td>85.5 ± 9.1 (n = 74/N = 30)</td>
<td>30.5 ± 2.0 (n = 112/N = 45)</td>
<td>p &lt; 0.001 (W)</td>
</tr>
<tr>
<td>ΔF\textsubscript{avg} (a.u.; 5 Ca\textsuperscript{2+}/+BayK)</td>
<td>89.5 ± 9.0 (n = 53/N = 21)</td>
<td>46.9 ± 5.2 (n = 52/N = 20)</td>
<td>p &lt; 0.001 (W)</td>
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<tr>
<td>ΔF\textsubscript{avg} (a.u.; ±BayK)</td>
<td>p = 0.14 (W)</td>
<td>p &lt; 0.01 (W)</td>
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<td>Exocytosis</td>
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<tr>
<td>ΔC\textsubscript{mem,20 ms} (fF) − pp</td>
<td>13.2 ± 1.1 (N = 38)</td>
<td>7.9 ± 0.9 (N = 37)</td>
<td>p &lt; 0.001 (W)</td>
</tr>
<tr>
<td>Q\textsubscript{Ca,20 ms} (pC) − pp</td>
<td>4.4 ± 0.3 (N = 38)</td>
<td>3.0 ± 0.2 (N = 37)</td>
<td>p &lt; 0.001 (W)</td>
</tr>
<tr>
<td>ΔC\textsubscript{mem,20 ms} (fF) − pp</td>
<td>8.1 ± 1.0 (N = 17)</td>
<td>4.7 ± 0.8 (N = 16)</td>
<td>p &lt; 0.01 (W)</td>
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<tr>
<td>Q\textsubscript{Ca,100 ms} (pC) − pp</td>
<td>3.3 ± 0.2 (N = 17)</td>
<td>2.6 ± 0.1 (N = 16)</td>
<td>p &lt; 0.05 (T)</td>
</tr>
<tr>
<td>ΔC\textsubscript{mem,100 ms} (fF) − pp</td>
<td>39.6 ± 5.2 (N = 41)</td>
<td>21.9 ± 4.3 (N = 40)</td>
<td>p &lt; 0.001 (W)</td>
</tr>
<tr>
<td>Q\textsubscript{Ca,100 ms} (pC) − pp</td>
<td>20.4 ± 1.4 (N = 41)</td>
<td>13.2 ± 0.9 (N = 40)</td>
<td>p &lt; 0.001 (W)</td>
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<tr>
<td>ΔC\textsubscript{mem,100 ms} (fF) − pp</td>
<td>35.1 ± 5.3 (N = 13)</td>
<td>19.5 ± 5.8 (N = 11)</td>
<td>p &lt; 0.01 (W)</td>
</tr>
<tr>
<td>ΔC mem,100 ms (fF) − pp</td>
<td>15.5 ± 0.1 (N = 13)</td>
<td>11.2 ± 0.9 (N = 11)</td>
<td>p &lt; 0.01 (T)</td>
</tr>
<tr>
<td>sustained ΔC\textsubscript{mem} (ΔC\textsubscript{mem,100 ms} − ΔC\textsubscript{mem,20 ms})</td>
<td>26.8 ± 4.6 (N = 38)</td>
<td>15.1 ± 4.4 (N = 35)</td>
<td>p &lt; 0.001 (W)</td>
</tr>
</tbody>
</table>

n denotes number of synapses (Ca\textsubscript{1.3} immunofluorescence and synaptic Ca\textsuperscript{2+} microdomains) and N number of IHCs (whole-cell Ca\textsuperscript{2+} current, Ca\textsuperscript{2+} imaging, and capacitance measurements). n.s. denotes not significant. For immunofluorescence, Bsn mutant data were separated into ribbonless and ribbon-occupied synapses. Statistical comparisons were made with an independent two-sample t test (T) or a Mann-Whitney-Wilcoxon (W) test (Experimental Procedures). a.u., arbitrary units; FWHM, full width at half-maximum; I\textsubscript{Ca}, whole-cell Ca\textsuperscript{2+} current; N\textsubscript{Ca}, number of Ca\textsuperscript{2+} channels; ΔC\textsubscript{mem} average Ca\textsuperscript{2+} current integral; pp, perforated-patch configuration; rp, ruptured-patch configuration. Sustained ΔC\textsubscript{mem} was calculated cell-wise, by subtracting the average ΔC\textsubscript{mem} response to 20 ms from the average ΔC\textsubscript{mem} response to 100 ms. Data are presented as mean ± SEM.
probability ($p_{\text{open}}$), we performed a nonstationary fluctuation analysis on Ca$^{2+}$ tail-currents ([BayK8644]$_{\text{e}}$ = 5 μM; Brandt et al., 2005). In line with the observed reduction in ICa amplitude, both variance and mean were reduced in Bsn$^{\text{ex4/5}}$ IHCs (Figures 3F and 3G). The analysis indicated a ~20% decrease in the number of functional Ca$^{2+}$ channels but statistically indistinguishable single-channel currents and maximal open probabilities in the presence of BayK8644 (Table 1 and Table S1). We note that due to uncertainties associated with the channel property estimates from fluctuation analysis (Tables 1 and Table S1), which also deviate from those obtained from single-channel recordings in immature IHCs (Zampini et al., 2010), emphasis is on comparison between the genotypes rather than on absolute values (see also Supplemental Experimental Procedures).

**Figure 3. Biophysical Properties of Voltage-Dependent Whole-Cell Ca$^{2+}$ Current (ICa)**

(A) Average steady-state ICa-V for Bsn$^{\text{wt}}$ and Bsn$^{\text{ex4/5}}$ IHCs in 5 mM [Ca$^{2+}$]$_{\text{e}}$ in [BayK8644]$_{\text{e}}$ = 5 mM [BayK8644] (n [Bsn$^{\text{wt}}$] = 29 IHCs, n [Bsn$^{\text{ex4/5}}$] = 19 IHCs). Note the smaller difference in max. ICa between the two genotypes (Bsn$^{\text{ex4/5}}$: ~80% of Bsn$^{\text{wt}}$ level; Table 1).

(B) As in (A) but in 10 mM [Ca$^{2+}$]$_{\text{e}}$ and presence of 5 μM BayK8644 (n [Bsn$^{\text{wt}}$] = 29 IHCs, n [Bsn$^{\text{ex4/5}}$] = 19 IHCs). Note the smaller difference in max. ICa between the two genotypes (Bsn$^{\text{ex4/5}}$: ~80% of Bsn$^{\text{wt}}$ level; Table 1).

(C) Average time-constant of ICa activation in 5 mM [Ca$^{2+}$]$_{\text{e}}$, as a function of membrane voltage ($V_m$), derived from single exponential fits to the initial 3.5 ms of ICa (see Supplemental Experimental Procedures; n [Bsn$^{\text{wt}}$]: ≤ 30 IHCs, n [Bsn$^{\text{ex4/5}}$]: ≤ 35 IHCs). Asterisks indicate $V_m$ at which differences between genotypes were statistically significant ($\alpha = 0.05$; Bonferroni correction). Average series resistance (R$_S$) was 6.0 ± 2.3 MΩ for Bsn$^{\text{wt}}$ IHCs, and 6.1 ± 2.3 MΩ for Bsn$^{\text{ex4/5}}$ IHCs (mean ± SD), respectively.

(D) Average ICa recorded in 10 mM [Ca$^{2+}$]$_{\text{e}}$ and presence of 5 μM BayK8644 (n [Bsn$^{\text{wt}}$] = 29 IHCs, n [Bsn$^{\text{ex4/5}}$] = 19 IHCs). Average R$_S$ was 4.7 ± 3.1 MΩ for Bsn$^{\text{wt}}$ IHCs, and 5.3 ± 3.3 MΩ for Bsn$^{\text{ex4/5}}$ IHCs (mean ± SD), respectively. The error bars in (A–D) represent SEM.

(E1 and E2) Average paired-pulse ICa traces (depolarization to $V_m$ of maximum ICa [Ca$^{2+}$]$_{\text{e}}$ = 10 mM) illustrate stronger inactivation in Bsn$^{\text{ex4/5}}$ IHCs, being more evident for longer (100 ms; E2) than for shorter depolarizations (20 ms; E1).

(F) Example mean Ca$^{2+}$ tail-currents (lower) used for nonstationary fluctuation analysis (Tables 1 and Table S1), elicited by repolarizing IHCs from −57 mV to −68 mV, and corresponding mean trial-to-trial variance (upper), [Ca$^{2+}$]$_{\text{e}}$ = 10 mM, [BayK8644]$_{\text{e}}$ = 5 μM.

(G) Grand average (lines with filled circles) of variance versus mean relationships (n [Bsn$^{\text{wt}}$] = 27 IHCs, n [Bsn$^{\text{ex4/5}}$] = 22 IHCs). Filled areas depict SD of grand average of variance. Broken lines represent grand average of parabolic fits (Supplemental Experimental Procedures).

Synaptic Ca$^{2+}$ microdomains, primarily reflecting Ca$^{2+}$ influx at the AZ (Frank et al., 2009), were visualized with the low-affinity Ca$^{2+}$ indicator Fluo-4N (400 μM, K$_D$ = 95 μM) in conjunction with the slow Ca$^{2+}$ chelator EGTA (2 mM). The Ca$^{2+}$ microdomain amplitude ($\Delta F$) measured under these conditions probably reflects a linear summation of the Ca$^{2+}$ influx contributed by the individual synaptic Ca$^{2+}$ channels (Frank et al., 2009). Consistent with the finding of Ca$_{\text{R1,3}}$ channel clusters in immunohistochemistry, we readily observed Ca$^{2+}$ microdomains also in Bsn$^{\text{ex4/5}}$ IHCs (Figure 4A). However, their average amplitude ($\Delta F_{\text{avg}}$; Table 1), measured at −7 mV in spot-detection experiments at the center of the Ca$^{2+}$ microdomains, was reduced to 36% of control (Figures 4B and 4C), exceeding the reduction of whole-cell ICa (to 60%–70%;...
Figure 4. Reduced Presynaptic Ca2+ Influx

(A) Exemplary localized Ca2+ influx sites in optical sections through the basal part of IHCs (Experimental Procedures). Resting fluorescence (F0) was subtracted (ΔF) images. Ca2+ microdomains at A2Zs are present in BsnΔEx4/5 IHCs, albeit of smaller amplitude.

(B) Exemplary spot-detection responses during depolarization to −7 mV (bar, lower; all reported responses were from the Ca2+ microdomain center) and simultaneously acquired whole-cell Ica (upper). Note the out-of-proportion reduction of synaptic Ca2+ influx.

(C) Grand average of spot-detection responses from Ca2+ microdomains (n [Bsnwt] = 74 AZs/30 IHCs, n [BsnΔEx4/5] = 112 AZs/45 IHCs); shaded areas indicate SD.

(D) Same as (C) but in presence of 5 μM BayK8644 (n [Bsnwt] = 53 AZs/21 IHCs, n [BsnΔEx4/5] = 52 AZs/20 IHCs). Note the peak at the end of the stimulation, corresponding to tail current-mediated Ca2+ influx (prolonged due to BayK8644).

(E and F) Cumulative frequency distributions of Bsnwt (E) and BsnΔEx4/5 (F) Ca2+ microdomain amplitudes (averaged over the second half of the stimulus) in either absence (black/grey) or presence (red/light red) of 5 μM BayK8644. CV denotes coefficient of variation (SD/mean).

(G) Normalized steady-state fluorescence-voltage relationships (n [Bsnwt] = 19 AZs, n [BsnΔEx4/5] = 27 AZs). Relative fluorescence changes were averaged over the last 14.6 ms of the 20 ms stimulus.

(H) Representative line scans across the Ca2+ microdomain center (5 mM [Ca2+]o). Bar indicates period of depolarization to −7 mV.

(I) Grand average of Bsnwt (I1) and BsnΔEx4/5 (I2) spot-detection responses, sorted according to the presence/absence of a localized ribbon (Experimental Procedures). n [Bsnwt] = 104 AZs/32 IHCs, n [BsnΔEx4/5] = 96 AZs/37 IHCs.

Figure 3) and Ca1,3 immunofluorescence (to 58%; Figure 2). Augmenting influx through Ca1,3 Ca2+ channels (5 μM BayK8644) alleviated the amplitude reduction of synaptic Ca2+ influx in BsnΔEx4/5 IHCs (to 52% of control; Figure 4D and Table 1) and increased amplitude variability among the BsnΔEx4/5, but not the Bsnwt synapses (Figures 4E and 4F). Kinetics (Figures 4C and 4D, Figure S3, and Table S1), voltage dependence (Figure 4G, Figure S3, and Table S1), and spatial extent (Figure 4H and Table S1) of the Ca2+ microdomains in BsnΔEx4/5 IHCs were similar to control. There was, however, a tendency toward faster kinetics and more negative activation of BsnΔEx4/5 Ca2+ microdomains (Table S1). While the former may reflect differences in Ca2+ buffering and/or diffusion, the latter may indicate an altered gating of synaptic Ca2+ channels in the absence of Bassoon and/or the ribbon.

In a second set of experiments, we studied Ca2+ signaling at ribbonless and ribbon-occupied A2Zs in separation with a fluorescent RIBEYE-binding peptide to identify ribbon-occupied A2Zs (Frank et al., 2009; Zenisek et al., 2004) in both BsnΔEx4/5 and Bsnwt IHCs. While Ca2+ microdomains at ribbon-occupied A2Zs had larger amplitudes than ribbonless synapses in Bsnwt IHCs, there was no significant difference between ribbonless and ribbon-occupied A2Zs in BsnΔEx4/5 IHCs (Figure 4I). The latter finding was unexpected given their difference in Ca1,3 immunofluorescence but could reflect limited sensitivity of functional Ca2+ imaging, precluding detection of very dim Ca2+ signals at BsnΔEx4/5 ribbonless synapses. In summary, the reduced amplitude of Ca2+ microdomains and its partial alleviation upon the
BayK8644-mediated increase in open probability led us to conclude that \textit{Bsn}\textsubscript{4Ex4/5} synapses contain fewer Ca\textsuperscript{2+} channels with a lower open probability. The reduction of synaptic Ca\textsuperscript{2+} influx beyond the decrease observed in whole-cell \textit{l}_{\text{Ca}} indicates a higher proportion of extrasynaptic channels in \textit{Bsn}\textsubscript{4Ex4/5} IHCs.

**Reduced RRP and Sustained Exocytosis but Intact Ca\textsuperscript{2+} Influx-Exocytosis coupling**

How does the reduction of Ca\textsuperscript{2+} channels and membrane-proximal vesicles—as well as a potential mislocalization of these two elements—affect hair cell exocytosis? We addressed this question in \textit{Bsn}\textsubscript{4Ex4/5} IHCs by measuring exocytic membrane capacitance changes (\(\Delta C_{\text{m},20\text{ ms}}\)) and Ca\textsuperscript{2+} currents (\(I_{\text{Ca}}\)) in response to 20 ms depolarizations at peak-\(I_{\text{Ca}} V_{\text{m}}\).

(D) Normalized conductance (G)-voltage relationships for both genotypes. (E1) Summary of exocytic \(\Delta C_{\text{m}}\) responses to 20 ms depolarizations to the five test potentials depicted in (D) plotted versus the corresponding peak \(I_{\text{Ca}}\) current integrals (\(Q_{\text{Ca}}\)). Pulsed were applied to 21 \textit{Bsn}\textsuperscript{wt} IHCs and 23 \textit{Bsn}\textsubscript{4Ex4/5} IHCs in random order at intervals of > 20 ms. Note the larger responses at \(+18\text{ mV}\) compared to \(-27\text{ mV}\), despite similar \(Q_{\text{Ca}}\) values, coinciding with a larger \(p_{\text{open}}\) of Ca\textsuperscript{2+} channels (see D). (E2) To compare the relation between \(p_{\text{open}}\) and the efficiency of synaptic vesicle release in the two genotypes, we applied 3 transformations to the plot shown in (E1): (1) assuming a certain extrasynaptic \(N_{\text{Ca}}\) (Brandt et al., 2005), we estimated the fraction of synaptic Ca\textsuperscript{2+} channels (out of total \(N_{\text{Ca}}\); Table 1), and multiplied \(Q_{\text{Ca}}\) by the respective ratio (<1) to estimate “synaptic \(Q_{\text{Ca}}\)” (2) We then doubled the mutant \(\Delta C_{\text{m}}\) data to account for the halving of membrane-proximal synaptic vesicles seen at mutant AZs (Figure 1). (3) Last, we accounted for the apparently reduced number of synaptic Ca\textsuperscript{2+} channels at mutant AZs by multiplying mutant \(Q_{\text{Ca}}\) by 1/0.52 (assuming that the Ca\textsuperscript{2+} microdomain amplitude in the presence of BayK8644 presents the most reliable reflection of synaptic \(N_{\text{Ca}}\) Table 1).

(F) Ratio of exocytic responses (20 ms and 100 ms depolarizations to peak-\(I_{\text{Ca}} V_{\text{m}}\)) between perforated-patch (endogenous Ca\textsuperscript{2+} buffers) and ruptured-patch ([EGTA] = 5 mM) configurations for \textit{Bsn}\textsuperscript{wt} and \textit{Bsn}\textsubscript{4Ex4/5} IHCs (Table 1).

The error bars in (C, E, and F) represent SEM.
The observation that the reduction of Ca\textsuperscript{2+}-influx-triggered exocytosis did not exceed the reduction in the number of membrane-proximal and -tethered vesicles (Figure 1) suggests that the remaining docking sites are equipped with nearby Ca\textsuperscript{2+} channels (reduction of synaptic Ca\textsuperscript{2+} channels: ~50%; Table 1; estimated from Bsn\textsuperscript{wt} versus Bsn\textsuperscript{mut} synaptic Ca\textsuperscript{2+} microdomain amplitude in the presence of BayK8644). Yet, a slower coupling between Ca\textsuperscript{2+} channels and vesicle docking sites than implied for the Ca\textsuperscript{2+} nanodomain regime suggested for wild-type IHC AZs could not be excluded (Brandt et al., 2005; Goutman and Glowatzki, 2007; Moser et al., 2006). Therefore, we studied the sensitivity of exocytosis to the slow Ca\textsuperscript{2+} chelator EGTA (Figure 5F). Consistent with the preservation of nanodomain-controlled vesicle fusion in Bsn\textsuperscript{Ex4/5} IHCs, their \(\Delta C_m\), 20 ms in the presence of 5 mM [EGTA], was reduced to 58% of control levels (Table 1)—closely resembling the reduction in the presence of endogenous Ca\textsuperscript{2+} buffers (see above). Additionally, we probed RRP exocytosis as a function of Ca\textsuperscript{2+} influx at different membrane potentials (Figures 5D and 5E). Changing the membrane potential manipulates open probability and single-channel current in opposite directions. Thus, exocytosis can be tested for the same absolute Ca\textsuperscript{2+} influx through either few open channels with high single-channel current (mild depolarizations) or more open channels with low single-channel current (strong depolarizations). If exocytosis of a given vesicle was under control of a population of several Ca\textsuperscript{2+} channels (Ca\textsuperscript{2+} microdomain control), exocytosis should be identical for the same Ca\textsuperscript{2+} current independent of the membrane potential. In case of a Ca\textsuperscript{2+} nanodomain control, more exocytosis is expected for more open Ca\textsuperscript{2+} channels, i.e., at more depolarized potentials (hysteresis; Zucker and Fogelson, 1986). This was indeed observed in Bsn\textsuperscript{wt} IHCs (Figure 5E1 and Figure S5), as described before (Brandt et al., 2005), but also in Bsn\textsuperscript{Ex4/5} IHCs (Figure 5E1 and Figure S5), further arguing that Ca\textsuperscript{2+} nanodomain control of exocytosis is maintained at mutant AZs. As a further consistency check, we scaled the exocytosis-Ca\textsuperscript{2+} current integral relationship of Bsn\textsuperscript{Ex4/5} IHCs by experimentally derived factors to normalize the data to the lower number of membrane-proximal vesicles and synaptic Ca\textsuperscript{2+} channels. This resulted largely in an overlap with the wild-type data (Figure 5E2). In summary, the data indicate that the coupling of Ca\textsuperscript{2+} channels to release sites remains intact despite Bassoon disruption but that the rates of initial and sustained exocytosis are reduced to a similar extent as the number of membrane-proximal vesicles.

**Figure 6. Slowed Vesicle Replenishment Kinetics**

(A) Example \(\Delta C_m\) responses and Ca\textsuperscript{2+} currents (IC\textsubscript{Ca}) from Bsn\textsuperscript{wt} and Bsn\textsuperscript{Ex4/5} IHCs upon two 20 ms depolarizations to maximum IC\textsubscript{Ca} potential, separated by 98 ms.

(B) Same as (A) but with 100 ms depolarizations.

(C) Summary of paired-pulse \(\Delta C_m\) recordings following 20 ms depolarizations. The graph shows the ratio of response magnitudes between the second and the first pulse (\(\Delta C_m^{\Delta C_m}\)) for different inter-pulse-intervals. Note the depression in both genotypes, which is, however, more pronounced in Bsn\textsuperscript{Ex4/5} IHCs (\(p < 0.01\) for IPI of 98 ms; \(n (Bsn^{wt}): 23\) to 32 IHCs; \(n (Bsn^{Ex4/5}): 20\) to 32 IHCs).

(D) Same as (C) but for 100 ms depolarizations.

Note the slight facilitation in Bsn\textsuperscript{wt} IHCs, but consistent depression in Bsn\textsuperscript{Ex4/5} IHCs for short IPIs, respectively (\(p < 0.01\) for IPI of 98, 198, and 398 ms; \(n (Bsn^{wt}) = 22\) to 35 IHCs; \(n (Bsn^{Ex4/5}) = 20\) to 39 IHCs).

The error bars in (C) and (D) represent SEM.

**In Vitro and In Vivo Analysis of Synaptic Vesicle Replenishment**

Traditionally, the synaptic ribbon has been assigned a conveyor belt and/or attractor function (Holt et al., 2004; Sterling and Matthews, 2005), according to which it is responsible for rapid supply of vesicles to the RRP and enables high rates of tonic neurotransmitter release (Gomis et al., 1999; Johnson et al., 2008; Moser and Beutner, 2000; Rutherford and Roberts, 2006; Schnee et al., 2005; Spassova et al., 2004). Hence, we tested whether the rate of RRP refilling was reduced in the absence of the ribbon and functional Bassoon protein. Here, we explored vesicle replenishment in vitro by measuring relative \(\Delta C_m\) in paired-pulse protocols, with the stimuli (20 ms or 100 ms long depolarizations) being separated by various time intervals (98, 198, and 398 ms; Figure 6). The ratio of Ca\textsuperscript{2+} current integrals was close to one in both genotypes (marginally smaller in Bsn\textsuperscript{Ex4/5} IHCs; Figures 6C and 6D and Table S1) indicating that the Ca\textsuperscript{2+} signals that drive exocytosis were mostly comparable between both pulses. For 20 ms stimuli at short inter-pulse-intervals (IPI: 98 ms) we observed stronger depression of the exocytic response in Bsn\textsuperscript{wt} IHCs, indicating a slower recovery of the RRP at Bsn\textsuperscript{wt} synapses (\(p < 0.01\)). For longer recovery times (IPI: 198, 398 ms), the difference did not reach statistical significance. While both Bsn\textsuperscript{wt} and Bsn\textsuperscript{Ex4/5} IHCs showed depression for short stimuli, Bsn\textsuperscript{wt} IHCs exhibited a tendency toward facilitation for long depolarizations (100 ms). In contrast, Bsn\textsuperscript{Ex4/5} IHCs also showed depression when challenged with long stimuli (\(p < 0.01\) for 98, 198, and 398 ms IPI).
Bassoon Organizes Hair Cell Active Zones

In vivo, we measured the recovery of the auditory nerve fiber response following a masking sound as a proxy of the recovery of the presynaptic RRP (Spassova et al., 2004). We used a forward masking paradigm (Harris and Dallos, 1979; Spassova et al., 2004) in which a 100 ms masking stimulus was separated from a 15 ms probe stimulus by a variable silent interval ranging between 2 and 512 ms (Figure 7A). Onset spike rates and adapted spike rates in response to the masking stimulus were reduced by a factor of 1.7 and 1.4, respectively, in Bsn<sup>−/−</sup> (poststimulus time histograms [PSTHs]; Figure 7B). We found an enhanced forward masking effect in Bsn<sup>−/−</sup> fibers when comparing each probe response to its masker response (lower ratio of spike rate for probe over spike rate for masker [averaged over the first 5 ms of the probe and the masker]) at 4-32 ms interval for mutants, p < 0.05 each). There was also a trend toward longer time of half-recovery from masking in Bsn<sup>−/−</sup> (34.9 ± 5.0 ms in mutant and 23.3 ± 4.9 ms in wild-type, p = 0.13).

Taken together, the in vitro and in vivo results suggest a disturbed replenishment of fusion-competent synaptic vesicles in Bsn<sup>−/−</sup> IHCs. To what degree is the impaired sound coding phenotype in Bsn<sup>−/−</sup> IHCs caused by a reduction in the number of release sites or by their deficient refilling? To answer this question, we quantified the forward masking data by a model of sound-dependent RRP fusion and replenishment combined with auditory nerve fiber refractoriness. The core parameters of this model are the number of release sites, sound-dependent rates (fusion rate constants and refilling rate constants per release site in the presence and absence of sound), and absolute and relative refractory periods (Supplemental Experimental Procedures). As those parameters are biophysically accessible quantities, the model can be used for quantitative, mechanistic data analysis of auditory nerve fiber responses in the context of cellular physiology.

A single set of parameters accurately reproduced PSTHs for all nine recovery periods (Figure 7B and Figure S6). The very same set of parameters also predicted the ratio of spike counts (probe/masker) for analysis windows of 5 ms and 13 ms following sound onset (Figure 7C). Importantly, the dominating difference between the parameter sets for the two genotypes was a 35% reduction in release site number for Bsn<sup>−/−</sup> (i.e., the maximal capacity of the RRP; see dotted line in Figure 7B for a simulation with wild-type release site number), while the fusion rates and vesicular release probability remained virtually unchanged (Table S2; consistent with capacitance measurements; Figure 5). Additionally, refilling rate constants were slightly reduced. When assuming wild-type vesicle replenishment kinetics for Bsn<sup>−/−</sup>—while keeping all other model parameters for this genotype—the adapted spike rates were accordingly slightly improved (see dashed line in Figure 7B).

Figure 7. Comparison of Sound-Evoked Spike Rates In Vivo for Bsn<sup>−/−</sup> and Bsn<sup>−/−Ex4/5</sup> Mice

Reduced in vivo action potential rates and minimally enhanced forward masking in auditory nerve fibers can be explained by a vesicle pool model combined with spike refractoriness.

(A) Illustration of the stimulus design: a 100 ms tone burst (masker) is separated by a 15 ms probe stimulus by a silent interval ranging between 2 and 512 ms (example: 64 ms). Both stimuli were presented at characteristic frequency, 30 dB above threshold and with 2.5 ms rise-fall times. The interval between two maskers was always 1 s. Each interval was repeated at least 50 times.

(B) Experimental results (blue [Bsn<sup>−/−</sup>], and red [Bsn<sup>−/−Ex4/5</sup>]) and model-predicted (black) spike rates for recovery periods of 8 (upper) and 64 (lower) ms in Bsn<sup>−/−</sup> (left, n = 15) and Bsn<sup>−/−Ex4/5</sup> (right, n = 7) auditory nerve fibers. Dotted line shows the prediction of a model with the set of Bsn<sup>−/−Ex4/5</sup> parameters except Bsn<sup>−/−</sup> release site number. Dashed line, instead, results from using Bsn<sup>−/−Ex4/5</sup> parameters with Bsn<sup>−/−</sup> vesicle replenishment kinetics.

(C) Experimental (dashed line, symbols representing means ± SEM) and model-predicted (line) relative spike counts during the first 5 (left) and the first 13 (right) ms of the auditory nerve fiber response to the probe stimulus normalized to the response to the masker stimulus. The error bars represent SEM.

(D) Schematic representation of tentative active zone structure at wild-type, ribbon-occupied Bsn mutant, and ribonless Bsn mutant afferent IHC synapses, respectively. (Top) Sections; (bottom) view as seen from the synaptic cleft. The illustration summarizes the findings of smaller Ca<sub>2+</sub> channel/membrane-proximal vesicle complements in the absence of a synaptic ribbon, altered substructure of Ca<sub>2+</sub> clusters in Bsn mutants.
In summary, using the model as a quantification of the in vivo results allowed us to draw conclusions about presynaptic quantities from postsynaptic measurements. Generally, the model validated our structural and functional findings, made independently in vitro. Additionally, it advanced our mechanistic understanding by permitting the discrimination between a reduction in the number of (1) generally available release sites and the reduction in the (2) occupancy of those release sites: the parameters suggest that the reduced response amplitude in Bsn<sup>ΔEx4/5</sup> fibers is primarily due to a reduction in the total number of release sites (35%) and to a lesser degree caused by a reduction in their occupancy. These two effects combine such that in the model the number of release sites occupied at rest is reduced to 50% of wild-type, which is in agreement with the 55% reduction in number of membrane-proximal vesicles observed in electron micrographs of Bsn<sup>ΔEx4/5</sup> AZs (Figure 1J).

**DISCUSSION**

In this study, we examined effects of genetic Bassoon disruption at several structural and functional levels. EM tomography revealed a spectrum of synapse morphologies from wild-type-like to loosely anchored ribbons to ribbonless. Intriguingly, we found that Bsn mutant synapses with a partially anchored ribbon (ribbon occupied) exhibited an intermediate phenotype between wild-type AZs and mutant ribbonless AZs. While fewest synaptic Ca<sup>2+</sup> channels were found at ribbonless AZs in Bsn<sup>ΔEx4/5</sup> IHCs, the ribbon-occupied Bsn<sup>ΔEx4/5</sup> AZs harbored more, but still fewer, Ca<sup>2+</sup> channels than wild-type AZs—similar to the quantification of membrane-proximal vesicle number. Fast and sustained exocytosis was reduced in proportion to the overall reduction in membrane-proximal vesicle and Ca<sup>2+</sup> channel number, while the Ca<sup>2+</sup> sensitivity of exocytosis remained normal. Moreover, vesicle replenishment was impaired. A mechanistic computational model of synaptic transfer, used to fit the in vivo data, independently supported morphological and functional in vitro findings. We conclude that Bassoon disruption and the associated ribbon loss reduces the number of functional release sites, impairs their refilling, and consequently lowers the RRP.

**Structural Consequences of Bassoon Disruption and Ribbon Loss**

The most prominent phenotype of Bassoon disruption is the loss of synaptic ribbons from a majority of AZs (Khimich et al., 2005; tom Dieck et al., 2005). In contrast to retinal photoreceptors (Specht et al., 2007), mature hair cells of Bsn mutants exhibited some (although few) ribbon-occupied synapses at typical locations (Figures 1 and 2). Together with observations at Bsn<sup>wt</sup> synapses without a ribbon, study of these ribbon-occupied AZs helped to test the role of the synaptic ribbon. Both semiquantitative immunofluorescence microscopy (Figures 2B and 2C) and confocal imaging of synaptic Ca<sup>2+</sup> influx (Figure 4I1) revealed that ribbon presence was associated with an increase in the number of Ca<sup>2+</sup> channels at Bsn<sup>wt</sup> AZs. Using STED microscopy, we furthermore observed a stripe-like arrangement of the Ca<sup>2+</sup> channel cluster(s) at ribbon-occupied Bsn<sup>wt</sup> AZs. These structures were reminiscent of the electron-dense material seen in electron tomograms of AZs in mouse IHCs (Figures 1E2–1H2) and frog saccular hair cells (Lenzi et al., 2002), and the row-like arrays of intramembrane particles observed in freeze-fracture electron micrographs (Roberts et al., 1990; Saito and Hama, 1984). At all Bsn<sup>ΔEx4/5</sup> synapses, this Ca<sub>1.3</sub> cluster geometry was dissolved into a pattern of small spots (Figure 2F), similar to alterations of presynaptic densities seen in electron tomography (Figures 1E2–1G2). This coincidence supports the view that the Ca<sub>1.3</sub> clusters are an integral part of the presynaptic density (Lenzi et al., 2002). While this difference in cluster geometry could, in principle, reflect a direct effect of Bassoon loss, we did not find evidence for direct interactions between Bassoon and Ca<sub>1.3</sub> (heterologous expression; Figure S4). The observation of spots rather than stripes at ribbon-occupied Bsn<sup>ΔEx4/5</sup> synapses might also reflect a decreased organizational impact of the ribbon when its anchorage is loosened. It is interesting to note that the CAST/ELKS1 homolog Bruchpilot has been implicated in clustering of presynaptic Ca<sup>2+</sup> channels at the Drosophila neuromuscular junction (Kittel et al., 2006). Bruchpilot is an integral component of presynaptic electron-dense projections (T-bars, which were absent in Bruchpilot mutants) and physically interacts with Ca<sup>2+</sup> channels, at least in vitro (Fouquet et al., 2009).

Our finding of abnormal clustering of synaptic Ca<sup>2+</sup> channels upon Bassoon disruption is supported by a comparison between the associated reduction in whole-cell (~20%) and synaptic Ca<sup>2+</sup> influx (~50%; both in the presence of BayK8644). The stronger decrease in synaptic Ca<sup>2+</sup> influx indicates an increased fraction of extrasynaptic Ca<sup>2+</sup> channels in mutant IHCs. Similar to Ca<sup>2+</sup> channels, the number of membrane-proximal vesicles appears to be greater when the ribbon is present. At least by trend, ribbonless mutant AZs showed the fewest vesicles, whereas the presence of a ribbon increased this figure, but not to wild-type levels.

**Functional Consequences of Bassoon Disruption and Ribbon Loss**

How do these findings relate to synaptic function? Specifically, how are the number of release sites—formed by vesicle docking sites and closely colocalized Ca<sup>2+</sup> channels—and synaptic exocytosis affected by Bassoon disruption? First, when probing fast and sustained exocytosis in Bsn<sup>ΔEx4/5</sup> IHCs, we observed a decrease in amplitude that was roughly comparable to the observed reduction in vesicle number and synaptic Ca<sup>2+</sup> channel number. Second, both intrinsic and apparent Ca<sup>2+</sup> cooperativity of exocytosis was normal in Bsn<sup>ΔEx4/5</sup> IHCs. Together, these observations suggest that the coupling between Ca<sup>2+</sup> influx through the remaining Ca<sup>2+</sup> channels and the fusion of the remaining vesicles was unaffected. Yet one faces the caveat that a static technique such as EM cannot distinguish between fewer physical docking sites or their lower occupancy due to impaired replenishment. Thus, distinguishing between these two scenarios is aided by probing vesicle resupply, which was slightly impaired in Bsn<sup>ΔEx4/5</sup> mice (Figure 6). This finding stands in agreement with the study of Hallermann and colleagues (Hallermann et al., 2010), which shows that vesicle reloading at a central synapse is impaired in Bsn mutants, evident by enhanced synaptic depression during sustained high-frequency activity.
Supplemental Information. Unless stated otherwise, all chemicals were obtained from Sigma-Aldrich.

### Toward Disentangling the Interplay between Bassoon and the Synaptic Ribbon

Bassoon, via interaction with RIBEYE (tom Dieck et al., 2005), contributes to ribbon anchorage. In hair cells, some residual and partial ribbon anchorage is observed, probably involving additional anchoring proteins. Those ribbon-occupied**Bsn** mutant synapses were inferior to their wild-type counterparts with regard to both Ca\(^{2+}\) channel clustering and membrane tethering of vesicles. These observations could either be explained by (1) a direct effect of functional Bassoon loss, or (2) by a limited capacity of “sick ribbons” to perform their task(s). Several recent studies at “conventional” synapses show that Bassoon is not required for synaptic transmission per se but is involved in clustering (Mukherjee et al., 2010) and replenishment of synaptic vesicles (Hallermann et al., 2010). Our results are generally consistent with these findings; however, ribbon synapses do require Bassoon also for basic synaptic transmission. It is likely that the more severe synaptic phenotype found in IHCs reflects the perturbation of ribbon-supported functions. For example, in contrast to Hallermann et al. (2010), we do find a substantial reduction in the number of release sites in **Bsn** mutant IHCs. Additionally, the trend toward fewer membrane-tethered vesicles in general but more vesicles at ribbon-occupied than ribbonless **Bsn** mutant synapses could, for example, be explained by a combinatorial effect of primary Bassoon loss and secondary Piccolo loss (Mukherjee et al., 2010) in the case of ribbonless synapses. Interestingly, no evidence for a reduced quantal content was found in **BSN** mutant cerebellar synapses by Hallermann et al. (2010) However, the complex nature of interactions between the numerous members of the cytomatrix of the active zone (Schoch and Gundelfinger, 2006) demand careful evaluation of “one-protein, one-function” hypotheses. The absence of detectable direct effects of Bassoon disruption on basal synaptic transmission at conventional synapses (Hallermann et al., 2010; Mukherjee et al., 2010) and the intermediate phenotypes seen in ribbon-occupied mutant synapses might favor a hypothesis of “sick ribbons” over direct Bassoon effects underlying the majority of observed synaptic and auditory phenotypes in **Bsn** mutants. Future studies, including silencing of ribbon components such as Piccolo and RIBEYE, are required to further our understanding of the roles of the synaptic ribbon and Bassoon for active zone structure and function as well as their dynamic regulation.

### EXPERIMENTAL PROCEDURES

A more detailed version of the Experimental Procedures is published in Supplemental Information. Unless stated otherwise, all chemicals were obtained from Sigma-Aldrich.

### Animals

Mice with deletion of exons 4 and 5 of the Bassoon gene (**Bsn**\(^{Ex4/5}\); Altrock et al., 2003) or carrying a gene-trapped allele (**Bsn**\(^{gt}\), Lexicon Pharmaceuticals, Inc.), and wild-type littermates were used. All experiments were approved by the University of Göttingen Board for Animal Welfare and the Animal Welfare Office of the State of Lower Saxony.

### Immunohistochemistry

Apical cochlear turns were fixed in methanol for 20 min at −20 °C and prepared as previously described (Khimich et al., 2005; Meyer et al., 2009). The following antibodies were used: mouse anti-CtBP2 (BD Biosciences), rabbit anti-GluR2/3 (Chemicon), rabbit anti-Ca星空{1.3 (Alomone Labs), mouse anti-GluR2 (Chemcon), mouse anti-Sap7407 to Bassoon (Abcam), and rabbit anti-BSN1.6 to Bassoon (provided by E.D. Gundelfinger).

### Confocal and STED Microscopy

Confocal image stacks were acquired with a Leica SP5 microscope and 100x oil immersion objective. For STED-imaging, two different microscopes were used: the Leica TCS STED (Figures 2D and 2E) and a custom apparatus (Harke et al., 2008) with a resolution of around 80 nm. For size and shape analysis of Ca\(^{2+}\) channel clusters, XY scans were acquired after finding the fluorescence maximum with a XZ-scan.

### Electron Microscopy and Tomography

Cochleae were processed for electron microscopy as described (Meyer et al., 2009 and Pangrsic et al., 2010). Thin sections were examined with a Philips CM120 BioTwin transmission electron microscope (Philips Inc.) with a TemCam F224A camera (TVIPS) at 20,000× magnification. Images were subsequently analyzed with iTEM software (Olympus). Tilt series from 250 nm sections were recorded at 27,500× magnification in the range of 129°, then calculated with Etomo (http://bio3d.colorado.edu).

### Patch-Clamp and Confocal Ca\(^{2+}\) Imaging of IHCs

IHCs from apical coils of freshly dissected organs of Corti (P20 through P31) were patch-clamped as described (Moser and Beutner, 2000) and fluctuation analysis (FA) was performed similarly as previously described (Meyer et al., 2009). Currents were low-pass filtered at 8.5 kHz or 5 kHz and sampled at 100 kHz (FA) or 40 kHz (Ca\(^{2+}\) currents, ΔCm measurements), respectively. Cells with holding current > −50 pA were discarded. Ca\(^{2+}\) currents were further isolated with a P/n protocol. In FA and Ca\(^{2+}\) current activation recordings, series resistance was compensated online (20%–50%; r = 10 μs). Residual series resistance averaged 4.4 ± 0.4 MΩ (Bsn\(^{Ex4/5}\); n = 35 ensembles) and 4.3 ± 0.3 MΩ (Bsn\(^{Ex4/5}\); n = 33 ensembles) in FA experiments. Flash photolysis was performed essentially as described in Beutner et al. (2001). Confocal Ca\(^{2+}\) imaging was performed as described (Frank et al., 2009).

### Single-Unit Recordings

Single-unit recordings from auditory nerve fibers of 6–10–week-old Bsn\(^{wt}\) and Bsn\(^{Ex4/5}\) mice (n = 7 each) were performed as described by Taberner and Liberman (2005) and Buran et al. (2010).

### Data Analysis

Data analysis was performed with Matlab (Mathworks), Igor Pro (Wavemetrix), and ImageJ software and is described in more detail in Supplemental Information. Two-tailed t tests or the Mann-Whitney-Wilcoxon test were used for statistical comparisons between two samples (∗p < 0.05, **p < 0.01, ***p < 0.001).

### SUPPLEMENTAL INFORMATION

Supplemental Information includes Supplemental Experimental Procedures, six figures, two tables, and two movies and can be found with this article online at doi:10.1016/j.neuron.2010.10.027.
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