Age-dependent structural reorganization of utricular ribbon synapses

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Abstract

In mammals, spatial orientation is synaptically-encoded by sensory hair cells of the vestibular labyrinth. Vestibular hair cells (VHCs) harbor synaptic ribbons at their presynaptic active zones (AZs), which play a critical role in molecular scaffolding and facilitate synaptic release and vesicular replenishment. With advancing age, the prevalence of vestibular deficits increases; yet, a direct link to the functional decline of VHC ribbon synapses remains to be demonstrated. To address this issue, we investigated the effects of aging on the ultrastructure of the ribbon-type AZs in murine utricles using various electron microscopic techniques and combined them with confocal and super-resolution light microscopy as well as metabolic imaging up to one year of age. In older animals, we detected predominantly in type I VHCs the formation of floating ribbon clusters. Our findings suggest that VHC ribbon-type AZs undergo dramatic structural alterations upon aging.

Keywords:

Aging/ribbon synapse/synaptogenesis/utricle/vestibular hair cells

Introduction

Sensory receptor cells in the eye and inner ear are equipped with specialized presynaptic organelles – synaptic ribbons – that tether dozens to hundreds of synaptic vesicles (SVs). This system enables indefatigable quantal release of the neurotransmitter glutamate (Matthews and Fuchs, 2010; Moser et al., 2006; Moser et al., 2019; Rutherford and Moser, 2016; Sterling and Matthews, 2005).

The mammalian vestibular system contains multiple sensory organs, the utricle and the saccule for sensing of linear acceleration and gravity, and the ampullae in the semicircular canals for the detection of head rotation (Li et al., 2016). Each of the vestibular organs harbors a sensory epithelium carrying two types of vestibular hair cells (VHCs) (Wersall, 1956). Ribbons decorate the active zones (AZs) of both, calyceal synapses of type I as well as conventional bouton synapses of type II VHCs. Morphologically, vestibular ribbons are highly variable in size, shape and number depending on the species, hair cell type and location (Eatock and Lysakowski, 2006). However, in comparison to auditory and visual ribbon synapses, relatively little is known about the synaptic architecture of vestibular ribbon synapses. Since the decline of vestibular function starts already at the age of 40 (Agrawal et al., 2009; Bermúdez Rey et al., 2016) and vestibular deficits can significantly impair the quality of life of affected patients, detailed longitudinal analyses of VHC ultrastructure upon maturation and aging are essential, but to date surprisingly scarce.

As evident by decreased amplitudes alongside increased latencies and thresholds of ocular vestibular-evoked myogenic potentials (oVEMPs) in humans (Agrawal et al., 2012; Iwasaki et al., 2008; Piker et al., 2011; Rosengren et al., 2011), vestibular function declines with age. This is in line with the observation of increased occurrences of vertigo, recurrent dizziness, nausea, disorientation, blurred

93 vision, imbalance, and increased risk of falling in the elderly (Agrawal et al., 2012;
94 Neuhauser, 2007; Neuhauser et al., 2005). In this context, it remains to be clarified,
95 which cells or subcellular structures undergo age-related deterioration in the utricular
96 system as well as other vestibular organs.

97 As suggested by several studies in rodent cochlear hair cells, ribbon synapse
98 abundance, size and shape are connected to functional changes (Michanski et al.,
99 2019; Peineau et al., 2021; Sobkowicz et al., 1982; Sobkowicz et al., 1986;
100 Stamataki et al., 2006; Wong et al., 2014), as well as the morphology of AZ-proximal
101 mitochondria (Stamataki et al., 2006). Furthermore, ribbon synapse dysfunction or
102 degeneration appear to present major factors underlying age-related hearing loss
103 (Huang et al., 2012; Peineau et al., 2021; Stamataki et al., 2006; Wong et al., 2014).
104 Whether a similar correlation exists in VHCs is largely unknown, but it is tempting to
105 speculate that disruption of the presynaptic energy supply, for example during aging,
106 may negatively influence synaptic performance. Mitochondria are important for
107 energy supply and Ca^{2+} buffering at synapses and thus play a prominent role in
108 neuronal homeostasis (Devine and Kittler, 2018). Moreover, recent work in zebrafish
109 lateral line neuromast hair cells indicated an important role of synaptic mitochondria
110 in ribbon size regulation during development and synaptic function as well as
111 structural integrity upon maturation (Wong et al., 2019).

112 Therefore, we set out to analyze the impact of maturation and aging on the
113 murine utricular VHC synapses by quantifying various aspects of presynaptic AZ
114 structure at ages ranging from 1 week to 11 months and beyond. To do so, we
115 combined transmission electron microscopy (TEM) with nanoscale secondary ion
116 mass spectrometry (NanoSIMS), electron tomography, focused ion beam-scanning

electron microscopy (FIB-SEM) as well as confocal and stimulated emission depletion (STED) microscopy.

We found that, while AZs of both types of VHCs contained ribbons of various shapes and sizes, type I VHCs were dominated by elongated ribbons upon maturation. Moreover, type I VHCs progressively formed complex cytosolic ribbon clusters that often were void of any physical contact to the AZ membrane along with enlarged mitochondria. In contrast to previous reports describing such ribbon clusters in VHCs (Favre et al., 1986; Park et al., 1987; Sans and Scarfone, 1996; Woods and Park, 1987), we now combined a detailed presynaptic morphometric analysis with isotopic labeling to additionally establish ribbon turnover rates in elderly VHCs. This analysis revealed that floating ribbons seem to be newly-synthesized in comparison to attached ribbons, while both ribbon populations appeared much 'younger' than other VHC organelles such as mitochondria. In contrast, type II VHCs showed a relatively stable AZ architecture with comparable ribbon counts and shapes, as well as mitochondrial sizes independent of aging.

In summary, our data suggests that the presynaptic architecture of VHCs – in particular in type I VHCs – retains its ability to undergo active morphological changes in adult stages. Our findings support the hypothesis that new VHC ribbons are continuously formed, but fail to attach at the presynaptic AZ and hence accumulate in its proximity. Forming clusters, they could serve as a reserve pool of ribbons when needed, or alternatively, may represent pathological accumulation of ribbons rendered incapable of attaching at the presynaptic membrane.

Results

143

144 **Upon maturation, the number of membrane-attached synaptic ribbons per**
145 **presynaptic AZ decreases**

146 To investigate maturation and aging of utricular VHC ribbon synapses, we performed
147 a detailed ultrastructural analysis of type I and type II VHCs in mice of five age
148 groups (postnatal day (P)9, P15, P20, 3 months, and 9-11 months). This covered the
149 developmental period of synaptogenesis, through maturation and ultimately to the
150 beginning of a functional decline. Behavioral studies of balance reflexes in rats
151 revealed that the vestibular system is functionally mature around postnatal day (P)16
152 (Hård and Larsson, 1975; Lannou et al., 1979). However, data from detailed
153 physiological and morphological examinations of rodents suggest that VHC synaptic
154 connectivity does not reach a fully mature state until 3-4 weeks of age (Curthoys,
155 1979; Dechesne et al., 1986; Jamon, 2014; Kawamura et al., 1998). In order to
156 distinguish between type I and II VHCs in immature P9 animals (with not yet fully
157 developed calyces), only VHCs with clearly identifiable surrounding calyx or afferent
158 bouton contacts were considered for analysis.

159 We first analyzed AZs of conventionally-embedded utricles that contain
160 ribbons with clear attachments via a presynaptic density (PD) to the AZ membrane.
161 In random 2D ultrathin sections, we determined the abundance, shape, and size of
162 membrane-attached ribbons, analogous to our previous study on cochlear inner hair
163 cells (IHCs) (Michanski et al., 2019). In immature VHCs of both types, we frequently
164 found more than one ribbon per AZ (Fig. 1A-G), whereas, in mature VHCs, typically,
165 a single ribbon was attached at the AZ plasma membrane (Fig. 1A, D). Additionally,
166 we categorized synaptic ribbon shapes (Fig. 1H), since it is well established in
167 auditory IHCs that ribbon morphology changes drastically from rather round shapes
168 prior to hearing onset to elongated and droplet-shaped ribbons in adulthood

169 (Michanski et al., 2019; Sobkowicz et al., 1982; Wong et al., 2014). Interestingly,
170 upon maturation, type I VHCs displayed an apparent increase in the abundance of
171 elongated ribbons (on the expense of droplet, wedge and round-flat ribbons).
172 Conversely, ribbon shape distributions in type II VHCs remained with some variability
173 much more uniform across different ages (Fig. 1H; Table S1). Our 2D TEM data
174 further revealed remarkably stable and largely comparable mean synaptic ribbon
175 areas in the membrane-attached ribbon populations of type I and type II VHCs as
176 well as between the different age groups (for quantification parameters see materials
177 and methods; Fig. S1A). Nevertheless, there was a trend of type I VHC ribbons
178 towards enlarged heights across all age groups in comparison to type II VHC
179 ribbons, which goes along with the observation of higher percentage of elongated
180 ribbons in type I VHCs (Fig. 1I-K; Table S2).

181 To our surprise, in contrast to mature auditory IHCs, we observed several
182 'floating' synaptic ribbons – i.e., ribbons lacking any apparent contact with the
183 plasma membrane – throughout all age groups and in both VHC types (Fig. S1B;
184 Table S3). While no major systematic differences in floating ribbon size could be
185 observed in either VHC type or between age groups, floating ribbons from 3-month-
186 old type I VHCs displayed significantly larger ribbon areas in comparison to type I
187 VHCs in other age groups (Fig. 1L; Table S2).

188

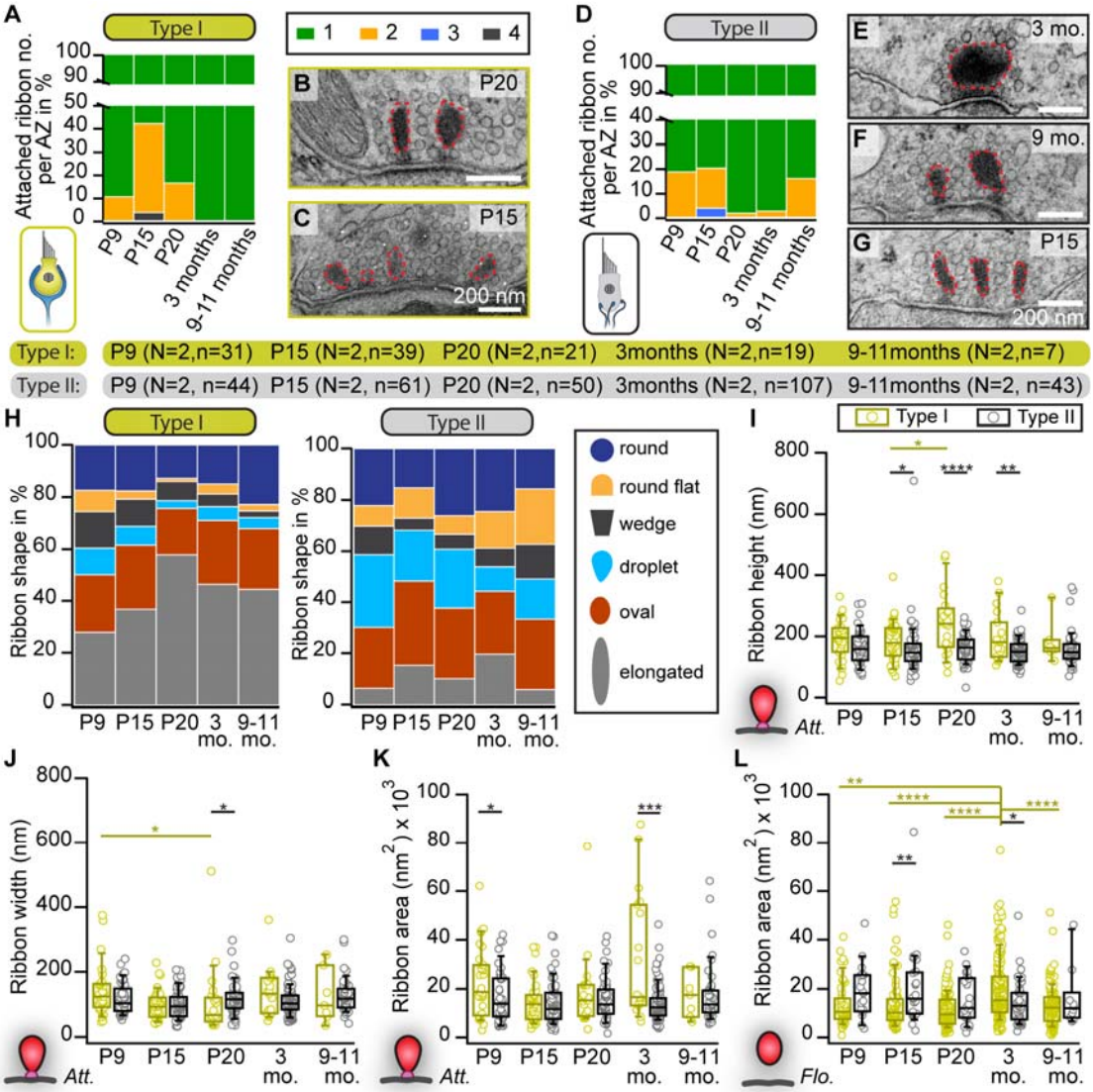

Fig. 1: Changes in the abundance and shape of VHC ribbons upon maturation and aging.

(A, D) Analysis of the number of membrane-attached synaptic ribbons reveals a larger proportion of multiple ribbons per AZ in immature type I (A) and type II (D) VHCs compared to mature ages. (B, C, E-G) Examples of electron micrographs depicting either multiple or single synaptic ribbons (highlighted with red dashed lines) in type I (B, C) and type II (E-G) VHCs. (H) Proportion of ribbon synapses categorized into different types of shapes, respectively for all quantified ages. (I-L) Box plots show random section quantifications of the ribbon size measurements for all age groups. No prominent changes regarding the ribbon area, height, and width could be observed in older animals. N = animal number, n = ribbon number. Significant differences between two groups were analyzed with a two-tailed t-test or Mann–Whitney Wilcoxon test. For multiple comparisons, ANOVA followed by the post-hoc Tukey or Kruskal-Wallis (KW) test followed by NPMC test was performed. For more detailed information see also Table S2.

206

207 **Progressive increase in the number of floating ribbons and ribbon cluster**
208 **formation in type I VHCs**

209 A characteristic feature of immaturity in cochlear IHCs is the presence of round, free-
210 floating ribbon precursors, which attach to the AZ membrane or might fuse with
211 membrane-anchored ribbons during maturation (Michanski et al., 2019; Sobkowicz
212 et al., 1982; Wong et al., 2014). Conversely, floating ribbons appear to persist in
213 VHCs independent of their developmental stage (Fig. 2A, B).

214 To now further characterize these organelles in greater detail in both VHC
215 types, a range of different ultrastructural approaches was employed. In 2D random
216 sections, we observed that, while the fraction of floating ribbons in type I VHCs
217 dramatically increased with age – ultimately constituting ~95% of all AZ-proximal
218 ribbons in the 9-11 months age group – (Fig. 2C; Table S3), the proportion of floating
219 ribbons in type II VHCs remained remarkably stable at ~20-30%. When measuring
220 the nearest distance between these floating ribbons and the plasma membrane, we
221 found appositions predominantly ~200 nm from the AZ membrane, with generally
222 tighter spacing in type II VHCs for almost all age groups (Fig. S1D; Table S8). This
223 finding may be indicative of a progressive age-dependent anchoring deficit –
224 especially in type I VHCs.

225 These floating ribbons then further accumulated into clusters (≥ 2 ribbons)
226 with increasing complexity and often – but not always – resided in direct proximity to
227 membrane-attached ribbons at the AZs of both VHC types. In type I VHCs, such
228 clusters were composed of up to nine ribbons (Fig. 2A, D; Fig. S1C), whereas a
229 maximum of four – but mostly a pair of ribbons per cluster – were observed in type II
230 VHCs analyzed from random sections (Fig. 2B, D; Table S3). In type I VHCs, the

231 ribbon clusters grew continuously from P20 onwards, while a similar trend was not
232 observed in type II VHCs. There, we even detected a decrease of cluster ribbon
233 content at P20 followed by a slight increase at older ages (Fig. 2D). A striking
234 increase in the proportion of single ribbons was observed in type II VHCs, whereas
235 in type I VHCs, synaptic ribbons in clusters clearly dominated at older ages (Fig.
236 S1E; Table S3).

237 Since floating and attached ribbons alike exhibited a translucent core in some
238 cases (Fig. 2A, B and Fig. S1C), we quantified the frequency of occurrence of this
239 phenomenon. This special ribbon morphology has previously been described in
240 several vestibular and cochlear studies, but mostly in adult tissue (Favre and Sans,
241 1979; Liberman, 1980; Michanski et al., 2019; Park et al., 1987; Sjöbäck and Gulley,
242 1979; Sobkowicz et al., 1982; Stamataki et al., 2006). Thus, it has been proposed
243 that this feature might indicate 'old' ribbons prior to their degradation. Evaluation of
244 floating and attached VHC ribbon counts with a translucent core in our study
245 revealed no age-dependent increase in this ribbon feature (Fig. S1F). Therefore, we
246 hypothesize that ribbon size – not structural 'age' – determines the formation of a
247 translucent core.

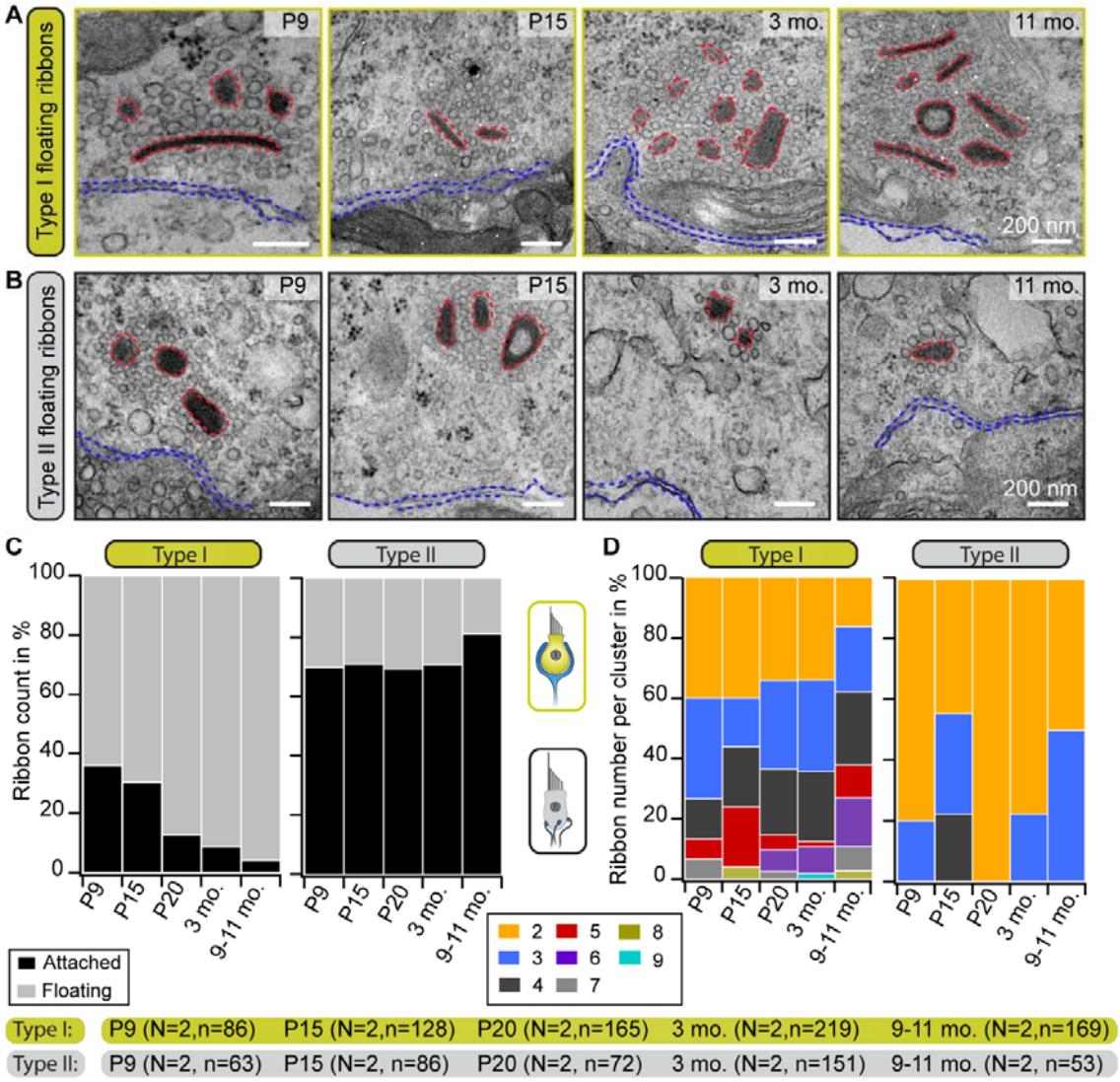

Fig. 2: Progressive accumulation of floating ribbons in type I VHCs.
(A, B) Representative electron micrographs showing single and multiple floating ribbons (red dashed lines) in type I (A) and type II (B) VHCs. All depicted ribbons appear to lack the presynaptic contact to the HC membrane (blue dashed lines). (C) Graphs representing the proportions of floating and membrane-attached ribbons during maturation and aging. Mature type I VHCs possess predominantly floating ribbons, whereas type II VHCs exhibit mainly attached ribbons throughout all investigated ages. (D) Quantification of the total number of ribbons per cluster reveals the occurrence of large ribbon clusters in both VHC types. With advancing age, the ribbon count per cluster increases in type I VHCs but slightly decreases in type II VHCs. N = animal number, n = ribbon number.

Since the nature of 2D-analysis may lead to a systematic under-estimation of ribbon attachment, we next verified the abundance of free-floating ribbons and their accumulation in larger clusters, by performing electron tomography. This 3D approach confirmed the occurrence of ribbon clusters consisting either of exclusively floating ribbons (Movie 1) or floating together with some membrane-attached ribbons (Movie 2), predominantly in type I VHCs vs. mainly attached single ribbons in mature type II VHCs (Movie 3).

Movie 1-3: Electron tomography with 3D segmentation of ribbon cluster and single attached ribbons.
Tomograms and their corresponding 3D models of mature (3 months) type I/II VHCs depicting several disc-shaped floating ribbons in type I VHCs and typical single attached ribbons in type II VHCs. The different SV pools, membrane-proximal (MP)-SVs and ribbon-associated (RA)-SVs, are defined as displayed in Fig. S1A.
Red: ribbon, magenta: presynaptic density, dark blue: postsynaptic density, light blue: HC membrane, yellow: SVs, orange: MP-SVs, green: RA-SVs, brown: SV not associated with the ribbon but part of the cluster "cloud". Scale bars: 200 nm.

Movie 1: https://owncloud.gwdg.de/index.php/s/bRolkq37mZxUCy5
Movie 2: https://owncloud.gwdg.de/index.php/s/DfmaoBIUGUHHJiA
Movie 3 https://owncloud.gwdg.de/index.php/s/u4rJwBpUWlzGJj3

To exclude the possibility that the preparation method of the delicate tissue could have caused putative artifacts leading to a higher frequency of floating ribbons, we applied an alternative sample preparation method. Here, slow fixative perfusion of the inner ear followed by utricle dissection yielded similar results as with our standard preparation method. Moreover, the previously reported age-related progressive genetic hearing loss in C57BL/6J mice (Henry and Chole, 1980; Hequembourg and Liberman, 2001; Ison and Allen, 2003; Johnson et al., 1997; Li and Borg, 1991; Mikaelian et al., 1974; Shnerson and Pujol, 1981; White et al., 2000; Willott, 1986) due to the Cdh23 mutation, did not strongly affect vestibular function and morphology does not seem to be altered (Mock et al., 2016). Nevertheless, to exclude the possibility of mouse background related structural changes in VHCs, we

299 further examined utricles of adult (6-month-old) CBA/J wild-type mice. These mice
300 maintain relatively normal hearing until they reach senescence (Shone et al., 1991;
301 Spongr et al., 1997; Walton et al., 1995; Willott, 1986; Willott et al., 1988a; Willott et
302 al., 1988b). Electron micrographs of random and serial sections from CBA/J mice
303 confirmed an age-dependent increase in the frequency of floating ribbons as well as
304 the presence of complex ribbon clusters in type I VHCs (Fig. S2).
305

306 **Molecular constituents of ribbon clusters**

307 The extremely high percentages of cytoplasmically floating ribbons – especially in
308 type I VHCs (~90% already at P20; Fig. 2C) – led to the question whether floating
309 and membrane-attached ribbons in type I and II VHCs are different in their molecular
310 composition from attached ribbons in other HC types. For example, in cochlear IHCs,
311 it could previously be shown that genetic loss of the large structural scaffold bassoon
312 prevented adequate ribbon anchoring at the AZ membrane (Khimich et al., 2005).
313 Therefore, immunohistochemical stainings of the main ribbon components RIBEYE
314 and piccolino as well as the molecular anchor bassoon were performed in order to
315 determine the localization of these proteins. We detected piccolino on floating
316 ribbons as well as membrane-attached ribbons being part of a ribbon cluster in both
317 VHC types (Fig. 3). A qualitative assessment of the images suggested the presence
318 of clusters in both young and older animals, while some very large clusters were only
319 observed in the older animals. Fig. 3F and L illustrate large ribbon clusters, which
320 were only observed in 32-week-old animals. It is also noteworthy that panel F and
321 the first image in lower panel D (Fig. 3; asterisks) depict ribbons from 32-week-old
322 mice without a nearby bassoon spot, which could indicate that these ribbons lack a
323 membrane-anchor and thus likely represent floating ribbons.

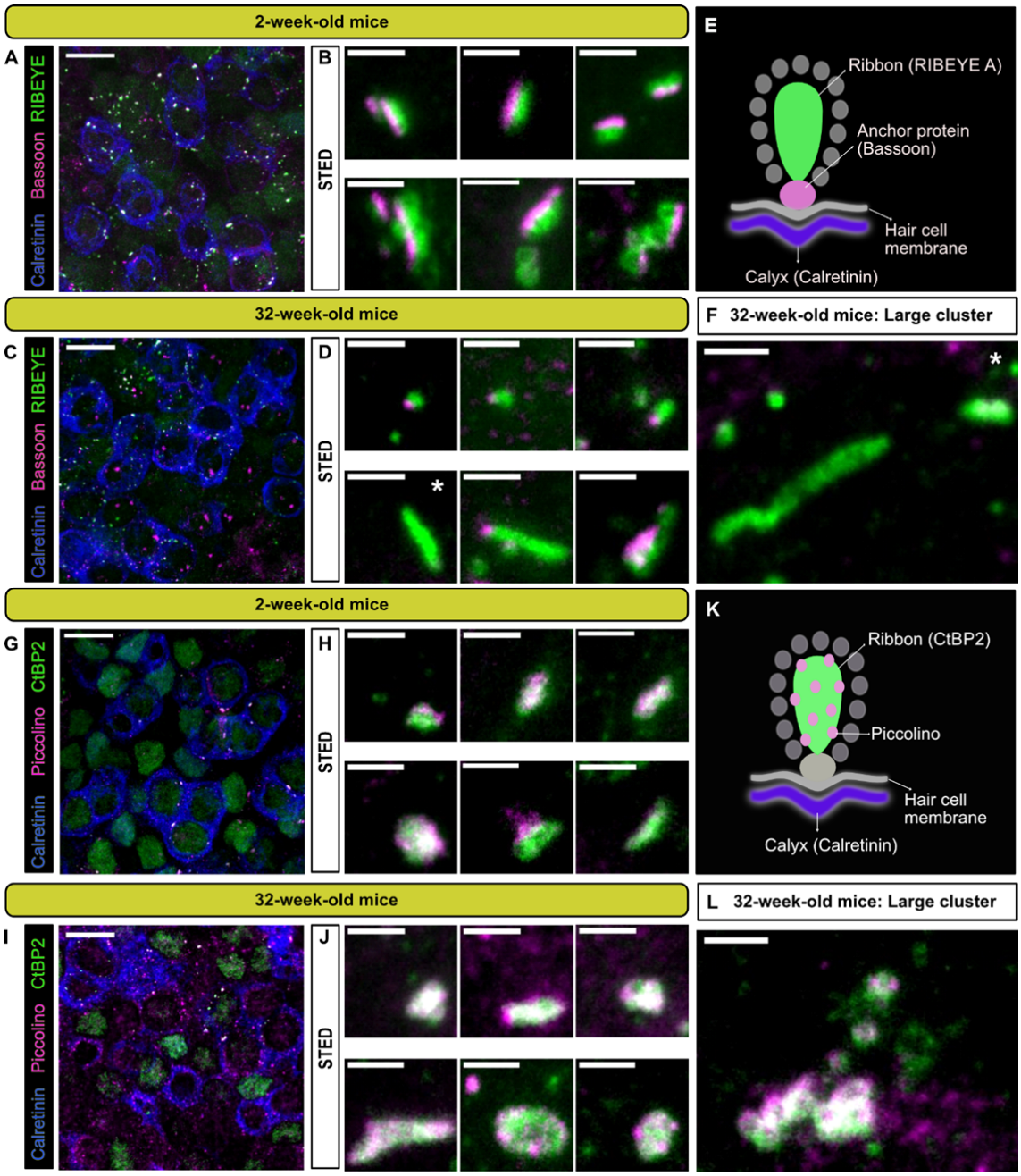

Fig. 3: Localization of the presynaptic AZ proteins bassoon and piccolino at type I VHCs ribbon synapses.

(A,C,G,I) 100x confocal images of the utricular striola, identified here by the presence of calretinin (blue) stained single and complex calyces, with combinations of various synaptic proteins. Ribbon proteins RIBEYE A (A,C) and CtBP2 (G,I) shown in green. Anchoring protein bassoon (B,D) and ribbon-associated protein piccolino (G,I) shown in magenta. Scale bars in A, C, G, and I: 10 µm. (B,D,H,J) Top panels show representative STED images of putative single ribbons or small ribbon clusters and lower panels show images of complex clusters near the type I VHC membranes, with the respective antibody combinations (scale bars for STED images: 1 µm). (E,K) Schematic representation of the antibody combinations used for each panel in young (2 weeks) and old mice (32 weeks). (F

and L) Representative STED images of particularly large ribbon clusters (scale bar: 1 µm). N = 3 animals for each combination. Note that 100x confocal images represent maximal projections of only a few optical planes for better visibility. White asterisks mark the panels depicting ribbons that seemingly lack bassoon (even in the optical planes that were omitted from projections).

The utricle is a remarkably dense tissue, with multiple layers of tightly-packed type I and type II VHCs. Especially for EM sectioning, this poses an intrinsic problem, as distinction between striolar and extrastriolar VHCs – even of the same VHC type – can be immensely difficult, but may influence our results and interpretation. Hence, we decided to perform a basic comparative analysis of ribbon parameters across aging type I VHCs from striolar and extrastriolar regions (Fig. S3). For this purpose, we bred *Ai14-Neurog1-creER*^{T2} knock-in mice (Druckenbrod and Goodrich, 2015; Koundakjian et al., 2007; Madisen et al., 2010) to sparsely express the red fluorescent protein tdTomato in a small subset of vestibular ganglion neurons. This strategy enabled us to trace individual neurons and clearly identify their afferent fibers with VHCs. By combining this with immunohistochemistry, we could then – apart from cellular location in the fixed utricle – either select individual VHCs with calretinin-positive and tdTomato-positive calyces (as indicative for calyx-only fibers in the striola) or calretinin-negative, but tdTomato-positive calyces (representative of the calyceal endings of dimorphic fibers in the extra-striolar region) and subject them to quantitative analysis. Using this approach, we could unambiguously and accurately establish ribbon counts and volumes of individual type I VHCs that were randomly labeled in either the striolar or extrastriolar regions and extended this analysis for three cohorts covering a time period from 2-3 weeks to 1.5 years of age. In line with previous work (Wan et al., 2019) and independent of location, we observed a decline in overall ribbon spots (Fig. S3B, D). Yet, while we found a statistically significant increase in ribbon spot size with advancing age in the striolar

365 region, such effects could not be detected in the extrastriolar region in these
366 experiments (Fig. S3B', D').

367 While this approach offers interesting new insights, it also comes with a range
368 of drawbacks: for example, one has to take into account that due to the limited lateral
369 and axial resolution of confocal microscopy (the latter also applies to 2D-STED),
370 small and dense ribbon clusters – as observed in our 2D TEM data – will appear as
371 one large ribbon in immunofluorescence experiments. Therefore, the true ribbon
372 number per VHC is likely to be underestimated. Even in our STED analysis, large
373 appearing ribbons or clusters might still not be completely resolved as separated
374 entities, and this way might escape correct quantification under confocal microscope.
375 Moreover, due to the very close spacing between floating ribbons and the AZ plasma
376 membrane – predominantly below the diffraction limit of ~200 nm – parameters such
377 as synaptic engagement could not reliably be investigated using this method.

378 Taken together, while all employed 2D techniques revealed a range of novel
379 insights into VHC synaptic morphology and age-dependent structural alterations at
380 VHC AZs, it became clear that a large-scale high-resolution 3D method is required to
381 determine the number of ribbons and ribbon clusters per cell and their true
382 dimensions in 3D.

383
384
385 **3D volume analysis of the basolateral compartment of VHCs reveals larger**
386 **ribbons in mature type I VHCs**

387 In order to investigate ribbon clusters in more contextual detail, we performed
388 focused ion beam – scanning electron microscopy (FIB-SEM) to obtain 3D
389 information of the entire basolateral compartment of individual VHCs. These data
390 could then be used to assess various ultrastructural parameters, including

391 presynaptic ribbons alongside individual SVs and the postsynaptic innervation – at
392 lateral resolutions of 3 or 5 nm and an axial resolution of 5 nm. Thereby,
393 morphological parameters including ribbon number, size and the location of ribbon
394 clusters can be visualized (Fig. 4). Since FIB-SEM is a very complex, expensive and
395 time-consuming method, we focused only on two representative ages: P15 for a
396 maturing age group, and 8-month-old for an adult stage.

397 Consistent with the 2D random section analyses and electron tomography
398 data, 3D segmentations from FIB-SEM volumes revealed the occurrence of ribbon
399 clusters with increased frequencies in type I VHCs (Fig. 4A, B; Movie 4 and 5) –
400 particularly in mature animals. In fact, the clusters were larger than expected from
401 the 2D TEM data or the immunohistochemistry and most ribbons within a cluster
402 were indeed floating in the 8-months age group (Fig. 4C; Table S4). Conversely,
403 type II VHCs exhibited predominantly attached ribbons in both age groups, which
404 might be a result of a clear assignment – floating vs. attached – which is only
405 possible in large 3D reconstructions (Fig. 4C; Table S4; Movie 6 and 7). Moreover,
406 both VHC types showed higher numbers of multi-ribbon (attached and floating
407 together) AZs per synaptic contact site in older ages. The AZs typically contain one
408 to two (in type II VHCs) attached ribbons, which, in particular in old type I VHCs, are
409 often surrounded by a cluster of floating ribbons. As mentioned above, in comparison
410 to our 2D analysis, the ribbon counts per cluster were larger at both investigated
411 ages (Fig. 4C'; Table S4) – with a maximum of 14 ribbons per cluster observed in a
412 type I VHC (Fig. 4D; Table S4). While type II VHCs had on average more ribbon
413 clusters per cell (P15 = 9 clusters, n = 1 VHC; 8 months = 5 clusters, n = 2 VHCs),
414 fewer but more complex ribbon clusters were found in type I VHCs (P15 = 2 clusters,
415 n = 3 VHCs; 8 months = 1.5 clusters per VHC; n = 4 VHCs). The average number of

416 ribbons per cluster rose from P15 with 4.8 to 7.2 at 8 months in type I VHCs. In
417 contrast, the average ribbon number per cluster remained constant in type II VHCs
418 with advancing age (P15 = 2.4 ribbons, 8 months = 2.5 ribbons), largely consistent
419 with our 2D data (Fig. 2). In addition, there are fewer ribbons per cell observed in
420 type I VHCs at both age groups (Table S5) with fewer single membrane-attached
421 ribbons (on average 2 ribbons) in 8-month-old type I VHCs compared to 8-month-old
422 type II VHCs (on average 15 ribbons). For more detailed information about ribbon
423 counts per individual VHC please refer to Table S5, which provides absolute ribbon
424 counts for the different ribbon categories.
425 Quantifications of ribbon volumes revealed an age-dependent increase in individual
426 ribbon volumes as well as the total ribbon volume per VHC (Fig. 4E-G; Table S6).
427 Thus, the FIB-SEM data provide new insights into the differences between type I and
428 II VHCs regarding the dimensions and counts of clusters as well as ribbon volumes.
429 The 2D random section data showed only a slight trend towards larger ribbons in
430 type I VHCs (Fig. 1I), while large volume data enabled accurate quantification of
431 individual ribbon volumes (Fig. 4E-G; Table S6) alongside the number of cluster-
432 associated SVs. In fact, a single cluster could accumulate >1000 SVs in our data
433 sets (Fig. 4H). Moreover, the summed ribbon volume per VHC indicated an age-
434 dependent increase in ribbon material in both VHC types, with type I VHCs
435 containing strikingly more ribbon material than type II VHCs in both age groups (Fig.
436 4G).
437
438 **Movie 4 and 5: FIB-SEM visualizations of type I VHCs with 3D segmentation of single**
439 **ribbons and ribbon clusters.**
440 Movies scanning through the representative FIB-SEM z-stacks of P15 (Movie 4) and 8-
441 month-old (Movie 5) type I VHCs, showing 3D reconstruction of the VHC contours
442 (transparent gray), part of the nuclei (dark gray), synaptic ribbons (red) and their
443 corresponding SVs (yellow). Movie 4 displays two neighboring P15 type I VHCs likely from
444 the striola that are enclosed by one complex calyx.

Movie 4: https://owncloud.gwdg.de/index.php/s/p7cMu7Vn56Eo8IJ
Movie 5: https://owncloud.gwdg.de/index.php/s/3FBCClQn8qRYcpo

Movie 6 and 7: FIB-SEM visualizations of the basolateral compartment of type II VHCs with corresponding 3D segmentations.
Movies scanning through the FIB-SEM z-stacks of P15 (Movie 6) and 8-month-old (Movie 7) type II VHCs. The corresponding 3D models depict VHC contours (transparent gray), part of the nuclei (dark gray), innervating nerve fibers (blue), mainly single attached ribbon synapses (red) and their corresponding SVs (yellow).
Movie 6: https://owncloud.gwdg.de/index.php/s/7oOyQvonWaDtOc5
Movie 7: https://owncloud.gwdg.de/index.php/s/6IlGWtXMvdHVri5

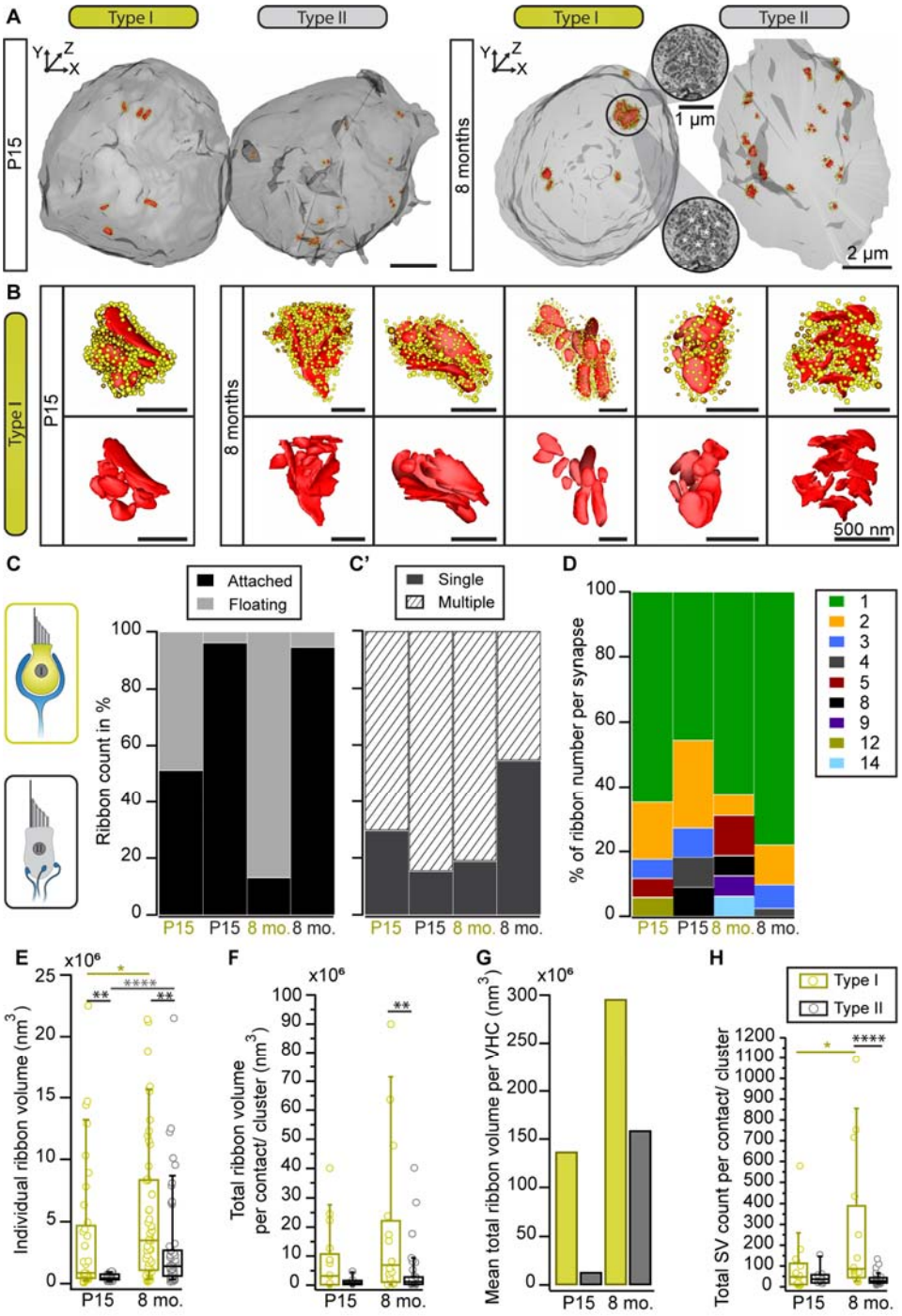

Fig. 4: Ribbon cluster abundance in large volume data.
(A) 3D models of type I and II VHCs from P15 and 8-month-old mice using FIB-SEM (top view). The P15 data set derived from type I VHCs with a complex calyx. The VHC bodies (light gray) contain numerous ribbon synapses (red) tethering SVs (yellow: ribbon-associated, orange: peripheral SVs). (B) Individual ribbon cluster segmentations of immature and mature type I VHCs. Upper row shows ribbon clusters with SVs, lower row displays the respective segmentation without SVs. (C, C') Bar graphs showing the distribution of attached vs. floating and single vs. multiple ribbons per VHC type and age group. (D) Analysis of the total ribbon count per cluster reveals more ribbon numbers in type I VHCs. (E-G) Ribbon size measurement data from both VHC types and age groups (Student's t-test or Mann-

468 Whitney-Wilcoxon test). (H) Boxplot illustrating the quantification of SV numbers per contact
469 or cluster (*P = 0.0178, ****P<0.0001 Mann-Whitney-Wilcoxon test). Note, the high SV count
470 in type I VHCs with >1000 SVs associated with a single cluster. For more detailed
471 information see also Table S6.
472
473
474 **Ribbon clusters may act as SV reservoirs**

475 Our observation that ribbon clusters bind a large reservoir of SVs raised the
476 question, if these SVs can actually still contribute to the SV cycle. The large number
477 of SVs associated with the ribbon clusters – especially in type I VHCs – might either
478 indicate (i) on-demand recruitable SV reservoirs or (ii) SV buffers that hold SVs that
479 were removed from the release cycle (Fig. 4B, H). To examine SVs in more detail,
480 we first analyzed the total amount of SVs within 80 nm distance from the ribbons in
481 our high resolution 2D TEM data set. For attached ribbons, we additionally
482 distinguished between SVs in membrane proximity (MP-SVs: ≤ 25 nm distance from
483 the AZ membrane and ≤ 80 nm from the presynaptic density) and ribbon-associated
484 vesicles (RA-SVs: first row of SVs around the ribbon within 80 nm) (Fig. S4), which
485 represent two well-studied morphological SV pools at auditory ribbon synapses and
486 might give some indications about SV availability (Chakrabarti et al., 2018; Jean et
487 al., 2018; Lenzi et al., 1999; Lenzi et al., 2002; Strenzke et al., 2016). Due to the
488 missing attachment point of floating ribbons to the membrane, the ribbon orientation
489 could not be identified and hence, only the total SV pool was analyzed for floating
490 ribbons.

491 Results from floating and attached ribbons revealed a tendency towards
492 higher numbers of total SV, MP- and RA-SV counts in type I VHCs especially at
493 older ages, similar to the data from FIB-SEM (Fig. 5A, D; Fig. S4A, B; Table S7).
494 However, the SV density of attached and floating ribbons, normalized to the ribbon
495 surface area (Fig. 5B, E), as well as the fraction of MP-SV and RA-SV pools
496 revealed no age-related differences for both VHC types (Fig. S4C, D; Table S7).

Finally, SV diameters were measured since our previous study revealed a decrease in SV diameters upon development of IHC ribbon synapses (Michanski et al., 2019). Interestingly, SV diameters from attached and floating ribbons showed significantly larger SVs in type II VHCs compared to type I VHCs with no age-dependent effects (Fig. 5C, F; Table S8).

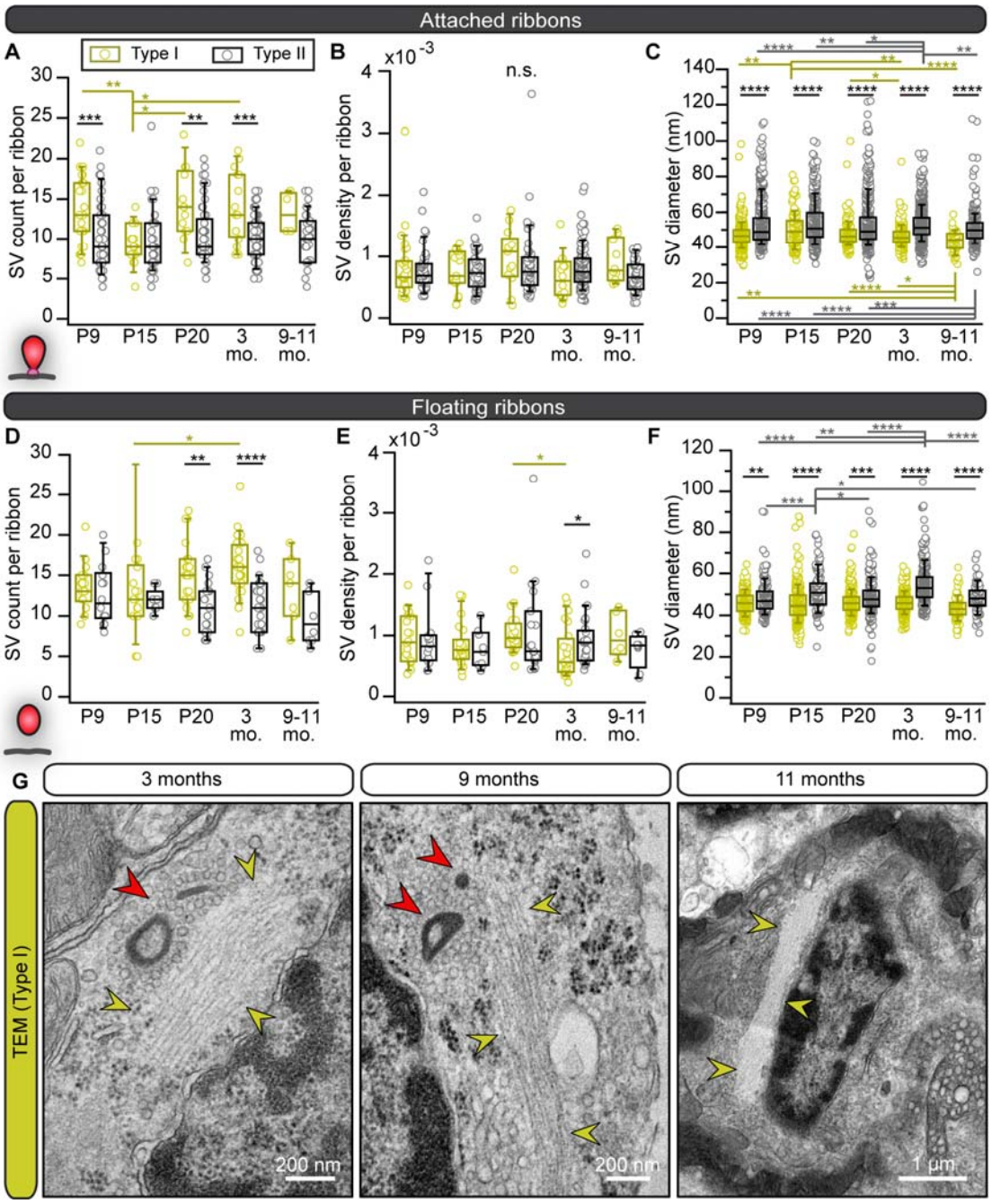

Fig. 5: Smaller SV diameters but larger SV clouds around type I VHC ribbons close to filamentous structures.
(A-F) Boxplots present the data for SV counts, their density and diameter measurements for attached and floating ribbons, respectively. (G) Electron micrographs from 2D TEM data display accumulations of filamentous cytoskeletal structures (yellow arrowheads) in type I VHCs of older ages, which were regularly detected nearby ribbon clusters (red arrowheads) with their corresponding excessive clouds of SVs. For more detailed information see Table S7 and S8.

An extensive apico-basal microfilament network may stabilize aged type I VHCs

Lastly, from ~3 months on, type I VHCs exhibited prominent strands of longitudinal filaments that could represent cytoskeletal elements such as microtubules or actin filaments (Fig. 5G). These strands were partially wrapped around the nucleus in a spiral manner, as segmented in our FIB-SEM data sets (Movie 8, Fig. S4F). About the function of these large cytoskeletal strands, likewise actin, can only be speculated; however, similar structures – previously termed 'microfilaments' – have been described in Heywood et al. (Heywood et al., 1975). If these microfilaments support ribbon (cluster) transport to the AZ remains elusive to date. Yet, in our previous study in cochlear IHCs, we described a potential involvement of microtubules in ribbon transport to the AZ due to close spatial proximity and co-localization of floating ribbon precursors with the microtubule-based motor protein KIF1a (Michanski et al., 2019). In addition, such microfilaments are likely also involved in maintaining cell shape and longitudinal stability.

Movie 8: Filamentous network at the nucleus level can be traced in a partial FIB-SEM z-stack of an 8-month-old type I VHC.
Movie displays a large microfilament network (highlighted with arrow heads) proximal to a ribbon cluster (highlighted by a red circle).
Movie 8: https://owncloud.gwdg.de/index.php/s/W3ZsvG5NWAqbCuM

Mature type I VHCs exhibit larger mitochondria that reside closer to the AZ

535 Ribbons are designed for release sites with large membrane turnover and commonly
536 operate in sensory systems characterized by tonic – probably high-rate – SV
537 release. Continuous synaptic SV cycling upon tonic transmission might require high
538 amounts of metabolic energy, i.e., ATP. Since production of ATP requires adequate
539 mitochondrial function, we next assessed mitochondria abundance and morphology
540 in our 3D FIB-SEM datasets. Indeed, in 8-month-old type I VHCs, the overall
541 mitochondrial volume was increased four-fold as compared to the young controls
542 with some mitochondria reaching sizes up to 100-fold larger than the average
543 mitochondrion in the young type I VHC (volume of 4.29 x 10^7 nm^3; Fig. 6A-E). In
544 contrast, much smaller differences were detected in young vs. aged type II VHCs
545 (Movie 9 and 10). The presence of enlarged mitochondria in type I VHCs could
546 further be observed with TEM already at 3 months of age (Fig. S5).

547 Since mitochondria were suggested to importantly influence presynaptic
548 activity through ATP, Ca^{2+} supply and buffering (Esterberg et al., 2016; Pickett et al.,
549 2018; Wong et al., 2019), their number and distance to ribbons might change during
550 maturation of synaptic machinery and structure. Indeed, our FIB-SEM data suggest a
551 developmental rise of mitochondrial counts nearby synaptically-engaged ribbons in
552 type I VHCs with a slight trend towards a closer proximity that was not observed in
553 type II VHCs (Fig. 6F, G). Here, the opposite was found with fewer mitochondria
554 nearby ribbons in older animals.

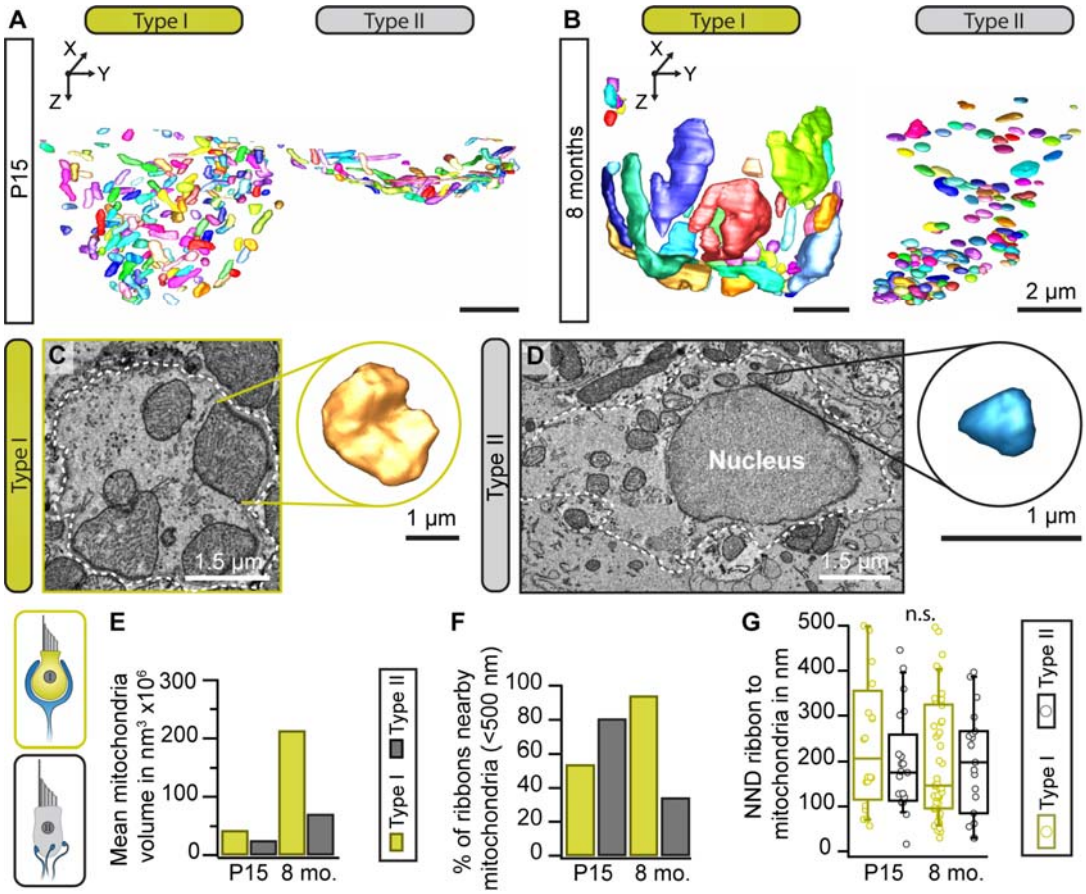

Fig. 6: Enlargement of mitochondrial volume in type I VHCs with advancing age. (A, B) Representative 3D segmentations of mitochondria in type I and II VHCs at indicated ages. (C, D) Single sections from the large FIB-SEM datasets illustrating the mitochondrial size difference between type I and type II VHCs. Insets illustrate 3D reconstruction of the individual mitochondrion. (E) Analysis of the mean mitochondria volume presents notably larger mitochondria in mature type I VHCs. (F) Percentage of mitochondria within <500 nm of the closest ribbon in both age groups and VHC types. (G) Nearest neighbor distance (NND) of mitochondria-proximal ribbons (located <500 nm to the closest mitochondrion), *n.s.* *P*>0.05 (t-test).

Movie 9 and 10: 3D visualization of mitochondria from FIB-SEM z-stacks of P15 and 8-month-old type I and type II VHCs.
Movies show 3D reconstructions of VHC contours (transparent gray), part of the nuclei (dark gray) and mitochondria (randomly colored).
Movie 9 (P15): https://owncloud.gwdg.de/index.php/s/ICNrhNwO27HTgzm
Movie 10 (8 months): https://owncloud.gwdg.de/index.php/s/ZyYt62UINSeGGHv

VHC ribbons show a high turnover

Since ribbon material seems to accumulate specifically in older type I VHCs, the question remains, where the observed floating ribbons originate from. Here, two

577 scenarios can be envisioned: (i) at the end of their lifetime, 'old' ribbons detach (with
578 SVs) for adequate degradation and resorption; however, this process might get
579 corrupted with advancing age resulting in accumulation of ribbons close to the AZ, or
580 (ii) new ribbons are constantly being formed in the cytosol and transported to the
581 AZs; however, once there, the membrane-anchoring at the AZ might fail or become
582 inefficient, thus leading to cluster formation. While given parts of our current data
583 may support either scenario, we next set out to clarify this issue directly via
584 metabolic labeling.

585 For this purpose, we made use of nanoscale secondary ion mass
586 spectrometry (NanoSIMS). In NanoSIMS, a primary Cs^+ beam irradiates the sample
587 and causes the sputtering of secondary particles from the sample surface. These
588 particles are partly ionized and subsequently identified by mass spectrometry. In
589 these experiments, mice were fed for 14 or 21 days with a modified diet, in which
590 every new nitrogen atom is provided as the heavy ^{15}N stable isotope variant that, in
591 concentrations higher than ~0.4% can be unambiguously distinguished from the far
592 more abundant (>99%) naturally-occurring ^{14}N. This approach provides unbiased
593 insights into the composition of the large majority of cellular structures, since not only
594 proteins, but also nucleic acids, lipids (e.g., phospholipids as phosphatidyl-choline, -
595 ethanol amine or –serine, and sphingolipids) and sugars (e.g., N-acetylated glycans)
596 contain nitrogen atoms. Hence, it serves as an excellent approach to identify "old"
597 vs. "new" material in VHCs by calculating the $^{15}N/^{14}N$ ratio. Typically, this approach
598 allows for lateral resolutions ~50-100 nm and axial resolutions of ~5-10 nm. To now
599 relate turnover rate to cellular ultrastructure, we combined NanoSIMS with 110 nm
600 ultrathin sections of conventionally-embedded samples (Fig. 7).

601 Surprisingly, we found that both the attached and floating ribbons contained
602 high levels of ^{15}N. In fact, the majority of the ribbons showed a higher turnover than
603 all other cellular components we analyzed (e.g., cytosol, nuclei or mitochondria; Fig.
604 7C, D). Several aged ribbons could be found exclusively in the membrane-attached
605 population when investigating animals fed with the isotopic diet for 14 days;
606 however, such ribbons were no longer identifiable after 21 days of feeding. Thus, our
607 data suggest that: (i) VHC ribbons exhibit considerable turnover dynamics exceeding
608 the ones observed for other organelles, (ii) different ribbons show different levels of
609 turnover, even in the same cell – with a full exchange taking place every 1-3 weeks
610 and importantly (iii) that floating ribbon clusters might be exclusively composed of
611 newly formed ribbons, therefore pointing to normal formation and transport, but a
612 faulty AZ membrane-attachment mechanism with advancing age.

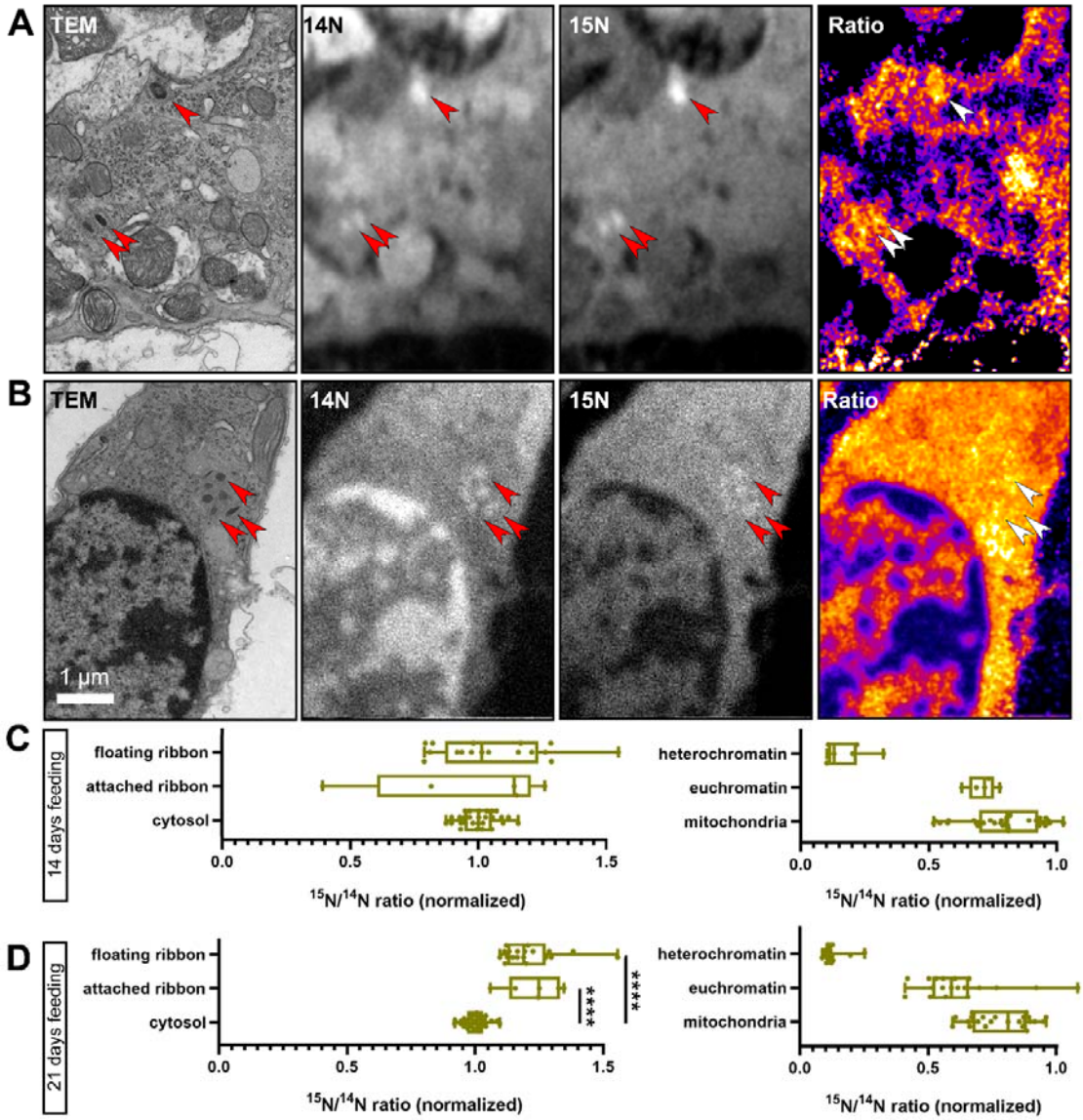

Fig. 7: NanoSIMS analysis of VHC ribbons.

(A-B) To measure protein turnover in VHCs, newly synthesized cellular components were labeled with ^{15}N via a modified diet. 10-14 months old wild-type mice were subjected to this diet for 14 days, before dissecting the VHCs and imaging them first in TEM and then in NanoSIMS. The arrowheads point to several ribbons, with (A) indicating a type II VHC and (B) a type I VHC. In (A) the topmost ribbon is membrane-attached, while the other two are floating. In (B), all six ribbons are floating in the cytosol. The ^{14}N and ^{15}N images indicate that the protein-dense ribbons are easily visualized in NanoSIMS, as bright (nitrogen-intense) spots. The ratio images show the ^{15}N (newly synthesized) signal divided by the ^{14}N (old) signal. The ribbons in (A) are considerably richer in ^{15}N than the ones in (B). (C) The analysis indicates the $^{15}N/^{14}N$ ratios for 13 attached ribbon and 40 floating ribbon measurements, from 15 series of NanoSIMS measurements, from 4 organs. The floating ribbons are substantially more enriched in ^{15}N than mitochondria or heterochromatin (P<0.0001, KW test). The attached ribbons are also significantly more enriched in ^{15}N than heterochromatin (P<0.0001, KW test). (D) A similar analysis for organs from animals fed with ^{15}N for 21 days. The ribbons are now substantially richer in ^{15}N than all other cellular components (P<0.0001; 6 to 25 measurements). The attached ribbons low in ^{15}N (old) have

now disappeared, suggesting that ribbons are replaced on a 1-3 week timeline. $N_{14\ days}$ = 2 animals; $N_{21\ days}$ = 2 animals.

Discussion

The present study describes several morphological alterations in aging utricular VHCs (Fig. 8). Specifically, we found that: (i) both, VHC ribbons and mitochondria (mainly in type I VHCs), undergo drastic changes in shape and increase in size/volume during the aging process. (ii) Ribbons, successively, are found detached from the AZs and these floating ribbons start to accumulate, particularly in type I VHCs, in an age-dependent manner, thereby causing a remarkable increase in the total amount of ribbon material per cell. (iii) These floating ribbons are decorated with full sets of seemingly mature SVs and form clusters within the cytoplasm, often in proximity to the AZ. (iv) Ribbons within these clusters are mainly composed of newly-synthesized proteins. Finally, (v) our data suggests that these 'new' ribbons might collectively fail at the final membrane-anchoring step, potentially due to an age-dependent AZ depletion of the ribbon-anchoring protein bassoon.

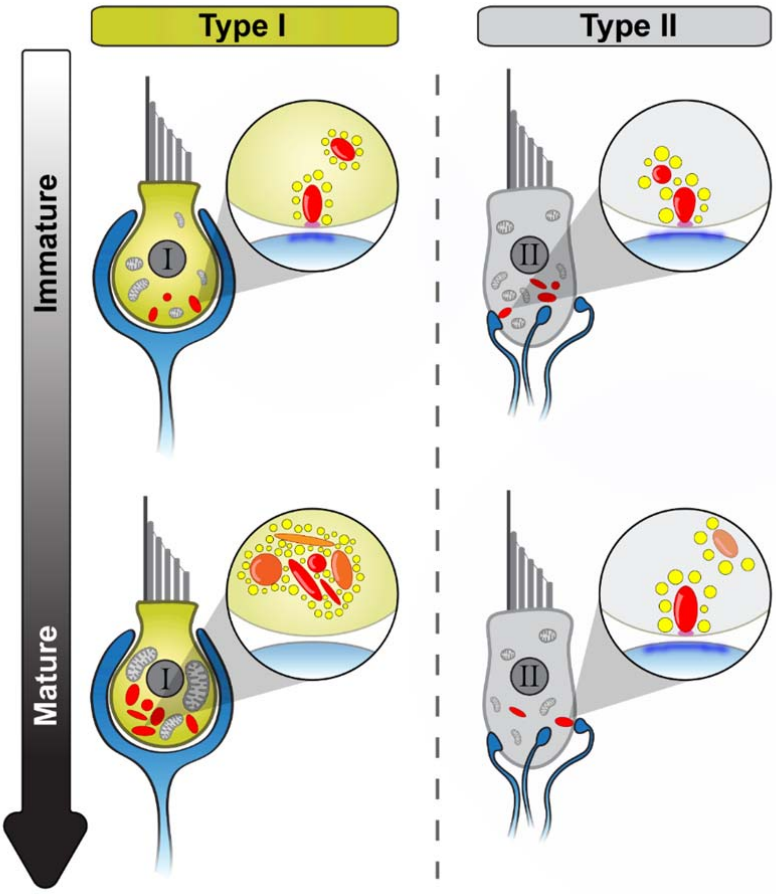

Fig. 8: Schematic overview of utricular VHC ribbon synapse maturation and with advancing age.
Summary of the main morphological observations during utricle development. While type II VHCs show very similar developmental events as detected in cochlear IHCs, type I VHCs undergo a distinct maturation process. Light blue: afferent contacts, red: synaptic ribbons (young/newly formed ribbons: transparent, old ribbons: opaque), yellow: SVs, magenta: presynaptic density, dark blue: PSD, light gray: mitochondria.

VHC ribbon synapses undergo structural alterations in an age- and cell-type-dependent manner

First and foremost, this study reveals striking differences in the ribbons decorating type I and type II VHCs, and even more so when compared to the ribbons in closely-related cochlear IHCs. For example, our most prominent finding is a progressive accumulation of ‘floating’, i.e., not membrane-attached ribbons that, instead of being degraded, form large clusters in type I VHCs. While previous studies already

reported on the occurrence of such cytosolic ribbon aggregates – for example, in VHCs of the murine *crista ampullaris*, ribbon clusters could be observed as early as P2-4 (Nordemar, 1983). Our study provides the first detailed longitudinal analysis of this phenomenon ranging from immature to late adult states.

Our observations in the maturing and older VHCs, approximately one year of age, stand in stark contrast to the developmental maturation of auditory IHCs, in which floating ribbons seem to present a characteristic, but transient, feature of cellular immaturity that disappears prior to hearing onset (Michanski et al., 2019; Sobkowicz et al., 1986; Wong et al., 2014). Consistent with our findings, floating ribbons were previously observed solely in adult type I VHCs, here specifically in female C57BL/6N mice; however, overall still more single ribbons than ribbon clusters were described (Park et al., 1987; Woods and Park, 1987). We assume that the ribbon number per cell and the ribbon number per cluster might have been underestimated due to the limitation of 2D data sets. The majority of estimates in our study, are thus based on the 3D volume reconstructions, which provide more accurate numbers of single ribbons as well as ribbons within clusters. At the age of 8 months, we found an average of approximately 2.5 single ribbons and ~11 ribbons within clusters in type I VHCs. In type II VHCs at the same age 15 ribbons were single and 12.5 organized in clusters. The latter were smaller, containing a maximum of 4 ribbons, whereas up to 14 ribbons were found in a single cluster in type I VHCs. Interestingly, ribbon accumulations and floating ribbons were also detected in type I VHCs from a human fetus (Favre et al., 1986; Sans and Scarfone, 1996), indicating a similar arrangement in humans.

691 **Morphological similarities and differences between type I and type II VHC**
692 **ribbons**

693 While ribbons in both VHC types are of various sizes and shapes, an age-dependent
694 transformation towards elongated ribbons was observed in type I VHCs only. In
695 contrast, ribbon shape distributions in type II VHCs remained much more uniform
696 across ages. Both cell types mostly anchor one to two ribbons per AZ and in both,
697 the number of membrane-attached ribbons per AZ peaks at P15, with a small
698 proportion of AZs involving three or four membrane-attached ribbons, in type II and I
699 VHCs, respectively. Our results in type II VHCs are in line with a previous study in
700 the vestibular end organs of adult guinea pigs showing that the AZs of type II VHCs
701 mainly harbor single synaptic ribbons, while ~20% of afferent terminals are opposed
702 by two or more ribbons (Sjöbäck and Gulley, 1979).

703 A morphological characteristic that seems to be conserved between distinct
704 hair cell types is the presence of translucent core ribbons (Liberman, 1980;
705 Michanski et al., 2019; Sobkowicz et al., 1982; Stamataki et al., 2006). While the
706 previous studies suggested that these 'fenestrated' ribbons represent the final stages
707 in the lifespan of a ribbon, we could not find any age-related differences in their
708 occurrence. This is in line with the work of Park et al. who observed such ribbons in
709 both VHC types in the saccule of young and old mice (Park et al., 1987; Woods and
710 Park, 1987), however, without the knowledge of the actual "ribbon age". Since
711 translucent cores tend to occur in larger ribbons, we hypothesize that such
712 morphological changes may appear due to RIBEYE packaging limitation. Future
713 studies employing NanoSIMS analysis might clarify whether the age of ribbons is a
714 defining factor in the occurrence of this phenomenon.

715

716 **Microfilaments may serve as a means of transport for VHC ribbons**

717 Ribbons were shown to form in the cytoplasm via auto-aggregation of the main
718 structural scaffold protein RIBEYE (Magupalli et al., 2008). These aggregates form
719 SV-tethering ribbon precursors that are then transported to the AZ in a microtubule
720 (MT) and kinesin-dependent process (Michanski et al., 2019). However, VHCs are
721 dominated by the occurrence of large strands of dense microfilaments that align with
722 the apico-basal axis of mature type I VHCs. These filaments were previously
723 described in otocyst VHCs during early development and were interpreted to be
724 involved in nucleus migration (Heywood et al., 1975). While the molecular nature of
725 these microfilaments remains elusive, functionally they might be involved in transport
726 of sub-cellular structures, as they occupy a large apico-basal volume in aged type I
727 VHCs. Moreover, since microfilament bundles were often observed in close proximity
728 to ribbon clusters, it is conceivable that they contribute to ribbon transport towards
729 the AZs. Alternatively, they might solely provide structural stability to VHCs. Future
730 studies will be required to establish the molecular identity and function of these
731 microfilaments.

732

733 **Aging affects mitochondrial volumes of utricular type I VHCs**

734 The production, transport, and maintenance of ribbons, together with continuous
735 tonic synaptic transmission are energy-demanding processes. Age-dependent
736 cellular alterations, which ultimately attenuate release efficiency, may lead to
737 energetic imbalance at the synapse that needs to be compensated. Such a scenario
738 may be supported by our findings of enlarged synaptic mitochondria observed in
739 aging type I VHCs. Mitochondria power the cells by producing ATP (Harris et al.,
740 2012), which is for example required to synthesize glutamate (Waagepetersen et al.,

741 2003), fill SVs with neurotransmitter molecules, prime SVs for release at the AZ
742 membrane (Forgac, 2007; Verhage and Sørensen, 2008; Yao and Bajjalieh, 2008) or
743 fuel local translation (Rossoll and Bassell, 2019). Moreover, an increased production
744 of mitochondrial ATP is required for exocytosis and the endocytosis-based recycling
745 of SVs, which even causes depletion of ATP during heavy cycling (Rangaraju et al.,
746 2014). Therefore, it is not surprising that mitochondria were described to play a key
747 role in quantal synaptic transmission (Smith et al., 2016; Wong et al., 2019). In the
748 present study, we found drastically enlarged mitochondria in older type I VHCs. A
749 similar observation of an performance- and age-dependent mitochondrial volume
750 increase was reported e.g., in the hippocampus (Cserép et al., 2018; Pathak et al.,
751 2015; Smith et al., 2016) and calyx of Held (Thomas et al., 2019), where
752 mitochondria were found to be larger after hearing onset. The size enlargement was
753 interpreted to support higher firing rates in auditory information processing to meet
754 the higher energy demands and, thus, was proposed to be functionally-coupled to
755 the release mode (Thomas et al., 2019). On one hand, since fewer ribbon synapses
756 are attached in older type I VHCs, these ribbons have to work harder to achieve the
757 same task, which might require higher energy. On the other hand, the accumulation
758 of relatively young synaptic ribbons, together with a large number of SVs, also
759 indicates an increased local translation of ribbon material, another energy
760 demanding process (Rossoll and Bassell, 2019). Finally, the mitochondria ultimately
761 supply energy for maintaining ionic homeostasis during continuous depolarization
762 and hyperpolarization of the hair cells, which may be essential for type I VHCs since
763 the supporting cells have no (or limited) access to the synaptic cleft.

764 Notably, in several studies, both vestibular and auditory disorders were
765 associated with mitochondrial dysfunction (Böttger and Schacht, 2013; Fischel-

766 Ghodsian et al., 2004; Iwasaki et al., 2011; Kokotas et al., 2007; Requena et al.,
767 2014), which might also cause enlarged mitochondria.
768
769 **The formation of ribbon clusters increases with age and is a striking hallmark**
770 **of older type I VHCs**
771 With increasing age, the proportion of membrane-attached ribbons steadily declines,
772 however, the prominence of large ribbon clusters, particularly in type I VHCs,
773 successively increases. Here, accumulations of more than 10 ribbons could be
774 shown to incapacitate several hundreds of SVs within a single cluster. While clusters
775 can also be found in type II VHCs, their size typically remains moderate, commonly
776 not exceeding 2-4 ribbons.
777 In our study, both types of VHCs have floating ribbons throughout adulthood, but
778 with different abundance. The proportion of floating ribbons in type II VHCs remains
779 mostly constant throughout the investigated ages (starting from early second
780 postnatal week) and decreases modestly by ~1 year of age, but detached ribbons
781 never fully disappear. It is important to note that in rodents new type II VHCs can be
782 formed throughout adulthood, although the regenerating potential is moderate (Burns
783 and Stone, 2017; Forge et al., 1993; Forge et al., 1998; Golub et al., 2012; Hicks et
784 al., 2020; Kawamoto et al., 2009; Lin et al., 2011; Slowik and Bermingham-
785 McDonogh, 2013). Therefore, we cannot completely rule out the possibility that type
786 II VHCs containing floating ribbons in old animals may represent (at least in part)
787 freshly-regenerated and hence immature hair cells. Future experiments will be
788 required to clarify this issue. Such a scenario is unlikely to take place in type I VHCs.
789 In these cells, floating ribbons represent the vast majority of the total ribbon
790 population in older mice, which additionally argues against these ribbons being a

791 signature of newly regenerated cells. A hallmark of type I VHCs is their use of two
792 modes of synaptic transmission, a classical quantal and a nonconventional non-
793 quantal mode of synaptic transmission (reviewed in (Eatock, 2018; Mukhopadhyay
794 and Pangrsic, 2022)). To what degree one or the other mode of transmission may
795 operate in individual cells, and possibly influence and support each other to enable
796 transmission of vestibular information, is under investigation (Contini et al., 2020;
797 Highstein et al., 2015; Songer and Eatock, 2013; Spaiardi et al., 2022). One might
798 like to speculate that floating ribbons may be a signature of "low" demand for quantal
799 synaptic transmission. If so, the questions to address in the future would be, whether
800 their abundance in cells changes dynamically upon demand or varies in distinct cell
801 subpopulations? While, due to different technical limitations, we could not assess the
802 absolute numbers of floating and attached ribbons in a large number of individual
803 VHCs, we so far found no indication of distinct subpopulations of type I VHCs with
804 different abundance of floating vs. attached ribbons, which remains an interesting
805 question to be revisited in the future.
806

807 **An age-dependent deficit in ribbon membrane-anchoring at the AZ gives rise**
808 **to floating ribbon clusters**

809 The high abundance of floating ribbons in adult VHCs is highly unusual for ribbon-
810 type synapses; however, various scenarios can be envisioned that may explain this
811 phenomenon: (i) in the process of synaptic degeneration, ribbons may detach from
812 the AZ membrane prior to degradation and/or recycling, thus accumulating in the
813 cytoplasm, (ii) the process of formation and attachment of ribbons may be activity-
814 dependent and highly dynamic, with cytosolic clusters in AZ proximity serving as a
815 'reserve pool' of ribbons that may be recruited to, or retracted from, the plasma

816 membrane upon a change in demand, thus rapidly adding or removing substantial
817 amounts of release-ready SVs, or (iii) 'new' ribbons may be formed continuously, but
818 with increasing age, lose the ability to attach adequately at the plasma membrane.

819 The first hypothesis may be supported by a recent study that reported a
820 selective loss of synaptic ribbons in extrastriolar type I VHCs of old mice (Wan et al.,
821 2019). This may possibly be associated with detachment and subsequent
822 degradation of synaptic ribbons, but such a mechanism remains to be
823 unambiguously demonstrated.

824 Regarding the second hypothesis, floating ribbons were found in mice that
825 were brought to space (Ross, 1994; Ross, 2000), suggesting a loss of attached
826 ribbons due to a reduction of synaptic activity in response to hypogravity. However,
827 since floating ribbons as well as ribbon clusters in rodents and humans are also
828 present under normal-gravitational conditions, this explanation appears rather
829 unlikely. Moreover, a significant decline of evoked vestibular potentials could only be
830 detected at an age of two years in mice (Wan et al., 2019). Alternatively, floating
831 ribbons may act as a dynamic reserve pool to supplement existing (or replace end-
832 of-lifetime) ribbons as well as SVs in case of changes in demand. In this context, our
833 NanoSIMS data revealed that some ribbons within a given cluster were slightly older
834 than others and ribbon were newly formed and possibly partially replaced within a
835 time line of 1.3 weeks. However, ribbons were considerably younger compared to
836 other subcellular structures such as mitochondria. The extent of structural plasticity
837 of ribbons is only partly understood to date; yet, in other sensory systems, such as
838 retinal photoreceptors or pinealocytes, ribbons were shown to adapt their shape in
839 response to the cellular state of activity (Fuchs et al., 2013; Spiwoks-Becker et al.,
840 2004; Vollrath and Seidel, 1989; Voorn and Vogl, 2020). Of particular interest here is

841 the observation that photoreceptor ribbons apparently regulate their size by pinching
842 off or fusing with SV-bearing precursor spheres upon changes in illumination
843 (Spiwoks-Becker et al., 2004). As discussed above, the abundance of VHC ribbon
844 synapses may change in hypogravity (Ross, 2000; Sultemeier et al., 2017). If floating
845 ribbons in VHCs also act as a reserve pool for SVs, is questionable. Judging from
846 the low amount of depolarization-evoked membrane capacitance change (reporting
847 SV fusion with the plasma membrane) in young type I cells (Spaiardi et al., 2022;
848 Vincent et al., 2014), it appears unlikely that hundreds of SVs were in place to
849 support very high rates of exocytosis and replenishment.

850 Finally – and in our opinion most likely – the occurrence of ribbon clusters
851 may be a result of an age-dependent degenerative processes that affect stable
852 ribbon anchoring at the AZ membrane. Our NanoSIMS data revealed that floating
853 ribbons in particular have a high protein turnover, thereby arguing in favor of the idea
854 that these ribbons represent the accumulation of 'new' ribbons. In support of this
855 hypothesis, our FIB-SEM data further indicate that throughout the VHC lifespan
856 additional ribbon material is formed and the total amount steadily increases. Either,
857 the protein synthesis of ribbon material is not controlled well enough anymore or, in
858 combination with the apparent reduction of bassoon levels at the AZ, render a
859 scenario most likely in which ribbon synthesis and transport remain intact, but
860 anchoring at the release site fails. Since bassoon is a well-characterized scaffold
861 essential for adequate ribbon attachment to the AZ membrane across different
862 sensory systems (Dick et al., 2003; Khimich et al., 2005), its involvement in this
863 process would be expected. While the exact molecular mechanisms remain to be
864 determined, our work adds important novel insights into VHC ribbon synapse
865 physiology and thus provides new directions for future studies

Materials and Methods

Animals

C57BL/6J, CBA/J and C57BL/6JRj (Janvier laboratory) wild-type mice of either sex between the postnatal day (P)9 and 11 months were deeply anesthetized with CO_2 and sacrificed by decapitation or cervical dislocation for immediate dissection of the utricle. For a subset of experiments, a Cre-dependent tdTomato reporter strain (*Ai14*; #007908 Jackson Laboratory; (Madisen et al., 2010) was crossbred with a *Neurog1*^{CreERT2} mouse line (#008529 Jackson Laboratory; (Koundakjian et al., 2007) to sparsely label random vestibular ganglion neurons. All experiments complied with national animal care guidelines and were approved by the University of Göttingen Board for Animal Welfare and the Animal Welfare Office of the State of Lower Saxony.

Immunohistochemistry, confocal and super-resolution STED microscopy

The inner ears were extracted from C57BL/6J mice at the age of 2 and 32 weeks. Small aperture was created in the bone just above the utricle with forceps. The pigmented membrane was carefully cut open to expose the utricle underneath. The entire inner ear was then transferred to 4% formaldehyde in phosphate buffer saline (PBS: Sigma #P4417, Germany) on ice for about 45 min to allow fixation of the tissues. Next, the sample was washed in deionized water for up to 1 minute and then briefly (ca. 30 s) transferred to TBD-1 (Shandon TBD-1™ Rapid Decalcifier, Ref# 6764001) for decalcification, which was followed by another brief wash in deionized water at room temperature. The fixed and decalcified utricle was then carefully extracted from the inner ear using fine forceps and transferred to PBS.

The utricles were washed 3 times in PBS for 10 minutes each, followed by blocking with Goat Serum Dilution Buffer (GSDB: 16% normal goat serum, 450 mM NaCl,

892 0.3% Triton X-100, and 20 mM phosphate buffer, at pH 7.4) for 1 hour at room
893 temperature in a wet chamber. The organs were then incubated overnight in a
894 primary antibody concoction prepared in GSDB at 4-8 ºC in a wet chamber. The
895 following day the organs were washed 3 times in wash buffer for 10 minutes each
896 and then incubated in a secondary antibody dilution in GSDB for 1 hour in a light
897 protected wet chamber at room temperature. Finally, they were washed again 3 times
898 in wash buffer before being mounted on microscopy slides with mounting medium
899 containing Mowiol 4–88 and DABCO (Carl Roth, Germany) or ProLong Glass
900 antifade mounting medium (ThermoFisher, Germany). The following antibodies and
901 dilutions were used: chicken-anti-calretinin (Synaptic Systems, Germany, dilution
902 1:200), rabbit-anti-piccolino (Synaptic Systems, Germany, dilution 1:200), rabbit-anti-
903 RIBEYE (Synaptic Systems, Germany, dilution 1:200), mouse-anti-bassoon (Abcam,
904 Germany, dilution 1:300), mouse-anti-CtBP2 (BD Biosciences, USA, dilution 1:200)
905 and rabbit anti-dsRed (Clontech, dilution 1:200) as well as a directly Atto-565
906 conjugated anti-RFP nanobody (NanoTag, dilution 1:100). The secondary antibodies
907 used were: Alexafluor488-labeled goat-anti-chicken (ThermoFisher Scientific, USA,
908 dilution 1:200), Alexafluor594 labeled goat-anti-mouse (ThermoFisher Scientific,
909 USA, dilution 1:200) or STAR635p-labeled cameloid anti-mouse IgG1 nanobodies
910 (NanoTag, dilution 1:100), as well as STAR580-labeled cameloid anti-rabbit
911 nanobodies (NanoTag, dilution 1:100) or STAR635p-labeled goat-anti-rabbit
912 (Abberior, Germany, dilution 1:200). Confocal and high resolution 2D-STED images
913 were acquired using an Abberior Instruments Expert Line STED microscope
914 equipped with 488, 561 and 640 nm excitation lasers, STED laser at 775 nm (1.2 W),
915 and a 100x oil immersion objective (1.4 NA, Olympus). Images were acquired using
916 the same microscope settings, analysed and processed in Fiji/ImageJ software

917 (Schindelin et al., 2012) or Bitplane Imaris 9.6.1 (Oxford Instruments), and
918 assembled in Affinity Designer 1.10.5 for display.
919
920

921 **Conventional embedding and transmission electron microscopy**

922 Utricle organs from mice ranging in age between P9 to 11 months were isolated in
923 HEPES Hank´s solution, which contained (in mM): 5.36 KCl (746436, Sigma,
924 Germany), 141.7 NaCl (746398, Sigma, Germany), 10 HEPES (H3375, 006K5424,
925 Sigma, Germany), 0.5 $MgSO_4$-$7H_2O$ (Sigma, Germany), 1 $MgCl_2$ (M2670, Sigma,
926 Germany), 2 mg/ml D-glucose (G8270-1KG, Sigma, Germany), and 0.5 mg/ml L-
927 glutamine (G3126-100G, #SLBS8600, Sigma, Germany) and was adjusted to pH 7.2,
928 ~300 mmol/kg. First the inner ear was dissected and the vestibular bone tissue was
929 carefully removed. The inner ear was afterwards placed into a petri dish filled with
930 proteinase XXIV solution (P 8038-100MG, Th. Geyer, Germany; 50 mg/ml in HEPES-
931 HANKS solution, w/v) for 5 min at room temperature to facilitate the removal of the
932 otolithic membrane overlying hair bundles of the utricle. In fresh HEPES Hank's
933 solution, the otolithic membrane was then removed and the exposed utricle was
934 excised.
935

936 Conventional embeddings of vestibular organs were performed according to our
937 previous studies (Michanski et al., 2019; Strenzke et al., 2016; Wong et al., 2014). In
938 brief, the organs were fixed immediately after dissection with 4% paraformaldehyde
939 (0335.1, Carl Roth, Germany) and 0.5% glutaraldehyde (G7651, Sigma, Germany) in
940 PBS (P4417, Sigma, Germany; pH 7.4) for 1 h on ice followed by a second fixation
941 step overnight with 2% glutaraldehyde in 0.1 M sodium cacodylate buffer (v/v, pH
942 7.2) at 4°C. Next, specimens were washed in 0.1 M sodium cacodylate buffer and

943 treated with 1% osmium tetroxide (75632.5ml, Sigma, Germany; v/v in 0.1 M sodium
944 cacodylate buffer) for 1 h on ice followed by further sodium cacodylate buffer and
945 distilled water washing steps. After the *en bloc* staining with 1% uranyl acetate (8473,
946 Merck, Germany; v/v in distilled water) for 1 h on ice, samples were briefly washed in
947 distilled water, dehydrated in an ascending concentration series of ethanol, infiltrated
948 and embedded in epoxy resin (R1140, AGAR-100, Plano, Germany) to get finally
949 polymerized for 48 h at 70°C. Subsequently, ultrathin sections (70-75 nm) from the
950 cured resin blocks were cut with an Ultracut E microtome (Leica Microsystems,
951 Germany) or an UC7 microtome (Leica Microsystems, Germany) equipped with a 35°
952 diamond knife (Diatome AG, Biel, Switzerland) and mounted on 1% formvar-coated
953 (w/v in water-free chloroform) copper slot grids (3.05 mm Ø, 1 mm x 2 mm; Plano,
954 Germany). Before imaging these sections at 80 kV using a JEM1011 transmission
955 electron microscope (JEOL, Japan), they were counterstained with uranyl acetate
956 and Reynold's lead citrate (Reynolds, 1963) or uranyl acetate replacement solution
957 (EMS, Science Services, USA). Micrographs of both hair cell types from the striolar
958 and extrastriolar region were acquired at 10,000-x magnification with a Gatan Orius
959 1200A camera (Gatan GmbH, using the Digital Micrograph software package, USA).
960

961 **Electron tomography**

962 Electron tomography was utilized as described previously in Strenzke *et al*. (Strenzke
963 et al., 2016). Semithin sections (250 nm) were cut from conventionally embedded
964 vestibular organs on an Ultracut E or an UC7 ultramicrotome (Leica Microsystems,
965 Germany) with a 35° diamond knife (Diatome AG, Biel, Switzerland). Sections were
966 placed on 1% formvar-coated (w/v in water-free chloroform) copper 100 mesh grids
967 (3.05 mm Ø; Plano, Germany) and counterstained as described above. 10 nm gold
968 beads (British Bio Cell, UK) were applied to both sides of the grids functioning as

969 fiducial markers. With the Serial-EM software (Mastronarde, 2005), tilt series image
970 acquisition was conducted mainly from -60 to +60° with 1° increments at 10,000-x
971 magnification and a pixel size of 1.43 nm using a JEM2100 (JEOL, Japan)
972 transmission electron microscope at 200 kV. For final tomogram alignments, the
973 IMOD software package etomo was used and tomographic reconstructions were
974 generated using 3dmod (Kremer et al., 1996). Movies were generated with IMOD, Fiji
975 (Schindelin et al., 2012) and Windows Movie Maker 2012.
976
977 **Focused ion beam (FIB)-scanning electron microscopy (SEM)**
978 Enhanced *en bloc* staining for FIB-SEM samples was performed according to
979 Deerinck *et al.* (Deerinck et al., 2018). After fixation (similar to the above-described
980 conventional embedding protocol), utricular organs were treated with a 1.5%
981 potassium ferrocyanide (EMS, USA) and 4% osmium tetroxide solution (v/v in 0.1 M
982 sodium cacodylate buffer) for 1 h on ice. Next, specimens were briefly washed in
983 distilled water and placed in a thiocarbohydrazide (w/v in distilled water) solution for
984 20 min followed by additional washing steps in distilled water. A second exposure to
985 2% osmium tetroxide (v/v in 0.1 M sodium cacodylate buffer) was conducted followed
986 by brief washing steps in distilled water before the samples were placed in 2.5%
987 uranyl acetate (v/v in distilled water) overnight at dark. Subsequently, samples were
988 washed in distilled water and contrasted with Reynold's lead citrate (Reynolds, 1963)
989 for 30 min at 60°C to be finally washed once again in distilled water, dehydrated in
990 increasing ethanol concentrations, infiltrated and embedded in Durcupan (25%, 50%,
991 75% Durcupan in acetone for 1 h each and 100% Durcupan overnight; 44610,
992 Sigma, Germany) to get polymerized for 48 h at 60°C. After trimming the cured
993 blocks with a 90° diamond trimming knife (Diatome AG, Biel, Switzerland), the blocks
994 were attached to SEM stubs (Science Services GmbH, Pin 12.7 mm x 3.1 mm) with a

995 silver filled epoxy (Epoxy Conductive Adhesive, EPO-TEK EE 129-4; EMS, USA) and
996 polymerized at 60° overnight. Samples were coated with a 10 nm platinum layer
997 using the sputter coating machine EM ACE600 (Leica, Germany) at 30 mA current to
998 be finally placed into the Crossbeam 540 focused ion beam scanning electron
999 microscope (Carl Zeiss Microscopy GmbH, Germany) and positioned at an angle of
1000 54°. A 400 nm platinum layer was deposited on top of the regions of interest and the
1001 Atlas 3D (Atlas 5.1, Fibics, Canada) software was used to collect the 3D data.
1002 Specimens were exposed to the ion beam driven with a 15 or 30 nA current while a 7
1003 or 15 nA current was applied to polish the cross-section. Images from both hair cell
1004 types of the striolar and extrastriolar region were acquired at 1.5 kV using the ESB
1005 detector (450 or 1500 V ESB grid, pixel size x/y 3 or 5 nm) in a continuous mill and
1006 acquire mode using 700 pA or 1.5 nA for the milling aperture (z-step 5 nm). For
1007 subsequent post processing, data were aligned using the Plugin "Linear Stack
1008 Alignment with SIFT", inverted and cropped in Fiji. Depending on the dataset
1009 properties, a smoothing function (3x3), local contrast enhancement using a CLAHE
1010 plugin in Fiji, and a binning by 2 in x/y was applied (Schindelin et al., 2012). Movies
1011 were generated with IMOD (Kremer et al., 1996), Fiji, Blender (www.blender.org) and
1012 Windows Movie Maker 2012.
1013

1014 **Nanoscale secondary ion mass spectrometry (NanoSIMS)**

1015 Mice were habituated to unlabeled ^{14}N-SILAM diet for 1 week before labeling
1016 (Silantes, Germany; cat. Num. 231,004,650). 10 and 14 months old C57BL/6JRj mice
1017 were then labeled with the ^{15}N-SILAM diet (Silantes, Germany; cat. Num.
1018 231,304,650), for 14 or 21 days before the inner ear perfusion with a 4%
1019 paraformaldehyde (0335.1, Carl Roth, Germany) and 0.5% glutaraldehyde (G7651,
1020 Sigma, Germany) in PBS (P4417, Sigma, Germany; pH 7.4) fixative for 1 h on ice.

1021 Afterwards, inner ears were placed in a second fixative solution overnight at 4°C (2%
1022 glutaraldehyde in 0.1 M sodium cacodylate buffer (v/v, pH 7.2)). On the second day
1023 fixed utricular organs were excised and the conventional embedding protocol was
1024 performed as described earlier in detail. The epoxy resin (R1140, AGAR-100, Plano,
1025 Germany) embedded samples were, similar to other conventional embedded
1026 specimens, further processed for transmission electron microscopy. 110 nm sections
1027 were cut using a 35° diamond knife (Diatome AG, Biel, Switzerland) with an UC7
1028 ultramicrotome (Leica Microsystems, Germany) and placed on 200-mesh copper
1029 finder grids (01910-F, Ted Pella). Regions of interest (ROIs) were selected with a
1030 JEM1011 (JEOL, Japan) at 80 kV using different magnifications (x80, x150x, x800,
1031 x2,500, x6,000, x8,000). Subsequently, NanoSIMS imaging was performed. The
1032 areas of interest, previously imaged by TEM, were imaged with a NanoSIMS 50 L
1033 (Cameca, Gennevilliers Cedex, France) using an 8 kV Cs^{+133} primary ion source. To
1034 reach the steady-state of the secondary ion yield, prior to each measurement, an
1035 area of 100x100 µm was implanted applying a current of 50 pA for ~3 min (primary
1036 aperture D1:1). Subsequently, a primary ion current of ~0.25 pA (primary aperture
1037 D1:5) was applied to obtain images of 256 x 256 pixels from areas of 10 x 10 or 5 x
1038 5 µm, resulting in a pixel size of 39.06 or 19.53 nm respectively. The dwell time was
1039 5 ms per pixel and three consecutive layers were accumulated for each final image.
1040 The detectors were set to collect the following ions: $^{12}C^{14}N^{-}$, $^{12}C^{15}N^{-}$, $^{31}P^{-}$ and $^{32}S^{-}$,
1041 and the mass resolving power of the instrument was adjusted to ensure the
1042 discrimination between isobaric interferences such as $^{12}C^{15}N^{-}$ from $^{13}C^{14}N^{-}$,
1043 or $^{12}C^{14}N^{-}$ from $^{12}C_2{}^{1}H_2{}^{-}$ by using an entrance slit of 20x140µm and an aperture slit
1044 of 200 x 200 µm. The images were then exported and processed using WinImage
1045 (Cameca, Gennevilliers Cedex, France). The following masses were collected for
1046 each run: $^{12}C^{14}N$ (referred to as ^{14}N in this report), $^{12}C^{15}N$ (referred to as ^{15}N in this

1047 report), and ^{31}P. ^{31}P peak was used to mark the location of cellular structures. Each
1048 image shown in this manuscript is the result of a summation of all three image layers
1049 taken during analysis.
1050

1051 **Data analysis and statistics**

1052 Quantitative analysis of electron microscopic random 2D sections was performed
1053 with ImageJ/Fiji (Schindelin et al., 2012) as follows:
1054 In order to quantify the occurrence of floating ribbons, we defined “floating ribbons”
1055 by a lack of any physical contact to the presynaptic density, while an “attached
1056 ribbon” exhibited a clear membrane anchorage via a presynaptic density. For the
1057 ribbon size of attached ribbon synapses, the height and width were measured taking
1058 the longest axis of the ribbon excluding the presynaptic density as well as manual
1059 tracing of the synaptic ribbon was performed in order to determine the ribbon area. In
1060 contrast, due to the lack of an attachment of floating ribbons to the membrane, no
1061 precise ribbon orientation could be identified and hence the size of floating ribbons
1062 could only be analyzed by measuring the ribbon area.
1063 For SVs of attached and floating ribbons, first the total amount of vesicles ≤ 80 nm
1064 from the ribbon surface were quantified in number and size. The latter analysis
1065 included the horizontal and vertical axis measurements, which were averaged to
1066 calculate the mean SV diameter. Next, the SV density was calculated by dividing the
1067 total number of SVs by the ribbon surface area.
1068 Exclusively for attached ribbon synapses, two distinct morphological vesicle pools
1069 were additionally quantified in terms of numbers (as previously characterized in
1070 (Strenzke et al., 2016)): (i) membrane-proximal synaptic vesicles (MP-SVs, ≤ 25 nm
1071 distance between SV membrane and AZ membrane and ≤ 80 nm from the
1072 presynaptic density); and (ii) ribbon-associated synaptic vesicles (RA-SVs, first layer

1073 of vesicles around the ribbon with a maximum distance of 80 nm from the ribbon
1074 surface to the vesicle membrane and not falling into the MP-SV pool).
1075
1076 Quantitative analysis of FIB-SEM data
1077 FIB-SEM datasets were segmented semi-automatically using the 3dmod package
1078 from the IMOD software (Kremer et al., 1996) and applying the imodinfo function,
1079 information about the ribbon and mitochondria volume was given. Distance
1080 measurements were performed with the measurement drawing tool along the x, y
1081 and z-axis.
1082
1083 Quantitative analysis of NanoSIMS data
1084 NanoSIMS images were analyzed using a custom-written Matlab macro (the
1085 Mathworks Inc., Natick, MA, USA). NanoSIMS images were overlaid with the EM
1086 images, using Adobe Photoshop CS6 (Adobe Systems Incorporated). ROIs were
1087 selected manually on the EM images, and the ^{15}N and ^{14}N counts were measured for
1088 all ROIs in the respective NanoSIMS images. The results were saved as text files
1089 and were then combined to provide the statistics shown in the NanoSIMS figure.
1090
1091 All data are mainly presented as boxplots with individual data points overlaid and as
1092 bar graphs highlighting the mean or percentages of the data, which were analyzed
1093 using Excel, Igor Pro 6 (Wavemetrics Inc., USA), R and Java, if not otherwise
1094 indicated. Normality was assessed with the Jarque-Bera test and equality of
1095 variances in normally distributed data was assessed with the F-test. In order to
1096 compare two samples, the two-tailed unpaired Student's t-test, or, when data were
1097 not normally distributed and/or variance was unequal between samples, the unpaired
1098 two-tailed Mann-Whitney-Wilcoxon test was used. One-way ANOVA test (followed by

Fischer post-hoc tests (NanoSIMS data)) followed by Tukey's test was used to calculate statistical significance in multiple comparisons for normally distributed data or in case of non-normally distributed data the Kruskal-Wallis (KW) test followed by non-parametric multiple comparisons test (NPMC) was used.

Employing the analysis tool based on Java Statistical Classes library (JSC) (Bertie, 2002) applied in our previous study (Jean et al., 2018), we performed the KW test for the identification of significant differences between SV diameters. Non-significant differences between samples are indicated as *n.s.*, significant differences are indicated as $*P < 0.05$, $**P < 0.01$, $***P < 0.001$, $****P < 0.0001$. Affinity Designer 1.10.5 and Adobe Illustrator 2023 (Adobe Inc., USA) were utilized for Figure assembling and display.

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Author Contributions:

S.M. and C.W. designed the study. S.M. performed electron microscopic work (conventional embeddings, enhanced en bloc stainings, TEM of random sections, electron tomography, FIB-SEM). T.H. contributed to 2D data analysis and acquisition. T.P., M.M. and C.V. performed immunohistochemistry as well as confocal microscopy and STED. S.M., M.G., T.H., M.M. and C.V. analyzed data. A.M.S. performed and W.M. supervised FIB-SEM. M.G. contributed to statistical analysis. P.I. made the Blender movies. S.J. and P.I. contributed to mitochondria data interpretation. E.F.F. prepared mice for the NanoSIMS experiments. S.M. and E.F.F. prepared tissue samples for NanoSIMS. C.W. performed TEM image acquisitions for NanoSIMS. P.A.G. and K.G. performed NanoSIMS and S.O.R. and P.A.G. analyzed NanoSIMS data. S.M. and C.W. prepared the manuscript with contributions from all other authors.

Conflict of interest

The authors declare no conflict of interest.

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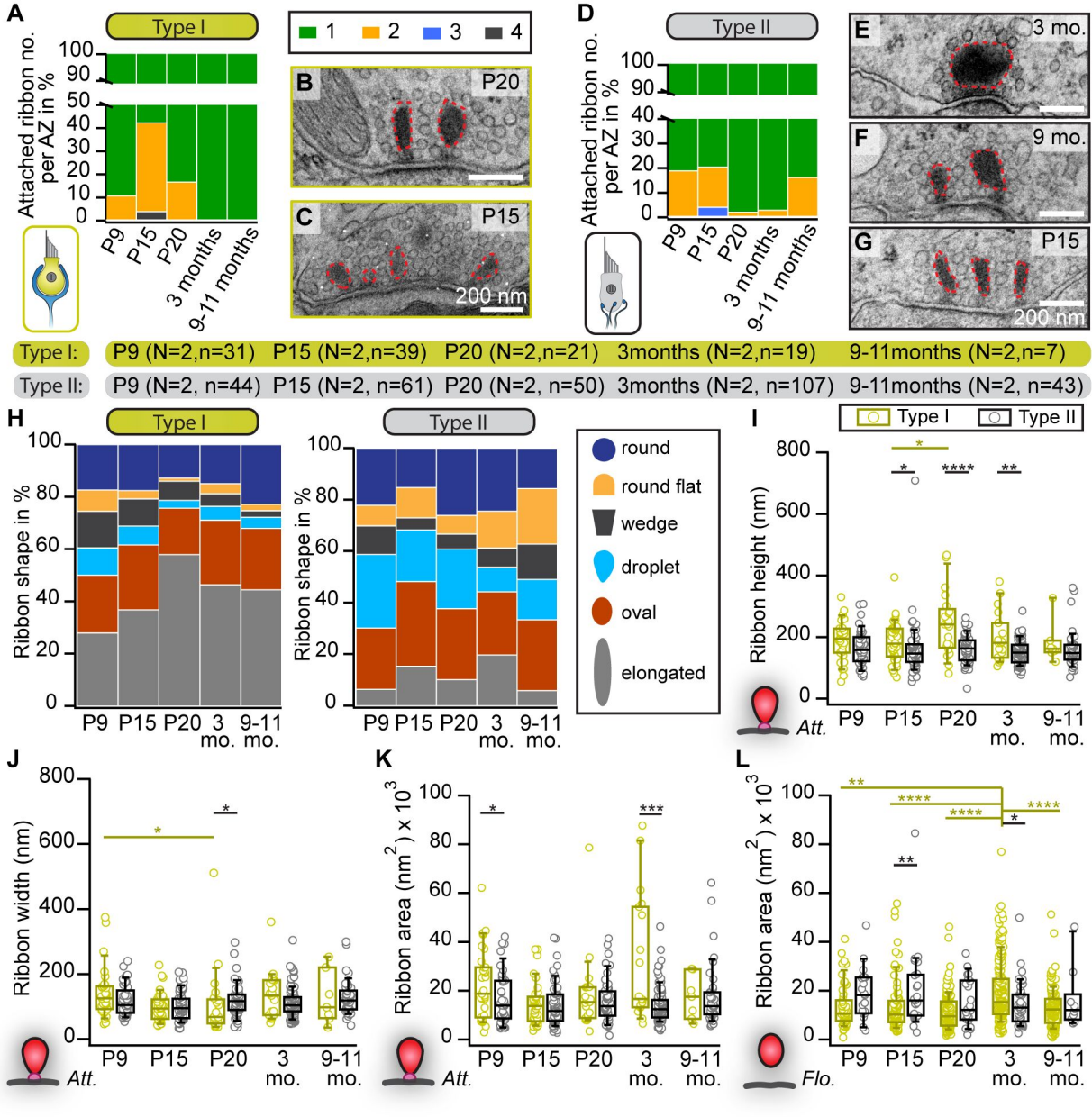
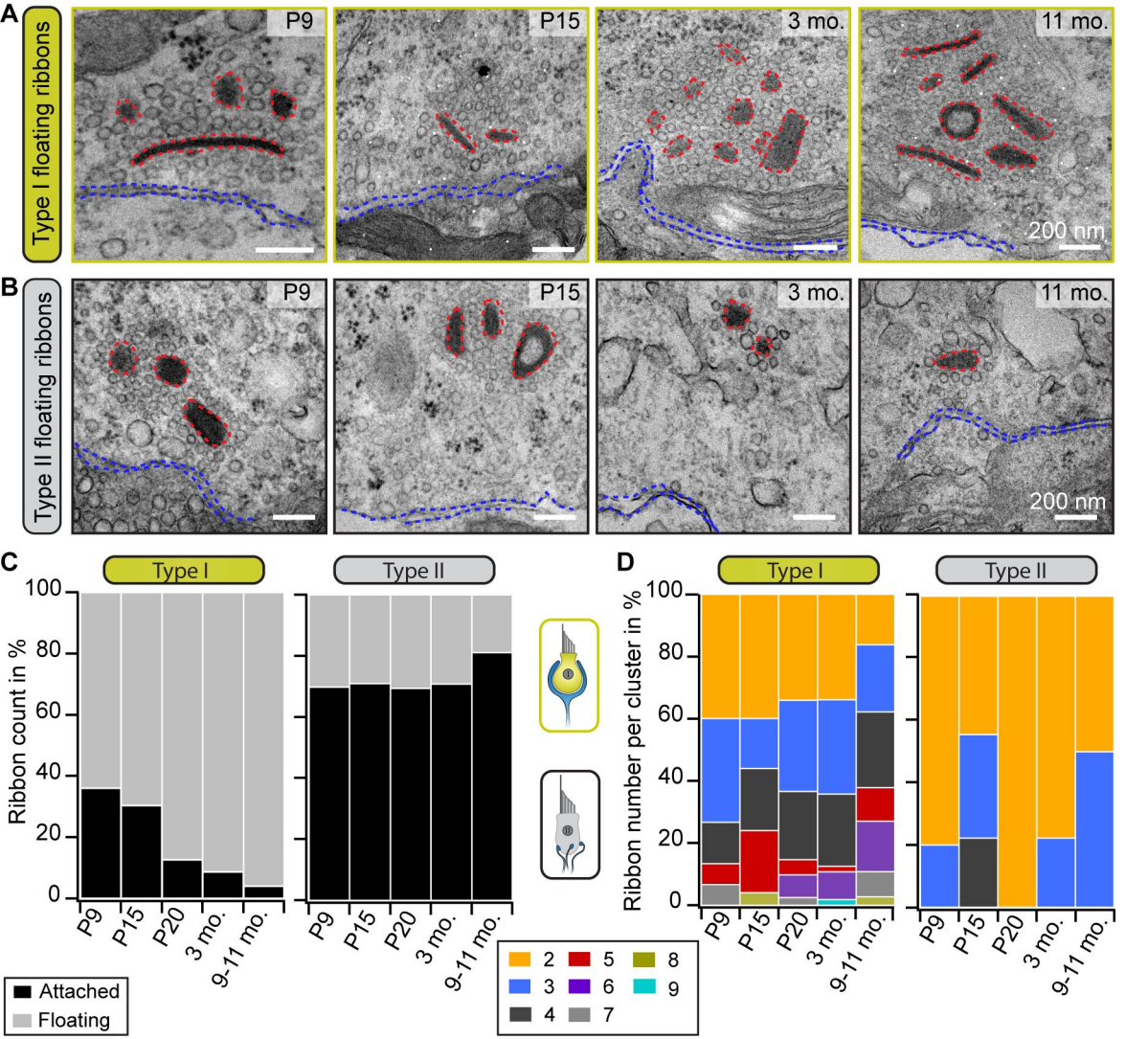
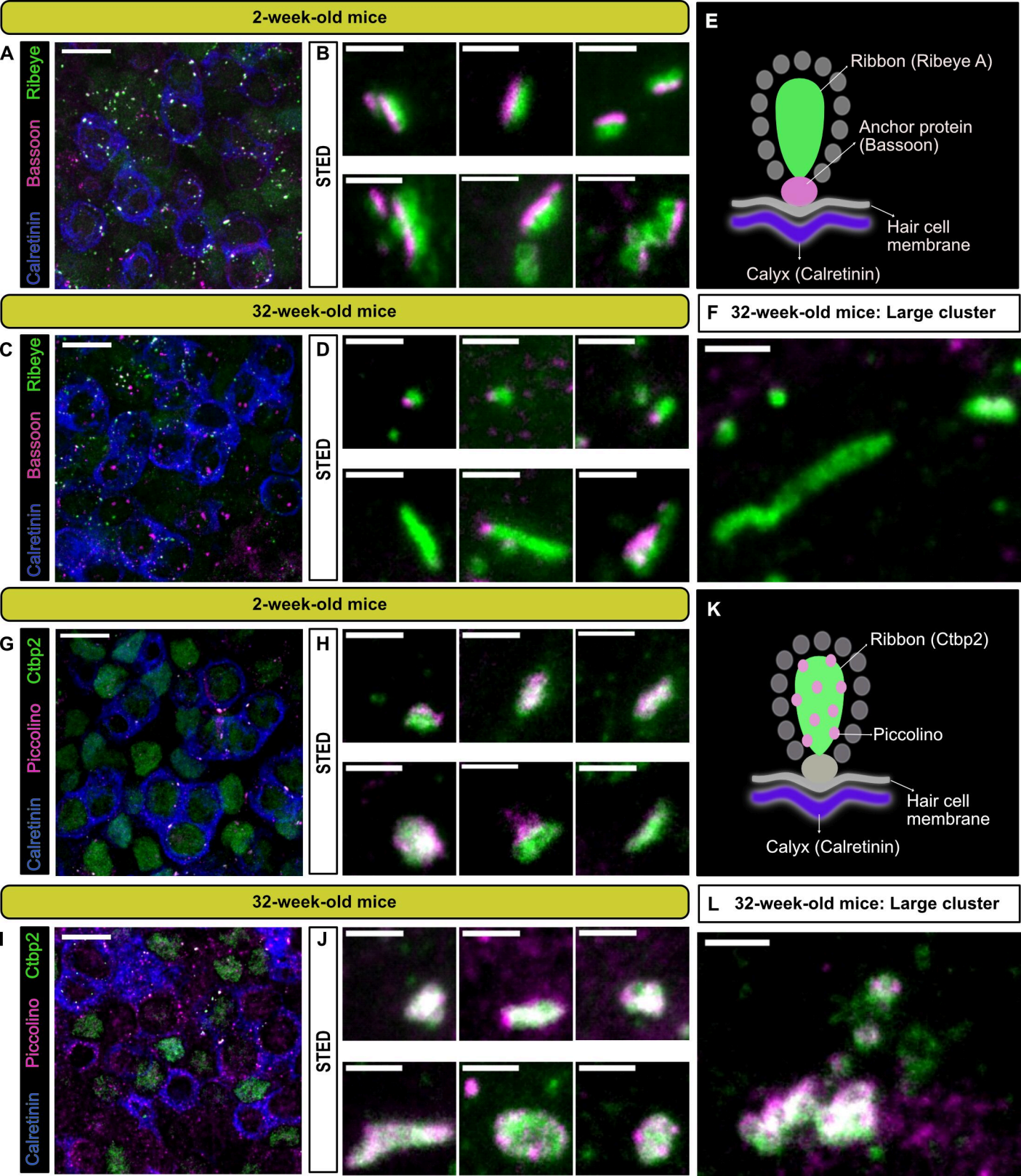
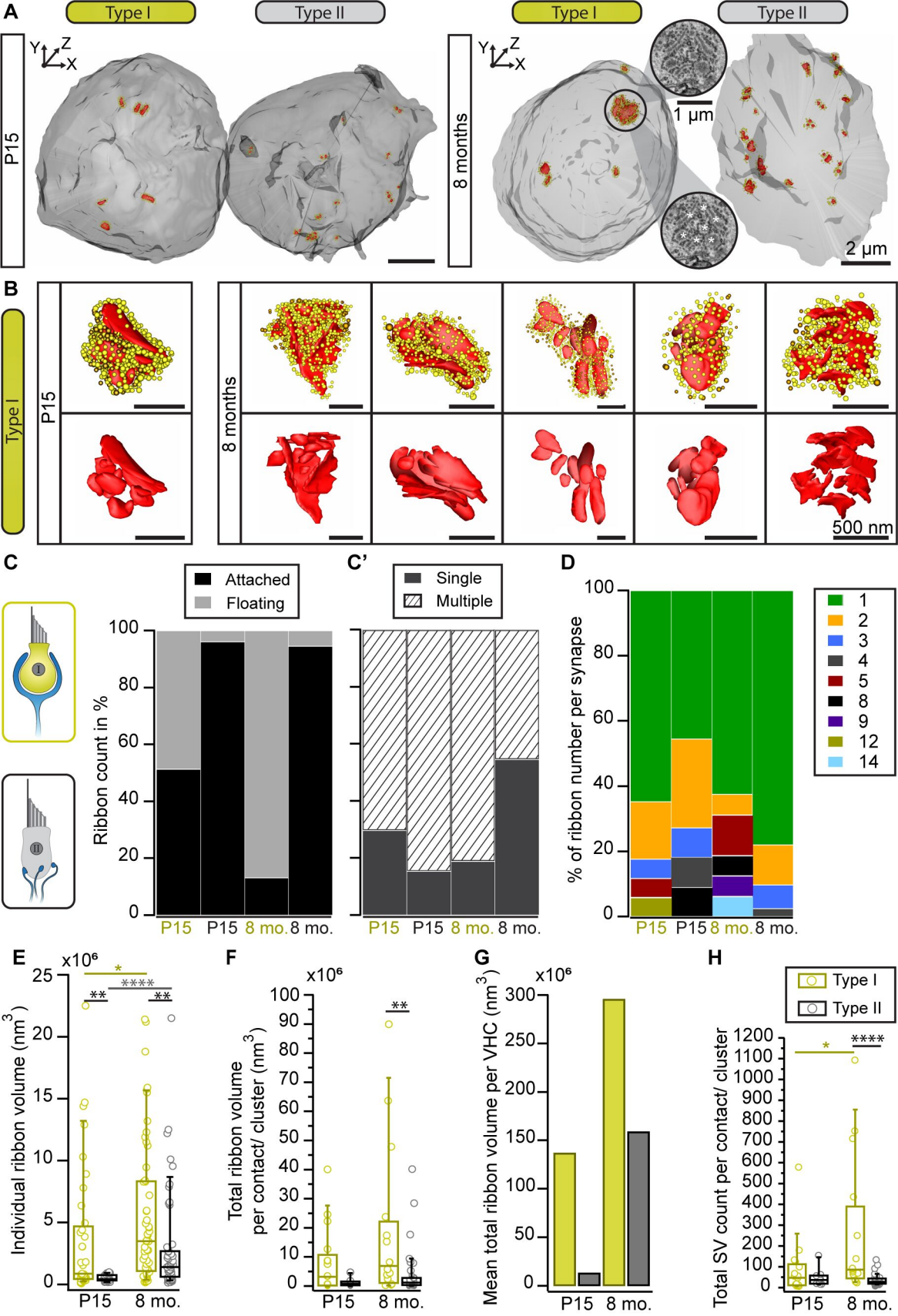
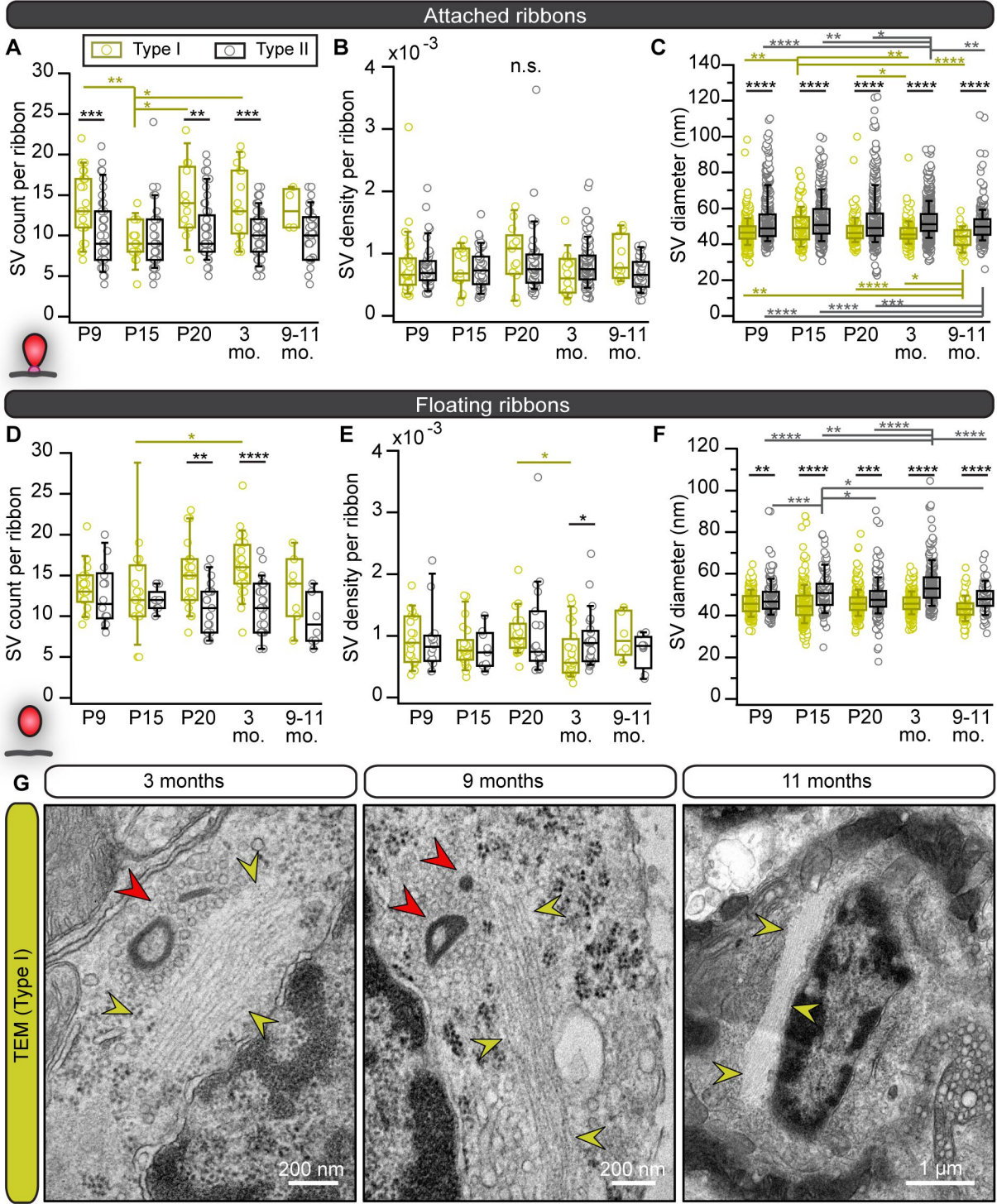

A
Type I
1 2 3 4
Attached ribbon no. per AZ in %
100
90
50
40
30
20
10
0
P9
P15
P20
3 months
9-11 months
B
P20
C
P15
200 nm
D
Type II
E
3 mo.
F
9 mo.
G
P15
200 nm
Type I: P9 (N=2,n=31) P15 (N=2,n=39) P20 (N=2,n=21) 3months (N=2,n=19) 9-11months (N=2,n=7)
Type II: P9 (N=2, n=44) P15 (N=2, n=61) P20 (N=2, n=50) 3months (N=2, n=107) 9-11months (N=2, n=43)
H
Ribbon shape in %
P9 P15 P20 3 mo. 9-11 mo.
round
round flat
wedge
droplet
oval
elongated
I
Type I
Type II
Ribbon height (nm)
800
600
400
200
0
Att.
P9 P15 P20 3 mo. 9-11 mo.
J
Ribbon width (nm)
Att.
K
Ribbon area (nm2) x 103
Att.
L
Ribbon area (nm2) x 103
Flo.

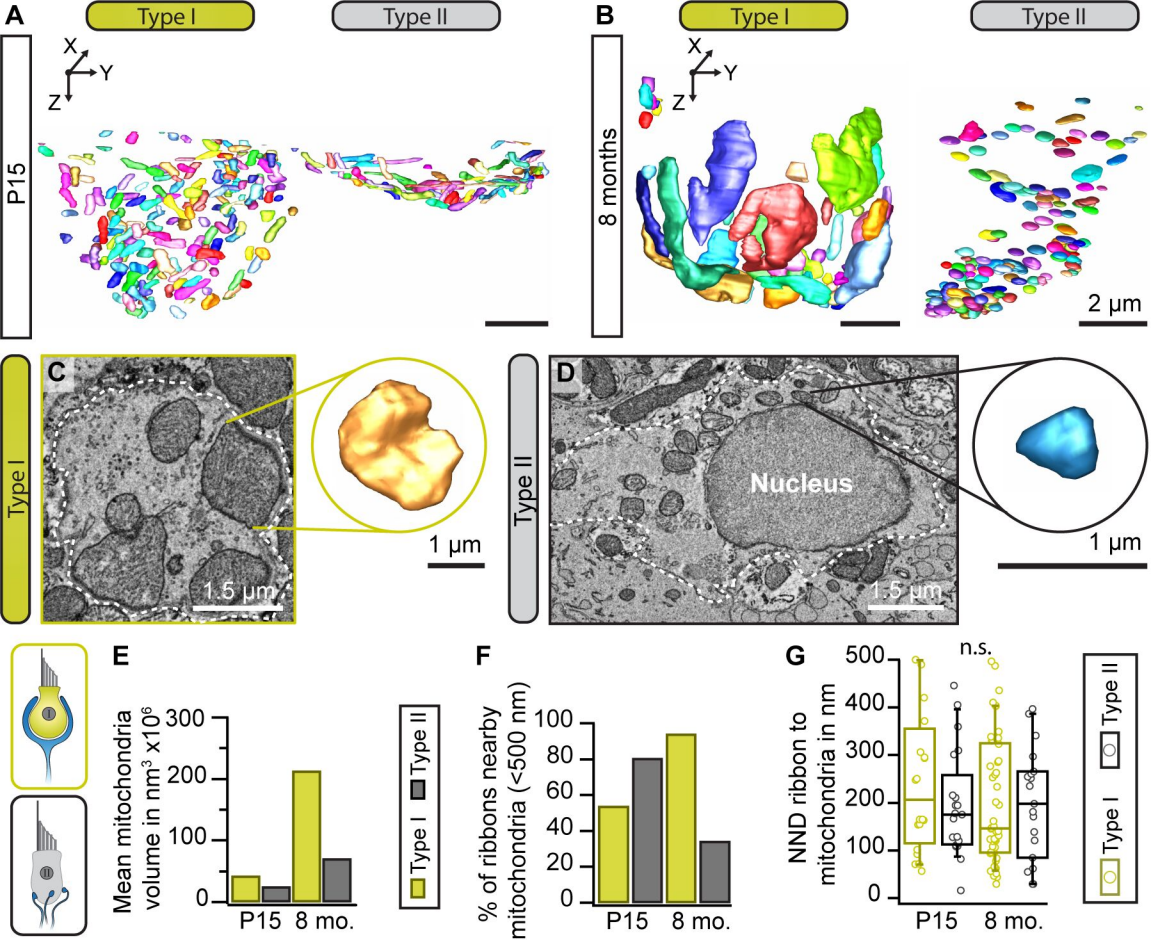


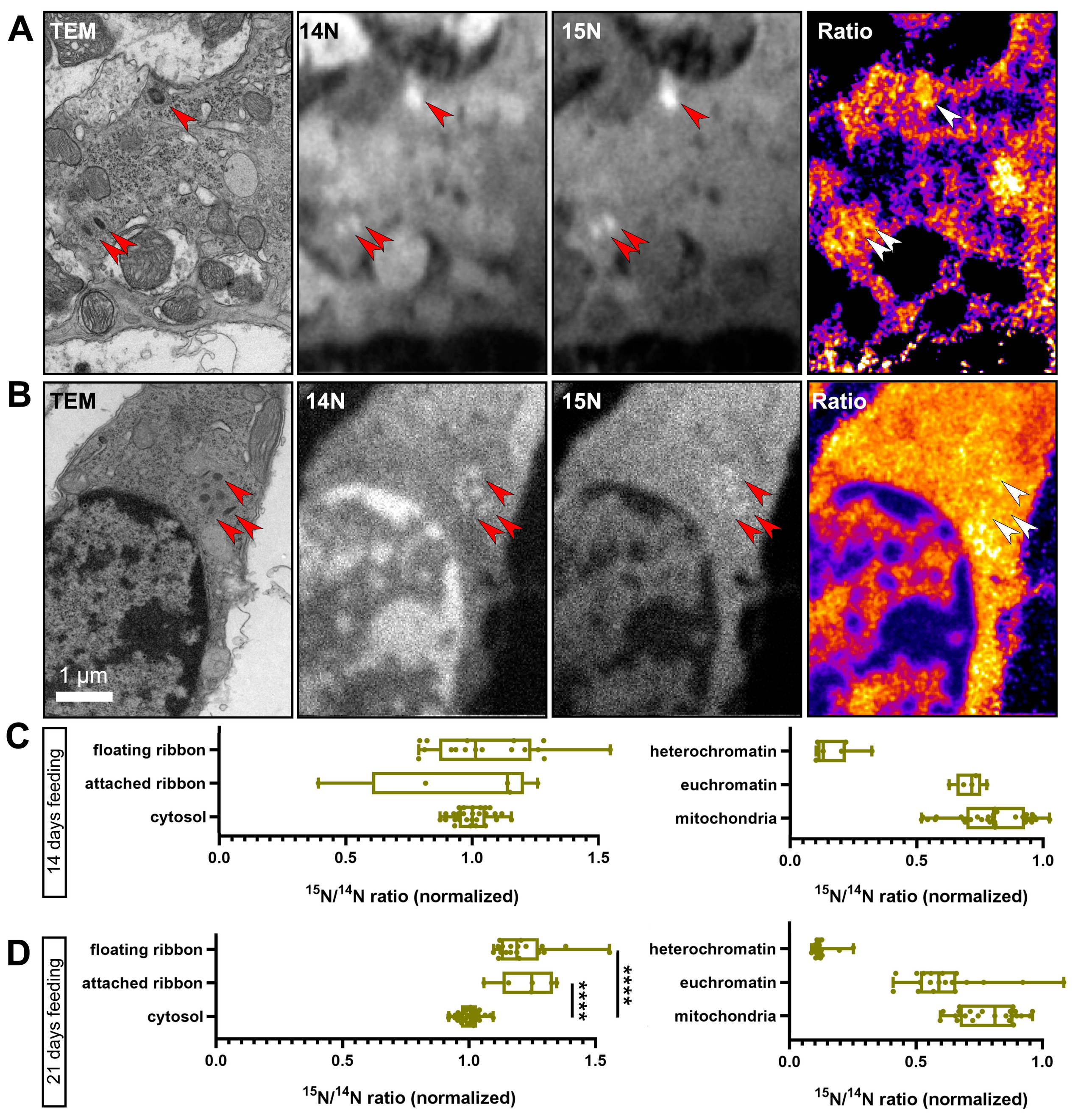
Type I:	P9 (N=2,n=86)	P15 (N=2,n=128)	P20 (N=2,n=165)	3 mo. (N=2,n=219)	9-11 mo. (N=2,n=169)
Type II:	P9 (N=2, n=63)	P15 (N=2, n=86)	P20 (N=2, n=72)	3 mo. (N=2, n=151)	9-11 mo. (N=2, n=53)

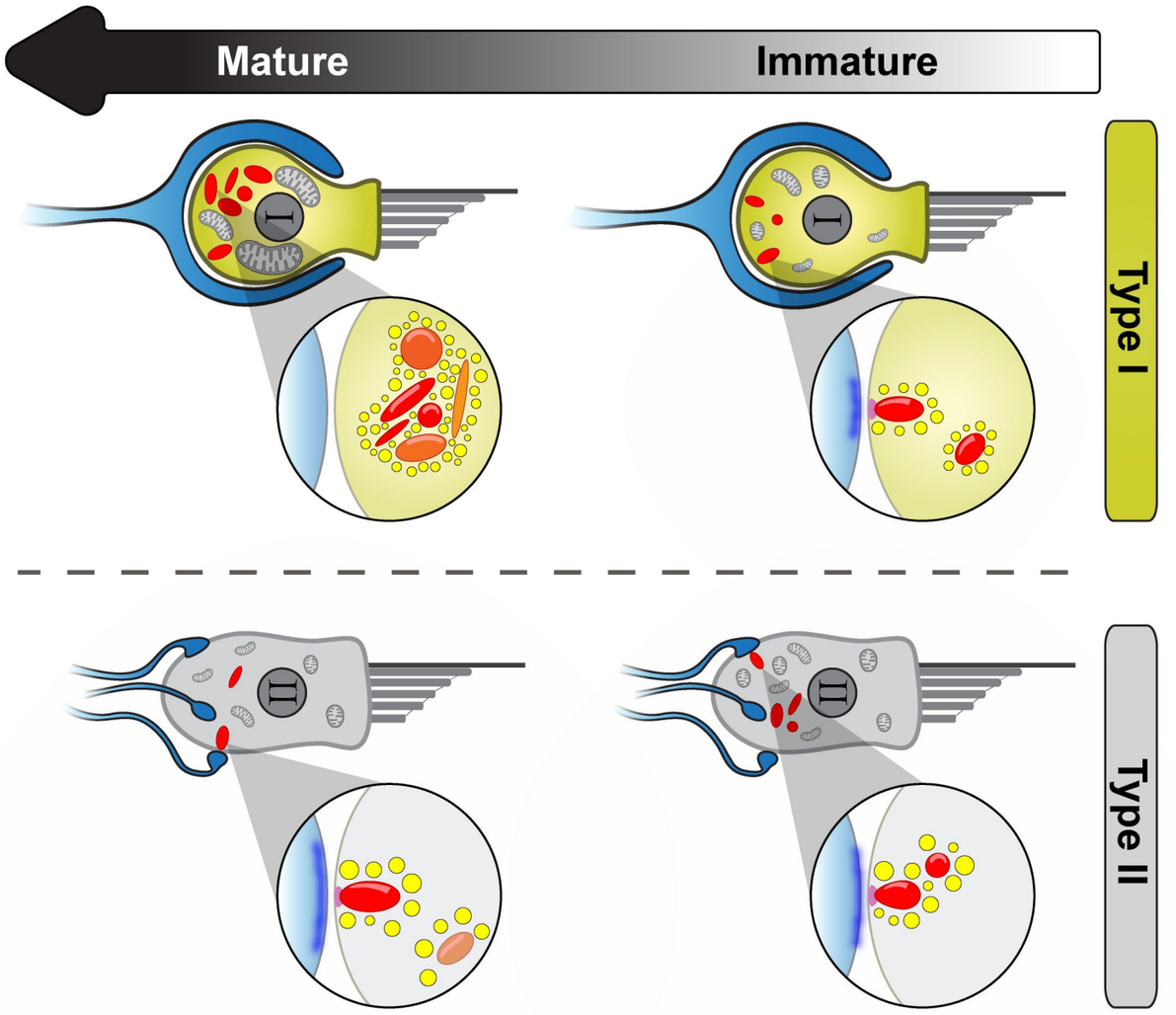

2-week-old mice
A
Ribeye
Bassoon
Calretinin
B
STED
E
Ribbon (Ribeye A)
Anchor protein
(Bassoon)
Hair cell
membrane
Calyx (Calretinin)
32-week-old mice
C
Ribeye
Bassoon
Calretinin
D
STED
F 32-week-old mice: Large cluster
2-week-old mice
G
Ctbp2
Piccolino
Calretinin
H
STED
K
Ribbon (Ctbp2)
Piccolino
Hair cell
membrane
Calyx (Calretinin)
32-week-old mice
I
Ctbp2
Piccolino
Calretinin
J
STED
L 32-week-old mice: Large cluster


A
Type I
Type II
Type I
Type II
P15
8 months
Y
Z
X
1 µm
2 µm
B
Type I
P15
8 months
500 nm
C
Attached
Floating
Ribbon count in %
P15
P15
8 mo.
8 mo.
C'
Single
Multiple
D
% of ribbon number per synapse
1
2
3
4
5
8
9
12
14
E
x10⁶
Individual ribbon volume (nm³)
P15
8 mo.
F
x10⁶
Total ribbon volume per contact/ cluster (nm³)
G
x10⁶
Mean total ribbon volume per VHC (nm³)
H
Type I
Type II
Total SV count per contact/ cluster


Attached ribbons
A
Type I
Type II
SV count per ribbon
P9
P15
P20
3 mo.
9-11 mo.
B
x10 -3
n.s.
SV density per ribbon
C
SV diameter (nm)
Floating ribbons
D
E
F
G
3 months
9 months
11 months
TEM (Type I)
200 nm
200 nm
1 µm


A
Type I
Type II
P15
X
Y
Z
B
8 months
2 µm
C
Type I
1.5 µm
1 µm
D
Type II
Nucleus
1.5 µm
1 µm
E
Mean mitochondria volume in nm³ x10⁶
300
200
100
0
P15
8 mo.
Type I
Type II
F
% of ribbons nearby mitochondria (<500 nm)
100
80
60
40
20
0
P15
8 mo.
G
n.s.
NND ribbon to mitochondria in nm
500
400
300
200
100
0
P15
8 mo.
Type I
Type II


A
TEM
14N
15N
Ratio
B
TEM
14N
15N
Ratio
1 µm
C
14 days feeding
floating ribbon
attached ribbon
cytosol
0.0
0.5
1.0
1.5
15N/14N ratio (normalized)
heterochromatin
euchromatin
mitochondria
0.0
0.5
1.0
15N/14N ratio (normalized)
D
21 days feeding
floating ribbon
attached ribbon
cytosol
****
****
0.0
0.5
1.0
1.5
15N/14N ratio (normalized)
heterochromatin
euchromatin
mitochondria
0.0
0.5
1.0
15N/14N ratio (normalized)


Mature
Immature
Type I
Type II