Piccolino regulates the architecture of the ribbon at cochlear inner hair cell synapses

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Abstract

Cochlear inner hair cells (IHCs) form specialized ribbon synapses with spiral ganglion neurons that tireless-ly transmit sound information at high rates over long time periods with extreme temporal precision. This functional specialization is essential for precise sound encoding and is attributed to a distinct molecular machinery with unique players or splice variants compared to conventional neuronal synapses. Among these is the active zone (AZ) scaffold protein piccolo/aczonin, which is represented by its short splice variant piccolino at cochlear and retinal ribbon synapses. While the function of piccolo at synapses of the central nervous system has been intensively investigated, the role of piccolino at IHC synapses remains unclear. In this study, we characterized the structure and function of IHC-synapses in piccolo gene-trap mutant rats ($Pclo^{gt/gt}$). We found a mild hearing deficit with elevated thresholds and reduced amplitudes of auditory brainstem responses. Ca^{2+} channel distribution and ribbon morphology were altered in apical IHCs, while their presynaptic function seemed unchanged. We conclude that piccolino contributes to the AZ organization in IHCs and is essential for normal synaptic transmission.

Keywords

Active zone/Ca^{2+} channel/cochlea/ hearing /synaptic vesicle

Introduction

Ribbon synapses are involved in vertebrate vision, hearing and balance. They are specialized in terms of function, morphology and molecular composition to enable indefatigable neurotransmission over long time periods. Depending on the ribbon synapse type, the electron-dense appearing synaptic ribbon can tether up to several hundreds of synaptic vesicles (SVs) (Matthews and Fuchs, 2010; Moser et al., 2019; Wichmann and Moser, 2015). In addition to employing the ribbon-specific protein RIBEYE, that

55 constitutes the main component of the synaptic ribbon (Becker et al., 2018; Jean et al., 2018; Maxeiner

56 et al., 2016; Schmitz et al., 2000), the molecular composition of ribbon-type AZs shows further

57 differences from conventional neuronal synapses (Chakrabarti and Wichmann, 2019; Moser et al.,

58 2019). For example, ribbon synapses employ different isoforms of presynaptic proteins such as rab3-

59 interacting molecule 2 (RIM2) instead of RIM1 (Grabner et al., 2015; Jung et al., 2015a). Moreover, the

60 presynaptic multi-domain protein piccolo/aczonin, is represented solely by its short splice variant

61 piccolino as shown for photoreceptor (Regus-Leidig et al., 2013) and cochlear inner hair cell (IHC) ribbon

62 synapses (Butola et al., 2017; Michanski et al., 2019; Regus-Leidig et al., 2013).

63 The function of piccolo at conventional synapses has been investigated intensely (Cases-Langhoff et al.,

64 1996; Fenster et al., 2003; Gundelfinger et al., 2015; Leal-Ortiz et al., 2008; Mukherjee et al., 2010).

65 Piccolo together with bassoon is involved in synapse assembly, SV clustering and maintaining synapse

66 integrity (Gundelfinger et al., 2015). Piccolo seems especially important for the SV pool organization. At

67 the calyx of Held, piccolo was found to organize the readily-releasable vesicle pool (RRP) (Parthier et al.,

68 2018) and at the endbulb of Held, piccolo is required for normal SV replenishment (Butola et al., 2017).

69 Furthermore, a reduction of SVs, specifically from the total recycling pool was found at hippocampal

70 neurons lacking piccolo (Ackermann et al., 2019). In contrast, piccolino's role at ribbon synapses remains

71 poorly understood. Importantly, in comparison to full-length piccolo, piccolino lacks a number of

72 interaction sites for presynaptic binding partners such as CAST/Munc13 and RIM, L-type Ca^{2+} channels as

73 well as the interaction site for its homologue bassoon (Regus-Leidig et al., 2013). Instead, piccolino was

74 found to interact with the ribbon component RIBEYE (Müller et al., 2019), in line with its localization

75 exclusively at the synaptic ribbon (Dick et al., 2001; Limbach et al., 2011; Michanski et al., 2019; Regus-

76 Leidig et al., 2013). While at conventional synapses, the functions of piccolo and bassoon seem partially

77 redundant (Altrock et al., 2003; Gundelfinger et al., 2015; Leal-Ortiz et al., 2008; Mukherjee et al., 2010;

78 Waites et al., 2013), piccolino's function at ribbon synapses might be unique and could differ from

79   piccolo's function at conventional synapses. Piccolino's absence or reduction had a striking structural

80   impact on photoreceptor ribbon synapses: the altered ribbon shape went along with a reduced number

81   of SVs around the ribbons (Müller et al., 2019; Regus-Leidig et al., 2014). A previous study of the

82   auditory system reported that piccolino KO mice lack functional deficits on the level of auditory brain-

83   stem response (ABR) thresholds (Li et al., 2021).

84   Given the visual phenotype and the prominent piccolino expression in IHCs, we performed a

85   comprehensive structural and functional study on piccolo gene trap mutant rats ($Pclo^{gt/gt}$) (Ackermann et

86   al., 2019; Medrano et al., 2020; Müller et al., 2019) by combining systems and cell physiology with

87   confocal, stimulated emission depletion (STED) and electron microscopy. We observed an altered Ca^{2+}

88   channel distribution and 3D reconstructions from electron microscopic data uncovered changes in

89   ribbon morphology for a subset of synapses, resulting in two morphologically distinguishable ribbon

90   categories in $Pclo^{gt/gt}$ IHCs. Category 1 ribbons appeared completely normal, while category 2

91   encompassed small, spherical ribbons that lacked SVs at their upper ribbon part. Recording ABRs, we

92   discovered a mild hearing phenotype despite our findings of normal IHC Ca^{2+} currents and exocytosis.

93   Our data suggest that piccolino is involved in the proper formation of synaptic ribbons, likely via

94   organizing RIBEYE, and potentially via the ribbon, normal clustering of Ca^{2+} channels. We conclude that

95   piccolino is essential for normal organization of ribbon type AZs and required for normal hearing.

96

Results

Reduced ABR wave I amplitudes and elevated ABR thresholds in $Pclo^{gt/gt}$ rats

99   To investigate the role of piccolino in the auditory system, we commenced our study by recording ABRs

100   in piccolino-deficient ($Pclo^{gt/gt}$) rats at the age of two months. We found a significant amplitude

101   reduction of ABR wave I , which represents the compound action potential of spiral ganglion neurons

102   (SGNs) to 73% of normal (1.68 ± 0.08 µV, $N_{animals} = 10$ for $Pclo^{gt/gt}$ rats *versus* 2.38 ± 0.16 µV, $N_{animals} = 10$

103   for $Pclo^{wt/wt}$ rats ($P < 0.01$, two-way repeated measures ANOVA with post-hoc Holm-Šidák correction for

104   multiple comparisons) at 80 dB (peak equivalent, pe, 20 Hz clicks) (Fig 1A, B). The successive ABR waves

105   were also slightly reduced in amplitude, but the difference was significant only for wave IV ($P < 0.05$, t-

106   test, Fig 1A). ABR thresholds in response to tone bursts showed a mild but significant elevation for

107   middle and high sound frequencies (approximately 10 - 20 dB; P_{12kHz}, P_{16kHz} and $P_{32kHz} < 0.01$, two-way

108   repeated measures ANOVA followed by Holm-Šidák multi-comparison test), but not for low frequency

109   tones and broadband click stimuli (Fig 1C). We also measured cochlear amplification mediated by outer

110   hair cells by recording the distortion product otoacoustic emissions (DPOAEs) which were unaltered (Fig

111   1D). Overall, we report a mild but significant impairment in synchronous sound onset responses of SGNs

112   despite intact cochlear amplification, which is consistent with a sound encoding deficit at the first

113   auditory synapse upon genetic disruption of piccolino. This hearing phenotype appears more

114   pronounced than that seen in piccolo mutant mice with maintained piccolino expression in IHCs (Butola

115   et al., 2017) or that of RIBEYE KO mice (Becker et al., 2018; Jean et al., 2018).
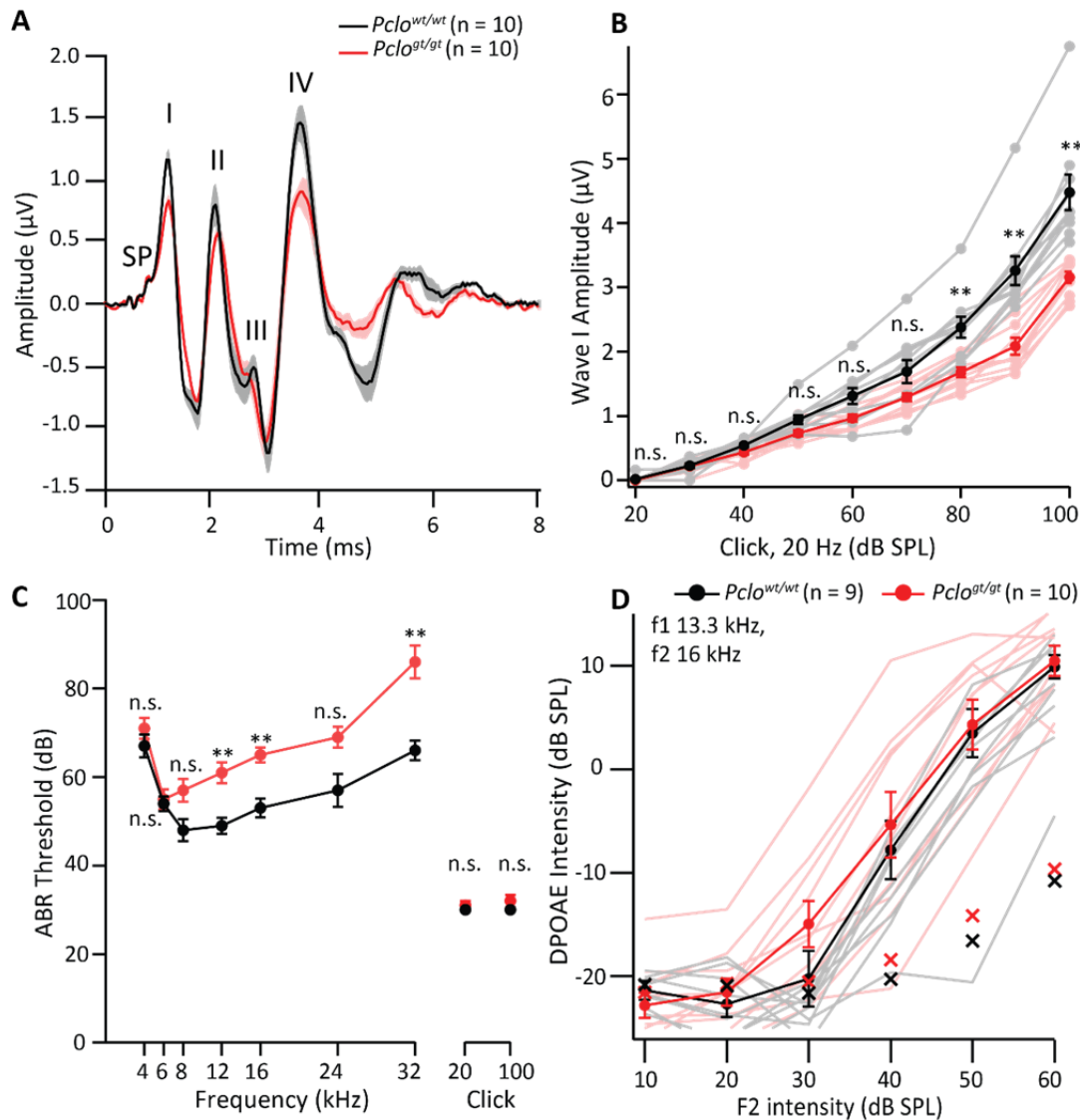
Figure 1. Reduced wave I amplitudes and elevated thresholds of auditory brainstem response. (A) Average ABR waveforms in response to 80 dB (pe) clicks (20 Hz) in 2 months old rats ($N_{animals}$ = 10 each for $Pclo^{wt/wt}$ and $Pclo^{gt/gt}$). Lines represent averages, shaded area represents ± SEM. SP: summating potential (hair cell receptor potential), roman numerals (I – IV): ABR waves generated along the auditory pathway. **(B)** ABR wave I amplitudes in response to 20 Hz clicks at different sound pressure levels show a significant decrease in $Pclo^{gt/gt}$ rats at 80dB and above, implying impaired synchronous sound evoked firing of their SGNs, ($P < 0.01$, two-way repeated measures ANOVA followed by post hoc Holm-Šidák multi-comparison correction). Dark lines represent averages, lighter ones represent individual traces. **(C)** $Pclo^{gt/gt}$ rats show elevated ABR thresholds for middle and high frequency tone bursts, whereas thresholds in response to low frequency tone bursts and click stimuli (applied at 20 and 100 Hz stimulation rate) appear comparable; P_{12kHz}, P_{16kHz}, and $P_{32kHz} < 0.01$, two-way repeated measures ANOVA followed by Holm-Šidák multi-comparison correction. **(D)** DPOAE amplitude in response to pairs of simultaneous sine waves (f_1 = 13.3 kHz, f_2 = 16 kHz) at increasing sound pressure levels (intensity of f_1 is 10 dB above f_2) appear comparable in $Pclo^{wt/wt}$ ($N_{animals}$ = 9) and $Pclo^{gt/gt}$ rats ($N_{animals}$ = 10), implying unaltered cochlear amplification. Dark lines represent mean ± SEM, lighter ones represent individual

132 traces, crosses represent noise floor. Asterisks indicate significance levels with *$P<0.05$, **$P<0.01$,
133 ***$P<0.001$.

134

135 Smaller ribbons but larger PSD areas at $Pclo^{gt/gt}$ active zones

136

137 We next investigated ribbon synapse abundance and morphology by performing immunohistochemistry

138 on whole-mounted apical portions of the organ of Corti acutely dissected from 2 months old $Pclo^{wt/wt}$

139 and $Pclo^{gt/gt}$ rats, followed by confocal and super resolution STED microscopy. We first used an antibody

140 that recognizes a peptide corresponding to amino acid 2012 to 2351 of rat piccolo, designed to bind to

141 full-length piccolo as well as the shorter splice variant piccolino. In $Pclo^{wt/wt}$ organs of Corti, we observed

142 immunofluorescent puncta at every afferent synaptic contact, colocalizing with the synaptic ribbon

143 (labeled with an antibody against CtBP2/RIBEYE), as well as at efferent synapses (immunofluorescent

144 spots not colocalized with ribbons). $Pclo^{gt/gt}$ organs of Corti, on the other hand, showed complete

145 absence of any piccolo/piccolino-specific immunofluorescence (Fig 2A, B). Next, we performed triple-

146 labeling using antibodies against CtBP2 (labeling the synaptic ribbon), bassoon (the presynaptic active

147 zone (AZ)) and homer1 (the postsynaptic density). The localization of the synaptic markers seemed

148 unaltered. We observed that the total number of synaptic ribbons (total count of CtBP2 spots) and the

149 number of anchored ribbons (number of CtBP2 and bassoon juxtapositions) stays intact upon piccolino

150 disruption (Fig 2D). CtBP2/piccolo co-labeled sections as shown in Fig 2B were included for estimation of

151 total ribbon counts.

152 We then acquired images of $Pclo^{wt/wt}$ and $Pclo^{gt/gt}$ synapses using 2D-STED (CtBP2) and confocal

153 (bassoon, homer1) imaging to assess their morphology (Fig 2C). Visually, a large proportion of synaptic

154 ribbons from $Pclo^{gt/gt}$ rats appeared smaller and more compact, in contrast to the typical ellipsoid-

155 shaped ribbons from $Pclo^{wt/wt}$ rats. A 2D-Gaussian function was fitted to raw images of randomly

156 selected synapses to estimate the area of the ribbon and the PSD. We found that on average, the size of

157 the synaptic ribbon appeared to be reduced for $Pclo^{gt/gt}$ rats ($38.34 \times 10^3 \pm 2.36 \times 10^3$ nm^2, S.D. = 19.71×10^3,

158    $n_{ribbons} = 71$, $N_{animals} = 3$) compared to $Pclo^{wt/wt}$ ($46.32 \times 10^3 \pm 1.77 \times 10^3$ nm^2, S.D. $= 15.12 \times 10^3$, $n_{ribbons} = 74$,

159    $N_{animals} = 3$; $P < 0.001$, Mann-Whitney-Wilcoxon Test, Fig 2E). Images with more than one ribbon per

160   synapse (observed only in $Pclo^{wt/wt}$ IHCs) were excluded from analysis to avoid overestimation of ribbon

161   size in $Pclo^{wt/wt}$ IHCs. On the other hand, the PSD size appeared to be larger for $Pclo^{gt/gt}$ rats ($22.46 \times 10^4 \pm$

162   1.20×10^4 nm^2, S.D. $= 9.56 \times 10^4$, $n_{PSD} = 65$, $N_{animals} = 3$) compared to $Pclo^{wt/wt}$ rats ($17.25 \times 10^4 \pm 1.04 \times 10^4$

163   nm^2, S.D. $= 8.61 \times 10^4$, $n_{PSD} = 70$, $N_{animals} = 3$; $P < 0.001$, Mann-Whitney-Wilcoxon Test, Fig 2F).

164
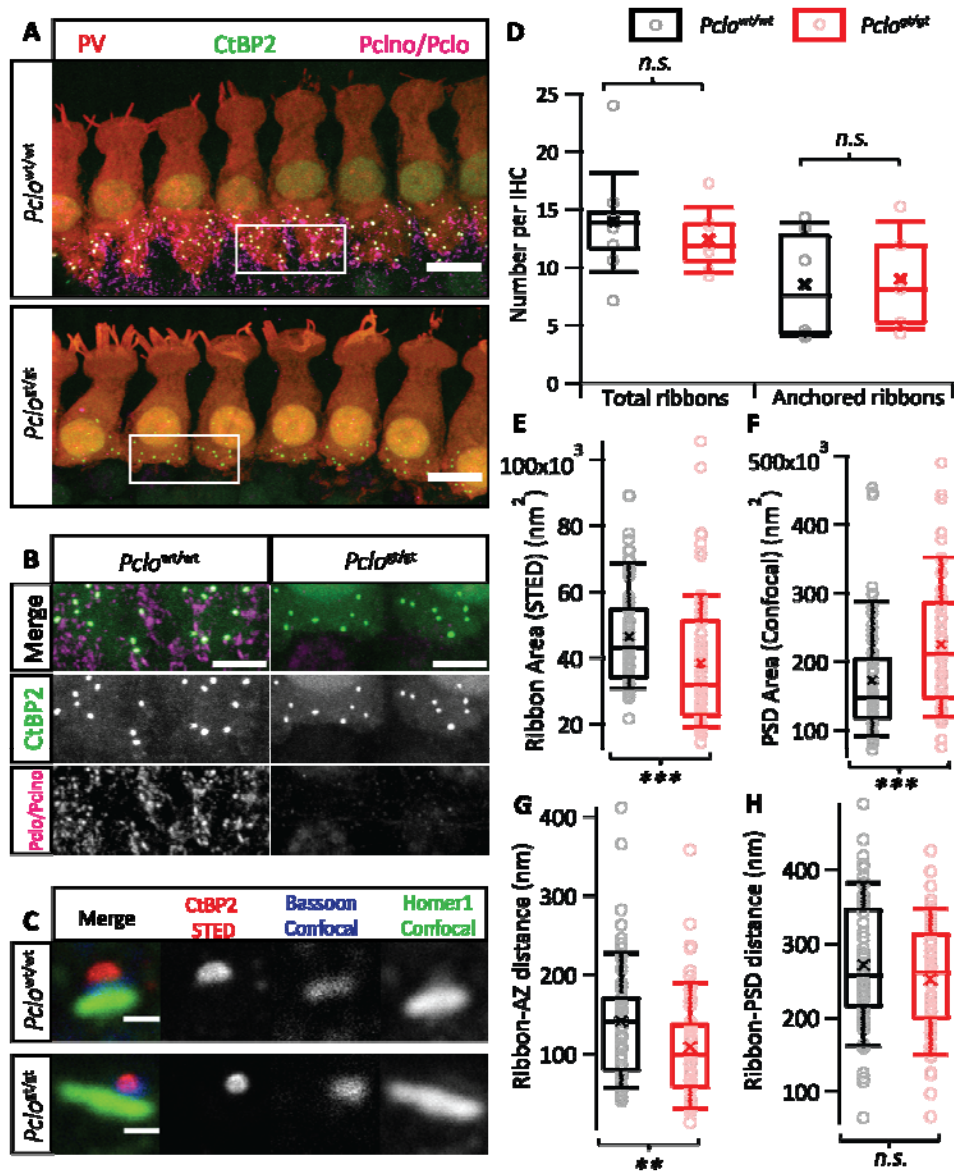
Figure 2. Absence of piccolino results in smaller synaptic ribbons and larger PSDs

166 **(A)** Maximal projections of confocal sections of apical organs of Corti from 2 months old *Pclo^{gt/gt}* and
167 *Pclo^{wt/wt}* rats. The whole mounts were co-labeled for IHC cytosol marker parvalbumin (PV, red), synaptic
168 ribbon marker CtBP2 (green) and piccolo/piccolino (magenta). Scale bars = 10 μm. **(B)** Zoom-ins from
169 insets in (A), (here removing the parvalbumin-channel for clarity) highlight the absence of piccolo-
170 specific immunofluorescence puncta in and around *Pclo^{gt/gt}* IHCs, in contrast to colocalizing CtBP2-
171 piccolino spots for *Pclo^{wt/wt}* IHCs and efferent piccolo immunofluorescence (not colocalising with the
172 synaptic ribbon). Scale bar = 5 μm. **(C)** Triple labeling of homer1 (green), bassoon (blue) and CtBP2 (red),
173 imaged in 2D-STED (CtBP2) and confocal mode (homer1 and bassoon). The morphology of afferent
174 synapses of piccolino deficient IHCs appears to be altered with small "compact" ribbons and larger PSDs.
175 Scale bars = 0.5 μm. **(D)** Box plot representing unaltered total number of ribbons (number of CtBP2
176 puncta; n_{IHC} = 81, n_{Corti} = 8, $N_{animals}$ = 6 for *Pclo^{wt/wt}*; and n_{IHC} = 79, n_{Corti} = 7, $N_{animals}$ = 4 for *Pclo^{gt/gt}*; P =
177 0.466, t-test) and number of anchored ribbons (number of CtBP2-bassoon juxtapositions; n_{IHC} = 52, n_{Corti}
178 = 6, $N_{animals}$ = 4 for *Pclo^{wt/wt}* and n_{IHC} = 51, n_{Corti} = 5, $N_{animals}$ = 3 for *Pclo^{gt/gt}*; P = 0.681, t-test). Inlaid points
179 represent average numbers/IHC drawn from individual organs of Corti. **(E, F)** Box plots of the 2D area of
180 synaptic ribbons and PSDs respectively, derived by fitting a 2D-Gaussian function to
181 immunofluorescence data as represented in (C). **(E)** $n_{ribbons}$ = 74, $N_{animals}$ = 3 for *Pclo^{wt/wt}* and $n_{ribbons}$= 71,
182 $N_{animals}$ = 3 for *Pclo^{gt/gt}*; P < 0.001, Mann-Whitney-Wilcoxon test. **(F)** n_{PSDs} =70, $N_{animals}$ = 3 for *Pclo^{wt/wt}* and
183 n_{PSDs} = 65, $N_{animals}$ = 3 for *Pclo^{gt/gt}*; P < 0.001, Mann-Whitney-Wilcoxon test. **(G, H)** The distance between
184 the centres of mass of CtBP2 and bassoon spots shows a reduction for piccolino-deficient ribbons (P <
185 0.01, Mann-Whitney-Wilcoxon test), while the distance between the centres of mass of CtBP2 and
186 homer1 was comparable (P > 0.05, Mann-Whitney-Wilcoxon test). n_{pairs} =75, $N_{animals}$ = 3 for *Pclo^{wt/wt}*;
187 n_{pairs}= 69, $N_{animals}$ = 3 for *Pclo^{gt/gt}*. Throughout, box and whisker plots present median, lower/upper
188 quartiles and 10–90th percentiles with individual data points overlaid and means shown as crosses.

189

190 Previously, Jing and co-workers have reported ribbon anchorage defects in bassoon gene-trap mutant

191 mice (Jing et al., 2013). To check for any analogous phenotype upon piccolino disruption, we estimated

192 the centers of mass of CtBP2, bassoon and homer1 signals and calculated the ribbon-AZ and the ribbon-

193 PSD center of mass distance. We found a reduction in the ribbon-AZ center of mass distance (n_{pairs} = 69,

194 $N_{animals}$ = 3 for both groups; P < 0.01, Mann-Whitney-Wilcoxon Test) in *Pclo^{gt/gt}* synapses, whereas the

195 ribbon-PSD center of mass distance appeared comparable (n_{pairs} = 64, $N_{animals}$ = 3 for both groups; P >

196 0.05, t-test) (Fig 2G, H). We speculate that the smaller ribbon size in *Pclo^{gt/gt}* IHCs may have resulted in

197 the smaller ribbon-AZ center of mass distance, while this might not have become obvious for ribbon-PSD

198 due to their larger and more variable center of mass distance.

199

200 Large 3D electron microscopic volume reconstructions revealed two distinct ribbon morphologies in
201 mutant IHCs

202 In a next step, we performed an ultrastructural analysis in order to determine fine structural changes

203 beyond those amenable to light microscopy. Focused ion beam-scanning electron microscopy (FIB-SEM)

204 enabled us to quantify morphological parameters such as ribbon and PSD sizes as well as SV numbers

205 and their localization within IHCs.

206 We compared the ribbon volume and localization in 2-3 months old adult $Pclo^{gt/gt}$ and $Pclo^{wt/wt}$ IHCs (Fig

207 3A, B; Movie 1,2). While our immunohistochemical data revealed ribbons of $Pclo^{gt/gt}$ AZs to be smaller on

208 average, FIB-SEM could distinguish two categories of ribbon-type AZs in $Pclo^{gt/gt}$ IHCs. They could be

209 clearly separated by several morphological features: category 1 encompassed ribbons that appeared

210 comparable to $Pclo^{wt/wt}$ littermate control ribbons regarding size, shape and SV occupancy. Ribbons

211 sorted in category 2 appeared smaller, more round in shape and lacked SVs at their membrane-distal

212 side (Fig 3C). A first guess that these categories might simply reflect the previously described ribbon

213 number and size difference of modiolar and pillar AZs of IHCs (Hua et al., 2021; Liberman et al., 2011;

214 Michanski et al., 2019; Ohn et al., 2016; Payne et al., 2021), did not match our data. Category 2 ribbons

215 seemed to be equally present at the modiolar and the pillar sides of $Pclo^{gt/gt}$ IHCs. Due to the low IHC

216 number for the large 3D volume data set we refrained from a statistical analysis. Since the two ribbon

217 categories robustly differ in size and SV numbers, we analyzed all mutant ribbons together and

218 additionally analyzed both categories separately. We found that ribbons of category 2 were indeed

219 smaller with fewer SVs (Fig 3D-F; Appendix Table 1) in contrast to category 1 and $Pclo^{wt/wt}$ ribbons

220 (2.23×10^6 nm^3 vs. 3.07×10^6 nm^3 in $Pclo^{gt/gt}$ category 1 and 6.74×10^6 nm^3 in $Pclo^{wt/wt}$, i.e., category 2

221 ribbons being 3 times smaller compared to $Pclo^{wt/wt}$ ribbons). The PSD area appeared unchanged for all

222 mutant synapses compared to $Pclo^{wt/wt}$ littermate controls (Fig 3G; Appendix Table 1), which contrasts

223 our immunofluorescence analysis. Notably, no double ribbons per synaptic contact were observed in

224    $Pclo^{gt/gt}$ rats, while in both FIB-SEM data sets of $Pclo^{wt/wt}$ IHCs double ribbons appeared frequently (Fig

225   3C), in accordance with previous observations for mouse IHCs (Hua et al., 2021; Michanski et al., 2019;

226   Payne et al., 2021; Sobkowicz et al., 1982; Sobkowicz et al., 1986; Stamataki et al., 2006; Wong et al.,

227   2014) and our immunofluorescence results.

228   Next to ribbon morphology, the overall distribution of ribbons within an IHC might be changed

229   in the mutants. Therefore, we quantified the nearest neighbor distance between ribbons in both

230   genotypes in our FIB-SEM data (Fig EV1E). For $Pclo^{wt/wt}$ IHCs, we included the distance measurements

231   between ribbons of the same synaptic contact (indicated as filled points in Fig EV1E). However, no

232   differences between all $Pclo^{gt/gt}$ compared to $Pclo^{wt/wt}$ ribbons could be observed, suggesting that the

233   distribution of synaptic contacts is normal in $Pclo^{gt/gt}$ IHCs (Fig EV1E; Appendix Table 1). An analogous

234   analysis of nearest neighbor distances between ribbons from our immunolabeled confocal sections of

235   the organs of Corti confirmed these findings since no difference in distances could be found (Fig EV1G,

236   H). We further noted that mitochondria often appeared in close vicinity to category 2 mutant ribbon

237   synapses (Fig EV1A-C). Indeed, apposition of mitochondria to category 2 ribbons was closer compared to

238    $Pclo^{wt/wt}$ and $Pclo^{gt/gt}$ category 1 ribbons (Fig EV1D; Appendix Table 1). However, the overall frequency of

239   mitochondria being in close vicinity to ribbons was comparable between $Pclo^{gt/gt}$ and $Pclo^{wt/wt}$ IHCs (Fig

240   EV1C). Finally, FIB-SEM revealed that larger ribbons frequently harbor a translucent core, regardless of

241   the genotype (Fig 3C, Fig EV1F), which is in line with previous findings (Liberman, 1980; Michanski et al.,

242   2019; Sobkowicz et al., 1982; Stamataki et al., 2006). Interestingly, no translucent core could be found in

243   the category 2 ribbons of $Pclo^{gt/gt}$ rats, likely owing to their smaller size.

244   Transmission electron microscopy of ultrathin sections from conventional embeddings further

245   corroborated these findings: Translucent cores are present in larger ribbons, while smaller ribbons in

246    $Pclo^{gt/gt}$ IHCs showed a uniform electron-density and lacked SVs at the upper ribbon part (Fig EV2A,B)

247   and were thus characterized as category 2. Using an anti-piccolino immunogold labeling with the same

248   antibody as for our immunofluorescence analysis, we found that piccolino was almost equally

249   distributed along individual *Pclo^wt/wt* ribbons of 10-11 months of age, while it seems that ribbons are less

250   frequently labeled in *Pclo^wt/gt* IHCs suggesting a reduction of piccolino molecules in heterozygotes (Fig

251   EV2C,D).
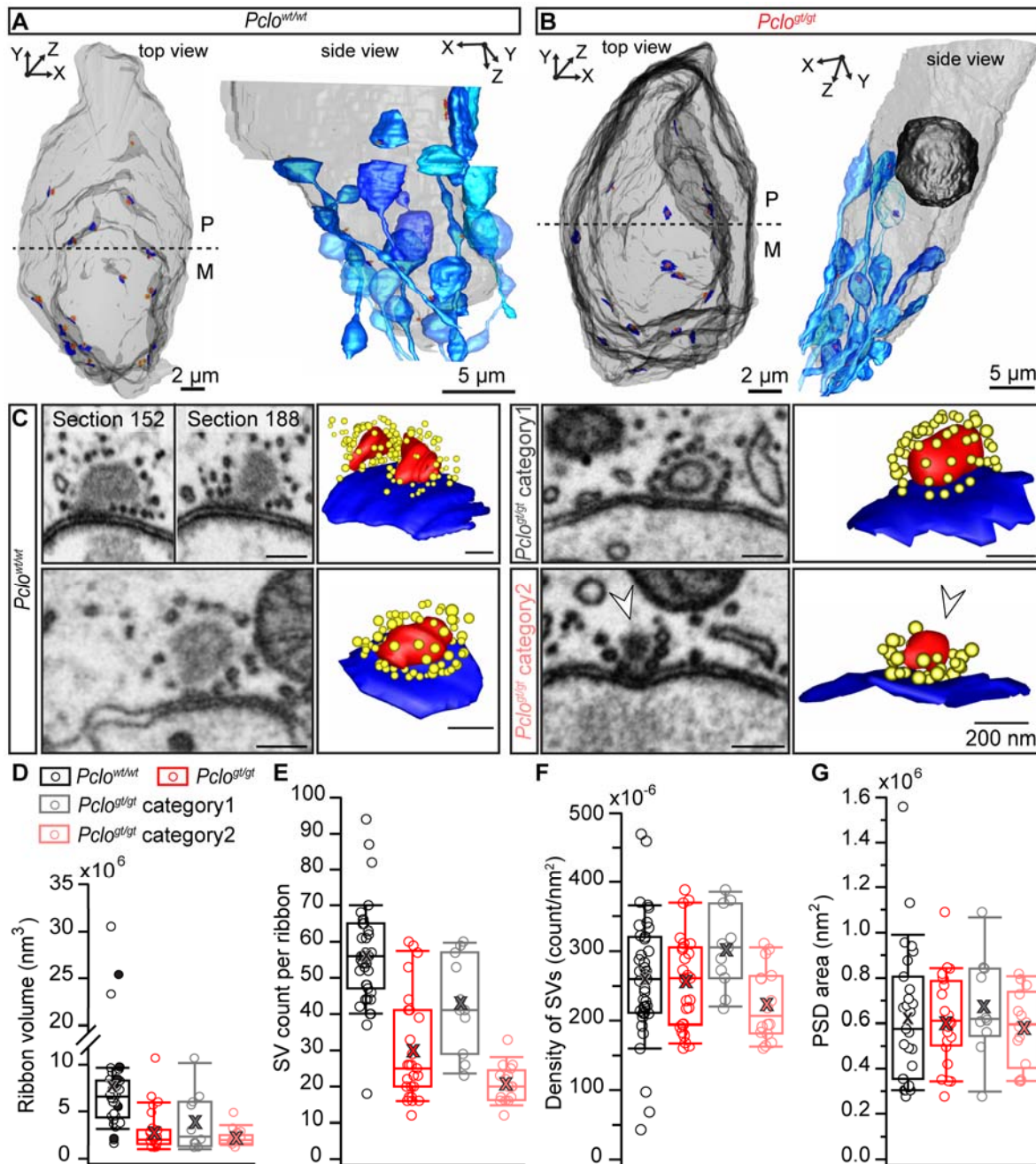
252

253   **Figure 3: FIB-SEM revealed two morphological distinct ribbon categories in *Pclo^gt/gt* inner hair cells.**

254 **(A, B)** 3D visualizations of $Pclo^{wt/wt}$ and $Pclo^{gt/gt}$ (2-3 months old) rat IHCs using FIB-SEM displayed in top
255 view and side view, without or with afferent nerve fiber contacts (different shades of light blue). Based
256 on the tissue context and the cellular apical-basal axis, the separation of pillar (P) and modiolar (M) sides
257 was determined. Light gray: IHC membrane, dark gray: nucleus, red: ribbons, dark blue: postsynaptic
258 density (PSD). **(C)** Representative single sections (left panels) of the reconstructed (right panels) ribbons
259 (red) with their surrounding synaptic vesicles (SVs, yellow) and opposing PSDs (dark blue). Multi-ribbon
260 AZs were observed only in $Pclo^{wt/wt}$ AZs (left upper panel), while $Pclo^{gt/gt}$ IHCs revealed two
261 morphological distinct AZ categories (right panel). In contrast to the $Pclo^{wt/wt}$-resembling ribbon synapse
262 architecture in category 1, category 2 ribbon synapses take a roundish shape and lack SVs at the upper
263 side of the ribbon (white arrowheads). **(D-G)** Box plots with the mean values (cross) and individual data
264 points display the quantification of ribbon volumes (D), SV counts (E), SV densities (F, SV count values
265 normalized to the ribbon area) and the PSD area (G), for $Pclo^{wt/wt}$ and $Pclo^{gt/gt}$ samples, respectively as
266 well as an additional separation of the $Pclo^{gt/gt}$ data into category 1 and category 2 synapses. Black filled
267 circles in D represent measurements from multi-ribbons AZs. $N_{animals}$ = 2, n_{IHCs} = 2 respectively for
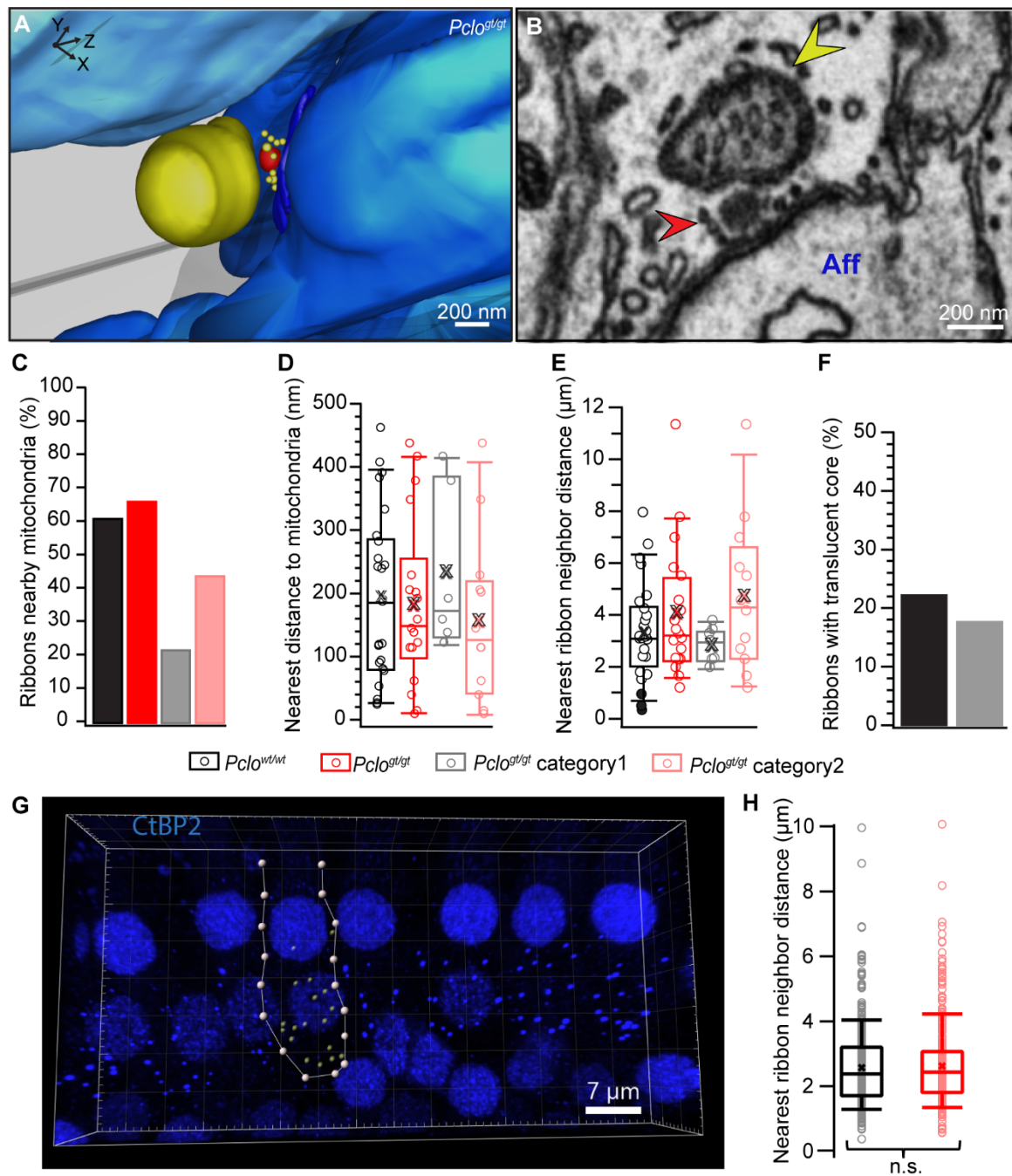268 $Pclo^{wt/wt}$ and $Pclo^{gt/gt}$ condition.

269

270 **Movie 1 and 2: FIB-SEM visualizations of the nuclear and basal region of $Pclo^{wt/wt}$ and $Pclo^{gt/gt}$ cochlear**
271 **IHCs with corresponding 3D segmentations.**
272 Movies scanning through the FIB-SEM z-stacks of $Pclo^{wt/wt}$ (Movie 1, 2 months and 4 days old) and
273 $Pclo^{gt/gt}$ (Movie 2, 3 months old) IHCs. The displayed 3D models depict IHC contours (transparent gray),
274 part of the nuclei (dark gray), innervating afferent nerve fibers (blue), ribbon synapses (red), their
275 corresponding SVs (yellow) and PSDs (dark blue). While multiribbon AZs (highlighted with red arrow) can
276 be detected in $Pclo^{wt/wt}$ IHCs, exclusively single ribbons that can be divided into two morphological
277 distinct categories (highlighted with white arrows) are found in $Pclo^{gt/gt}$ IHCs.

278 Movie1: https://owncloud.gwdg.de/index.php/s/lLuJangpAovxArc

279 Movie2: https://owncloud.gwdg.de/index.php/s/XFPfoGcwWaMH4H3

280

281

Fig EV1: Mitochondria tend to be closer to the ribbons of category 2 *Pclo^gt/gt* AZs

(A) FIB-SEM 3D model and an exemplary section **(B)** of a representative *Pclo^gt/gt* category 2 ribbon (red/arrowhead) with a mitochondrion (dark yellow/arrowhead) nearly touching the upper side of the ribbon (devoid of SVs) with an afferent bouton (Aff). **(C)** Analysis of mitochondria-proximal ribbons in a range of 500 nm around the ribbon. **(D)** Measurements of the shortest distance between synaptic ribbon and mitochondria surfaces. **(E)** Box plot of distance measurements, measured from the surfaces of the respective structure, between nearest neighboring synaptic ribbons depicted with individual data points and mean values highlighted as a cross. Black filled circles represent the distance measurements between the individual ribbons of multi-ribbon AZs. **(F)** Quantification of ribbon counts exhibiting a

291 translucent core reveals this feature only in $Pclo^{wt/wt}$ ribbons and the larger sized category 1 $Pclo^{gt/gt}$
292 ribbons (gray) but not in category 2 $Pclo^{gt/gt}$ ribbons. $N_{animals}$ = 2, n_{IHCs} = 2 respectively for $Pclo^{wt/wt}$ and
293 $Pclo^{gt/gt}$ condition. **(G)** Immunofluorescent spots corresponding to synaptic ribbons (labeled with an
294 antibody against CtBP2/RIBEYE) were detected using Imaris 9.6 (Oxford Instruments) in confocal 3D
295 projections of apical organs of Corti from 2 months old $Pclo^{wt/wt}$ and $Pclo^{gt/gt}$ rats. Yellow spots in
296 exemplary image shown here represent detected ribbons within one IHC. **(H)** Nearest ribbon neighbor
297 distance was measured between spot centers and appears comparable between $Pclo^{wt/wt}$ and $Pclo^{gt/gt}$
298 conditions, ($P > 0.05$, Mann-Whitney-Wilcoxon test), in agreement with FIB-SEM data shown in (E).
299 $n_{ribbons}$ = 435, n_{IHCs} = 33, $N_{animals}$ = 3 for $Pclo^{wt/wt}$ and $n_{ribbons}$ = 396, n_{IHCs} = 32, $N_{animals}$ = 3 for $Pclo^{gt/gt}$.

300

301
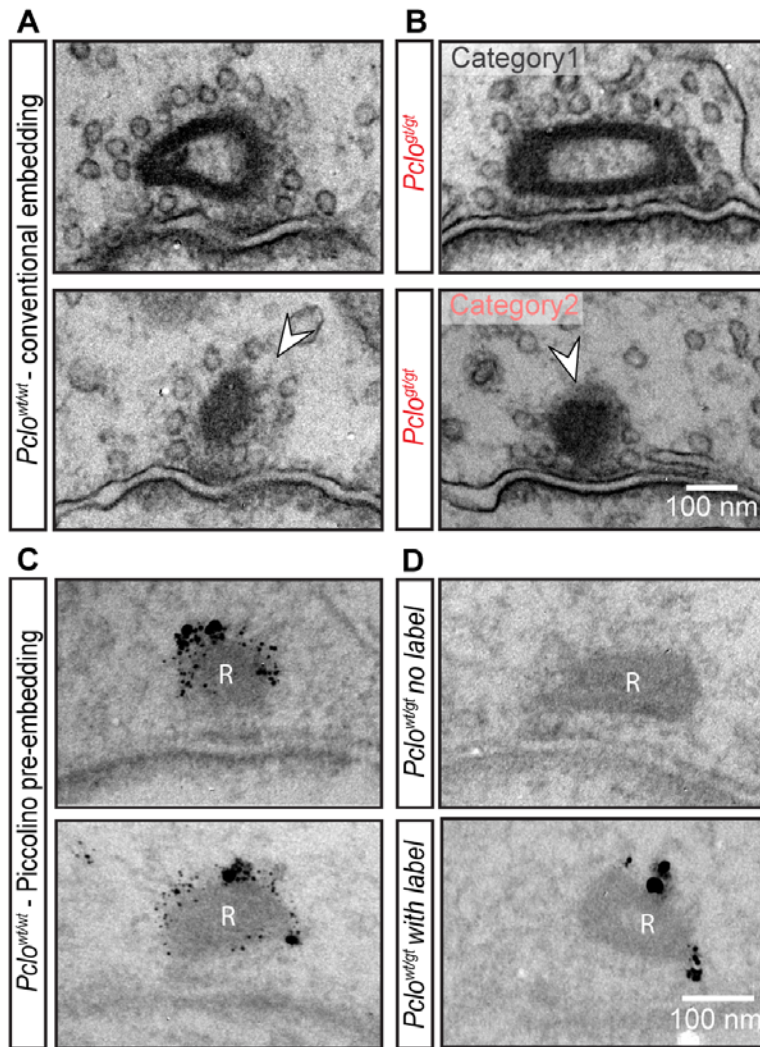
Fig EV2: 2D Transmission electron microscopy corroborates the notion of 2 ribbon categories in
$Pclo^{gt/gt}$ as well as in $Pclo^{wt/wt}$ inner hair cells – immuno-electron microscopical detection of piccolino
(A) $Pclo^{wt/wt}$ ribbons with (upper panel) and without a translucent core (lower panel). Ribbons show a SV
distribution along the full ribbon surface (arrowhead). **(B)** $Pclo^{gt/gt}$ category 1 (upper panel) with a
translucent core and category 2 (lower panel) with a lack of SVs at the upper ribbon side (arrowhead).
(C, D) Representative examples of an anti-piccolino pre-embedding immunogold labeling, which can be
found around the synaptic ribbons (R) in $Pclo^{wt/wt}$ (C) and $Pclo^{wt/gt}$ (D) IHCs. (D) In $Pclo^{wt/gt}$ IHCs, most

309 synaptic ribbons lacked anti-piccolino labeling (upper panel). However, for few ribbons the occurrence
310 of silver enhanced gold particles could be observed (lower panel).
311

	$Pclo^{wt/wt}$	$Pclo^{gt/gt}$	$Pclo^{gt/gt}$ category 1	$Pclo^{gt/gt}$ category 2
Ribbon volume (nm^3) x10^6	7.82 ± 0.95	3.09 ± 0.42	4.00 ± 0.92	2.37 ± 0.21
SV count	56.05 ± 2.22	29.78 ± 2.76	42.91 ± 3.99	20.75 ±1.31
SV density x10^{-6}	259.96 ± 14.29	261.05 ± 12.76	305.91 ± 17.18	206.32 ± 13.03
PSD area (nm^2) x10^6	0.63 ± 0.06	0.60 ± 0.04	0.68 ± 0.07	0.58 ± 0.04
NND mitochondria (nm)	198.04 ± 27.13	185.16 ± 31.27	234.79 ± 52.74	160.34 ± 38.29
NND ribbons (µm)	3.40 ± 0.38	4.08 ± 0.58	2.89 ± 0.26	4.77 ± 0.85

312 **Appendix Table 1: FIB-SEM data on count, size and distance measurements of AZ parameters.**
313 Data are presented as mean ± SEM. Abbreviations: SV = synaptic vesicles, PSD = postsynaptic density,
314 NND = nearest neighbor distance.
315
316
317 Electron tomography reveals fewer ribbon-associated SVs at category 2 mutant AZs but a normal fraction
318 of ribbon-SV filaments

319 Next, we analyzed the morphologically distinct SV pools of ribbon synapses and SV tethering as piccolino

320 was shown to interact with RIBEYE and proposed to support the SV trafficking via interacting with other

321 presynaptic proteins. We studied ribbon-associated (RA) and membrane-proximal (MP) SV pools, as well

322 as their tethering using electron tomography combined with high-pressure freezing and freeze

323 substitution (HPF/FS) which enables high resolution with a close-to-native structural preservation of

324 ribbons, SVs and filaments (Chakrabarti et al., 2018).

325 We could verify the two ribbon categories in $Pclo^{gt/gt}$ IHCs, with normal $Pclo^{wt/wt}$ resembling ribbons

326 (category 1) and altered, small and spherical ribbons, void of membrane-distal SVs (category 2) (Fig 4A-

327 C). Consequently, as for the FIB-SEM data, we determined all morphometric parameters (shown in Fig

328 4D) for all $Pclo^{gt/gt}$ AZs combined and separated into category 1 and 2. For the grouping of the

329 reconstructed ribbons of $Pclo^{gt/gt}$ rats, we performed the K-means clustering analysis, which confirmed

330 our blinded and manual annotation of AZs into category 1 and 2, including all $Pclo^{gt/gt}$ and $Pclo^{wt/wt}$

331 tomograms. Notably, in the manual analysis, none of the $Pclo^{wt/wt}$ AZs were sorted in category 2,

332 confirming that this is a morphological category only found in the mutants. Considering all mutant

333   ribbons ($Pclo^{gt/gt}$), most of the parameters were comparable to $Pclo^{wt/wt}$ (Fig 4E-G; Appendix Table 2), but

334   significantly fewer SVs were observed at $Pclo^{gt/gt}$ AZs (Fig 4H), while the differences in SV density did not

335   reach significance (Fig 4I). Furthermore, we observed a consistent difference in all our morphometric

336   parameters between category 1 and 2 $Pclo^{gt/gt}$ AZs. Interestingly, the ribbon volume, the PSD area, the

337   total SV count per ribbon as well as the RA-SV count were even increased in category 1 AZs in

338   comparison to $Pclo^{wt/wt}$ AZs (Fig 4E,G,H,K; Appendix Table 2). However, the PSD area difference did not

339   reach significance in contrast to our immunofluorescence data. This discrepancy might arise from the

340   limited section thickness of 250 nm for electron tomography, which does not allow for a full

341   reconstruction of the PSD extent.

342   Ultrastructural quantification of the two morphological SV pools (described in (Chakrabarti et

343   al., 2018)) revealed a reduction in the number of MP-SVs in category 1 and 2 AZs separately as well as in

344   the combined mutant dataset (Fig 4J; Appendix Table 3). Consistent with our FIB-SEM data, ribbons of

345   category 2 AZs lacked SVs at their membrane-distal side (Fig 4K). However, this did not affect the SV

346   density per ribbon surface area (Fig 4I). Moreover, RA-SV seemed to be further away from the synaptic

347   ribbon in both mutant AZ categories (Fig 4L; Appendix Table 3). Next, we investigated the fractions of

348   the SV-pools following our previously published approach (Chakrabarti et al., 2018). The MP- and the

349   RA-SV pool fractions were comparable between the combined mutant ribbon synapses and the

350   littermate controls (Fig 4M; Appendix Table 3). As expected from the above results, category 1 and

351   category 2 AZs showed opposing effects: while in category 1, the fraction of MP-SVs was significantly

352   reduced, it was strikingly increased in $Pclo^{gt/gt}$ category 2 AZs, which was significant when compared to

353    $Pclo^{gt/gt}$ category 1 and $Pclo^{wt/wt}$ AZs. Such an opposite effect was also found for the RA-SVs that were

354   fewer in category 2 but increased in category 1 compared to $Pclo^{wt/wt}$ AZs, explaining why we did not

355   observe overall changes when combining all mutant AZs (Fig 4M; Appendix Table 3). The analysis of SV

356   sizes from the different pools resulted in uniformly sized diameters for all groups (Fig 4N; Appendix

357   Table 3).

358   As tethering of SVs to the ribbon might involve ribbon-standing piccolino, we investigated the tethering

359   of the RA-SVs analyzing the filament number and to which structures the SVs connect to. These different

360   tethering states were previously stated as morphological SV subpools, which are present at the ribbon

361   as well as at the membrane (Chakrabarti et al., 2018). In this study, we focused on the RA-SVs since

362   piccolino was shown to localize exclusively to the ribbon (Dick et al., 2001, 20; Limbach et al., 2011;

363   Michanski et al., 2019). Filaments could be observed at both, $Pclo^{wt/wt}$ and $Pclo^{gt/gt}$ ribbon synapses (Fig

364   5A,B). When counting the number of filaments per ribbon (Fig 5D) or alternatively the numbers of all

365   filaments per tomogram (Fig 5E), we did not observe any significant differences (Appendix Table 4).

366   Subsequently, we quantified the different RA-SV-subpools as done in (Chakrabarti et al., 2018) but no

367   changes could be detected (Fig 5F; Appendix Table 4). Therefore, we conclude that tethering of the

368   remaining ribbon-associated SVs of piccolino-deficient IHC synapses is unchanged.

369   Finally, we found cisternal structures in close ribbon proximity at $Pclo^{gt/gt}$ ribbon synapses (Fig. 4C, Fig

370   EV3; Appendix Table 4). In category 2 $Pclo^{gt/gt}$ IHCs, these cisternal structures could frequently be

371   observed close to the SV lacking upper ribbon part (Fig EV3B). Similar observations were made in IHCs

372   upon disruption of bassoon (Khimich et al., 2005) and the endocytic proteins AP2 and endophilins (Jung

373   et al., 2015b; Kroll et al., 2019). Moreover, similar cisternal structures have been reported in

374   hippocampal synapses of $Pclo^{gt/gt}$ rats. The precise identity of these structures, which have been referred

375   to as endosome-like vacuoles remains to be determined.

376
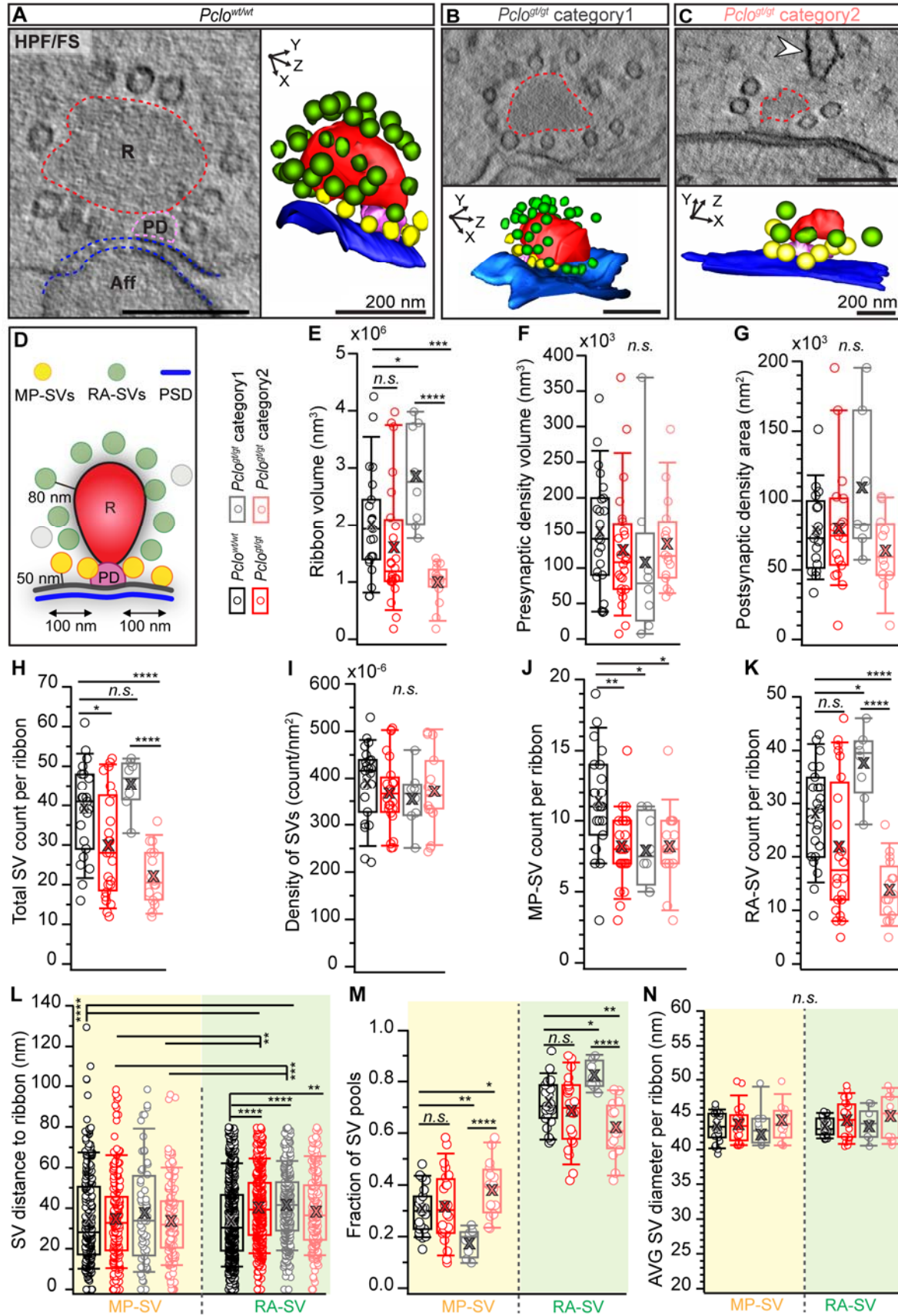
377 **Figure 4: Electron tomography reveals smaller ribbons and fewer RA-SVs in *Pclo^{gt/gt}* category 2 AZs**

378 **(A-C)** Representative virtual electron tomographic sections with their corresponding 3D models of
379 *Pclo^{wt/wt}* (A,) and *Pclo^{gt/gt}* IHCs (B, C) obtained after HPF/FS from 1-2 months old rats. Reconstructions
380 (right panels) show the ribbon (R in left panels) in red, the presynaptic density (PD) in magenta,
381 membrane-proximal synaptic vesicles (MP-SVs) in yellow, ribbon-associated synaptic vesicles (RA-SVs) in
382 green, the postsynaptic density (PSD) of the afferent bouton terminal (Aff) in blue. Based on an
383 unsupervised K-means clustering, *Pclo^{gt/gt}* ribbon synapses were also divided into category 1 and
384 category 2 AZs. Compared to *Pclo^{wt/wt}* and category 1 AZs, the upper (membrane-distal) ribbon side of
385 category 2 AZs was devoid of RA-SVs but frequently faced cisternal structures (arrowhead). **(D)**
386 Schematic illustration of tomogram analysis. **(E-N)** Box plots with individual data points and mean values
387 highlighted as crosses show the quantification of the specific parameters. While smaller ribbons were
388 observed for *Pclo^{gt/gt}* category 2 AZs and larger sized ribbons for *Pclo^{gt/gt}* category 1 (E), no significant
389 differences were detected for PD (F) and PSD (G) sizes. Significantly less SVs per ribbon were observed in
390 *Pclo^{gt/gt}* deriving mainly from the category 2 (H). The SV density (SV counts normalized to the ribbon
391 area) was unaltered in *Pclo^{gt/gt}* AZs (pooled or per category). Analysis of the two different SV pools
392 showed equally reduced MP-SV counts for all *Pclo^{gt/gt}* analysis groups (J). Significantly fewer RA-SVs were
393 found in the *Pclo^{gt/gt}* category 2, while more were present in *Pclo^{gt/gt}* category 1 (K). Nearest distance
394 measurements from the SV to the ribbon demonstrated increased distances for the RA-SV pool in all
395 three *Pclo^{gt/gt}* analysis groups (L). Opposing results regarding the SV pool fraction between category 1
396 and category 2 *Pclo^{gt/gt}* AZs were detected (M). While a greater fraction of MP-SVs was found in the
397 category 2 than in category 1, the RA-SV fraction was significantly decreased in category 2 in comparison
398 to category 1. **(N)** No differences between *Pclo^{wt/wt}* and *Pclo^{gt/gt}* in the SV diameter were observed for
399 either SV pool. *Pclo^{wt/wt}*: $N_{animals} = 3$, $n_{ribbons} = 23$, $n_{PSDs} = 17$; *Pclo^{gt/gt}*: $N_{animals} = 4$, $n_{ribbons} = 24$, $n_{PSDs} = 19$;
400 Category 1: $n_{ribbons} = 8$, $n_{PSDs} = 7$; Category 2: $n_{ribbons} = 16$, $n_{PSDs} = 12$. Significant differences between two
401 groups were analysed with the t-test or the Mann–Whitney Wilcoxon test (E-K, M). For multiple
402 comparisons, ANOVA followed by the post-hoc Tukey (E-K, M, N) or KW test followed by NPMC test (L)
403 was performed. For more detailed information see also Appendix Table 2,3.
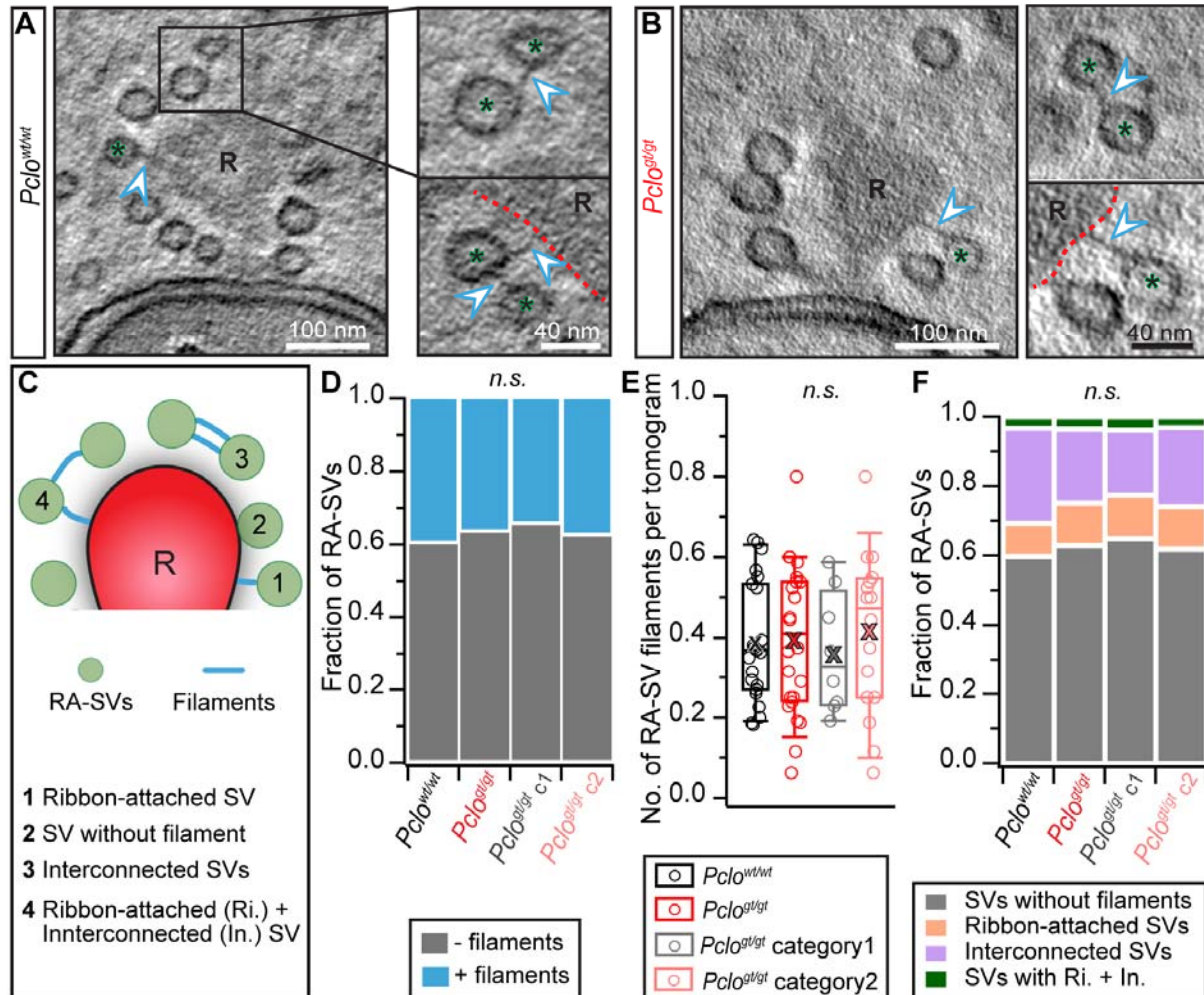
Figure 5: Normal tethering of synaptic vesicles at *Pclo^gt/gt* ribbons

(A,B) Exemplary virtual electron tomographic sections of *Pclo^wt/wt* and *Pclo^gt/gt* ribbon synapses (ribbon highlighted with "R" and/or red dotted line). Proteinaceous filaments of different types (Ri: ribbon-attached, In: interconnected, and both Ri and In; filaments are marked with arrowheads) were detected at RA-SVs (asterisks) in both *Pclo^wt/wt* and *Pclo^gt/gt* IHCs. **(C)** Illustration of the various tethering states of RA-SVs. **(D,E)** The fraction of RA-SVs with and without filaments (D) as well as the total number of RA-SV filaments per tomogram (E) in *Pclo^gt/gt* was comparable to *Pclo^wt/wt* (t-test and ANOVA with post-hoc Tukey test) . **(F)** The graph represents the fraction of RA-SVs around the ribbon regarding the filament type. No changes in the occurrence of individual filament types could be found. Multiple comparison tests of the filament types were performed using ANOVA with the post-hoc Tukey test (without filaments, Ri., In.) or KW test followed by NPMC test (Ri. + In.). *Pclo^wt/wt*: $N_{animals}$ = 3, $n_{ribbons}$ = 23; *Pclo^gt/gt*: $N_{animals}$ = 4, $n_{ribbons}$ = 24; Category 1: $n_{ribbons}$ = 8; Category 2: $n_{ribbons}$ = 16. For more detailed information see Appendix Table 4.
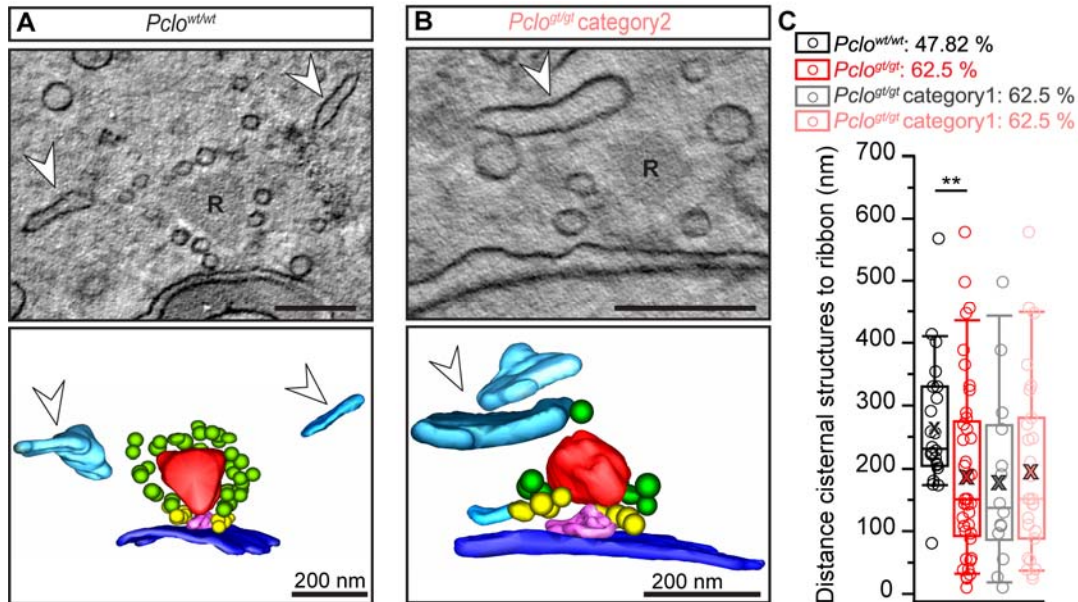
Fig EV3: Cisternal structures close to ribbon synapses frequently occur in *Pclo^gt/gt* IHCs

(A,B) Individual virtual electron tomographic sections of HPF/FS IHCs depicting a *Pclo^wt/wt* (A) and a *Pclo^gt/gt* (B) ribbon synapse (upper panel) with their corresponding 3D models (lower panel). Ribbons (R) are represented in red, presynaptic densities in magenta, MP-SVs in yellow, RA-SVs in green, postsynaptic densities in dark blue and cisternal structures in cyan, which are additionally highlighted with arrowheads. **(C)** Cisternal structures within a 1 µm range are closer to ribbons at *Pclo^gt/gt* AZs (P = 0.009, Mann–Whitney Wilcoxon test, for multiple comparison KW followed by NPMC test). *Pclo^wt/wt*: $N_{animals}$ = 3, $n_{ribbons}$ = 23; *Pclo^gt/gt*: $N_{animals}$ = 4, $n_{ribbons}$ = 24; Category 1: $n_{ribbons}$ = 8; Category 2: $n_{ribbons}$ = 16. For more detailed information see Appendix Table 4.

	Ribbon volume (nm³) x10⁶	PD volume (nm³) x10³	PSD area (nm) x10³	Total SV count	SV density x10⁻⁶
Pclo^wt/wt	2.00 ± 0.19	151.75 ± 16.79	77.55 ± 7.28	38.91 ± 2.41	391.91 ± 16.68
Pclo^gt/gt	1.61 ± 0.22	125.56 ± 17.06	80.15 ± 9.92	29.92 ± 2.68	368.75 ± 15.39
***Pclo^gt/gt* category1 (c1)**	2.86 ± 0.31	107.75 ± 41.28	109.62 ± 19.56	45.50 ± 2.27	356.94 ± 21.56
***Pclo^gt/gt* category2 (c2)**	0.99 ± 0.84	134.46 ± 15.99	62.96 ± 7.78	22.13 ± 1.78	374.67 ± 20.71
P-values	****P c1 vs. c2 ***P=0.0005 *Pclo^wt/wt* vs. c2 *P=0.031 *Pclo^wt/wt* vs. c1 n.s. *Pclo^wt/wt* vs. *Pclo^gt/gt*	n.s.	n.s.	****P *Pclo^wt/wt* vs. c2 ****P c1 vs. c2 *P=0.016 *Pclo^wt/wt* vs. *Pclo^gt/gt* n.s. *Pclo^wt/wt* vs. c1	n.s.

430 **Appendix Table 2: Counts and size measurements of AZ structures observed by HPF/FS electron**
431 **tomography.**
432 Data are presented as mean ± SEM. *P*-values between two groups were calculated using the t-test or the
433 Mann–Whitney Wilcoxon test. For multiple comparisons, the ANOVA test with post-hoc Tukey test or
434 KW followed by NPMC test was performed. Non-significant differences are indicated as *n.s.*, significant
435 differences as *P* < 0.05, **P* < 0.01, ***P* < 0.001, ****P* < 0.0001. Abbreviations: PD = presynaptic
436 density, PSD = postsynaptic density, SV = synaptic vesicle.

437

	Count		SV distance (nm)		SV fraction		SV diameter (nm)	
	MP-SV	RA-SV	MP-SV	RA-SV	MP-SV	RA-SV	MP-SV	RA-SV
$Pclo^{wt/wt}$	11.30 ± 0.75	28.04 ± 1.90	34.72 ± 1.42	33.52 ± 0.76	0.30 ± 0.02	0.72 ± 0.02	43.17 ± 0.43	43.35 ± 0.27
$Pclo^{gt/gt}$	8.17 ± 0.53	21.75 ± 2.60	35.07 ± 1.53	40.28 ± 0.77	0.32 ± 0.03	0.69 ± 0.03	43.54 ± 0.52	44.27 ± 0.53
$Pclo^{gt/gt}$ category1 (c1)	8.00 ± 0.87	37.50 ± 2.29	37.76 ± 3.18	41.50 ± 0.98	0.18 ± 0.02	0.82 ± 0.02	42.21 ± 0.51	43.26 ± 0.70
$Pclo^{gt/gt}$ category2 (c2)	8.25 ± 0.68	13.88 ± 1.40	33.76 ± 1.68	38.62 ± 1.21	0.38 ± 0.03	0.62 ± 0.03	44.21 ± 0.69	44.78 ± 0.70
P-values	**P=0.001 $Pclo^{wt/wt}$ vs. $Pclo^{gt/gt}$ *P=0.037 $Pclo^{wt/wt}$ vs. c1 *P=0.013 $Pclo^{wt/wt}$ vs. c2	****P c2 vs. $Pclo^{wt/wt}$ ****P c1 vs. c2 *P=0.012 $Pclo^{wt/wt}$ vs. c1 n.s. $Pclo^{wt/wt}$ vs. $Pclo^{gt/gt}$	****P $Pclo^{wt/wt}$ MP-SV vs. $Pclo^{gt/gt}$ & c1 RA-SV ***P=0.0007 c2 MP-SV vs. c1 RA-SV ***P=0.0003 $Pclo^{gt/gt}$ MP-SV vs. c1 RA-SV **P=0.003 $Pclo^{gt/gt}$ MP-SV vs. $Pclo^{gt/gt}$ RA-SV **P=0.004 c2 MP-SV vs. $Pclo^{gt/gt}$ RA-SV RA-SV: ****P $Pclo^{wt/wt}$ vs. $Pclo^{gt/gt}$ & c1 **P= 0.016 $Pclo^{wt/wt}$ vs. c2	MP-SV: ****P c1 vs. c2 **P=0. 006 $Pclo^{wt/wt}$ vs. c1 *P=0.019 $Pclo^{wt/wt}$ vs. c2 n.s. $Pclo^{wt/wt}$ vs. $Pclo^{gt/gt}$ RA-SV: ****P c1 vs. c2 *P=0.022 $Pclo^{wt/wt}$ vs. c1 **P=0.004 $Pclo^{wt/wt}$ vs. c2 n.s. $Pclo^{wt/wt}$ vs. $Pclo^{gt/gt}$		n.s.	

438 **Appendix Table 3: Counts, size and distance measurements of MP and RA-SV pools from HPF/FS**
439 **electron tomography.**
440 Data are presented as mean ± SEM. Depending on the normality and equality of variances, significant
441 differences between two groups were analyzed with the t-test or the Mann–Whitney Wilcoxon test. For
442 multiple comparisons, the ANOVA test with post-hoc Tukey test or KW followed by NPMC test was
443 performed. Non-significant differences are indicated as *n.s.*, significant differences as *P* < 0.05,
444 **P* < 0.01, ***P* < 0.001, ****P* < 0.0001. Abbreviations: MP-SV = membrane-proximal synaptic vesicle,
445 RA-SV = ribbon-associated synaptic vesicle.

446

	Fraction of tethers		No. of tethers per tomogram	Fraction of filament types				Distance cisternal structures
	-	+		-	Ri.	In.	Ri.+In.	
Pclo$^{wt/wt}$	0.60	0.40	0.39 ± 0.03	0.60	0.10	0.27	0.03	266.54 ± 22.25
Pclo$^{gt/gt}$	0.63	0.37	0.40 ± 0.04	0.63	0.12	0.21	0.03	191.82 ± 22.15
Pclo$^{gt/gt}$ category1 (c1)	0.65	0.35	0.36 ± 0.05	0.65	0.12	0.19	0.04	179.70 ± 37.24
Pclo$^{gt/gt}$ category2 (c2)	0.62	0.38	0.41 ± 0.05	0.62	0.12	0.23	0.03	198.10 ± 27.96
P-values	n.s.		n.s.	n.s.				**P=0.009 *Pclo$^{wt/wt}$* vs. *Pclo$^{gt/gt}$*

447 **Appendix Table 4: Electron tomographical tether analysis for RA-SVs.**
448 Data are presented as mean ± SEM. Depending on the normality and equality of variances tests,
449 significant differences between two groups were analyzed with the t-test or the Mann–Whitney
450 Wilcoxon test. For multiple comparisons, the ANOVA test followed by post-hoc Tukey test or the KW
451 followed by NPMC test was performed. Non-significant differences are indicated as *n.s.*, significant
452 differences as *$P < 0.05$, **$P < 0.01$, **$P < 0.001$, ****$P < 0.0001$. The abbreviation - stands for without,
453 + for with, Ri. for ribbon attached, In. for interconnected.

454

455 Impaired Ca^{2+} channel clustering in mutant animals
456

457 Previous studies often reported altered Ca^{2+} channel clustering upon the disruption of presynaptic

458 scaffold proteins at ribbon-type AZs (reviews in Moser et al., 2019; Pangrsic et al., 2018). For instance,

459 disruption of bassoon, RIM, RIM-BP2 as well as RIBEYE impairs Ca^{2+} channel clustering at IHC AZs (Frank

460 et al., 2010; Jean et al., 2018; Jung et al., 2015a; Krinner et al., 2017; Neef et al., 2018). To investigate

461 the impact of piccolino disruption on Ca^{2+} channel clustering, we stained acutely dissected organs of

462 Corti from 2-3 weeks old rats, as employed for IHC physiology (see below), using antibodies against

463 Ca$_V$1.3 channels, bassoon and CtBP2 and performed 2D-STED imaging (Fig 6A,B). We randomly selected

464 and imaged 176 synapses from *Pclo$^{wt/wt}$* (N$_{animals}$ = 3) and 191 synapses from *Pclo$^{gt/gt}$* (N$_{animals}$ = 3) and

465 categorized them based on the morphology of Ca^{2+} channel clusters into single-line, double-line, spot-

466 like, and complex clusters (Fig 6B), as has been previously described (Jean et al., 2018; Krinner et al.,

467 2017; Neef et al., 2018). The subjective categorization generated comparable outcomes when

468   performed by different observers. In control littermates, up to 70.5% of $Ca_V1.3$ clusters appeared as a

469   single line, while smaller fractions appeared as double lines (10.8%) and spot-like (round) clusters

470   (18.7%). Of the $Pclo^{gt/gt}$ $Ca_V1.3$ clusters, on the contrary, only about 47.7% appeared as single lines and

471   10.5% as double lines, while 28.8% of them showed a spot-like shape. Additionally, several $Ca_V1.3$

472   clusters in $Pclo^{gt/gt}$ synapses appeared as fragmented lines and rings seemingly composed of multiple

473   smaller substructures, which we categorized as complex clusters (remaining 13.1%; Fig 6C). Likely as a

474   corollary, we noted more $Ca_V1.3$ structures at $Pclo^{gt/gt}$ AZs than in wild-type (Fig 6D). We cannot rule out

475   that an artificial aggregation of untethered Ca^{2+} channels by the fixative or antibodies contributes to the

476    $Ca_V1.3$ clusters remaining as fragments and single spot-like $Ca_V1.3$ clusters (Neef et al., 2018).

477   Similar to our observations in 2 months old rats, we saw a clear reduction in the size of the synaptic

478   ribbon in younger $Pclo^{gt/gt}$ rats; $57.65 \times 10^3 \pm 1.36 \times 10^3$ nm^2, S.D. = 18.75×10^3, $n_{ribbons}$ = 191, $N_{animals}$ = 3 for

479    $Pclo^{gt/gt}$ vs. $67.81 \times 10^3 \pm 1.98 \times 10^3$ nm^2, S.D. = 26.26×10^3, $n_{ribbons}$ = 176, $N_{animals}$ = 3 for $Pclo^{wt/wt}$; $P < 0.0001$,

480   Mann-Whitney-Wilcoxon test. This difference remains significant ($P < 0.0001$, Mann-Whitney-Wilcoxon

481   test) when removing the two outliers from the control data set. The area of single-line and single spot-

482   like $Ca_V1.3$ clusters was also estimated by fitting a 2D-Gaussian function and was found to be smaller in

483    $Pclo^{gt/gt}$ IHCs (P = 0.026 for single-line clusters, P = 0.028 for single spot-like clusters and P = 0.027 for all

484   single structures; Mann-Whitney-Wilcoxon test; lines and spots pooled, represented in box plot in Fig

485   6E). This remained significant when removing the outlier from the control group (P = 0.03, Mann-

486   Whitney-Wilcoxon test). The areas of ribbons and $Ca_V1.3$ clusters showed a positive correlation for both

487    $Pclo^{wt/wt}$ and $Pclo^{gt/gt}$ (P_r = 0.53, $P < 0.0001$ for $Pclo^{wt/wt}$ and P_r = 0.44, $P < 0.0001$ for $Pclo^{gt/gt}$, considering

488   only synapses with single $Ca_V1.3$ structures, Fig. 6E).

489
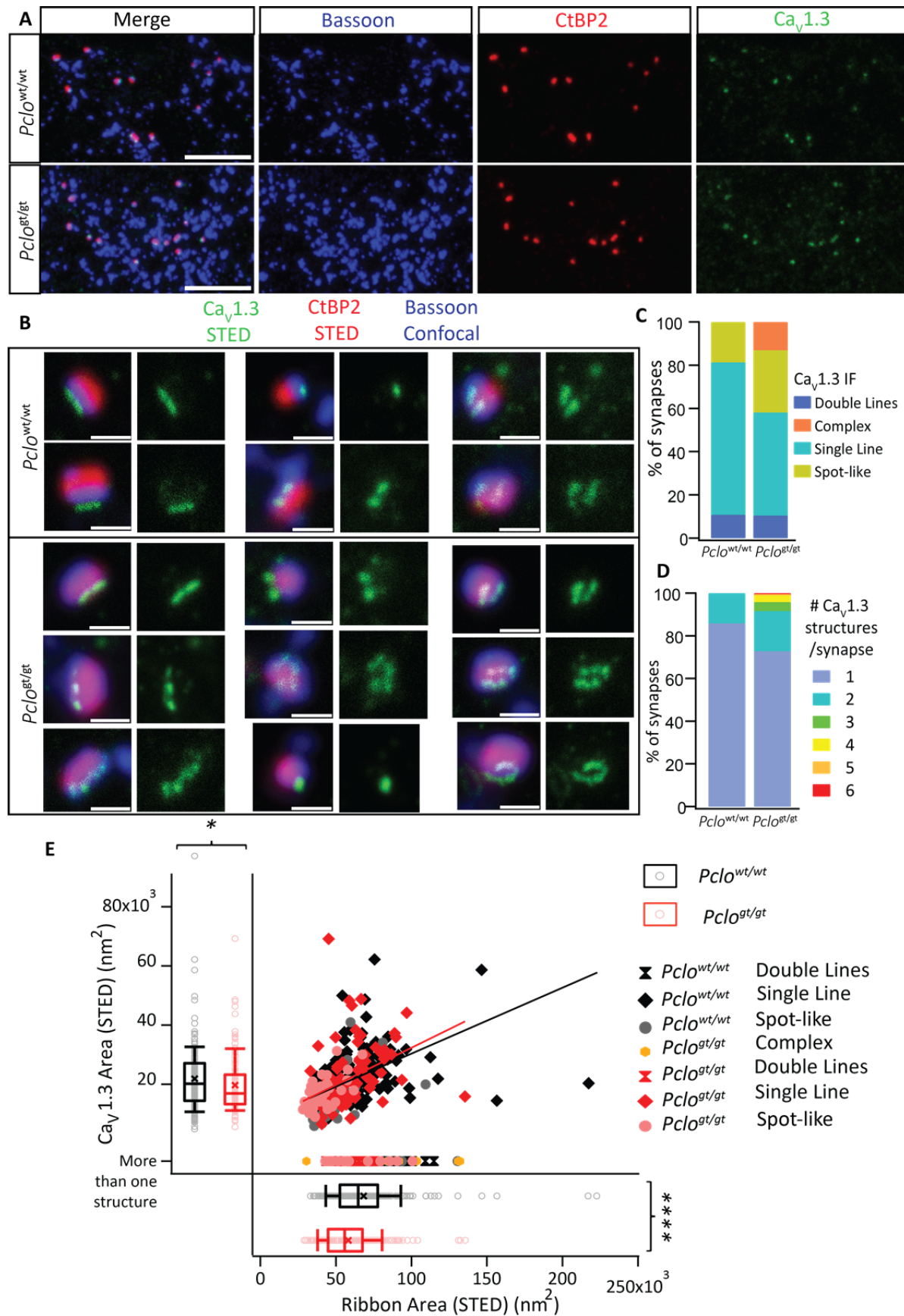
490 **Figure 6. STED imaging reveals altered morphology of Ca$_V$1.3 channel clusters upon piccolino**
491 **disruption**
492 **(A)** Maximum projections of confocal sections of organ of Corti from 2-3 weeks old rats, stained for the
493 presynaptic scaffold protein bassoon (blue), CtBP2 labeling the synaptic ribbon (red) and Ca$_V$1.3 channel
494 clusters (green). Juxtaposition of the three proteins appears comparable. Scale bars = 5 μm. **(B)**
495 Randomly selected synapses from the triple-labeling represented in (A) were further imaged using 2D-
496 STED (Ca$_V$1.3 and CtBP2) and confocal (bassoon) modes. Scale bars = 0.5 μm. Most synapses in *Pclo$^{wt/wt}$*
497 IHCs showed a single line-shaped Ca$_V$1.3 channel cluster, while some showed spot-like clusters and
498 double lines (top panel). On the contrary, *Pclo$^{gt/gt}$* synapses additionally showed Ca$_V$1.3 clusters in
499 complex arrangements, with some clusters seemingly broken into several smaller spots (lower panel).
500 **(C, D)** 176 *Pclo$^{wt/wt}$* (N$_{animals}$ = 3) and 191 *Pclo$^{gt/gt}$* (N$_{animals}$ = 3) were categorized based on the apparent
501 morphology of their Ca$_V$1.3 channel clusters into line-, double line-, spot-like and complex-clusters.
502 *Pclo$^{gt/gt}$* IHCs evidently show a higher percentage of synapses with spot-like morphology and complex
503 clusters with multiple substructures. **(E)** The 2D areas of the synaptic ribbon and of corresponding
504 Ca$_V$1.3 clusters (only ones with a single structure) were estimated by fitting a 2D-Gaussian function to
505 the STED data obtained. The ribbons in IHCs of 2-3 weeks old *Pclo$^{gt/gt}$* rats appear significantly smaller (P
506 < 0.0001, Mann-Whitney-Wilcoxon test), even after excluding the outliers in the *Pclo$^{wt/wt}$* dataset. The
507 area of Ca$_V$1.3 clusters with single structures (single line/spot; 151 *Pclo$^{wt/wt}$* synapses, 129 *Pclo$^{gt/gt}$*
508 synapses) also appears smaller (P < 0.05, Mann-Whitney-Wilcoxon test). Note the positive correlation
509 between ribbon and size of Ca$_V$1.3 clusters (P$_r$ = 0.53, P < 0.0001 for *Pclo$^{wt/wt}$* and P$_r$ = 0.44, P < 0.0001 for
510 *Pclo$^{gt/gt}$*). Ribbon areas corresponding to clusters with multiple structures have also been plotted. Box
511 plots depict individual data points overlaid with mean values shown as crosses.

512

513 Ca^{2+} current amplitude, voltage-dependence and kinetics remain unchanged upon piccolino disruption

514 We next investigated if piccolino disruption, in addition to altering the morphology of Ca^{2+} channel

515 clusters, also affects Ca$_V$1.3 Ca^{2+} channel physiology. We performed whole-cell perforated patch-clamp

516 recordings of Ca^{2+} currents from IHCs of 2-4 weeks old rats in the presence of 5 mM [Ca]$_e$ for enhanced

517 signal to noise ratio. Step depolarizations of 20 ms from -87 to 68 mV in 5 mV step increments were

518 applied to obtain current-voltage (IV) relations (Fig 7A). Ca^{2+} current amplitudes in *Pclo$^{gt/gt}$* IHCs (-175.48

519 ± 12.87 pA, n$_{IHC}$ = 20, N$_{animals}$ = 13) appeared comparable to those in *Pclo$^{wt/wt}$* IHCs (-182.23 ± 10.21 pA,

520 n$_{IHC}$ = 20, N$_{animals}$ = 13; P = 0.67, t-test, Fig 7A, Appendix Table 5). We did not find any statistically

521 significant changes in the voltage-dependence of Ca^{2+} channel activation in piccolino deficient IHCs in

522 contrast to what was observed in IHCs lacking synaptic ribbons (Jean et al., 2018) (Fig 7C). Activation and

523 inactivation kinetics of Ca^{2+} channels also appeared unaltered (Fig 7B, D; see figure legend for

524 description).

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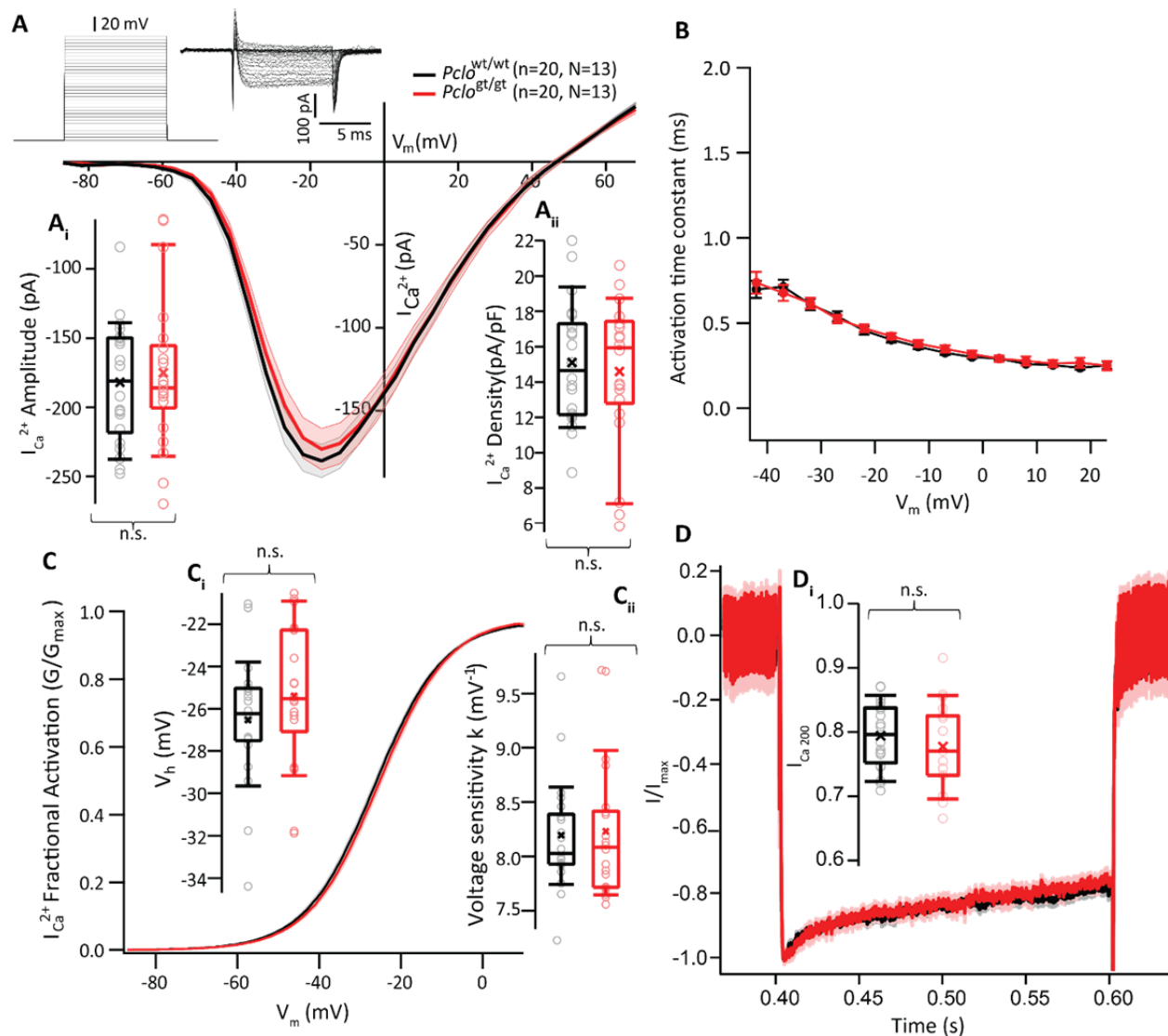
526
527 **Figure 7. $Pclo^{gt/gt}$ IHCs show normal Ca^{2+} currents**

528 **(A)** Current-voltage (IV) relations from whole-cell perforated patch clamp recordings of IHCs, $[Ca]_e = 5$
529 mM. Line represents mean current traces, shaded area represents ± SEM. **(A_i)** A comparable whole-cell
530 Ca^{2+} current amplitude ($P = 0.67$, t-test), and **(A_{ii})** current density ($P = 0.68$, t-test) was observed in IHCs
531 of 2-4 weeks old $Pclo^{wt/wt}$ ($n_{IHC} = 13$, $N_{animals} = 20$) $Pclo^{gt/gt}$ ($n_{IHC} = 13$, $N_{animals} = 20$) rats. **(B)** A power
532 exponential function was fitted on the first 5 ms of the current traces to obtain the activation time
533 constant (mean ± SEM) of Ca^{2+} current at different voltages, which was unaltered. Estimation becomes
534 less certain below -42 mV and above 23 mV, and hence these extremes were excluded from analysis. **(C)**
535 A Boltzmann function was fitted to the current traces from **(A)** to derive the fractional activation of the
536 whole-cell Ca^{2+} current. **(C_i)** Voltage of half-maximal activation (V_h) and **(C_{ii})** slope (k) of the Boltzmann fit
537 do not show any statistically significant differences ($P = 0.28$ and $P = 0.71$ respectively, t-test). **(D)**
538 Average peak amplitude-normalised Ca^{2+} currents in response to 200 ms depolarisations from -87 mV to
539 -17 mV (shaded area represents ± SEM). Residual Ca^{2+} current **(D_i)** show comparable inactivation in

540 piccolino deficient IHCs; P = 0.22, Mann-Whitney-Wilcoxon test (n_{IHC} = 18, $N_{animals}$ = 12 for $Pclo^{wt/wt}$ and

541 n_{IHC} = 17, $N_{animals}$ = 11 for $Pclo^{gt/gt}$). Box plots show individual data points overlaid; mean values are

542 shown as crosses.

543

Parameter	$Pclo^{gt/gt}$			$Pclo^{wt/wt}$			P-value
	Mean ± SEM	S.D.	Sample size	Mean ± SEM	S.D.	Sample size	
I_{Ca}^{2+}	-175.48 ± 12.87 pA	56.08 pA	n_{IHC} =20, $N_{animals}$=13	-182.23 ± 10.21 pA,	44.51 pA	n_{IHC} =20, $N_{animals}$ =13	P>0.05 (T)
I_{Ca}^{2+}density	14.57 ± 0.97 pA/pF	4.22 pA/pF		15.08 ± 0.80 pA/pF	3.50 pA/pF		P>0.05 (T)
V_h	-25.39 ± 0.78 mV	3.40 mV		-26.53 ± 0.71 mV	3.09 mV		P>0.05 (T)
k	8.23 ± 0.15 mV^{-1}	0.66 mV^{-1}		8.19 ± 0.12 mV^{-1}	0.51 mV^{-1}		P>0.05 (W)
$I_{Ca, 200}$	0.77 ± 0.02	0.09	n_{IHC} =17, $N_{animals}$ =11	0.79 ± 0.01	0.05	n_{IHC} =18, $N_{animals}$ =12	P>0.05 (W)
ΔC_m, 20 ms	12.06 ± 2.48 fF	8.23 fF	n_{IHC} =12, $N_{animals}$ =6	12.48 ± 1.94 fF	6.13 fF	n_{IHC} =11, $N_{animals}$ =8	P>0.05 (W)
Q_{Ca}, 20 ms	3.21 ± 0.38 pC	1.27 pC		3.65 ± 0.27 pC	0.85 pC		P>0.05 (W)
ΔC_m, 100 ms	35.38 ± 6.15 fF	20.41 fF		36.82 ± 7.07 fF	22.36 fF		P>0.05 (W)
Q_{Ca}, 100 ms	15.15 ± 1.80 pC	5.97 pC		17.52 ± 1.35 pC	4.27 pC		P>0.05 (W)

544 **Appendix Table 5. IHC physiology**

545 Data is represented as mean ± SEM; n_{IHC} denotes number of IHCs, $N_{animals}$ is number of rats used.

546 Statistical analysis was performed using an unpaired two-sample t test (T), or using Mann-Whitney-

547 Wilcoxon test (W). I_{Ca}^{2+} = whole cell Ca^{2+} current, V_h = voltage of half maximal activation, k = voltage

548 sensitivity of activation, $I_{Ca, 200}$ = residual Ca^{2+} current at 200 ms depolarization, ΔC_m = change in exocytic

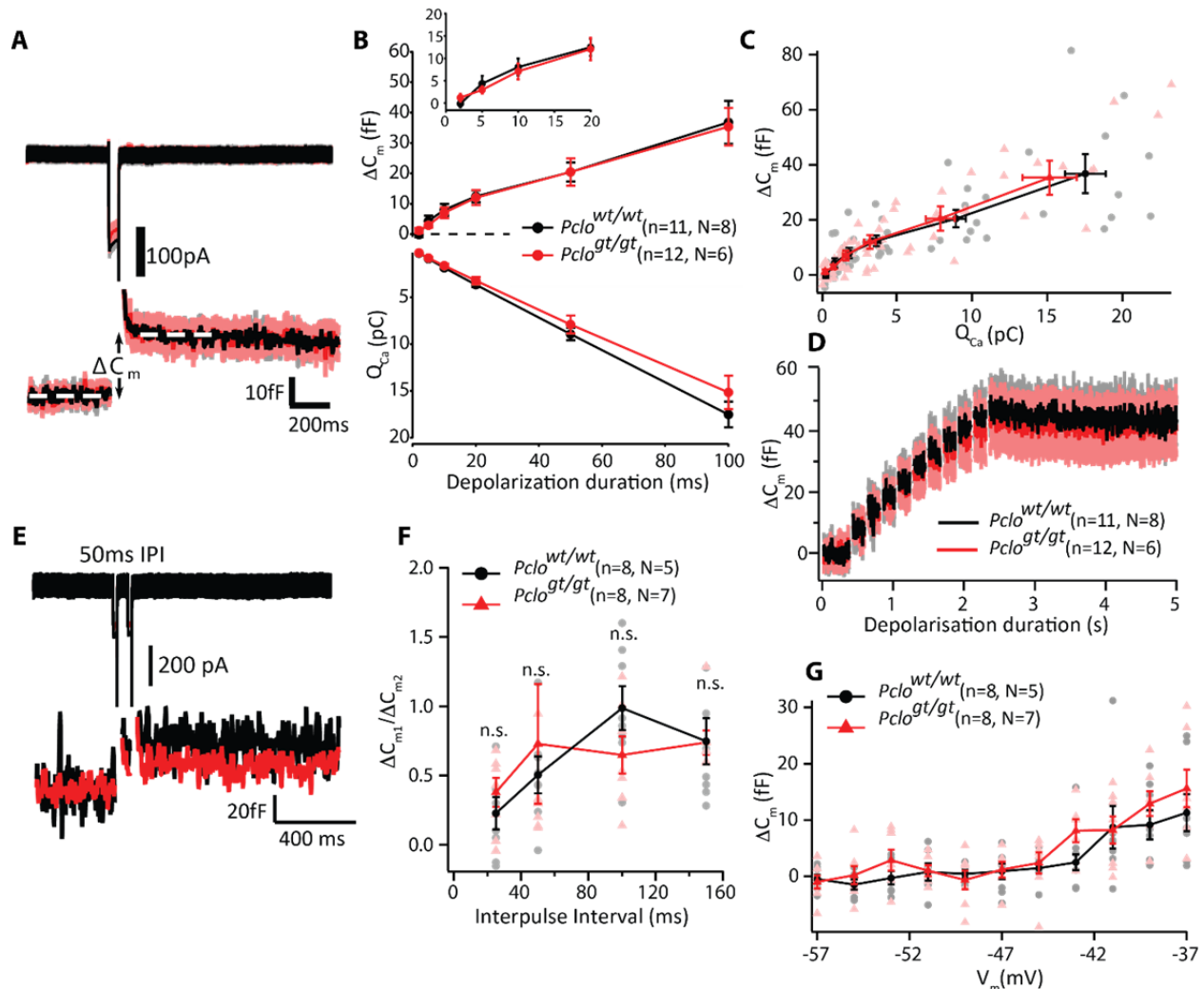549 membrane capacitance, Q_{Ca} = Ca^{2+} current integral.

550

551

552 SV exocytosis and replenishment are unaltered in $Pclo^{gt/gt}$ IHCs

553 The synaptic ribbon plays a critical role in the exocytosis of the readily releasable pool of SVs (RRP) in

554 retinal bipolar cells (Hull et al., 2006; Maxeiner et al., 2016; Snellman et al., 2011) and in rod

555 photoreceptors (Grabner and Moser, 2021). The situation is more complex for cochlear IHCs lacking

556   synaptic ribbons (ribbon-less): while the RRP was reduced in bassoon deficient IHCs (Buran et al., 2010;

557   Khimich et al., 2005) it was normal in RIBEYE deficient IHCs (Becker et al., 2018; Jean et al., 2018). Some

558   of this discrepancy can be attributed to additional effects of bassoon disruption e.g. on Ca^{2+} channel

559   abundance and topography (Frank et al., 2010; Khimich et al., 2005; Neef et al., 2018) and

560   compensatory synaptic transformations in the case of RIBEYE-deficiency (Jean et al., 2018). Here, we

561   investigated if piccolino disruption impairs SV exocytosis and replenishment. We recorded exocytic

562   membrane capacitance (ΔC_m) in response to step depolarizations (to near the potential of maximal Ca^{2+}

563   currents) using perforated patch clamp recordings in IHCs of 2-4 week old rats with 5 mM $[Ca]_e$. ΔC_m in

564   response to short and long depolarizations (recruiting the fast and sustained components of exocytosis

565   respectively, Moser and Beutner, 2000) did not show any significant differences (Fig 8B, Appendix Table

566   5). To estimate the SV replenishment, we applied ten consecutive trains of 10 ms depolarization pulses

567   (Fig 8D) and also performed paired pulse recordings (Fig 8E,F) as have been described before (Jean et al.,

568   2018; Krinner et al., 2017). Again, we did not observe alterations in $Pclo^{gt/gt}$ IHCs which seems

569   reminiscent of what was observed in ribbon-less IHCs of RIBEYE KO mice (Becker et al., 2018; Jean et al.,

570   2018). Next, we analyzed the voltage-dependence of ΔC_m by stimulating IHCs with weak physiological

571   depolarization pulses as RIBEYE KO IHCs showed a mild depolarized shift of exocytosis (Jean et al., 2018).

572   In contrast, we do not observe any such alterations in exocytosis upon piccolino disruption (Fig 8G).

573

Figure 8. SV exocytosis and replenishment appear unaltered at the synapses of piccolino-deficient IHCs.

(A) Average Ca^{2+} currents (upper panel) and corresponding changes in membrane capacitance (ΔC_m, lower panel) in response to 50 ms depolarisations from -87 mV to -17 mV (shaded area represents ± SEM) from $Pclo^{wt/wt}$ IHCs (n_{IHC} = 11, $N_{animals}$ = 8) and $Pclo^{gt/gt}$ IHCs (n_{IHC} = 12, $N_{animals}$ = 6). **(B)** Mean ± SEM ΔC_m and the corresponding Ca^{2+} current integral (Q_{Ca}) obtained in response to depolarisations of variable durations (2 to 100 ms) to -17 mV: normal phasic (≤ 20 ms) and sustained exocytosis in $Pclo^{gt/gt}$ IHCs (n_{IHC} = 12, $N_{animals}$ = 6) in comparison to $Pclo^{wt/wt}$ IHCs (n_{IHC} = 11, $N_{animals}$ = 8). Inset shows zoom-in for the first 20 ms. **(C)** ΔC_m vs. the corresponding Q_{Ca}: normal apparent Ca^{2+} dependence of exocytosis (Mean ± SEM for each pulse duration are represented as darkened points, lightly shaded points represent individual IHCs). **(D)** Mean ΔC_m traces (shaded area represents ± SEM) in response to trains (10 stimuli) of 10 ms depolarisations from -87 to -17 mV: similar responses indicate intact SV replenishment for $Pclo^{gt/gt}$ IHCs (n_{IHC} = 12, $N_{animals}$ = 6) and $Pclo^{wt/wt}$ (n_{IHC} = 11, $N_{animals}$ = 8) IHCs. **(E)** Representative ΔC_m traces in response to pairs of 20 ms pulses separated by an inter-pulse interval (IPI) of 50 ms. **(F)** Paired-pulse ratios ($\Delta C_{m2}/\Delta C_{m1}$) at varying IPIs (25, 50, 100, and 150 ms) also indicate comparable rates of SV replenishment for $Pclo^{gt/gt}$ (n_{IHC} = 8, $N_{animals}$ = 7) and $Pclo^{wt/wt}$ (n_{IHC} = 8, $N_{animals}$ = 5) IHCs. **(G)** ΔC_m in response to 100 ms

591 depolarisations of different potentials (Mean ± SEM and individual (lightly shaded points): no statistically

592 significant difference, (n_{IHC} = 8, $N_{animals}$ = 5 for $Pclo^{wt/wt}$; n_{IHC} = 8, $N_{animals}$ = 7 for $Pclo^{gt/gt}$).

593

Discussion

595 In the present study we analyzed the impact of genetic disruption of piccolino, the short isoform of the

596 multidomain protein piccolo/aczonin on the structure and function of IHC ribbon synapses. Using a

597 multidisciplinary approach, we identified a mild synaptic hearing impairment and roles of piccolino for

598 ribbon morphology, SV complement and Ca^{2+} organization at the IHC AZ. Intriguingly the observed

599 subtle structural alterations seemed to affect only a subset of AZs that, however, distributed throughout

600 the synaptic IHC pole regardless of position along the modiolar-pillar axis. Moreover, we failed to detect

601 alterations of presynaptic function at least on the whole IHC level which contrasts with a mild sound

602 encoding deficit observed on the systems level. Together our results indicate that piccolino is required

603 for normal structure and function of IHC synapses.

Mild functional and structural impairment upon piccolino disruption

605 Genetic disruption of piccolino in rats (10-weeks-old $Pclo^{gt/gt}$) resulted in a mild hearing deficit with

606 elevated ABR thresholds for middle and high frequency tone bursts and a significant reduction of the

607 amplitude of the first ABR wave reflecting an impairment in synchronous firing of SGNs. Previous studies

608 in piccolo KO mice (Mukherjee et al., 2010) reported normal sound thresholds and a reduced wave II

609 amplitude (Butola et al., 2017). In these mutants, the piccolino splice variant was not affected (Butola et

610 al., 2017), thus normal sound encoding on the level of ribbon synapses was expected, given that

611 piccolino is the only isoform present at ribbon synapses (Regus-Leidig et al., 2013). Recently, Li and co-

612 workers reported findings in mice with complete loss of piccolo and piccolino demonstrating functional

613 impairments of the retina while hearing seemed unaffected with normal ARBs up to 8 months of age (Li

614   et al., 2021). Our findings on piccolino gene-trap rats contrast the normal hearing in KO mice for which

615   we currently do not have an explanation.

616   Moreover, using STED and electron microscopy, we observed several alterations of ribbon synapse

617   morphology in cochlear IHCs of $Pclo^{gt/gt}$ rats. A fraction of the $Ca_V1.3$ Ca^{2+} channel clusters appeared

618   fragmented into smaller patches. Furthermore, focusing on single spot-like and single line-shaped $Ca_V1.3$

619   Ca^{2+} channel clusters we found them to be smaller, as were synaptic ribbons, while PSDs were enlarged.

620   Electron microscopy uncovered two morphologically distinct AZ categories at mutant synapses: category

621   1 closely resembled control AZ, while ribbons of category 2 AZs were strikingly smaller and completely

622   lacked SVs at the membrane-distal part of the ribbon. To our surprise, these structural alterations were

623   not accompanied by functional changes on the whole IHC level: Ca^{2+} currents and exocytosis of $Pclo^{gt/gt}$

624   IHCs were comparable to the controls. It will be interesting in future studies to study synaptic function

625   at the level of single synapses in order to test the hypothesis that postulated functional deficits of

626   category 2 AZs were masked by category 1 AZs. Indeed, category 1 AZs were found to be even larger

627   compared to littermate controls with an elevated number of SVs. Together, the presence of both,

628   enlarged category 1 AZs and the small category 2 AZs, yielded normal Ca^{2+} currents and exocytic

629   function at the whole IHC level. Further, the enlarged PSDs, which we observed in the $Pclo^{gt/gt}$ rats might

630   contribute to compensate for presynaptic structural deficits. Such enlarged PSDs were previously

631   observed in piccolo and bassoon mutants in conventional synapses (Mukherjee et al., 2010). *Ex vivo*

632   patch-clamp recordings from IHCs of higher frequency places of the cochlea as well as *in vivo* recordings

633   of single SGNs in future studies might help to further elucidate the relationship of exocytosis, which

634   could differ in its dependence on piccolino along the tonotopic axis, and neural sound encoding.

635   A role of piccolino as a structural determinant for ribbon synapses

636   Previously, it was shown for several species that morphological and functional attributes of IHC ribbon

637   synapses display a spatial gradient along the modiolar (or neural) – pillar (or abneural) axis. IHC AZs vary

638   in ribbon size (Kantardzhieva et al., 2013; Liberman, 1980; Meyer et al., 2009; Michanski et al., 2019;

639   Ohn et al., 2016; Payne et al., 2021), number, voltage-dependence of activation and coupling of SVs to

640   Ca^{2+} channels (Frank et al., 2009; Ohn et al., 2016; Özçete and Moser, 2021). Such synaptic diversity

641   offers an exciting candidate mechanism for how IHCs might decompose the full range of sound intensity

642   information into complementary neural codes in SGNs that have long been known to differ in

643   spontaneous and sound-evoked firing (Moser et al., 2019; Rutherford and Moser, 2016). Classically SGNs

644   have been categorized into three functional subtypes: low, medium, and high spontaneous rate (SR)

645   SGNs (Kiang et al., 1965; Liberman, 1978; Sachs and Abbas, 1974; Taberner and Liberman, 2005; Winter

646   et al., 1990). Recently, analysis of molecular SGN profiles has led to the identification of three molecular

647   SGN subtypes type 1a-c which were suggested to relate to the functional SGN subtypes (Li et al., 2020;

648   Petitpré et al., 2018; Shrestha et al., 2018; Sun et al., 2018) and might also instruct properties of the

649   corresponding presynaptic IHC AZ (Sherrill et al., 2019). Last but not least, differences in efferent

650   innervation of the peripheral SGNs neurites (e.g. Hua et al., 2021; Liberman et al., 1990) might

651   contribute to the synaptic and neurophysiological differences of SGNs (Ruel et al., 2001; Yin et al., 2014).

652   How the various mechanisms interplay to the collective sound encoding of SGNs has yet to be

653   elucidated. Specifically, how IHCs diversify their AZs remains an exciting research question. Specifically,

654   identifying molecular pathways and determinants setting the specific structure and function is a key

655   task.

656   The present finding of two categories of presynaptic AZs in *Pclo^{gt/gt}* IHCs despite the given

657   overall AZ variance provides an interesting insight into how individual AZ proteins can determine

658   structure. A structural role of piccolino at ribbon synapses was previously demonstrated in the retina

659   (Regus-Leidig et al., 2014). It is conceivable that its interaction with RIBEYE plays an important role in

660   maintaining synapse architecture (Müller et al., 2019). Already the reduction of piccolino resulted in

661   structural changes of plate-like photoreceptor ribbons to smaller, more oval shaped ones (Regus-Leidig

662   et al., 2014). Müller et al. discussed the option that RIBEYE together with piccolino organizes the plate-

663   like structure of the synaptic ribbon in the retina, specifically for rod photoreceptors and rod bipolar

664   cells. Since IHC ribbon synapses are more oval in their shape, the authors suggested that probably a

665   different RIBEYE-piccolino ratio might exist at IHC ribbon synapses, which would result in their oval

666   structure (Müller et al., 2019). Grading piccolino abundance across the AZs of individual IHCs might

667   likewise contribute to different morphologies of their ribbons. However, whether different ratios of

668   piccolino/RIBEYE prevail at the AZs of individual IHCs remains to be investigated in future experiments.

669   The present findings on piccolino deficient IHCs did not point to preferred position along the modiolar-

670   pillar axis of the AZ categories, which would not seem to support a major role of piccolino in shaping

671   synaptic heterogeneity in IHCs. We speculate that the ribbon of category 2 AZ found upon disrupting

672   piccolino might represent a sort of basic IHC ribbon version, which is then modulated by the amount of

673   piccolino, while category 1 might reflect a compensatory potential specific to a subset of synapses.

674   Addressing functional differences between the AZ categories in $Pclo^{gt/g}$ IHCs will require future single

675   synapse analysis (Goutman and Glowatzki, 2007; Özçete and Moser, 2021).

676   Normal synaptic vesicle tethering at piccolino-deficient AZs

677   Piccolino was shown to bind to RIBEYE, while its N-terminus faces the cytoplasm and was suggested to

678   regulate steps in the SV cycle by binding to other presynaptic proteins (Müller et al., 2019). In line with

679   previous studies for photoreceptor ribbon synapses (Limbach et al., 2011; Müller et al., 2019), our

680   immunogold labelings of rodent ribbon synapses confirmed that piccolino localizes to synaptic ribbons

681   of IHCs (the present study, (Michanski et al., 2019)). Moreover, ribbons of $Pclo^{gt/gt}$category 2 AZs were

682   void of membrane distal SVs, which could point either to a role of piccolino in SV tethering or in enabling

683   a tethering-relevant ribbon part. Importantly, normal ribbon-tether number of those remaining RA-SVs

684   at category 2 AZs argue against an essential role of piccolino in tethering SVs to the ribbon. Nonetheless,

685   the increased distance of SVs to the ribbon surface of AZs could indicate a contribution of piccolino in

686   attracting SVs. Moreover, a previous study has shown that a 110 kDa piccolino fragment is still

687   detectable in $Pclo^{gt/gt}$ rat retina (Müller et al., 2019), which could exert some residual function. For

688   example, the interaction site for the actin/dynamin-binding protein Abp1 is likely still present in the

689    $Pclo^{gt/gt}$ mutant rats, while the RIBEYE interaction site, which is located in the C-term region of piccolino,

690   is eliminated (Müller et al., 2019). Fenster et al. showed the interaction between piccolo and Abp1,

691   which also binds to both F-actin and the GTPase dynamin. This led to the hypothesis that piccolo

692   localizes Abp1 to AZs to create a functional connection between the dynamic actin cytoskeleton and SV

693   recycling at conventional synapses (Fenster et al., 2003), the relevance of which for ribbon synapses

694   remains to be tested.

695   Key molecular organizers of the ribbon-type AZs in IHCs

696   Bassoon and piccolo are important scaffold proteins at AZs with partially overlapping functions (review

697   in Gundelfinger et al., 2015). Disruption of piccolo resulted in normal sized AZs but fewer SVs in AZs of

698   endbulb of Held synapses (Butola et al., 2017). In AZs of rat IHCs, the disruption of piccolino had several

699   structural consequences (Fig 9). Mutant AZs showed only single ribbons and generally fewer MP-SVs.

700   Moreover, a subset of AZs with smaller ribbons was found, which lack SVs at their membrane distal side.

701   The Ca^{2+} channel clusters appeared fragmented, resembling what has been shown for IHC AZs in RIBEYE

702   mutants (Jean et al., 2018). Altered localization and reduced number of Ca^{2+} channels were previously

703   reported for RIM2 (Jung et al., 2015a) and RIM-BP2 (Krinner et al., 2017) deficient IHC AZs. RIM2 and

704   RIM-BP2 positively regulate the number of synaptic $Ca_V1.3$ channels at IHC AZs and promote fast SV

705   recruitment to the RRP. Loss of the ribbon due to disruption of RIBEYE resulted in subtle functional

706   impairments in IHCs and hearing (Fig 9) (Becker et al., 2018; Jean et al., 2018), while in rod

707   photoreceptors RIBEYE disruption dwarfed the RRP to a third (Grabner and Moser, 2021). Maintained

708   presynaptic IHC function in ribbonless IHCs was attributed to partial compensation that encompasses

709   several small bassoon-positive presynaptic densities at ribbon-less AZs tethering a large number of SVs

710   (Jean et al., 2018). This raised the question if ribbons are dispensable for the function of IHC AZs as long

711   as bassoon remains present. Piccolino lacks the interactions site to bassoon (Regus-Leidig et al., 2013),

712   and different from conventional synapses, piccolino and bassoon segregate at ribbon-type AZs (Dick et

713   al., 2001; Khimich et al., 2005; Limbach et al., 2011; Michanski et al., 2019; tom Dieck et al., 1998; Wong

714   et al., 2014). In contrast to the mild sensory coding phenotypes of piccolino mutants (the present study,

715   (Li et al., 2021; Müller et al., 2019)), disruption of bassoon strongly alters transmission at afferent

716   synapses of retina and cochlea (Fig 9). The deletion of exon 4 and 5 ($Bsn^{\Delta Ex4/5}$) resulted in detached

717   synaptic ribbons in photoreceptors and IHCs and impaired sensory coding assayed at the systems and

718   synaptic levels (Dick et al., 2003; Khimich et al., 2005). In addition, to the reduced RRP, $Bsn^{\Delta Ex4/5}$ IHC AZs

719   showed fewer and mislocalized Ca^{2+} channels (Frank et al., 2010; Khimich et al., 2005; Neef et al., 2018).

720   It has remained challenging to disentangle effects of ribbon-loss and overall AZ alterations upon bassoon

721   disruption (Frank et al., 2010; Jing et al., 2013), in particular given the mild functional deficits in ribbon-

722   less IHCs of RIBEYE KOs (Becker et al., 2018; Jean et al., 2018). We suggest that bassoon - as the major

723   component of the presynaptic density (Wong et al., 2014) - is the main organizer at IHC ribbon synapse

724   AZs and might even be able to tether SVs to the presynaptic density. Piccolino and RIBEYE likely jointly

725   determine ribbon shape and size and might thus finetune exocytosis at IHC ribbon synapses.

726

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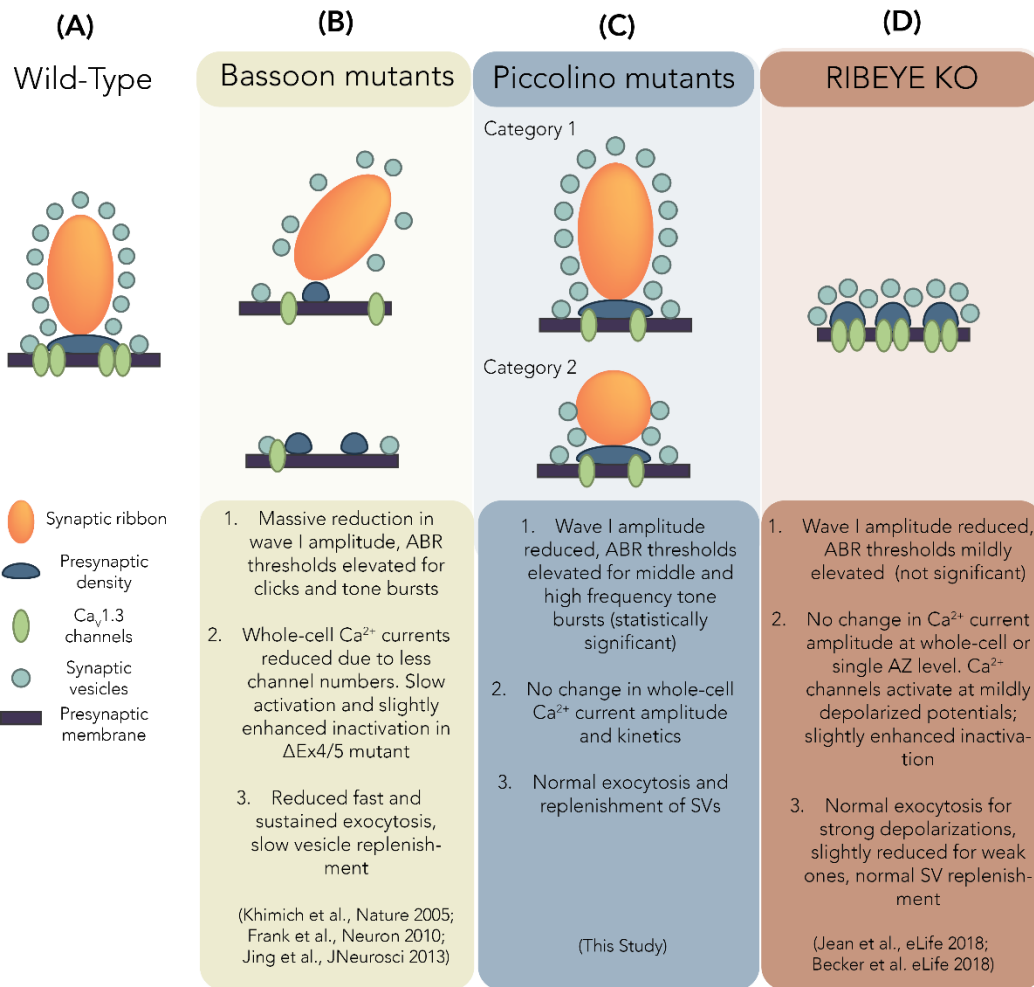
Figure 9: Summary of key structural and functional observations at IHC AZs upon genetic perturbation of bassoon, piccolino and RIBEYE. (A) Wild-type IHC synaptic ribbons drawn for reference; these are typically ellipsoid shaped and anchored to the presynaptic membrane via the presynaptic density. **(B)** Disruption of bassoon causes ribbon anchorage defects, resulting in ribbon-less AZs (lower panel, representing partial deletion ΔEx4/5 mutants) or loosely anchored ribbons (top panel, representing the predominant form in bassoon gene-trap mutants). **(C)** Disruption of piccolino results in two categories of AZs: category 1 wild-type-like ribbons and roundish, small ribbons for category 2 AZs that lack SVs at their membrane-distal apex. **(D)** RIBEYE deficient IHCs show ribbon-less AZs with multiple smaller presynaptic densities and SV assemblies feeding into the same postsynapse. Ca^{2+} channel clusters appear smaller and fragmented in IHCs of bassoon, piccolino and RIBEYE mutants.

Material and Methods

Animals

740

741

742 We used a piccolo gene trap ($Pclo^{gt/gt}$) rat strain, which is described in detail in (Medrano et al., 2020).

743 Briefly, transposon mutagenesis resulted in the integration of a transposon element into exon 3 of the

744 *PCLO* gene, leading to a stop in the reading frame. $Pclo^{gt/gt}$ rats and wild-type littermate controls

745 ($Pclo^{wt/wt}$), from heterozygous breeding of either sex between 2 weeks and 11 months, were deeply

746 anesthetized with CO_2 and sacrificed by decapitation for immediate dissection of the cochleae. All

747 experiments complied with national animal care guidelines and were approved by the University of

748 Göttingen Board for Animal Welfare and the Animal Welfare Office of the State of Lower Saxony.

749

Systems physiology

750

751 ABR and DPOAE were performed for 2-months old rats as described before (Jing et al., 2013; Strenzke et

752 al., 2016). Animals were anesthetized intraperitoneally with a combination of ketamine (125 mg/kg) and

753 xylazine (2.5 mg/kg), and the heart rate was constantly monitored to control the depth of anesthesia.

754 The core temperature was maintained constant at 37°C using a rectal temperature-controlled heat

755 blanket (Hugo Sachs Elektronik–Harvard Apparatus). For stimulus generation, presentation, and data

756 acquisition, we used the TDT III Systems (Tucker Davis Technologies) run by BioSig32 software (Tucker

757 Davis Technologies). Sound pressure levels (SPL) are provided in decibels SPL root mean square (RMS)

758 (tonal stimuli) or decibels SPL peak equivalent (clicks) and were calibrated using a ¼ inch Brüel and Kjær

759 microphone (model 4939). Tone bursts (4/8/12/16/24/32 kHz, 10 ms plateau, 1 ms cos^2 rise/fall) or

760 clicks of 0.03 ms were presented at 20 Hz in the free field ipsilaterally using a JBL 2402 speaker. The

761 difference potential between vertex and mastoid subdermal needles was amplified (50,000 times),

762 filtered (low pass, 4 kHz; high pass, 100 Hz) and sampled at a rate of 50 kHz for 20 ms, 2 × 2000 times to

763   obtain two mean ABRs for each sound intensity. Hearing threshold was determined with 10 dB precision

764   as the lowest stimulus intensity that evoked a reproducible response waveform in both traces by visual

765   inspection. For DPOAEs, a 24-bit sound card and the MF1 speaker system (Tucker David Technologies)

766   were used to generate two primary tones (f2/f1 ratio: 1.2). Primary tones were coupled into the ear

767   canal by a custom-made probe containing an MKE-2 microphone (Sennheiser) and adjusted to an

768   intensity of 60 dB sound pressure level at the position of the ear drum as mimicked in a mouse ear

769   coupler. The microphone signal was amplified (DMX6Fire; Terratec) and analyzed by fast Fourier

770   transformation using custom software (Matlab, mathworks).

771

772   Immunohistochemistry and Imaging

773   Cochleae from 2-3 weeks old (postnatal day P14 - P27) and 2 months old rats were dissected in ice-cold

774   HEPES Hank's solution containing (in mM): 5.26 KCl, 141.7 NaCl, 0.5 $MgSO_4$-7H_2O, 10 HEPES, 1 $MgCl_2$,

775   11.1 D-glucose and 3.42 L-glutamine, pH adjusted to around 7.2, osmolality ~300 mOsm/kg. Fixation was

776   performed by perfusing the cochleae with 4% formaldehyde (in PBS) for 60 min on ice, while for staining

777   Ca^{2+} channel clusters, a shorter fixation of 5-10 min was performed. For cochleae from 2 months old

778   rats, decalcification was additionally performed using Morse's solution (10% sodium citrate, 22.5%

779   formic acid) for 15-20 min. The organ of Corti was dissected and washed three times in PBS at room

780   temperature. Blocking and permeabilisation of the tissue was performed with GSDB (goat serum dilution

781   buffer: 16% normal goat serum, 450 mM NaCl, 0.3% Triton X100, 20 mM phosphate buffer, pH ~7.4) for

782   1 h at room temperature. Samples were then incubated with primary antibodies (diluted in GSDB, refer

783   to table 1) overnight at 4°C and were washed three times for 10 min in wash buffer (450 mM NaCl, 0.3%

784   Triton X 100, 20 mM phosphate buffer, pH ~ 7.4). This was followed by incubation with respective

785   secondary antibodies (diluted in GSDB, refer to table 1) for 1 h in a light-protected wet chamber. Finally,

786 the samples were washed three times for 10 min in wash buffer and in 5 mM phosphate buffer for 5

787 min, before mounting onto glass slides with a drop of fluorescence mounting medium (*Mowiol* 4-88,

788 Carl Roth) and covered with thin glass coverslips. Images were acquired in confocal/STED mode using an

789 Olympus IX83 inverted microscope combined with an Abberior Instruments Expert Line STED

790 microscope (Abberior Instruments GmbH). We employed lasers at 488, 561, and 633 nm for excitation

791 and a 775 nm (1.2W) laser for stimulated emission depletion. 1.4 NA 100X or 20X oil immersion

792 objectives were used. Confocal stacks were acquired using *Imspector* Software (pixel size = 80 X 80 nm

793 along xy, 200 nm along z). For 2D-STED images, a pixel size of 15 X 15 nm (in xy) was used. The acquired

794 images were z-projected with NIH ImageJ software and adjusted for brightness and contrast. Organs of

795 Corti from both *Pclo$^{wt/wt}$* and *Pclo$^{gt/gt}$* were always processed in parallel using identical staining protocols,

796 laser excitation powers and microscope settings.

797 **Table 1:** List of antibodies:

Antibody	Host specie	Company	Dilution	Identifier
Anti-parvalbumin	Guinea pig (polyclonal)	Synaptic Systems	1:200	195 004
Anti-CtBP2	Mouse (monoclonal IgG1)	BD Biosciences	1:200	612044
Anti-piccolo	Rabbit (polyclonal)	Synaptic Systems	1:200	142 113
Anti-bassoon	Chicken (polyclonal)	Synaptic Systems	1:200	141 016
Anti-homer1	Rabbit (polyclonal)	Synaptic Systems	1:200	160 002
Anti-Ca$_V$1.3 (CACNA1D)	Rabbit (polyclonal)	Alomone Labs	1:100	ACC 005
Alexa Fluor 488 conjugated anti-guinea pig	Goat (polyclonal)	Invitrogen	1:200	A11073
Alexa Fluor 568 conjugated anti-rabbit	Goat (polyclonal)	Thermo Fisher	1:200	A11011
STAR635p conjugated anti-mouse	Goat (polyclonal)	Abberior	1:200	2-0002-007-5
Alexa Fluor 488 conjugated anti-rabbit	Goat (polyclonal)	Invitrogen (MoBiTec)	1:200	A11008
Alexa Fluor 568 conjugated anti-chicken	Goat (polyclonal)	Abcam	1:200	Ab175711
Alexa Fluor 488 conjugated anti-chicken	Goat (polyclonal)	Invitrogen	1:200	A11039
STAR580 conjugated anti-	Goat (polyclonal)	Abberior	1:200	2-0002-005-1

mouse				
STAR635p conjugated anti-rabbit	Goat (polyclonal)	Abberior	1:200	2-0012-007-2

798

Focused ion beam-scanning electron microscopy (FIB-SEM)

800 Enhanced en bloc staining for FIB-SEM samples of 2 $Pclo^{wt/wt}$ and 2 $Pclo^{gt/gt}$ rats was performed according

801 to Deerinck et al. (Deerinck et al., 2018) and to our previous studies (Jean et al., 2020; Michanski et al.,

802 2019). Organs of Corti, within the apical turn of the cochlea from 2-3 months old rats, were isolated in

803 ice-cold HEPES Hank´s solution (5.36 mM KCl (746436, Sigma-Aldrich, Germany), 141.7 mM NaCl

804 (746398, Sigma-Aldrich, Germany), 10 mM HEPES (H3375, 006K5424, Sigma-Aldrich, Germany), 0.5 mM

805 $MgSO_4$-$7H_2O$ (Sigma-Aldrich, Germany), 1 mM $MgCl_2$ (M2670, Sigma-Aldrich, Germany), 2 mg/ml D-

806 glucose (G8270-1KG, Sigma-Aldrich, Germany), 0.5 mg/ml L-glutamine (G3126-100G, #SLBS8600, Sigma-

807 Aldrich, Germany) and was adjusted to pH 7.2, ~300 mmol/kg). After the dissection, organs were

808 immediately fixed with 4% paraformaldehyde (0335.1, Carl Roth, Germany) and 0.5% glutaraldehyde

809 (G7651, Sigma-Aldrich, Germany) in PBS (P4417, Sigma-Aldrich, Germany; pH 7.4) for 1 h on ice followed

810 by a second fixation step overnight with 2% glutaraldehyde in 0.1 M sodium cacodylate buffer (v/v, pH

811 7.2) at 4°C. The next day, fixed tissues were treated with a 1.5% potassium ferrocyanide (25154-10,

812 EMS) and 4% osmium tetroxide solution (75632.5ml, Sigma-Aldrich, Germany; v/v in 0.1 M sodium

813 cacodylate buffer) for 1 h on ice. Specimens were then briefly washed 5 times in distilled water and

814 placed in a thiocarbohydrazide (w/v in distilled water) solution for 20 min followed by additional 5 brief

815 washing steps in distilled water at room temperature (RT). Next, a second incubation into 2% osmium

816 tetroxide (v/v in 0.1 M sodium cacodylate buffer) for 2 h at RT followed with subsequent 5 brief washing

817 steps in distilled water before the samples were placed in 2.5% uranyl acetate (v/v in distilled water)

818 overnight at RT and in darkness. The following day, samples were briefly washed 5 times in distilled

819 water at RT and contrasted with Reynold's lead citrate (Reynolds, 1963) for 30 min at 60°C to be

820   subsequently washed once again in distilled water, dehydrated in increasing ethanol concentrations

821   (30%, 50%, 70%, 95% and 100%), infiltrated and finally embedded in Durcupan (25%, 50%, 75%

822   Durcupan in Acetone for 1 h each and 100% Durcupan overnight at RT and for another 2 h with fresh

823   100% Durcupan on the following day; 44610, Sigma-Aldrich, Germany) to get polymerized for at least 48

824   h at 60°C. The cured blocks were then trimmed with a 90° diamond trimming knife (Diatome AG, Biel,

825   Switzerland), attached to SEM stubs (Science Services GmbH, Pin 12.7 mm x 3.1 mm) using a silver filled

826   epoxy (Epoxy Conductive Adhesive, EPO-TEK EE 129-4; EMS) and polymerized at 60° overnight.

827   Afterwards, samples on SEM stubs were coated with a 10 or 15 nm platinum or gold layer using the

828   sputter coating machine EM ACE600 (Leica Microsystems) at 30 mA current to be finally placed into the

829   Crossbeam 540 FIB-SEM (Carl Zeiss Microscopy GmbH) and positioned at 54°. A 400 or 500 nm carbon or

830   platinum layer was deposited on top of the regions of interest and the Atlas 3D (Atlas 5.1, Fibics,

831   Canada) software was used to collect the acquired image dataset. Specimens were exposed to the ion

832   beam driven with a 30 nA current while a 7 or 15 nA current was applied to polish the surface. Images

833   were acquired at 1.5 kV (1000 pA) using the ESB detector (450 V ESB grid, pixel size x/y 3 or 5 nm) in a

834   continuous mill and acquire mode using 700 pA or 1.5 nA for the milling aperture (z-step 5 or 10 nm).

835   For subsequent post processing, data were aligned using the Plugin "Linear Stack Alignment with SIFT",

836   inverted and cropped in Fiji. Depending on the dataset properties, a Gaussian blur, local contrast

837   enhancement using a CLAHE plugin in Fiji, and a binning by 2 in x/y was applied (Schindelin et al., 2012).

838

839   High-pressure freezing (HPF) and Freeze-substitution (FS)

840   High-pressure freezing was essentially performed as described previously (Chapochnikov et al., 2014;

841   Jung et al., 2015a). In brief, the apical turn cochlear organs from 1-2 months old $Pclo^{wt/wt}$ and $Pclo^{gt/gt}$

842   rats were dissected in ice-cold HEPES Hank´s solution and mounted onto type A specimen carriers (Leica

843   Microsystems, Wetzlar, Germany; 3 mm in diameter and 0.2 mm in depth). The 1-hexadecene (Sigma-

844   Aldrich, Germany) coated flat side of the type B carriers (Leica Microsystems, Wetzlar, Germany; 3 mm

845   in diameter and 0.1 mm in depth) was then placed onto the sample containing type A carrier. The

846   assembled carriers were loaded into the middle plates of the high-pressure freezing sample holder and

847   excess liquid was removed with filter paper. Afterwards, the sample holder was assembled and loaded

848   into the HPM100 (Leica Microsystems, Wetzlar, Germany) to cryofix the tissues and store them in liquid

849   nitrogen until further processing. Subsequently, the high-pressure frozen samples were freeze-

850   substituted using the EM AFS2 (Leica Microsystems, Wetzlar, Germany) machine. In brief, organs were

851   incubated in 0.1% (w/v) tannic acid in acetone at -90°C for 4 days followed by 3 washing steps in

852   acetone at -90°C for 1 h, respectively. Next, 2% (w/v) osmium tetroxide in acetone was applied and

853   incubated at -90°C for 7 h. During the following 33.4 h the temperature gradually rose up to 4°C and

854   osmium tetroxide in acetone was removed, samples were washed in acetone 3 times (1 h each) and

855   brought to RT. Finally, samples were infiltrated and embedded in epoxy resin (Agar-100 kit, Plano,

856   Germany; epoxy/acetone 1:1 3-6 h; 100% epoxy resin overnight and 3-6 h on the next day) to get

857   polymerized for at least 48 h at 70°C.

858

859   Conventional embedding

860   Cochlea organs from 3 months old $Pclo^{wt/wt}$ and $Pclo^{gt/gt}$ rats (one animal each) were dissected as

861   described above. Subsequently, organs were fixed immediately after dissection with 4%

862   paraformaldehyde (0335.1, Carl Roth, Germany) and 0.5% glutaraldehyde (G7651, Sigma, Germany) in

863   PBS (P4417, Sigma, Germany; pH 7.4) for 1 h on ice followed by a second fixation step overnight with 2%

864   glutaraldehyde in 0.1 M sodium cacodylate buffer (v/v, pH 7.2) at 4°C. Next, specimens were washed in

865   0.1 M sodium cacodylate buffer and treated with 1% osmium tetroxide (75632.5ml, Sigma, Germany;

866   v/v in 0.1 M sodium cacodylate buffer) for 1 h on ice followed by further sodium cacodylate buffer and

867   distilled water washing steps. After the *en bloc* staining with 1% uranyl acetate (8473, Merck, Germany;

868   v/v in distilled water) for 1 h on ice, samples were briefly washed in distilled water, dehydrated in an

869   ascending concentration series of ethanol (30%, 50%, 70%, 95% and 100%), infiltrated and embedded in

870   epoxy resin (R1140, AGAR-100, Plano, Germany) to get finally polymerized for at least 48 h at 70°C.

871

872   Immunogold pre-embedding

873   The Triton X immunogold pre-embedding protocol was applied according to our previous study

874   (Michanski et al., 2019). In brief, cochlea organs from 10-11 months old $Pclo^{wt/wt}$ and $Pclo^{wt/gt}$ rats

875   ($N_{animals}$ = 2 for each genotype) were dissected as mentioned above and fixed with 2% paraformaldehyde

876   and 0.06% glutaraldehyde in PEM (0.1 M PIPES: P1851-500g, Sigma, Germany; 2 mM EGTA: E3889,

877   Sigma, Germany; 1 mM $MgSO_4$ x 7 H_2O, v/v) for 90 min on ice. Afterwards, samples were washed in PEM

878   and blocked for 1 h in 2% bovine serum albumin (BSA; 900099, Aurion)/ 3% normal horse serum (NHS;

879   VEC-S-200, Biozol, Germany) in 0.02% PBST (0.02% Triton X-100 (X100-500ml, Sigma, Germany) diluted

880   in PBS, v/v). Next, samples were incubated with the anti-rabbit piccolo primary antibody (polyclonal,

881   Synaptic Systems: 142113; 1:200 diluted in 0.02% PBST), detecting the long and the short (piccolino)

882   isoform of piccolo for 1 h at RT and overnight at 4°C. Subsequently, specimens were washed with 0.02%

883   PBST and incubated for 2 h with the 1.4 nm gold-coupled anti-rabbit secondary antibody (Nanogold-

884   anti-rabbit, Nanoprobes; 1:30 diluted in 0.02% PBST) followed by another washing step in 0.02% PBST

885   for 30 min and overnight at 4°C. Next day, after further washing steps in 0.02% PBST, samples were

886   post-fixed with 2% glutaraldehyde in PBS (v/v) for 30 min and briefly washed in distilled water. For silver

887   enhancement, the HQ Silver-enhancement kit (Nanoprobes) was used for 3 min in the dark and

888   specimens were briefly washed four times. Further fixation was obtained by the treatment with 2%

889   osmium tetroxide (v/v in 0.1 M cacodylate buffer) for 30 min followed by one washing step in distilled

890   water for 1 h and two washing steps in distilled water for 30 min, respectively. Finally, samples were

891   dehydrated in an ascending concentration series of ethanol (30%, 50%, 70%, 95% and 100%), infiltrated

892   and embedded in epoxy resin (R1140, AGAR-100, Plano, Germany) and polymerized for at least 48 h at

893   70°C.

894   Ultrathin-sectioning and post-staining

895   The polymerized blocks were trimmed into a pyramidal shape to remove excess resin and 70 nm

896   ultrathin sections were cut with a 35° diamond knife (Diatome AG, Biel, Switzerland) using an EM UC7

897   (Leica Microsystems, Wetzlar, Germany) ultramicrotome in order to check for the correct region as well

898   as the structural preservation. Ultrathin sections were collected on 1% formvar-coated copper slot

899   grids (Athene, 3.05 mm diameter, 1 x 2 mm; G2500C, Plano). For electron tomography, 250 nm semi-

900   thin sections were obtained and collected on 1% formvar-coated mesh grids (100 mesh; Athene, 3.05

901   mm diameter; G2410C, Plano). For both sectioning techniques, post-staining was performed using

902   UranyLess (22409, EMS, Hatfield, PA) for 20 min followed by several brief washing steps with distilled

903   water.

904

905   Transmission electron microscopy and electron tomography

906   For conventional embedded samples, immunogold pre-embedded samples and to first check for the

907   quality of the cryo-fixed tissues and the region of interest, 2D electron micrographs were taken from

908   70 nm ultrathin sections at 80 kV using a JEM1011 transmission electron microscope (JEOL, Freising,

909   Germany) equipped with a Gatan Orius 1200A camera (Gatan, Munich, Germany).

910   After prescreening the 250 nm semi-thin sections from HPF samples for ribbon synapses, electron

911   tomography was performed as described previously (Strenzke et al., 2016). First, 10 nm gold beads

912   (British Bio Cell) were applied to both sides of the post-stained grids functioning as fiducial markers.

913   With the Serial-EM software (Mastronarde, 2005), single axis tilt series were acquired mainly from -60°

914   to +60° with 1° increments at 12,000-x magnification and a pixel size of 1.19 nm using a JEM2100 (JEOL)

915   transmission electron microscope at 200 kV. For final tomogram generation, the IMOD software package

916   etomo was used and tomographic reconstructions were generated using 3dmod (Kremer et al., 1996).

917

918   Patch-clamp

919   Apical turns of the organs of Corti from 2-4 weeks old rats were isolated in ice-cold HEPES Hank's

920   solution containing (in mM): 5.26 KCl, 141.7 NaCl, 0.5 $MgSO_4 \cdot 7H_2O$, 10 HEPES, 1 $MgCl_2$, 11.1 D-glucose

921   and 3.42 L-glutamine, pH adjusted to around 7.2, osmolality ~300 mOsm/kg. The recording chamber was

922   perfused with modified Ringer's solution containing (in mM): 2.8 KCl, 111 NaCl, 35 TEA-Cl, 10 HEPES, 1

923   CsCl, 1 $MgCl_2$, 11.1 D-glucose, 5mM $CaCl_2$, pH adjusted to around 7.2 with NaOH and osmolality ~300

924   mOsm/kg. Cleaning of the tissue was performed to make the inner hair cells accessible for patch clamp

925   by removing the tectorial membrane and surrounding cells. The clean exposed basolateral surface of

926   IHCs was patch-clamped using EPC-10 amplifier (HEKA Electronics, Germany) controlled by *Patchmaster*

927   software at RT, as also described previously (Moser and Beutner, 2000). Pipettes solutions contained (in

928   mM): 137 Cs-gluconate, 10 TEA-Cl, 10 4- aminopyridine, 10 HEPES, 1 $MgCl_2$, and 300µg/ml of

929   amphotericin B, pH adjusted to 7.2 using HCl and CsOH and osmolality ~290 mOsm/kg.

930   Cells were kept at a holding potential of -87 mV. All voltages were corrected for liquid junction potential

931   offline (17 mV). Currents were leak corrected using a p/10 protocol. Recordings were discarded when

932   the leak current exceeded -50 pA, R_S exceeded 30 MΩ or Ca^{2+} current rundown exceeded 25%. Current-

933   voltage relationships (IVs) were recorded once the access resistance dropped below 30 MΩ, by applying

934   increasing 10 ms long step-depolarization pulses of voltage ranging from -87 mV to 65 mV, in steps of 5

935   mV. Exocytosis measurements were performed by measuring increments in membrane capacitance

936   using the Lindau-Neher technique (Lindau and Neher, 1988). Membrane capacitance changes (ΔC_m)

937   were recorded by stimulating the cells at the potential for maximal Ca^{2+} influx (-17 mV) for variable

938 durations. An interval of 10-90 s was given before the successive stimuli were used. Each protocol was

939 sequentially applied two- three times and only IHCs with reproducible measurements were included. For

940 analysis, traces were averaged 400 ms before and after the depolarisation (skipping the first 60 ms; or

941 first 5, 10, 25 ms in the case of dual pulse experiments with inter-pulse intervals of 25, 50, 100 ms

942 respectively). The traces were subjected to 5 or 10 pass binomial smoothing using Igor Pro 6

943 (WaveMetrics Inc.) for display.

944

945 Data analysis and statistics

946 Quantitative analysis of FIB-SEM and tomogram datasets

947 Data were segmented semi-automatically using 3dmod of the IMOD software (Kremer et al., 1996).

948 IHCs, nuclei, afferent nerve fibers, ribbon synapses, mitochondria, pre- and postsynaptic densities were

949 assigned as "closed" objects using the *sculpt* drawing tool. For SVs, first the total amount of vesicles ≤

950 80 nm from the ribbon surface were quantified in number and size using the spherical "scattered"

951 object at the maximum projection of the vesicle for spherical SVs. Non-spherical SVs were segmented

952 manually as "closed" objects. The number of SVs was presented as total number per ribbon as well as

953 normalized to the size of the ribbon area. Further, two distinct morphological vesicle pools were

954 additionally quantified in size and number (as previously characterized in (Strenzke et al., 2016)): (i)

955 membrane-proximal synaptic vesicles (MP-SVs, ≤ 50 nm distance between SV membrane and AZ

956 membrane and ≤ 100 nm from the presynaptic density); and (ii) ribbon-associated synaptic vesicles (RA-

957 SVs, first layer of vesicles around the ribbon with a maximum distance of 80 nm from the ribbon surface

958 to the vesicle membrane and not falling into the MP-SV pool). Using the *imodinfo* function of 3dmod,

959 information about the ribbon, mitochondria, pre- and postsynaptic density sizes was given as well as the

960 radii for all vesicle pools were determined with this function in order to calculate the average diameter

961 per tomogram. Distance measurements were performed with the measurement drawing tool along the

962   x, y and z-axis. Movies were generated with 3dmod, Fiji (Schindelin et al., 2012) and iMovie (Apple Inc.,

963   version 10.3.1).

964

965

966   <u>Quantitative analysis of confocal and STED images</u>

967   The size of the synaptic ribbon, PSD and Ca^{2+} channel clusters (lines and spots) were estimated by fitting

968   a 2D-Gaussian function to individual structures in 2D-STED or confocal images. This yielded values of full

969   width of half maximum (FWHM) along the long and short axes. The areas of the structures have been

970   reported as areas of ellipsoids, calculated as: Area = π X (Long Axis/2) X (Short Axis/2). For estimation of

971   centers   of   mass   of   the   immunofluorescent   spots,   the   following   formula   was   used:

$$CM_r = \sum_{i=1}^{n} \frac{m_i r_i}{M}$$

972   Where CM_r represents the xy coordinates of the center of mass, m_i is the intensity of individual pixel, r_i is

973   the xy coordinate of the pixel and M is the sum of all intensities.

974   All images were analyzed and z-projected using NIH ImageJ software, and further analysis was

975   performed using Igor Pro 6 (WaveMetrics Inc.). For analysis of nearest neighbor distance of synaptic

976   ribbons   from   immunolabelled   confocal   sections,   we   used   *Imaris   9.6*   (Oxford   Instruments).

977   Immunofluorescent ribbon spots were detected per IHC using the inbuilt spot-detection algorithm in a

978   region of interest marked within a z-stack. Spot parameters provided for ellipsoid detection were 0.44

979   μm and 1.13μm for estimated XY and Z diameters respectively. Thresholding was performed based on

980   quality of immunofluorescence to eliminate artefactual spots. A manual check was performed to include

981   undetected spots and to exclude falsely detected spots and spots not localizing within the cell of

982   interest. The nearest neighbor distance was computed as the minimum distance between the centers of

983   homogenous mass of the detected spots.

984

985   Statistical analysis

986   Data are mainly presented as box plots with the mean values highlighted as a cross and with individual

987   data points overlaid and were analysed using Excel, Igor Pro 6 and 7 (WaveMetrics Inc.) and R. In order

988   to characterize the subgroups of $Pclo^{gt/gt}$ ribbon synapses, acquired with electron tomography, in

989   addition to our manual grouping, we performed an unsupervised K-means clustering with R by

990   considering the following variables: ribbon, presynaptic density as well as SV pool counts and sizes. An

991   optimal number of k=2 clusters was selected based on the decrease of the total within-cluster sum of

992   squares observed.

993

994   Using Igor Pro, normality of data was assessed with the Jarque-Bera test or the Wald-Wolfowitz runs

995   test and equality of variances in normally distributed data was assessed with the F-test. Differences

996   between two groups were evaluated for significant differences using the two-tailed unpaired Student's

997   t-test, or, when not normally distributed and/or variance was unequal, the unpaired two-tailed Mann–

998   Whitney Wilcoxon test. For multiple comparisons, the one-way ANOVA with post-hoc Tukey's test or the

999   Kruskal-Wallis (KW) test with multiple comparison correction (NPMC: Non-parametric multiple

1000   comparison test) was utilized. For Fig 1B, C, we performed two-way repeated measures ANOVA with

1001   post-hoc Holm-Šidák correction for multiple comparisons using *GraphPad Prism* 9 (GraphPad Software).

1002   Non-significant differences between samples are indicated as *n.s.*, significant differences are indicated

1003   as *$P < 0.05$, **$P < 0.01$, ***$P < 0.001$, ****$P < 0.0001$.

Authors contributions

S.M., R.K., T.M. and C.W. designed the study. S.M. performed electron microscopic work (conventional embeddings, HPF/FS, enhanced en bloc stainings, TEM of random sections, electron tomography, FIB-SEM data acquisition and post-processing) with contribution from C.W., data analysis and supervised I.F. together with C.W. for pre-embedding immunogold labeling data acquisition. R.K. performed immunohistochemistry, confocal and STED microscopy, systems physiology, physiological cell data acquisition as well as data analysis with contribution from J.N.. A.M.S. performed and W.M. supervised FIB-SEM data acquisition and post-processing. J.N. supervised electrophysiology. F.A., F.K.H and C.C.G provided unpublished material and contributed to pilot experiment. N.S. supervised systems physiology data acquisition and analysis. M.G. contributed to statistical analysis. S.M., R.K., T.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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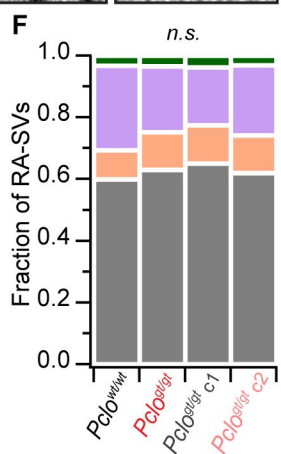1252   *Assoc. Res. Otolaryngol. JARO* **15**, 571–583.

1253

A *Pcloʷᵗ/ʷᵗ*

Y Z / X top view   X Y / Z side view

P
M

2 μm   5 μm

B *Pcloᵍᵗ/ᵍᵗ*

Y Z / X top view   X Z / Y side view

P
M

2 μm   5 μm

C *Pcloʷᵗ/ʷᵗ*

Section 152 | Section 188

Pcloᵍᵗ/ᵍᵗ category1

Pcloᵍᵗ/ᵍᵗ category2

200 nm

D

Legend:
- *Pcloʷᵗ/ʷᵗ* (black)
- *Pcloᵍᵗ/ᵍᵗ* (red)
- *Pcloᵍᵗ/ᵍᵗ* category1 (gray)
- *Pcloᵍᵗ/ᵍᵗ* category2 (pink)

Ribbon volume (nm^3) $\times 10^6$

E SV count per ribbon

F Density of SVs (count/nm^2) $\times 10^{-6}$

G PSD area (nm^2) $\times 10^6$

A $Pclo^{wt/wt}$

B $Pclo^{gt/gt}$

C

RA-SVs

Filaments

1 Ribbon-attached SV
2 SV without filament
3 Interconnected SVs
4 Ribbon-attached (Ri.) +
  Innterconnected (In.) SV
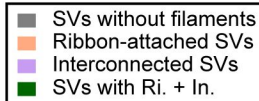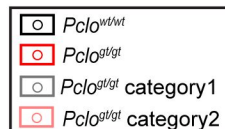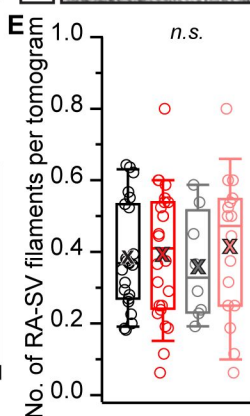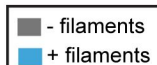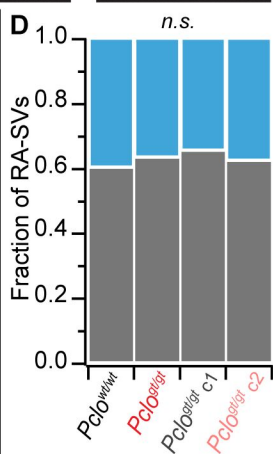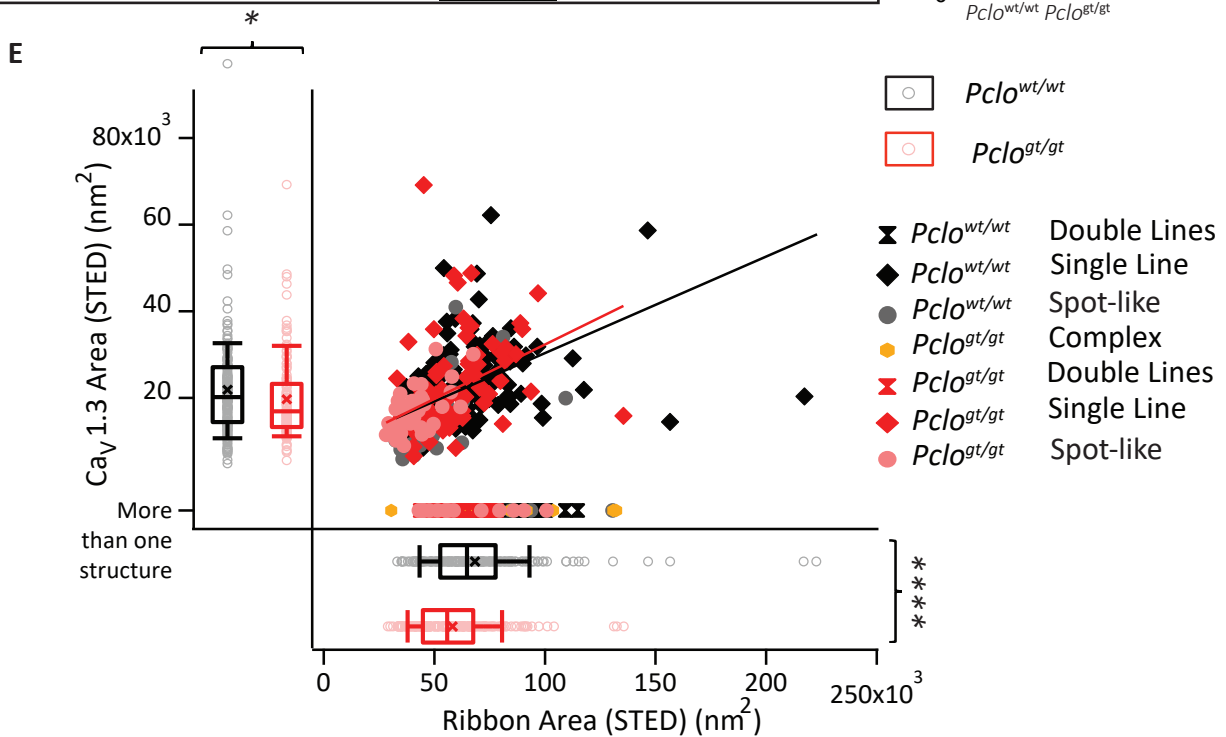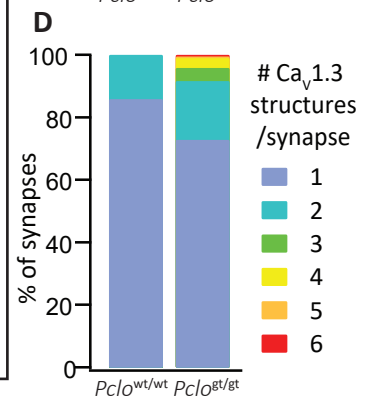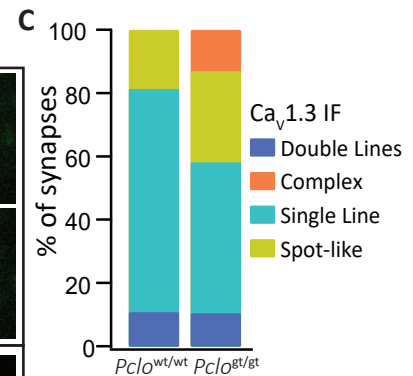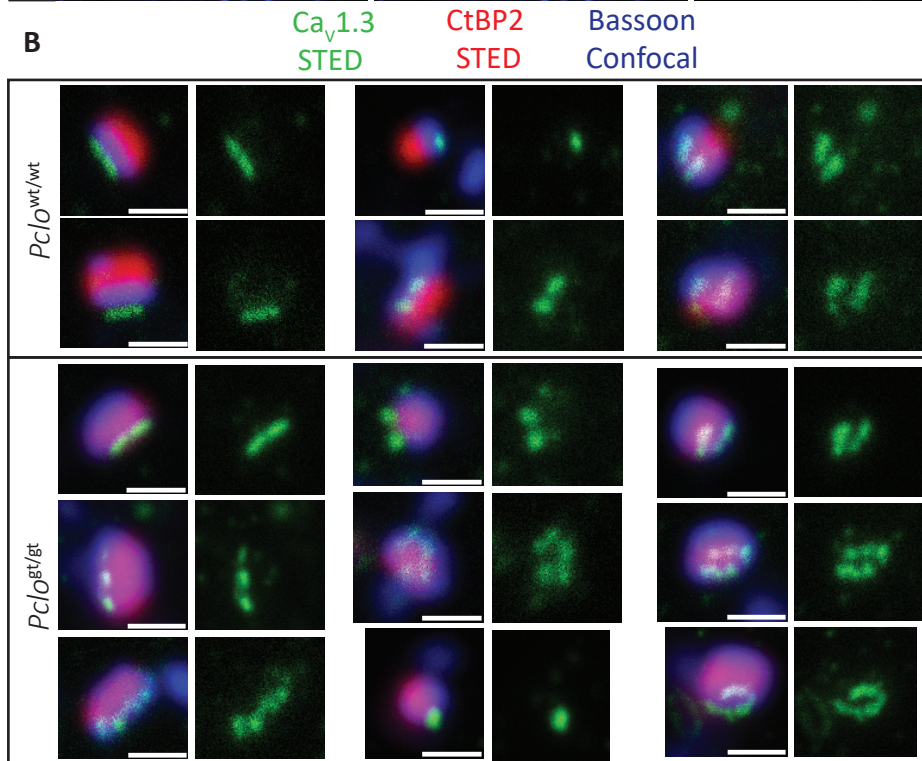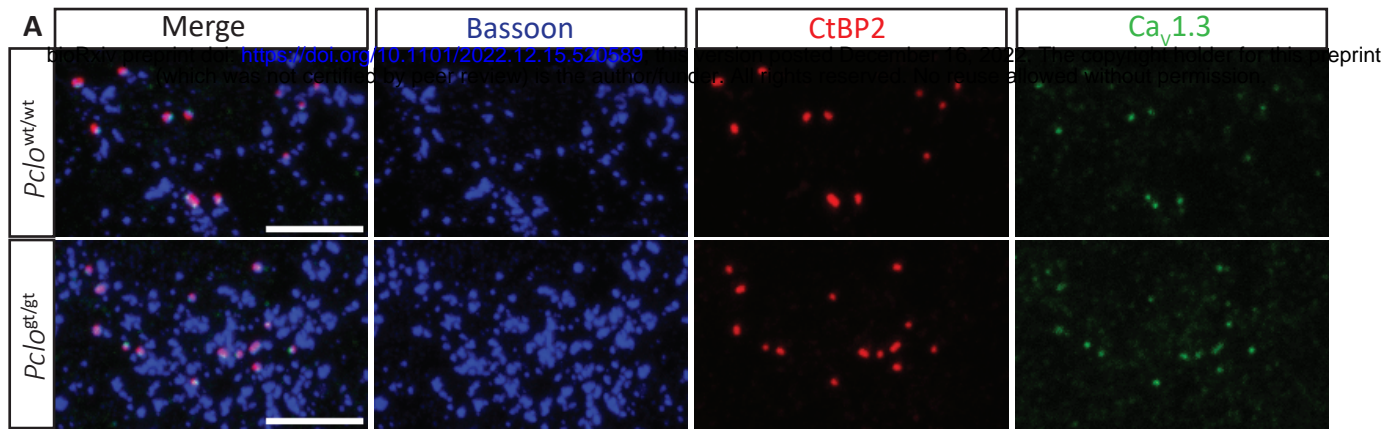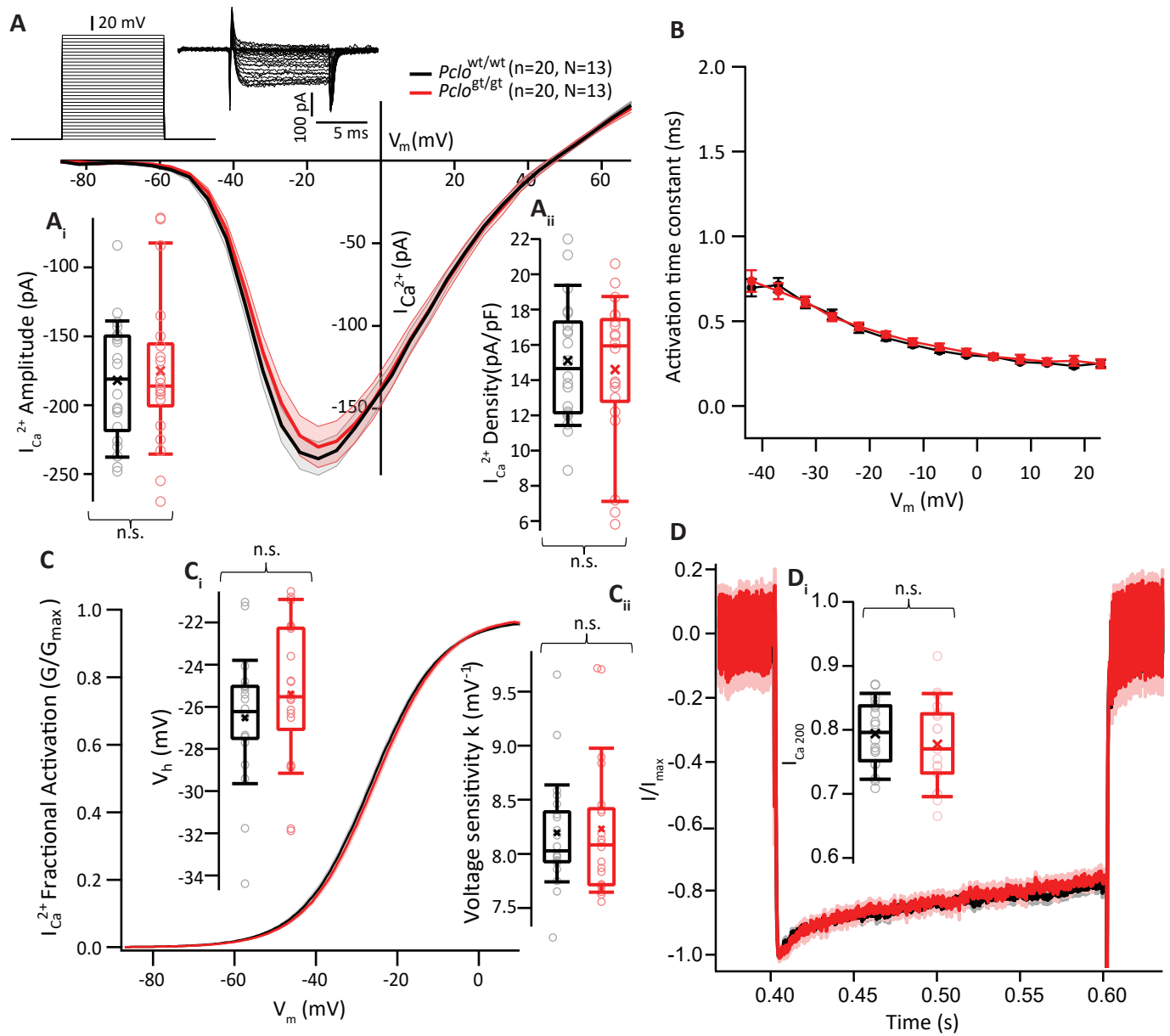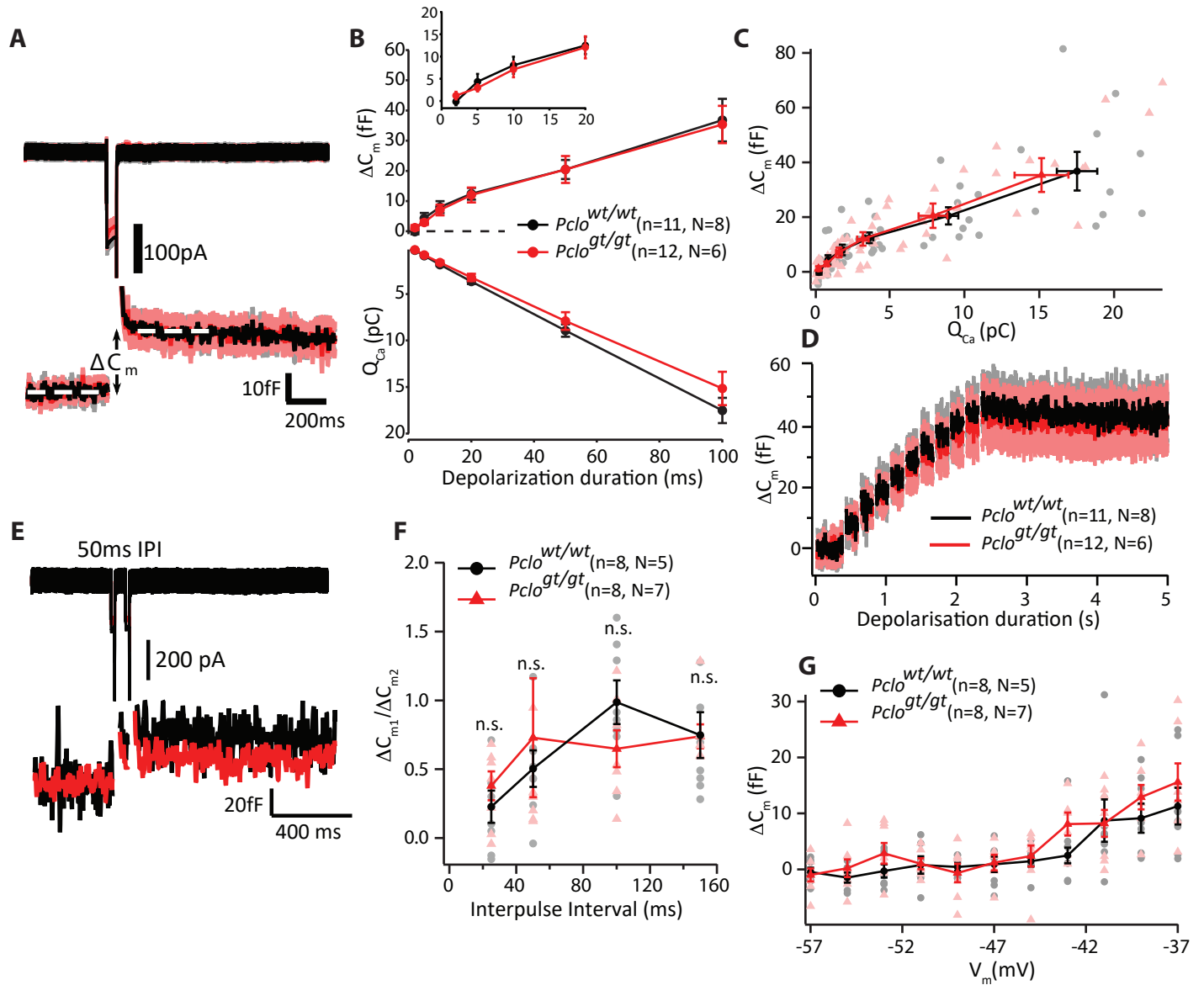
D *n.s.*

Fraction of RA-SVs

$Pclo^{wt/wt}$ $Pclo^{gt/gt}$ $Pclo^{gt/gt}$ c1 $Pclo^{gt/gt}$ c2

- filaments
+ filaments

E *n.s.*

No. of RA-SV filaments per tomogram

$Pclo^{wt/wt}$
$Pclo^{gt/gt}$
$Pclo^{gt/gt}$ category1
$Pclo^{gt/gt}$ category2

F *n.s.*

Fraction of RA-SVs

$Pclo^{wt/wt}$ $Pclo^{gt/gt}$ $Pclo^{gt/gt}$ c1 $Pclo^{gt/gt}$ c2

SVs without filaments
Ribbon-attached SVs
Interconnected SVs
SVs with Ri. + In.

(A) Wild Type

(B) Bassoon mutants

(C) Piccolino mutants

(D) RIBEYE-KO

Category 1

Category 2

Legend:

- Synaptic ribbon
- Presynaptic density
- $Ca_V1.3$ channels
- Synaptic vesicles
- Presynaptic membrane
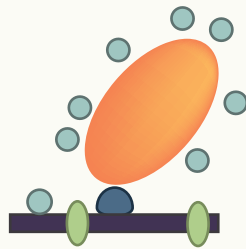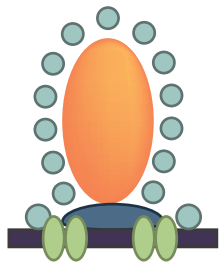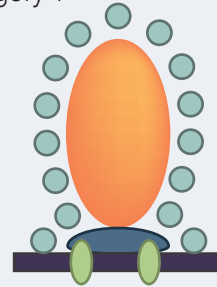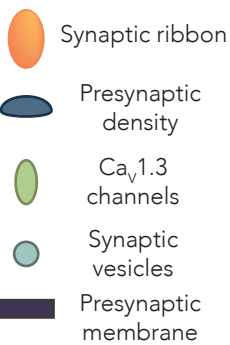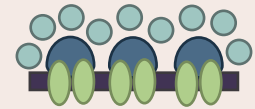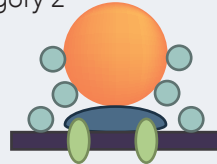
(B)
1. Massive reduction in wave I amplitude, ABR thresholds elevated for clicks and tone bursts

2. Whole-cell Ca^{2+} currents reduced due to less channel numbers. Slow activation and slightly enhanced inactivation in ΔEx4/5 mutant

3. Reduced fast and sustained exocytosis, slow vesicle replenishment

(Khimich et al., Nature 2005; Frank et al., Neuron 2010; Jing et al., JNeurosci 2013)

(C)
1. Wave I amplitude reduced, ABR thresholds elevated for middle and high frequency tone bursts (statistically significant)

2. No change in whole-cell Ca^{2+} current amplitude and kinetics
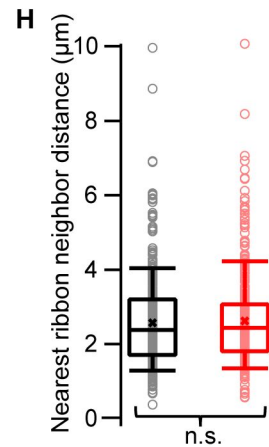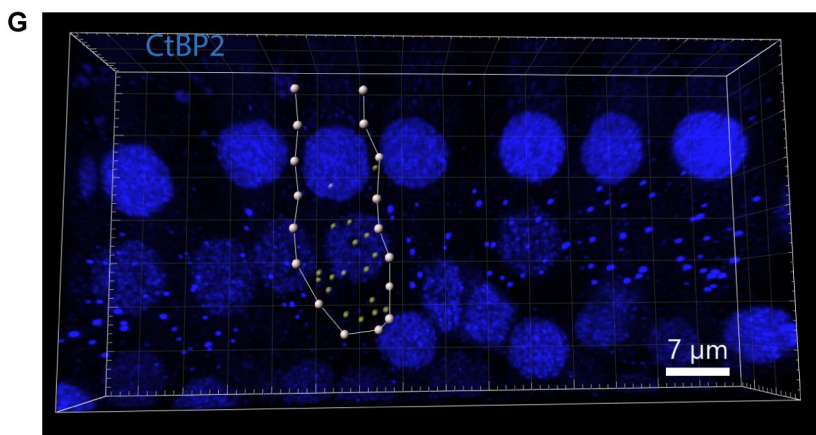
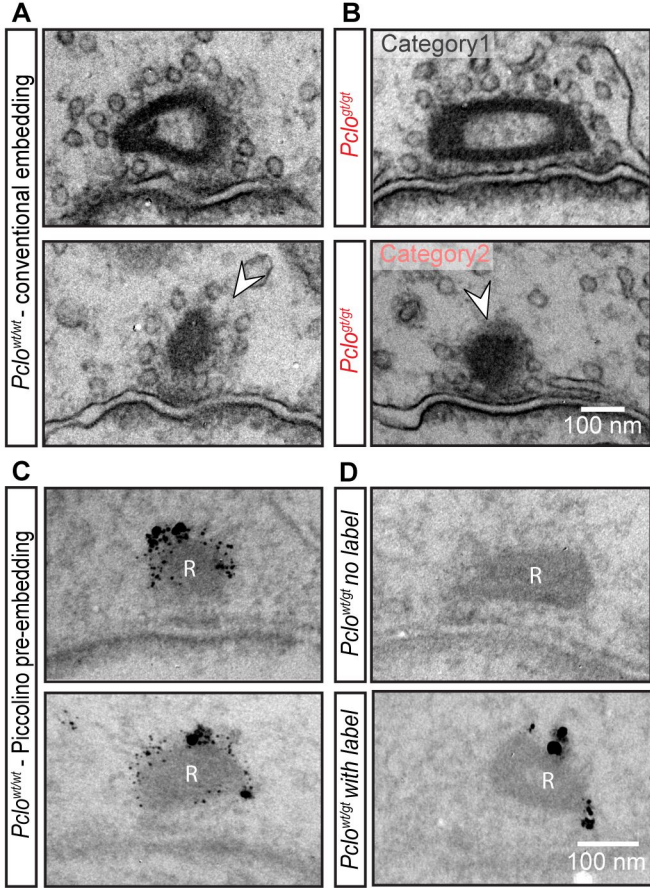3. Normal exocytosis and replenishment of SVs

(This Study)

(D)
1. Wave I amplitude reduced, ABR thresholds mildly elevated (not significant)

2. No change in Ca^{2+} current amplitude at whole-cell or single AZ level. Ca^{2+} channels activate at mildly depolarized potentials; slightly enhanced inactivation

3. Normal exocytosis for strong depolarizations, slightly reduced for weak ones, normal SV replenishment
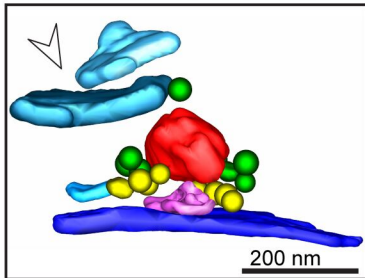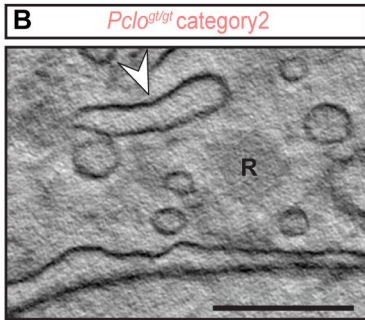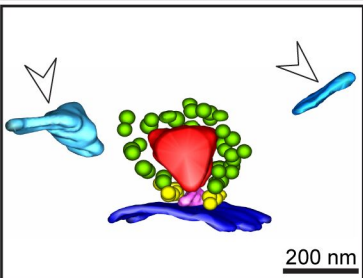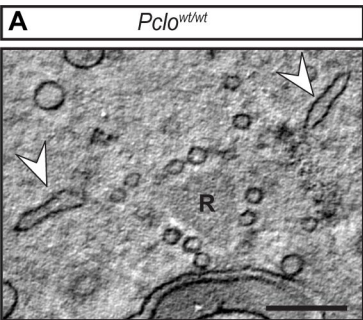
(Jean et al., eLife 2018; Becker et al. eLife 2018)

A Pclo^gt/gt

200 nm

B Aff

200 nm

C

Ribbons nearby mitochondria (%)

D

Nearest distance to mitochondria (nm)

E

Nearest ribbon neighbor distance (μm)

F

Ribbons with translucent core (%)

□ Pclo^wt/wt   □ Pclo^gt/gt   □ Pclo^gt/gt category1   □ Pclo^gt/gt category2

G CtBP2

7 μm

H

Nearest ribbon neighbor distance (μm)

n.s.

A *Pclo^{wt/wt}* - conventional embedding

B Category1 · *Pclo^{gt/gt}*

Category2 · *Pclo^{gt/gt}*

100 nm

C *Pclo^{wt/wt}* - Piccolino pre-embedding

R

R

D *Pclo^{wt/gt} no label*

R

Pclo^{wt/gt} with label

R

100 nm

A *Pclo^{wt/wt}*

B *Pclo^{gt/gt}* category2

C

Pclo^{wt/wt}: 47.82 %
Pclo^{gt/gt}: 62.5 %
Pclo^{gt/gt} category1: 62.5 %
Pclo^{gt/gt} category2: 62.5 %

Distance cisternal structures to ribbon (nm)

**