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Identification of a Munc13-sensitive step in chromaffin cell large dense-core vesicle exocytosis

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Short title: Munc13s in LDCV Priming and Docking

20   **Abstract**

21   It is currently unknown whether the molecular steps of large dense-core vesicle

22   (LDCV) docking and priming are identical to the corresponding reactions in synaptic

23   vesicle (SV) exocytosis. Munc13s are essential for SV docking and priming, and we

24   systematically analyzed their role in LDCV exocytosis using chromaffin cells lacking

25   individual isoforms. We show that particularly Munc13-2 plays a fundamental role in

26   LDCV exocytosis, but in contrast to synapses lacking Munc13s, the corresponding

27   chromaffin cells do not exhibit a vesicle docking defect. We further demonstrate that

28   ubMunc13-2 and Munc13-1 confer Ca^{2+}-dependent LDCV priming with similar

29   affinities, but distinct kinetics. Using a mathematical model, we identify an early

30   LDCV priming step that is strongly dependent upon Munc13s. Our data demonstrate

31   that the molecular steps of SV and LDCV priming are very similar while SV and

32   LDCV docking mechanisms are distinct.

33

Introduction

The regulated, Ca^{2+}-triggered secretion of catecholamines from chromaffin cell LDCVs is an integral part of the physiological adaption to environmental stressors. Like the exocytosis of neuronal SVs, LDCV exocytosis is mediated by SNARE complex formation, in concert with Ca^{2+} sensors and essential regulatory proteins (James and Martin, 2013; Neher, 2006; Ovsepian and Dolly, 2011; Pang and Sudhof, 2010).

Mammalian uncoordinated 13 (Munc13) proteins are essential SV priming factors in neurons (Augustin et al., 1999; Richmond et al., 1999; Rosenmund et al., 2002), and ultrastructural studies have shown that in synapses lacking Munc13s/Unc-13, SVs also fail to physically dock to synaptic active zones (Imig et al., 2014; Siksou et al., 2009; Weimer et al., 2006). At the molecular level, this morphological phenotype most likely corresponds to a role of Munc13s in mediating the formation of SNARE complexes at vesicular release sites (Hammarlund et al., 2007; Hammarlund et al., 2008; Imig et al., 2014; Ma et al., 2011; Ma et al., 2013; Yang et al., 2015).

The Munc13 family consists of five members, Munc13-1 (*Unc13a*), Munc13-2 (*Unc13b*), Munc13-3 (*Unc13c*), the brain specific angiogenesis inhibitor I-associated protein 3 (*Baiap3*), and the non-neuronal isoform Munc13-4 (*Unc13d*) (Koch et al., 2000). Genetic deletion of *Unc13a* and *Unc13b* completely eliminates SV exocytosis in hippocampal neurons (Varoqueaux et al., 2002), and selectively reduces synaptic vs. extrasynaptic exocytosis of neuronal LDCVs (van de Bospoort et al., 2012), which indicates that SV and LDCV exocytosis at active zones is mediated by similar molecular mechanisms. By contrast, studies in *C. elegans* and *Drosophila* have shown that Unc-13/dUnc-13 selectively regulate SV release, whereas the Ca^{2+}-dependent

58   activator proteins for secretion (CAPS/Unc-31) specifically regulate LDCV release

59   (Hammarlund et al., 2008; Renden et al., 2001; Speese et al., 2007; Zhou et al., 2007).

60   In mammals, Munc13s and CAPSs appear to perform non-redundant functions critical

61   for both SV and LDCV exocytosis in neurons (Jockusch et al., 2007; van de Bospoort

62   et al., 2012), as well as for LDCV exocytosis in neuroendocrine cells (Elhamdani et

63   al., 1999; Kabachinski et al., 2014; Kang et al., 2006; Kwan et al., 2006; Liu et al.,

64   2010; Liu et al., 2008; Speidel et al., 2008). Yet, to date, while CAPS-1 and CAPS-2

65   have been shown to be required for LDCV exocytosis in mammalian chromaffin cells

66   (Liu et al., 2010; Liu et al., 2008), evidence that endogenous Munc13s are required

67   for LDCV exocytosis is lacking. In fact, the role of Munc13-1 and ubMunc13-2 has

68   only been examined in the context of overexpression studies, and other isoforms have

69   not been investigated (Ashery et al., 2000; Bauer et al., 2007; Liu et al., 2010; Stevens

70   et al., 2005; Zikich et al., 2008).

71   In the present study, we performed the first comprehensive analysis of all neuronal

72   and neuroendocrine members of the Munc13 protein family in chromaffin cells,

73   defining their respective roles in LDCV exocytosis. We identify the Ca^{2+}-dependent

74   step in the priming process at which Munc13-1 and ubMunc13-2 operate, and

75   demonstrate that, although they are critical for LDCV priming and release, LDCV

76   docking can occur without them.

77

Results

Expression of Munc13 isoforms in the mouse adrenal gland

We first analyzed the expression of all Munc13 isoforms in the murine adrenal gland by Western blotting (Figure 1). In perinatal adrenal glands, we detected Munc13-1 (Figure 1A and Figure 1–figure supplement 1B), the ubiquitous isoform ubMunc13-2 (Figure 1B and Figure 1–figure supplement 1B), and Baiap3 (Figure 1D). Not detected were the brain-specific isoform of Munc13-2 (bMunc13-2), which is a splice variant expressed from the same gene as ubMunc13-2 (Figure 1B), Munc13-3 (Figure 1C), and the non-neuronal isoform Munc13-4 (Figure 1E). To directly compare the expression levels of Munc13-1, ubMunc13-2, bMunc13-2, and Munc13-3, we used knockin mice that express these proteins fused to enhanced yellow or green fluorescent protein (EYFP/EGFP) from the respective endogenous loci (Cooper et al., 2012; Kalla et al., 2006). We found that ubMunc13-2-EYFP is the only isoform readily detectable in the adrenal gland using an antibody to the GFP-derived tags (Figure 1–figure supplement 1A).

To assess whether the isoforms detected in whole gland homogenates are present in the adrenal medulla, and/or the adrenal cortex, we dissected adult wild-type (WT) adrenal glands, and used an antibody to the LDCV marker Chromogranin A (CgA) to monitor effective separation of the medullary tissue, which consists mostly of chromaffin cells, from cortical tissue (Figure 1F). The expression of Munc13-1 and Baiap3 in the adrenal gland is largely restricted to the medulla. Expression of ubMunc13-2 was detected in both adrenal medulla and cortex. Thus, a significant fraction of the ubMunc13-2 signal detected in whole gland homogenates (Figure 1B)

101   appears to originate from the adrenal cortex, possibly due to innervation of the cortex

102   by ubMunc13-2 positive synapses.

103

104   **Absence of Munc13-1, Munc13-3, or Baiap3 does not impair LDCV exocytosis in**

105   **chromaffin cells**

106   Next, we analyzed cultured chromaffin cells from knockout (KO) mice deficient for

107   the individual Munc13 isoforms (Figure 2). LDCV exocytosis was triggered using

108   flash photolysis of caged Ca^{2+}, which causes a sharp global increase in intracellular

109    $[Ca^{2+}]$ (Neher, 2006). Fusion of LDCVs with the plasma membrane was monitored by

110   measurement of the membrane capacitance change (ΔCm). Fitting a sum of three

111   exponentials to the exocytotic burst of each individual trace identifies the amplitudes

112   and time constants of release, which are generally interpreted as two kinetically

113   distinct vesicle pools, the fast burst as the Readily-Releasable Pool (RRP), and the

114   slow burst as the Slowly-Releasable Pool (SRP) (Sorensen et al., 2003a; Voets, 2000).

115   However, as will be discussed later, the slow burst component may in fact not be a

116   releasable pool, but instead represent the conversion from a Non-Releasable Pool

117   (NRP), to the RRP (Walter et al., 2013). The rate of sustained release was measured

118   as a linear component after the exocytotic burst, and reflects the ongoing recruitment

119   of LDCVs into the NRP/SRP and RRP. Deletion of Munc13-1 (*Unc13a*), the major

120   Munc13 isoform in SV exocytosis (Augustin et al., 1999; Varoqueaux et al., 2002),

121   did not markedly alter LDCV exocytosis compared to WT littermate controls (Figure

122   2A,D), nor did it affect the kinetics of the exocytotic burst (Figure 2D).

123   Although we did not detect Munc13-3 in the adrenal gland, we wanted to rule out

124   possible physiological effects of protein expression below the detection limit of

125 Western blot analysis (Figure 1C), and included $Unc13c^{KO}$ mice in our analysis.

126 However, as expected, LDCV exocytosis in $Unc13c^{KO}$ chromaffin cells was not

127 perturbed (Figure 2B,D).

128 We then investigated the possible role of Baiap3 in LDCV exocytosis, as this isoform

129 is prominently expressed in the adrenal medulla (Figure 1D,F). Surprisingly, LDCV

130 exocytosis in $Baiap3^{KO}$ cells was intact (Figure 2C,D). Furthermore, $Baiap3^{KO}$ cells

131 also did not show a release deficit when we stimulated the cells using a series of

132 depolarization steps (Figure 2–figure supplement, 1A-C), nor did overexpression of

133 Baiap3 in WT cells affect LDCV exocytosis (Figure 2–figure supplement 1D-F).

134

Absence of ubMunc13-2 dramatically reduces LDCV release

136 We then analyzed the role of ubMunc13-2 and Munc13-1 in chromaffin cell LDCV

137 exocytosis. For this purpose, we used an $Unc13a/b$ (DKO) mouse line. Heterozygous

138 (Het) animals of this line express ~50 % of WT levels of Munc13-1 and Munc13-2,

139 which does not affect neurotransmission (Augustin et al., 1999; Varoqueaux et al.,

140 2002). Data were collected from genotype groups available for a given litter and were

141 pooled for analysis. Because our breeding scheme did not produce littermate WT

142 animals in sufficient numbers, and because deletion of $Unc13a$ alone was without

143 effect, data from $Unc13a^{WT}Unc13b^{Het}$ and $Unc13a^{Het}Unc13b^{Het}$ cells were pooled and

144 used as control (Figure 2E-J, $Unc13a^{WT/Het}Unc13b^{Het}$).

145 Deletion of both $Unc13a$ alleles together with a single $Unc13b$ allele

146 ($Unc13a^{KO}Unc13b^{Het}$) did not reduce LDCV release (Figure 2E,F). By contrast,

147 abrogation of ubMunc13-2 expression alone, irrespective of the $Unc13a$ genotype,

148 drastically diminished release (Figure 2E,F). Furthermore, in the context of the

149 $Unc13b^{KO}$ background, cells with $Unc13a^{WT}$, $Unc13a^{Het}$, and $Unc13a^{KO}$ genotypes

150 showed a progressive reduction of LDCV release that depended on the number of

151 $Unc13a$ alleles present (Figure 2F,G). The fast and slow burst components were

152 reduced to 39%, 32%, and 27%, and to 54%, 52%, and 42% of control levels,

153 respectively (Figure 2F). The rate of sustained release was reduced even more

154 dramatically, to 26%, 19%, and 12% of control levels (Figure 2F). When one uses the

155 $Unc13a^{WT}Unc13b^{KO}$ genotype as a reference point (Figure 2G), the deletion of

156 $Unc13a$ caused a reduction of the sustained release component to 48%. The rate of

157 sustained release of $Unc13a^{KO}Unc13b^{KO}$ cells was also significantly reduced when

158 compared to $Unc13a^{Het}Unc13b^{KO}$ cells.

159 The deletion of both $Unc13a$ and $Unc13b$ significantly delayed the onset of vesicular

160 exocytosis triggered by flash photolysis, compared to control and

161 $Unc13a^{KO}Unc13b^{Het}$ cells (Figure 2H). $Unc13a^{WT}Unc13b^{KO}$ cells also showed a mild

162 increase in delay. However, this difference was significant only when compared to

163 $Unc13a^{KO}Unc13b^{Het}$ cells, but not compared to the other groups.

164 Thus, ubMunc13-2, the only isoform expressed from the $Unc13b$ gene in mouse

165 chromaffin cells, is the most critical isoform for LDCV release in this cell type.

166 Moreover, in its absence it becomes apparent that endogenous Munc13-1 also

167 regulates LDCV release in this cell type.

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Reduced IRP and RRP in the absence of ubMunc13-2

We next assessed whether ubMunc13-2 affects LDCV release in response to Ca^{2+}

entry through voltage-gated Ca^{2+} channels by stimulating the cells with a series of

depolarization steps (Figure 3). The first six short depolarizations of the train release

the Immediately-Releasable Pool (IRP), i.e. the subset of RRP vesicles located closest

to Ca^{2+}-channels (Schonn et al., 2010; Voets et al., 1999). We found a significant

reduction in LDCV release; the size of the RRP in $Unc13b^{KO}$ cells was reduced to

53% of WT levels (Figure 3C). This deficit is somewhat less pronounced than the

reduction seen in the flash photolysis experiment (reduction to 39%, Figure 2F), most

likely because the depolarization protocol used to obtain the data shown in Figure 3

lasts several seconds and therefore causes some ongoing recovery of the RRP. By

contrast, in flash photolysis experiments, the RRP is probed within ~60 ms (3 times

the time constant), which is much faster than the recovery of the RRP. Strikingly, in

the depolarization experiment, impaired release in $Unc13b^{KO}$ cells was already

evident in response to the first 10-ms depolarization (Figure 3B), which implies that

lack of ubMunc13-2 would even affect resting level catecholamine release driven by

low frequency stimulation (Zhou and Misler, 1995).

Reduced catecholamine release in the absence of ubMunc13-2

To understand how ubMunc13-2 affects the kinetics of single catecholamine release

events, we performed single spike amperometry while infusing the cells with a

solution with moderate (~4.6 µM) $[Ca^{2+}]$ (Figure 4). $Unc13b^{KO}$ cells showed a

dramatic reduction in spike frequency (Figure 4B,C), whereas basic spike parameters

such as duration, half-width, maximum amplitude, charge, rise time, and decay time

195   were unchanged (Figure 4D-I). Amplitude, duration and charge of the spike foot

196   signal, which is thought to reflect release during the initial formation of the fusion

197   pore prior to full fusion, were also unchanged (Figure 4J-L). However, we found that

198   the number of spikes that did show these foot signals was slightly reduced in

199   $Unc13b^{KO}$ cells (Figure 4M), which may indicate that fusion pore dynamics are

200   altered for some release events. However, overall, the LDCVs undergoing fusion in

201   the absence of ubMunc13-2 do so without major alterations in fusion kinetics or

202   vesicle content.

203

204   **Munc13 isoforms display different LDCV priming efficiencies**

205   As our experiments so far showed that, with the exception of Baiap3, the contribution

206   of the Munc13 isoforms to the regulation of LDCV release correlates with their level

207   of expression in perinatal adrenal glands, we next wanted to compare the intrinsic

208   properties of the different isoforms. To this end, we overexpressed Munc13-1,

209   ubMunc13-2, Baiap3, and its closest relative Munc13-4 using Semliki Forest Virus

210   (SFV) in $Unc13a^{KO}Unc13b^{KO}$ cells. Munc13-1 and ubMunc13-2 were expressed as

211   EGFP fusion constructs whose functions are identical to those of the respective WT

212   proteins (Rosenmund et al., 2002), whereas Baiap3 and Munc13-4 were expressed as

213   internal ribosome entry site (IRES)-EGFP constructs, to avoid possible confounding

214   effects of a fusion tag. $Unc13a^{KO}Unc13b^{KO}$ cells expressing only EGFP were used as

215   control. For the purpose of comparison, the averaged traces obtained from the rescue

216   experiments with the four isoforms were plotted in the same graph (Figure 5A). The

217   exocytotic burst was measured as the ΔCm within the first 0.5 s after the flash

218   stimulus, and the rate of sustained release was measured as the ΔCm between 0.5 s

219   and 4 s after the flash (Figure 5B). Interestingly, Munc13-1 and ubMunc13-2 were

220   both able to rescue the LDCV release deficit of $Unc13a^{KO}Unc13b^{KO}$ cells (Figure

221   5A,B). However, rescue with ubMunc13-2 resulted in an enormous enhancement of

222   LDCV exocytosis to levels that by far exceeded the amount of exocytosis typical of

223   WT cells, for both the exocytotic burst and the rate of sustained release (Figure

224   5A,B). The direct comparison of Munc13-1 and ubMunc13-2 expressing cells with

225   matching EGFP fluorescence intensity confirmed that the stronger enhancement of

226   burst size and rate of sustained release in ubMunc13-2 expressing cells was not due to

227   higher expression levels of ubMunc13-2 (Figure 5–figure supplement 1).

228   Overexpression of Baiap3 failed to rescue the LDCV release deficit of the

229   $Unc13a^{KO}Unc13b^{KO}$ chromaffin cells (Figure 5A,B). Yet, its closest relative,

230   Munc13-4, which regulates SNARE-mediated vesicular exocytosis in the

231   hematopoietic system (Feldmann et al., 2003; Shirakawa et al., 2004), was able to

232   rescue LDCV exocytosis in $Unc13a^{KO}Unc13b^{KO}$ cells, albeit less efficiently than

233   Munc13-1 or ubMunc13-2 (Figure 5A,B). To exclude the possibility that these

234   findings might be due to inefficient translation of Baiap3 and Munc13-4, protein

235   expression was confirmed with isoform-specific antibodies in SFV-infected neuronal

236   cultures, which provide enough material for Western blot analysis (Figure 5–figure

237   supplement 2). Thus, individual Munc13 isoforms appear to show inherent

238   differences in their ability to promote LDCV release in chromaffin cells.

239

240   **LDCV docking in the absence of ubMunc13-2 and Munc13-1**

241   We went on to investigate whether $Unc13a^{KO}Unc13b^{KO}$ chromaffin cells show an

242   LDCV   docking   defect   analogous   to   the   SV   docking   defect   seen   in

11

243 *Unc13a*^{KO}*Unc13b*^{KO} synapses (Siksou et al., 2009). SV docking deficits of

244 Munc13/Unc-13 deficient synapses in mice and *C. elegans* were previously only

245 detected when rapid cryo-fixation methods were employed instead of classical

246 chemical fixation for ultrastructural analysis (Siksou et al., 2009; Weimer et al.,

247 2006). Moreover, it has been shown that 3D electron tomography (ET) allows a more

248 accurate assessment of SV docking at the active zone (Imig et al., 2014; Siksou et al.,

249 2009). To study LDCV recruitment and docking in chromaffin cells, we therefore

250 combined high-pressure freezing (HPF) and freeze-substitution of acute adrenal gland

251 slices with classical 2D-EM (Figure 6A-E) and high-resolution 3D-ET analyses

252 (Figure 6F-N). Quantitative analysis of 2D-EM images of *Unc13a*^{Het}*Unc13b*^{Het}

253 (Figure 6A) and *Unc13a*^{KO}*Unc13b*^{KO} chromaffin cells (Figure 6B) did not reveal any

254 differences in LDCV distribution within 2 μm of the plasma membrane (PM) (Figure

255 6C), in the number of membrane-proximal LDCVs within 40 nm of the PM (Figure

256 6D), or in the total number of LDCVs (Figure 6E). LDCV docking and recruitment

257 into the vicinity of the PM were assessed using 3D-ET (Figure 6F-N). From all

258 LDCVs analyzed within 100 nm of the PM, the percentage of membrane-proximal

259 LDCVs (0-40 nm) (Figure 6M) and their distribution (Figure 6L) was unaltered

260 between both groups, indicating that LDCV recruitment to the PM is intact in

261 Munc13-deficient chromaffin cells. The number of docked LDCVs, defined as

262 LDCVs in physical contact with the PM and assigned to the 0-4 nm bin in Figure 6L,

263 and the number of docked LDCVs normalized to the number of membrane-proximal

264 LDCVs (Figure 6N) were unchanged. Furthermore, the average LDCV diameter of

265 docked or non-docked LDCVs, measured by 3D-ET, did not differ significantly

266 between genotypes, although *Unc13a*^{KO}*Unc13b*^{KO} LDCVs tended to be smaller

267 (Figure 6–figure supplement 1). Thus, in spite of the dramatic release deficit seen in

268 *Unc13a*[KO]*Unc13b*[KO] chromaffin cells and the dramatic SV docking deficit seen in

269 neurons of this genotype (Siksou et al., 2009), we did not detect any changes in

270 LDCV docking, nor a loss or accumulation of LDCVs in the vicinity of the PM. Thus,

271 chromaffin cells can generate what appears to be a full-sized pool of morphologically

272 docked LDCVs in the absence of Munc13-1 and Munc13-2, which implies that the

273 molecular requirements of morphological LDCV and SV docking are distinct.

274 Additionally, this could either indicate that the mechanism of functional docking, i.e.

275 priming, differs between LDCV and SVs as well, or else, that the primed LDCVs (i.e.

276 those that belong to the RRP) are in the minority among the docked vesicles and

277 therefore cannot be detected.

278 To distinguish between these two possibilities, we estimated the total number of

279 docked LDCVs per cell. To this end, we re-calculated the percentage of docked

280 vesicles identified using 3D-ET, as the percentage of membrane proximal vesicles (0-

281 40 nm) identified in the 2D-EM analysis and converted LDCVs/µm PM to

282 LDCVs/cell as described (Parsons et al., 1995). This conversion was necessary due to

283 the limited volume sizes analyzed by 3D-ET and the uneven distribution of LDCVs

284 within the cells. The estimated size of the morphologically docked pool was ~662

285 LDCVs per chromaffin cell in control cells. For *Unc13a*[KO]*Unc13b*[KO] cells we

286 calculated ~865 docked LDCVs per cell, which can be accounted for by two factors

287 used in the calculation: *Unc13a*[KO]*Unc13b*[KO] cells are slightly larger, and their LDCVs

288 are slightly smaller (Figure 6–figure supplement 1). Both values are lower than the

289 previously reported ~1607 for embryonic day (E)18 murine chromaffin cells (de Wit,

290 2010), presumably reflecting improved discrimination between docked and undocked

291 vesicles by 3D-ET. With a diameter of a docked LDCV of ~170 nm (Figure 6–figure

292 supplement 1E) and assuming a specific membrane capacitance of 1 µF/cm², this

13

293   corresponds to a vesicular capacitance of 0.91 fF, in excellent agreement with recent

294   electrophysiological measurements of 0.94 fF (Pinheiro et al., 2014). Thereby, the

295   size of the RRP, which is <40 fF at resting $[Ca^{2+}]$ (Voets, 2000), corresponds to <44

296   vesicles from the total of ~662 in control cells, indicating that even with 3D-ET, the

297   RRP will be very hard or even impossible to distinguish morphologically from other

298   docked vesicles in adrenal chromaffin cells.

299

300   **Identification of a Munc13-sensitive step in LDCV priming**

301   Thus far, our data indicate that although morphological docking of LDCVs does not

302   require Munc13s, the priming of a functional RRP does. We therefore wanted to

303   identify the Munc13-sensitive step in the LDCV priming process, and compare the

304   intrinsic properties of Munc13-1 and ubMunc13-2, the two most relevant isoforms in

305   the adrenal medulla. In a recently published mathematical model for LDCV fusion,

306   we showed that the fast and slow bursts of release originate from two serially

307   arranged pools of vesicles, the RRP and the non-releasable NRP, respectively (Walter

308   et al., 2013) (Figure 7). The NRP in turn is refilled from a larger depot pool. Thus, the

309   model features two separate priming steps (Liu et al., 2010), but only one fusion

310   pathway (Figure 7E). Since the deletion of *Unc13a* and *Unc13b* changed the fast and

311   slow burst to nearly the same degree (Figure 2F), within the framework of this model,

312   Munc13s must act upstream of the NRP. Furthermore, since the sustained release rate

313   is changed proportionally as well, Munc13s likely act to accelerate the forward

314   priming rate, k_1 (Ashery et al., 2000). In most models, this rate constant k_1 is Ca^{2+}-

315   dependent (Voets, 2000; Walter et al., 2013), and confers overall Ca^{2+}-dependence to

14

316   the primed vesicle pool. We next investigated how the two relevant Munc13-isoforms

317   in chromaffin cells (ubMunc13-2, Munc13-1) affect this priming step.

318   The Ca^{2+}-dependence of LDCV-priming (essentially k_1 in Figure 7E) can be assessed

319   in an experiment by varying the pre-flash intracellular $[Ca^{2+}]$, before an uncaging

320   flash is used to probe the size of the primed vesicle pool (Voets, 2000). We expressed

321   either Munc13-1 or ubMunc13-2 in $Unc13a^{KO}Unc13b^{KO}$ cells and extended the range

322   of pre-flash $[Ca^{2+}]$ values from the previously used 300-600 nM (Figure 2 and Figure

323   5) to 250-1200 nM (Figure 7A and Figure 7–figure supplement 1). In order to

324   compare the respective Ca^{2+}-sensitivities rather than the absolute priming rates of

325   Munc13-1 and ubMunc13-2 – and to overcome cell-to-cell variability – we

326   normalized capacitance traces to their value after 3 s (Figure 7A, left and middle

327   panels). Using the fractional increase in capacitance after 30 ms as a read-out of the

328   primed vesicle pool, we identified the characteristic Ca^{2+}-dependence of priming.

329   Strikingly, the Ca^{2+}-dependence was almost identical for the two isoforms and could

330   be fitted with a single Hill equation (Figure 7A right-hand panel, Table 1).

331   Thus, Ca^{2+}-dependent priming is supported with identical steady-state affinities in the

332   presence of Munc13-1 or ubMunc13-2. However, when applying Ca^{2+}-uncaging

333   flashes from a relatively low pre-flash $[Ca^{2+}]$, the two isoforms induce quite different

334   secretion kinetics (Figure 7B,C). For ubMunc13-2, secretion shows a clear sigmoid

335   shape with acceleration after ~0.5 s (Figure 7C), which is absent when the pre-flash

336    $[Ca^{2+}]$ is higher. This sigmoid shape of ubMunc13-2 driven secretion was noted

337   before and was attributed to a slow association of ubMunc13-2 with Calmodulin and

338   Ca^{2+}, resulting in a slow "priming switch" (Zikich et al., 2008). In contrast, Munc13-1

339   does not show this secondary acceleration (Figure 7B), regardless of the pre-flash

340    $[Ca^{2+}]$. To understand the origin of this behavior, we modeled the Ca^{2+} association

341   with the priming sensor (PS) explicitly - in our previous model (Walter et al., 2013),

342   this step had been assumed to be always in equilibrium. In accordance with the

343   observed identical steady state Ca^{2+} dependencies (Figure 7A), we used identical

344   dissociation constant (K_D) values for both isoforms, and varied only the on-rate, k_{on},

345   (the off-rate was changed simultaneously, $k_{off} = K_D*k_{on}$, with constant K_D). This led

346   to a very satisfactory fit to both Munc13-1 and ubMunc13-2 data from both low and

347   high pre-flash $[Ca^{2+}]$ (Figure 7B,C). Also, this model made it possible to fit both the

348   control trace, and the $Unc13b^{KO}$ trace (Figure 7D). Importantly, the fit was performed

349   simultaneously to all traces, so that we could ensure consistency between fits,

350   simplify the interpretation of parameter changes, and ensure that all conditions could

351   be reproduced by one version of our model (for fitted parameters, refer to Table 1).

352   Our model (Figure 7E) assumes that the NRP vesicles can only fuse after maturing to

353   the RRP state (Walter et al., 2013). Earlier models assumed that the 'NRP pool' can

354   fuse directly via an alternative pathway; in those cases, the corresponding pool was

355   called 'Slowly Releasable' (SRP) (Voets, 2000). We note that our conclusion that

356   Munc13-1/ubMunc13-2 exert their main effects upstream of both pools (NRP/SRP

357   and RRP), i.e. on k_1, is consistent with both ideas (see also Ashery et al., 2000).

358   Therefore our observations here do not necessarily distinguish between the parallel

359   pool model (SRP and RRP are both releasable) and the sequential pool model (only

360   the RRP is releasable); but see (Walter et al., 2013) for data supporting the sequential

361   pool model.

362   The modeling resulted in two main conclusions (Table 1): First, as expected, the

363   action of Munc13 (either isoform) is consistent with an increase in k_1 – the forward

364   rate of priming within the first priming step (Figure 7E). This is seen both by the

365   increase in the fitted k_1 upon overexpression of either isoform, and by the decrease of

366 k_1 in the *Unc13b*[KO] cells. Second, ubMunc13-2 increases k_1 4.5-fold more than

367 Munc13-1, but it does so after a longer delay. In the model, this delay is due to slower

368 kinetics of Ca^{2+} binding to the priming sensor (Zikich et al., 2008). Note that this step

369 might coincide with the translocation of Munc13-1 or ubMunc13-2 to the membrane

370 as a prerequisite for the priming action of the protein. Thus, the different delays might

371 reflect differences in the membrane translocation step. As a minor note, we also

372 noticed that k_2, the forward rate of downstream priming ("priming 2", Figure 7E)

373 always changed in the opposite direction of k_1. The reason for this is unclear, but one

374 explanation could be that the protein(s) driving downstream priming compete with

375 Munc13 for association with the fusion machinery.

376
377

Discussion

Our study provides a comprehensive analysis of the Munc13 protein family in LDCV docking and priming, and shows that genetic deletion of Munc13-1 (*Unc13a*) and Munc13-2 (*Unc13b*) severely impairs LDCV release in chromaffin cells. Yet surprisingly, LDCV docking, unlike SV docking, does not require Munc13s. We furthermore identify the step most sensitive to Munc13s in the LDCV priming process, and show that ubMunc13-2 and Munc13-1 accelerate this step with identical Ca^{2+} affinities but distinct Ca^{2+} binding rates.

The essential role of Munc13s in vesicular release appears to lie in the opening of Syntaxin (Stx-1)/Munc18-1 complexes, to permit the formation of Stx-1/SNAP-25 heterodimers that act as docking platforms for the vesicular SNARE protein Synaptobrevin-2 (Syb-2) (Hammarlund et al., 2007; Ma et al., 2011; Ma et al., 2013; Richmond et al., 1999; Sassa et al., 1999; Yang et al., 2015). SNARE complex assembly is thought to proceed in an N- to C-terminal zipper-like fashion (Fasshauer and Margittai, 2004; Pobbati et al., 2006; Sorensen et al., 2006; Walter et al., 2010), and, at least for SVs, this assembly seems to be the molecular correlate of both the physical docking process, and acquisition of fusion competence, which is referred to as priming (Imig et al., 2014). However, as will be discussed below, morphological docking of LDCVs does not require Munc13s (Figure 6), yet the priming of a full-sized RRP does (Figure 2), indicating that LDCV docking and functional priming do not represent a one-step process in neuroendocrine cells.

LDCV and SV docking have distinct requirements

401   Our data demonstrate that although Munc13s are critical for functional priming of

402   LDCVs in chromaffin cells, morphological LDCV docking, even when assessed by

403   3D-ET at unprecedented resolution, is not impaired in the absence of Munc13s

404   (Figure 6). Thus, in contrast to synapses, where most, if not all docked SVs are part of

405   the RRP, the majority of docked LDCVs in chromaffin cells are not primed, and the

406   functional RRP therefore cannot be distinguished from other docked LDCVs by

407   current ultrastructural methods. Although we cannot completely exclude the

408   possibility that Munc13-3 and Munc13-4 may be present at very low levels, that we

409   were unable to detect (Figure 1), it seems unlikely that their presence could account

410   for the full-sized pool of docked LDCVs in $Unc13a^{KO}Unc13b^{KO}$ cells.

411   This raises the question of how the non-primed LDCVs are docked. SV docking

412   requires the SNARE proteins Stx-1, SNAP-25, and Syb-2, as well as Munc13s and

413   CAPSs, but not necessarily the Ca^{2+} sensor of fusion, Synaptotagmin-1 (Syt-1) (Imig

414   et al., 2014). By contrast, current models imply that LDCV docking is mediated by

415   Syt-1, possibly via interaction with the Stx-1/SNAP-25 acceptor complex (de Wit et

416   al., 2006; de Wit et al., 2009; Parisotto et al., 2012). Additionally, Munc18-1 docks

417   LDCVs via its interaction with the closed form of Stx-1 (Gandasi and Barg, 2014;

418   Gerber et al., 2008; Gulyas-Kovacs et al., 2007; Han et al., 2011; Voets et al., 2001)

419   and is also involved in an additional tethering step (Toonen et al., 2006). The

420   vesicular SNAREs seem to be dispensable for docking in chromaffin cells

421   (Borisovska et al., 2005; Gerber et al., 2008), although they have been implicated in

422   PC12 cells (Wu et al., 2012). Some of these discrepancies are most likely due to

423   methodological and terminological differences as well as to limitations in assessing

424   true membrane attachment. However, since we used the same experimental approach

426 previously employed to detect SV docking deficits (Imig et al., 2014; Siksou et al.,

427 2009), our data clearly show that the molecular requirements of SV and LDCV

428 docking are distinct. More specifically, while the formation of the docked/primed

429 RRP requires Munc13s in both cases, and thus appears to be mechanistically quite

430 similar for SVs and LDCVs, the non-primed LDCVs in chromaffin cells appear to

431 dock via a separate, Munc13-independent mechanism.

432

433 Our findings are therefore consistent with the following model of LDCV docking and

434 priming: (i) LDCV docking mediated by Munc18-1/Stx-1, this configuration would

435 be the starting point for Munc13-mediated SNARE complex assembly, i.e. priming

436 (Ma et al., 2011; Ma et al., 2013), and in parallel, (ii) LDCV docking mediated by a

437 second configuration, that would not be expected to progress to SNARE complex

438 assembly directly or as efficiently, and thus be consistent with the large unprimed, but

439 docked LDCV pool. What this second configuration would look like in terms of

440 molecular interactions is less clear. Docking via Syt-1/Stx-1/SNAP-25 complexes

441 would be consistent with un-primed docking (de Wit et al., 2009). This mode of

442 docking would require the assumption that in chromaffin cells, Stx-1/SNAP-25

443 complexes can escape NSF/SNAP mediated disassembly. An additional or alternative

444 mode of un-primed docking may involve the recruitment of vesicles based on the

445 interaction of Syt-1 with phosphatidylinositol 4,5-bisphosphate (PIP2)/Stx-1 clusters

446 (Honigmann et al., 2013; Park et al., 2015), although further interactions may be

447 required to achieve close membrane apposition.

448 In the model suggested above, we explicitly included only molecular components for

449 which docking deficits have been demonstrated in chromaffin cells. However, LDCV

450 docking most likely involves additional factors. For instance, the docking of LDCVs

451 to Munc18-1/Stx-1 complexes probably requires the interaction between Munc18 and

452 the vesicle-associated small GTPases Rab3 and Rab27 (Graham et al., 2008; Tsuboi

453 and Fukuda, 2006; van Weering et al., 2007), and additional docking/tethering factors

454 may be involved in docking both primed and un-primed LDCVs to the membrane.

455

456 **Release kinetics of LDCV pools as correlates of SNARE complex assembly**

457 Our analysis of how Munc13s prime LDCVs for fusion identifies the earliest phase of

458 the priming process as the step most sensitive to Munc13s. We interpret our data

459 according to a model that features Munc13 as a Ca^{2+}-sensitive priming protein in a

460 single pathway to LDCV fusion with two serially arranged vesicle states or pools

461 (NRP and RRP) (Figure 7E). This essentially allows us to describe what was

462 previously interpreted as the release of two kinetically distinct LDCV pools (SRP and

463 RRP), as two sequential priming processes, priming 1 and priming 2, resulting in only

464 one releasable pool, the RRP (Walter et al., 2013). According to previous data, the

465 step most sensitive to Munc13s – priming 1 – is also the step affected by mutations

466 designed to interfere with the initiation of N-terminal SNARE complex assembly

467 (Walter et al., 2013; Walter et al., 2010). This is in line with a function of Munc13s in

468 initiating SNARE-complex assembly (Yang et al., 2015). The second priming step

469 may involve a downstream, presumably more C-terminal phase of SNARE-complex

470 assembly, although other options remain open. Thus, in the model (Figure 7E), the

471 formation of the NRP, i.e. the step most sensitive to Munc13s, most likely represents

472 the initiation of N-terminal SNARE complex assembly.

473

Catalysis of fast and slow LDCV priming by Munc13-1 and ubMunc13-2

As our study demonstrates, Munc13 isoforms differ in their ability to facilitate LDCV priming (Figure 5). We detected three endogenously expressed isoforms in murine chromaffin cells, Munc13-1, ubMunc13-2, and Baiap3 (Figure 1). Baiap3, somewhat surprisingly given its prominent expression and ability to translocate to membranes in a Ca^{2+}-dependent manner (Lecat et al., 2015), does not appear to be involved in LDCV priming in this cell type. However, Munc13-4, which regulates SNARE-mediated vesicle exocytosis in the hematopoietic system (Boswell et al., 2012; Feldmann et al., 2003; Shirakawa et al., 2004), and is the closest relative of Baiap3 (Koch et al., 2000), can promote LDCV priming, albeit less efficiently than Munc13-1 and ubMunc13-2.

The two most relevant isoforms, Munc13-1 and ubMunc13-2, promote LDCV priming with very similar steady-state Ca^{2+}-affinities, but nonetheless confer unique release kinetics depending on the pre-stimulus $[Ca^{2+}]$. Modeling of the secretion kinetics produced by overexpression of Munc13-1 and ubMunc13-2 in $Unc13a^{KO}Unc13b^{KO}$ cells allowed us to isolate the intrinsic properties of each isoform (Table 1). Secretion driven solely by the dominant isoform ubMunc13-2 shows a characteristic sigmoid shape at low pre-stimulus $[Ca^{2+}]$ (Figure 7C) (Zikich et al., 2008). In our secretion model, the best-fit parameters indicate 2.5-fold slower sensing of $[Ca^{2+}]$ for ubMunc13-2, which can however accelerate priming dramatically when $[Ca^{2+}]$ increases. Thus, the fitted maximum priming rate (k_1) for ubMunc13-2 is 4.5-fold higher than for Munc13-1. However, although Munc13-1 is unable to support the same maximum priming rate as ubMunc13-2, it reacts faster to a change in the $[Ca^{2+}]$ concentration (Figure 7B), which may reflect distinct conformational changes in response to $[Ca^{2+}]$, and/or differences in a membrane translocation step.

22

499

Thus, neuroendocrine cells can fundamentally modify the kinetics of secretion by expressing different Munc13 isoforms. Previous data from autaptic neurons showed that Munc13-1 causes short term depression, whereas ubMunc13-2 causes short-term facilitation (Rosenmund et al., 2002), which parallels our findings in chromaffin cells from low basal $[Ca^{2+}]$, raising the possibility that the functions of different Munc13 isoforms in priming LDCVs and SVs are conserved, even though their role in docking is not. Our model therefore provides a theoretical framework for how the molecular properties of priming factors may be linked to the kinetics of exocytosis.

508

Sequential actions of upstream and downstream priming catalysts

Although Munc13s have the strongest effect on the priming step 1, i.e. the formation of the NRP vesicle state, they also influence priming step 2, i.e. the formation of the RRP. Remarkably, overexpression and deletion of Munc13s change the rate constants k_1 (priming 1) and k_2 (priming 2) in opposite directions (Figure 7D). One possible reason for this effect could be that the interaction of Munc13s with the SNARE fusion machinery may compete with that of another priming protein, which mainly catalyzes priming step 2. A likely candidate for this second catalyst appears to be CAPS, as deletion of CAPS1 and CAPS2 leads to a significant reduction of the RRP, but has little effect on the NRP/SRP, placing Munc13 upstream of CAPS (Liu et al., 2010; Liu et al., 2008).

Furthermore, in PC12 cells, strong stimulation bypasses the need for CAPS-1 in LDCV exocytosis, but not the need for ubMunc13-2 (Kabachinski et al., 2014), and

522   the ability of CAPS to promote membrane fusion is impaired by C-terminal mutations

523   in Stx-1 (Daily et al., 2010). We therefore propose an LDCV priming model, in which

524   the Munc13-driven priming step 1 corresponds to the initiation of N-terminal

525   SNARE-complex assembly, and the CAPS-driven priming step 2 represents a more

526   C-terminal, and presumably more easily completed phase of zippering (Gao et al.,

527   2012). Assuming that priming step 2 is not catalyzed by CAPS alone but also

528   influenced by Munc13 and possibly Syt-1, such a model would also offer an

529   explanation as to why in both SV and LDCV exocytosis, lack of CAPS can be

530   compensated for by an increase in Ca^{2+}, whereas lack of Munc13 cannot (Jockusch et

531   al., 2007; Kabachinski et al., 2014).

532

533   **Conclusion**

534   In summary, our data show that mammalian neurons and neuroendocrine cells both

535   require Munc13s to generate fusion-competent vesicles, although the molecular steps

536   leading to LDCV docking prior to SNARE complex assembly appear to be unique. In

537   LDCV priming, the step most sensitive to Munc13s is the initial phase (priming step

538   1), which most likely corresponds to the initiation of N-terminal SNARE-complex

539   assembly. Individual Munc13 isoforms accelerate this step at distinct rates, thereby

540   imparting distinct properties on the kinetics of LDCV release, which indicates that

541   they may have specialized functions in the fine-tuning of catecholamine release in

542   response to varying physiological stimuli.

543

Materials and methods

Animals

All experiments were performed in compliance with the regulations of the local Animal Care and Use Committee of Lower Saxony, Oldenburg, Germany. The generation and basic characterization of the KO lines of the Munc13 isoforms has been described previously (Augustin et al., 2001; Augustin et al., 1999; Varoqueaux et al., 2002; Wojcik et al., 2013). $Unc13d^{KO}$ ($Unc13d^{Jinx}$) mice (Crozat et al., 2007) were obtained from Jackson Laboratories. Adult and perinatal mice were killed by decapitation prior to the removal of adrenal glands and other tissues.

Western blotting and antibodies

Adrenal glands of perinatal animals were excised and stored at -80°C prior to use. Adrenal glands from around 20 perinatal animals were pooled for the preparation of homogenates. Homogenates of whole adrenal glands were prepared by homogenization in an ice-cold buffer (320 mM D-glucose, 20 mM HEPES, 2 mM EDTA, pH 7.4, with 0.5 µg/ml leupeptin, 1 µg/ml aprotinin and 0.1 mM PMSF added freshly prior to homogenization), using a Potter S homogenizer. For the preparation of adrenal cortical and medullary homogenates, adrenal glands from WT animals were dissected in ice-cold buffer containing 19 mM NaH_2PO_4 and 81 mM Na_2HPO_4 and material from 5-6 animals was pooled. Spleen homogenates were prepared similarly and the DNA in the samples subsequently digested with 0.66 U/µl benzonase (E1014, Sigma-Aldrich) in 3.86 mM $MgCl_2$ for 10 min at 37°C prior to denaturation. Whole brain homogenates were prepared using a Potter S homogenizer and centrifuged for

25

567 10 min at 1000 g at 4°C to remove the nuclear fraction. Homogenates were analyzed

568 by Western blotting with the following antibodies at the indicated dilutions: rabbit-

569 anti-Munc13-1 (1:500) (126103, Synaptic Systems), rabbit-anti-ubMunc13-2

570 (1:2000), rabbit-anti-bMunc13-2 (1:1000), rabbit-anti-Munc13-3 (1:500)

571 (Varoqueaux et al., 2005), rabbit-anti-Baiap3 (1:1000) (Wojcik et al., 2013), goat-

572 anti-Munc13-4 (1:250) (NB100-41385; Novus Biologicals), rabbit-anti-

573 Chromogranin A (1:8000) (259002; Synaptic Systems), mouse-anti-GFP (1:500)

574 (11814460001; Roche), mouse-anti-valosin containing protein (VCP) (1:1000)

575 (612182; BD Transduction Laboratories), and mouse-anti-GAPDH (1:25000)

576 (ab8245; Abcam). Secondary antibodies (goat anti-rabbit IgG, 111035144; goat anti-

577 mouse IgG, 115035146, donkey anti-goat, 705-035-147) were obtained from Jackson

578 ImmunoResearch.

579

580 **Chromaffin cell culture**

581 Chromaffin cell cultures were prepared as described in Sørensen et al. (Sorensen et

582 al., 2003b). Cultures from $Unc13a^{KO}$ and $Unc13a^{KO}Unc13b^{KO}$ mice were prepared on

583 embryonic day (E)18, and from $Unc13b^{KO}$, $Unc13c^{KO}$, and $Baiap3^{KO}$ mice on

584 postnatal day (P)0, in each case using littermates of the appropriate genotypes as

585 controls. For overexpression of Baiap3 in WT cells, chromaffin cells from P0 WT

586 C57Bl/6N mice were used. Briefly, adrenal glands were excised and placed into ice-

587 cold Locke's solution (154 mM NaCl, 5.6 mM KCl, 0.84 mM NaH_2PO_4, 2.14 mM

588 Na_2HPO_4 and 10 mM D-glucose, pH 7.0). The glands were then transferred to 300 µl

589 of a papain solution [20 U/ml papain (Worthington Biochemical), 200 mg/L L-

590 cysteine, 1 mM $CaCl_2$, 0.5 mM EDTA, in DMEM (Gibco)], which had been

591   equilibrated for 15 min with 95 % O_2 and 5 % CO_2, and incubated with gentle shaking

592   for 45 min at 37°C. To terminate the papain digestion, 300 µl of inactivating solution

593   [10 % fetal bovine serum (Gibco), 2.5 g/L trypsin inhibitor (Gibco) and 2.5 g/L

594   albumin in DMEM (Gibco)] were then added, followed by an incubation period of 5

595   min at 37°C. The mixture of solutions was then replaced by 160 µl of DMEM

596   (Linaris) supplemented with 1 % insulin-transferrin-selenium X (Gibco) and 200 U/L

597   penicillin-streptomycin (Gibco). The glands were triturated with a 200-µl pipette tip

598   and the cell suspension was placed as 50 µl drops on coverslips in a 6-well plate.

599   Following an incubation period of 30 min at 37°C at 8 % CO_2 to allow cells to settle,

600   2 ml of DMEM (Linaris) with the supplements described above were added per well

601   and the cells were kept at 37°C and 8 % CO_2. The cells were used for

602   electrophysiological recordings on days *in vitro* 2-3.

603

604   **Viral constructs**

605   Expression constructs based on the SFV plasmid (pSFV1) for Munc13-1 and

606   ubMunc13-2, both subcloned in frame with a C-terminal EGFP, have been described

607   previously (Rosenmund et al., 2002). Munc13-4 and Baiap3 pSFV1 expression

608   constructs were generated as IRES-EGFP constructs using the full-length mouse

609   cDNAs. Production of SFV particles was done according to published protocols

610   (Ashery et al., 1999). Briefly, pSFV1 constructs and pSFV-helper2 DNA were

611   linearized with Spe I and transcribed into RNA using SP6 RNA polymerase. RNA

612   from the pSFV1 constructs and the pSFV-helper2 construct, 10 µg each, were

613   electroporated (500 V, 0.957 mF) into baby hamster kidney 21 cells. Supernatant of

614   cell cultures containing the virus was collected after 24 h. In cases where the virus

27

615 titer was low, the supernatant was concentrated approximately 25-fold using a filter

616 unit with a nominal molecular weight limit of 100 kDa (UFC910024, AMICON).

617 SFV particles encoding the respective Munc13 isoforms with EGFP or only EGFP as

618 a control were added to chromaffin cell cultures, infected cells identified based on the

619 EGFP fluorescence, and electrophysiological recordings performed 4-6 h after

620 addition of the virus.

621

622 **Whole-cell capacitance measurements**

623 Whole cell patch-clamping was performed with Sylgard-coated 4-6 MΩ pipettes

624 (Science Products) at a setup equipped with a Zeiss Axiovert 200 microscope (Zeiss)

625 and a HEKA EPC-10 amplifier controlled by Patchmaster (HEKA). Capacitance

626 measurements were performed according to the Lindau-Neher technique using the

627 „sine+dc" mode in the Lockin Extension of Patchmaster. The frequency and peak-to-

628 peak amplitude of the sine wave were 1042 Hz and 70 mV, respectively, and the

629 holding potential was -70 mV. Recordings were sampled at 12.5 kHz and filtered at

630 2.9 kHz. Flash photolysis experiments were performed according to established

631 protocols (Voets, 2000; Walter et al., 2010). The extracellular solution contained 147

632 mM NaCl, 10 mM HEPES, 11.1 mM D-glucose, 2.8 mM KCl, 2 mM $CaCl_2$, 1 mM

633 $MgCl_2$ and 3 µM tetrodotoxin (pH 7.2, 300-310 mOsM). For flash experiments, the

634 intracellular solution contained 109 mM L-glutamic acid, 35 mM HEPES, 5 mM

635 nitrophenyl-EGTA (Synaptic Systems), 5.65 mM $CaCl_2$, 2 mM Mg-ATP, 0.3 mM

636 Na-GTP, 0.205 mM fura-4F (Invitrogen), 0.3 mM furaptura (Invitrogen) and 1 mM

637 ascorbic acid (titrated to pH 7.2 with CsOH, osmolarity 290-295 mOsM). The flash

638 stimulus was applied approximately 80 s after the whole-cell configuration was

639   established using a xenon lamp (Rapp OptoElectronics). Unless otherwise specified,

640   only cells with pre-flash $[Ca^{2+}]$ in the range of 300-600 nM were used for analysis. In

641   flash photolysis experiments requiring pre-flash $[Ca^{2+}]$ concentrations higher than 600

642   nM (Figure 7 and Figure 7–figure supplement 1), pulses of light at wavelengths of

643   340 and 380 nm were applied at varying frequencies to release Ca^{2+} from nitrophenyl-

644   EGTA and the cell was kept at the target $[Ca^{2+}]$ for ~20 s before the flash stimulus

645   was given. The pre-flash $[Ca^{2+}]$ was taken as the averaged measured $[Ca^{2+}]$ during the

646   20 s period. In depolarization experiments, the same extracellular solution was used

647   except that tetrodotoxin was omitted. The intracellular solution contained 111 mM L-

648   glutamic acid, 35.5 mM HEPES, 17 mM NaCl, 1 mM $MgCl_2$, 2 mM Mg-ATP, 0.3

649   mM Na-GTP (titrated to pH 7.2 with CsOH, osmolarity 290-295 mOsM), and fura-4F

650   and furaptra at the same concentration used in flash experiments. In flash photolysis

651   experiments, pool sizes (fast and slow bursts) and their time constants were obtained

652   by fitting a sum of exponential functions to the capacitance traces (Sorensen et al.,

653   2003b), using a custom macro (Three-Exponential-Fit-Macro-Igor) with the software

654   IgorPro (WaveMetrics). The near-linear rate of release of the sustained component is

655   measured as a linear component with the unit capacitance increase per second. The

656   exocytotic delay was defined as the time point where the exponential fit meets the

657   pre-flash capacitance.

658

659   **Ca^{2+} measurements**

660   In flash experiments, exocytosis was stimulated by a sudden elevation of intracellular

661    $[Ca^{2+}]$ using UV flash stimuli given by a xenon flash lamp (Rapp OptoElectronics).

662    $[Ca^{2+}]$ measurements were performed according to established protocols (Voets,

663   2000; Walter et al., 2010). The ratiometric Ca^{2+} indicator dyes fura-4F and furaptra

664   were alternately excited at 340 and 380 nm using a Polychrome V monochromator

665   (TILL Photonics), and the emitted light was detected with a photomultiplier. The area

666   of fluorescence measurement was limited to the diameter of the cell. The 340/380 nm

667   fluorescence ratio was independently calibrated at the same dye concentrations with a

668   range of intracellular solutions with known $[Ca^{2+}]$, buffered with Ca^{2+} buffers 1,2-

669   bis(o-aminophenoxy)ethane-N,N,N',N'-tetraacetic acid (BAPTA, Invitrogen) and

670   diethylenetriaminepentaacetic acid (DPTA, Sigma-Aldrich). The $[Ca^{2+}]$ of the

671   calibration solutions was calculated using K_D of BAPTA = 0.222 μM and K_D of

672   DPTA = 80 μM.

673

674   **Amperometry**

675   Amperometric recordings were performed using carbon fibers of 5 μm diameter

676   (Thornel P-650/42; Cytec, NJ, USA), insulated using the polyethylene method

677   (Bruns, 2004). Vesicle fusion was triggered by infusing the cells through the patch

678   pipette with a solution containing 4.6 μM free Ca^{2+}. The fibers were clamped at 700

679   mV and currents were hardware filtered at 3 kHz using an EPC-7 patch clamp

680   amplifier (HEKA). Currents were digitized at 25 kHz and filtered off-line using a

681   Gaussian filter with a cut-off set at 1 kHz. Filtering, spike detection, and analysis

682   were performed using a freely available, custom-written macro (Mosharov and

683   Sulzer, 2005) running under IgorPro (Wavemetrics, Lake Oswego, OR). A spike

684   detection threshold of 5 pA and a foot detection threshold of 2 pA were imposed. For

685   each analyzed cell, the median of each parameter (duration, halftime, amplitude,

686   charge, rise time, decay time, foot amplitude, foot duration, foot charge) was

687 calculated from all spikes, and this value was used for averaging between cells

688 (giving the mean of cell medians).

689

High-pressure freezing of adrenal gland slices for EM analysis

691 Adrenal glands from E18 animals were embedded in 3% low gelling agarose (Sigma-

692 Aldrich) and adrenal gland slices were prepared according to published protocols

693 (Moser and Neher, 1997). Slices were allowed to recover in bicarbonate-buffered

694 saline (125 mM NaCl, 26 mM $NaHCO_3$, 2.5 mM KCl, 1.25 mM NaH_2PO_4, 2 mM

695 $CaCl_2$, 1 mM $MgCl_2$, 10 mM D-glucose and 0.2 mM (+)-tubocurarine) at 37°C for 15

696 min and were subsequently kept in the same solution at RT before cryofixation. Slices

697 were rapidly frozen in external cryoprotectant (20% BSA in bicarbonate-buffered

698 saline) using a HPM100 HPF device (Leica). After freezing, samples were stored in

699 liquid nitrogen until further processing. Freeze substitution was performed as

700 previously published (Rostaing et al., 2006). Briefly, samples were substituted in

701 anhydrous acetone, fixed by 2% OsO_4 in acetone for 7h at -90°C prior to a

702 temperature ramp (5°C/h) to -20°C, an incubation for 16 h at -20°C, and a final ramp

703 (10°C/h) to 4°C. Samples were washed in acetone and infiltrated with EPON resin at

704 room temperature (acetone/EPON 1:1 for 3 h, 90% EPON in acetone overnight, and

705 pure EPON for 36 h). Finally specimen carriers containing infiltrated samples were

706 incubated for 24 h at 60°C to polymerize. Aluminum sample carriers were trimmed

707 off the EPON block with a specimen trimming device (EM TRIM2, Leica) to expose

708 the surface of the embedded tissue for ultramicrotomy.

709

Sectioning, contrasting, and fiducial marker application for EM

710 **Sectioning, contrasting, and fiducial marker application for EM**

711 An Ultracut UCT ultramicrotome (Leica) was used to cut 500 nm-thick sections until

712 the first tissue appeared. Ultrathin (50 nm) and semithin (400 nm) sections were then

713 collected onto Formvar-filmed, carbon-coated copper slot or mesh grids for 2D and

714 3D ultrastructural analysis, respectively. Vitrified samples were subjected to rigorous

715 quality control (Mobius et al., 2010) and samples exhibiting indications of ice-crystal

716 damage were excluded from the analysis. Ultrathin sections were post-stained with

717 1% uranyl acetate in ddH$_2$O for 30 min, washed several times in ddH$_2$O, stained with

718 0.3% lead citrate for 2 min, washed, and dried with filter paper. For 3D-tomographic

719 analysis, 400 nm-thick sections were briefly incubated in a solution of Protein A

720 conjugated to 10 nm gold particles (Cell Microscopy Center, Utrecht, The

721 Netherlands) to introduce fiducial markers.

722

723 **2D-EM analysis of chromaffin cells**

724 Electron micrographs (2048 x 2048 pixels) were acquired with a sharp:eye CCD

725 camera (Tröndle, TRS) at 5,000 fold magnification using the multiple image

726 acquisition and alignment feature of iTEM software (version 5.1, Olympus Soft

727 Imaging Solutions GmbH). Assembled montages had dimensions of approximately 21

728 x 21 μm and typically contained 1-3 randomly chosen chromaffin cells. Only

729 chromaffin cells with a clearly visible plasma membrane were included in the

730 analysis. iTEM software was used to measure chromaffin cell plasma membrane

731 circumference and the cytoplasmic area (calculated by subtraction of the nuclear area

732 from the total cell area). Additional parameters including the number of LDCVs and

733 the shortest distance of each LDCV to the plasma membrane were quantified using

734   ImageJ software. In regions where the plasma membrane did not appear as a clear cut,

735   the shortest distance from the vesicle membrane to the middle of the membrane

736   projection was measured. For this reason and due to the fact that LDCVs (mean

737   diameter of CTRL LDCVs ~ 160 nm; ~ 170 nm for docked CTRL LDCVs) might

738   have their centers outside of the imaged ultrathin (50 nm) section, we did not quantify

739   LDCV docking as defined by physical membrane-attachment in these 2D projection

740   images, but rather calculated the number of membrane-proximal LDCVs (within 0-40

741   nm of the plasma membrane). All vesicles with electron-dense cargo were included in

742   the analysis. Secretory vesicles of both genotypes exhibited heterogeneity in size (see

743   Figure 6–figure supplement 1) and appearance (e.g. compactness of the dense-core),

744   possibly reflecting distinct LDCV types (e.g. adrenaline vs. noradrenaline) or

745   different levels of maturity present in immature (E18) chromaffin cells. Data are

746   presented as LDCV density (number of LDCVs per μm^2 area cytoplasm), the number

747   of LDCVs within 40 nm of the plasma membrane normalized to the cell perimeter,

748   and the mean frequency distribution of LDCVs from the plasma membrane in 40 nm

749   bins.

750

751   **3D-EM analysis of LDCV docking in chromaffin cells**

752   For high-resolution electron tomographic analysis of LDCV docking in adrenal

753   chromaffin cells, we randomly selected regions between two neighboring cells that

754   exhibited a high density of LDCVs in proximity of the plasma membrane in thick

755   (400 nm) sections. Single-axis tilt series were acquired from -60° to +60° with 1°

756   increments and binned by the factor two at 10,000 fold magnification using an Orius

757   SC1000 camera (Gatan) and the SerialEM software for automated tilt series

758   acquisition (Mastronarde, 2005). Tomograms were reconstructed using the IMOD

759   package (Kremer et al., 1996), and exported as z-stacks for analysis with ImageJ

760   (National Institutes of Health). All analyses were performed blindly and manually.

761   The smallest distance between the plasma membrane and the outer leaflet of each

762   LDCV membrane was measured at its vesicular midline using the straight-line tool of

763   ImageJ on individual virtual z-slices. Only vesicles observed in physical contact with

764   the plasma membrane in tomographic volumes were considered 'docked'. In

765   distribution analyses docked LDCVs were assigned to the 0-4 nm bin to account for

766   the voxel dimensions of reconstructed tomograms (isotropic voxel size = 2.86 nm).

767   The number of membrane-attached (0-4 nm, 'docked') LDCVs identified in a

768   tomographically reconstructed volume was normalized to the number of membrane-

769   proximal (0-40 nm of plasma membrane) LDCVs. The number of membrane-

770   proximal (0-40 nm) LDCVs was expressed as a percentage of all LDCVs within 100

771   nm of the plasma membrane and compared across genotypes to test for potential

772   differences in the ability of recruiting LDCVs close to the plasma membrane. The

773   frequency distribution displays the number of docked LDCVs (0-4 nm, first bin) and

774   subsequently the distances of LDCVs from the plasma membrane in 2 nm bins.

775   Statistical analyses were performed using Student's *t*-test. The mean LDCV diameter

776   was calculated from the area of the LDCV measured at its midline including the

777   vesicular phospholipid bilayer in electron tomograms by using the elliptical selection

778   tool in ImageJ. All LDCVs in the randomly chosen field of view that contained their

779   midline within the tomographic volume were analyzed (Figure 6–figure supplement

780   1).

781

34

782　For illustrative purposes, figures depicting tomographic subvolumes represent an

783　overlay of 3 consecutive slices produced by the slicer tool of the 3dmod software of

784　the IMOD package to generate a ~ 8.6 nm thick subvolume.

785

786　**Calculation of the number of docked LDCVs per chromaffin cell**

787　The size of the pool of docked LDCVs per cell can be calculated from the number of

788　LDCVs per µm² PM area (n_a) (Parsons et al., 1995; Plattner et al., 1997). We

789　measured the number of membrane-proximal LDCVs (0-40 nm of PM) per µm PM

790　length (n_l) in ultrathin sections of 0.05 µm thickness. Our 3D-ET approach permitted

791　us to accurately measure LDCV diameters (d_v in µm) in chromaffin cells (Figure 6–

792　figure supplement 1). The number of LDCVs per µm² PM area could then be

793　calculated as $n_a = n_l/(d_v + 0.05)$ (Parsons et al., 1995; Plattner et al., 1997). The

794　average cell surface area (a_c in µm^2) per genotype was estimated based on the average

795　cell capacitance measured in cultured cells assuming 1 µF/cm^2. We chose this

796　method, rather than using the cell circumference measured in ultrathin sections,

797　because the chromaffin cells in adrenal slices are not round, but have rather complex

798　shapes (Figure 6–figure supplement 1). Using either measurement, the

799　$Unc13a^{KO}Unc13b^{KO}$ cells were slightly larger, by 5% based on capacitance, and by

800　15% based on cell circumference measurements.

801　_$Unc13a^{Het}Unc13b^{Het}$ control cells:_

802　n_l: 0.984 ± 0.075 vesicles per µm length

803　d_v: 0.1627 ± 0.005 µm vesicle diameter

804　n_a: 4.627 vesicles per µm² PM area

805　a_c: 421.23 µm² cell membrane area

35

806   The estimated number of LDCVs within 40 nm of the PM in $Unc13a^{Het}Unc13b^{Het}$

807   chromaffin cells is therefore ~ 1949. Our 3D analysis of LDCV docking revealed that

808   only 33.97% of LDCVs within 40 nm of the PM are physically attached to the PM,

809   therefore we estimated the pool of docked vesicles to contain ~ 662 LDCVs in our

810   acute adrenal slice preparation.

811   _$Unc13a^{KO}Unc13b^{KO}$ cells:_

812   n_l: 1.150 ± 0.083 vesicles per μm length

813   d_v: 0.1525 ± 0.003 μm vesicle diameter

814   n_a: 5.679 vesicles per μm² PM area

815   a_c: 442.26 μm² cell membrane area

816   The estimated number of LDCVs within 40 nm of the PM in $Unc13a^{KO}Unc13b^{KO}$

817   chromaffin cells is therefore ~ 2511. Our 3D analysis of LDCV docking revealed that

818   only 34.43% of LDCVs within 40 nm of the PM are physically attached to the PM,

819   therefore we estimated the pool of docked vesicles to contain ~ 865 LDCVs in our

820   acute adrenal slice preparation.

821

822   **Modeling the Ca^{2+}-dependence of vesicle priming**

823   We simulated capacitance traces in Ca^{2+} uncaging experiments with an exocytosis

824   model that was adapted from a previous study of ours (Walter et al., 2013) to

825   explicitly describe the Ca^{2+} dependent vesicle priming reaction. Parameters (Table 1)

826   were either taken from the literature, directly from experiments (Figure 7A), or

827   determined by fitting the model to experimental capacitance traces.

828   We assume that Munc13 proteins act on the Ca^{2+}-dependent priming reaction, which

829   ensures the refilling of vesicles from a large depot pool (Voets, 2000). We wanted to

830   explicitly describe this reaction in terms of its thermodynamic steady state binding

831   properties (i.e. its dissociation constant K_D and cooperativity n), and in terms of its

832   Ca^{2+} binding kinetics. Let the chemical equation for Ca^{2+} binding to the priming

833   sensor (PS) be:

$$n * Ca^{2+} + PS \underset{k_{off}}{\overset{k_{on}}{\rightleftharpoons}} (Ca_n PS)$$
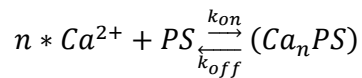
834   Then its overall dissociation constant K_D is defined as:

835    $K_D \quad = \frac{[Ca^{2+}]^n [PS]}{[Ca_n PS]}$

836   In order to obtain the values of the K_D and n experimentally, we pre-equilibrated

837   chromaffin cells at different pre-flash $[Ca^{2+}]$ prior to uncaging. The secretion

838   responses were averaged in bins and normalized to their respective values 3 s after the

839   flash. Fitting the fraction of release 30 ms after the uncaging stimulus as a function of

840   pre flash $[Ca^{2+}]$ with the following Hill equation (non-linear curve fitting routine of

841   Origin Pro 8 G, OriginLab Corporation) allowed us to estimate K_D and n:

842    $Fraction([Ca^{2+}]) = \frac{[Ca^{2+}]^n}{K_D + [Ca^{2+}]^n} Fmax$

843   Where $Fraction([Ca^{2+}])$ is the relative release at 30 ms, and $[Ca^{2+}]$ is the pre-flash

844   Ca^{2+} concentration. $Fmax$, n and K_D are free parameters. The best fit is shown as

845   solid line in Figure 7 and the values of n and K_D can be found in Table 1.

846   We assume that priming is only increased when the proper number (n) of Ca^{2+} ions

847   are bound to the PS. Therefore, the rate of priming is proportional to the fraction (f)

848   of PS that has bound the correct number of Ca^{2+} ions divided by the total amount of

849   PS:

$$f(Ca^{2+}) = \frac{[Ca_nPS]}{[PS_{tot}]}$$

$$k_1(Ca^{2+}) = f(Ca^{2+}) \, k_{1Max}$$

850   Where k_{1Max} is the asymptotic value of the priming rate for $[Ca^{2+}] \gg K_D$. Since the

851   total amount of PS is the sum of Ca^{2+}-free and Ca^{2+}-bound PS:

$$f(Ca^{2+}) = \frac{[Ca_nPS]}{[Ca_nPS] + [PS]}$$

852   At steady state, the following relationships hold:

$$f(Ca^{2+})_{SteadyState} = \frac{[Ca^{2+}]^n}{K_D + [Ca^{2+}]^n}$$

$$k_{1SteadyState}(Ca^{2+}) = \frac{[Ca^{2+}]^n}{K_D + [Ca^{2+}]^n} \, k_{1Max}$$

853   In order to describe temporal changes in this fraction at non-equilibrium conditions,

854   the Ca^{2+} concentrations ($[Ca^{2+}]$) were interpolated from the experimental values and

855   the concentration of $[Ca_nPS]$ was calculated at all time points by numerical

856   integration using the "ode15s" function in Matlab (version R2013a, Mathworks) of

857   the kinetic equation:

$$\frac{d[Ca_nPS]}{dt} = k_{on}[PS][Ca^{2+}]^n - k_{off}[Ca_nPS]$$

858   We assume that the amount of PS is not limiting (i.e. that each vesicle contains a PS).

859   Then, by investing the relationships

$$[V_{tot}] = [Ca_nPS] + [PS]$$

860 and

$$K_D = \frac{k_{off}}{k_{on}}$$

861 we obtain

$$\frac{d[Ca_nPS]}{dt} = k_{on}([V_{tot}] - [Ca_nPS])\,[Ca^{2+}]^n - K_D k_{on}[Ca_nPS]$$

862 Such that the fraction of activated PS can be calculated at time t:

$$f(Ca^{2+}, t) = \frac{[Ca_nPS](t)}{[V_{tot}]}$$

863

864 This allows us to calculate k_1 at all times t.

865

$$k_1(Ca^{2+}, t) = f(Ca^{2+}, t)\,k_{1Max}$$

866

867 Our model consists of a sequence of mandatory steps for vesicle maturation and

868 fusion (Walter et al., 2013). Vesicles from the depot enter a non-releasable state

869 (NRP, Figure 7E) from which they cannot fuse directly. Instead, these vesicles first

870 need to mature into the readily releasable pool (RRP), a transition that is governed by

871 a Ca^{2+}-dependent rate constant ($k_2(Ca^{2+})$). This Ca^{2+}-dependence is modeled by a

872 Ca^{2+}-dependent catalyst as described in Walter et al., 2013:

873

874    $k_2(Ca^{2+})=k_{20}+g(Ca^{2+})k_{2cat}$

875    $k_{-2}(Ca^{2+})=k_{-20}+g(Ca^{2+})k_{-2cat}$

$$k_{-2cat} = \frac{k_{-20}}{k_{20}} k_{2cat}$$

876

877   As in our previous study, we assume that binding of one Ca^{2+} ion activates the

878   catalyst and that the catalyst is in equilibrium with Ca^{2+}, which allows us to calculate

879    $g(Ca^{2+})$:

880

$$g(Ca^{2+}) = \frac{[Ca^{2+}]}{K_{D,cat} + [Ca^{2+}]}$$

881

882   **Kinetic equations of the exocytosis model**

$$\frac{d[Depot]}{dt} = -k_1(Ca^{2+})[Depot] + k_{-1}[NRP]$$

$$\frac{d[NRP]}{dt} = k_1(Ca^{2+})[Depot] - \left(k_{-1} + k_2(Ca^{2+})\right)[NRP] + k_{-2}(Ca^{2+})[RRP]$$

$$\frac{d[RRP]}{dt} = k_2(Ca^{2+})[NRP] - (k_{-2}(Ca^{2+}) + 3k_3[Ca^{2+}])[RRP] + k_{-3}[RRPCa]$$

$$\frac{d[RRPCa]}{dt} = 3k_3[Ca^{2+}][RRP] - (k_{-3} + 2k_3[Ca^{2+}])[RRPCa] + 2k_{-3}[RRPCa_2]$$

$$\frac{d[RRPCa_2]}{dt} = 2k_3[Ca^{2+}][RRPCa] - (2k_{-3} + k_3[Ca^{2+}])[RRPCa_2]$$
$$+ 3k_{-3}[RRPCa_3]$$

40

$$\frac{d[RRPCa_3]}{dt} = k_3[Ca^{2+}][RRPCa_2] - (3k_{-3} + k_4)[RRPCa_3]$$

$$\frac{d[F]}{dt} = k_4[RRPCa_3]$$

883

Modeling of exocytosis upon Ca^{2+} uncaging

885   To find the steady state occupation of the system $k_1(Ca^{2+})$, $k_2(Ca^{2+})$ and

886    $k_{-2}(Ca^{2+})$ were calculated for the experimental pre-flash Ca^{2+} values, the first five

887   kinetic equations were taken and set to zero, and mass conservation of vesicles was

888   obeyed:

$$0 = [Depot]_0 + [NRP]_0 + [RRP]_0 + [RRPCa]_0 + [RRPCa_2]_0 + [RRPCa_3]_0$$
$$- [V_{tot}]$$

889   This system of 6 equations was solved using the function "fsolve" of Matlab (version

890   R2013a, Mathworks). The steady state values were used as starting values for the

891   numerical integration of the kinetic equations using the "ode15s" function of Matlab

892   (version R2013a, Mathworks).

893   For parameter optimization, model simulations were compared to experimental

894   capacitance data. The parameters of the model were varied and the goodness of fit

895   was determined in a cost function, which was the sum of squared deviations between

896   data and model prediction. In order to avoid bias towards data with larger secretion,

897   the cost function used relative deviations: deviations were normalized to the maximal

898   value of either the experimental capacitance trace or the simulated one, whichever

899   was smaller (led to a larger cost):

41

$$cost = \frac{weight(i)}{\max(y_{Experiment})} \sum_i \left(y_{Experiment}(i) \right.$$

$$\left. - y_{Model}(i) \right)^2 \quad for \; \max(y_{Experiment}) < \max(y_{Model})$$

$$cost = \frac{weight(i)}{\max(y_{Model})} \sum_i \left(y_{Experiment}(i) \right.$$

$$\left. - y_{Model}(i) \right)^2 \quad for \; \max(y_{Experiment}) \geq max\,(y_{Model})$$

900

901 Three principal kinetic components have been described in capacitance traces: a fast

902 component with a time constant of tens of milliseconds, a slower component with a

903 time constant of hundreds of milliseconds and a sustained component with a time

904 constant of several seconds (Voets, 2000). To account for the fact that more data

905 points exist for slower components (due to the constant sampling rate) which would

906 dominate the fit, deviations at shorter times after the uncaging flash were given larger

907 weight: the weight was 100 for all datapoints upto 80 ms after the uncaging stimulus;

908 the weight was 10 for all datapoints occurring later than 80 ms, but earlier than 1.2 s

909 after the stimulus; the weight was 1 for all datapoints thereafter. To ensure

910 consistency, all conditions depicted in Figure 7 were fitted simultaneously and the

911 cost values from all data were summed. Only the parameters labeled with "best fit" in

912 Table 1 were allowed to vary. Because the lowest bin of the capacitance traces of the

913 DKO overexpressing Munc13-1 and ubMunc13-2 in Figure 7B and C contained

914 relatively few cells, the total number of vesicles was also a free parameter under these

915 conditions (best fit values V_{tot}(Munc13-1 low Ca^{2+})=2120 fF, V_{tot}(Munc13-1 low

916 Ca^{2+})=3340 fF). All parameters were optimized by minimizing the cost values using a

917   Nelder-Mead Simplex algorithm implemented in the Matlab function "fminsearch"

918   (version R2013a, Mathworks).

919

920   **Statistics**

921   Statistical analyses were performed using two-tailed Student's *t*-test, ANOVA with

922   post-hoc Tukey's test, or Mann-Whitney U test as specified in the figure legends.

923

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935   M.M., C.I., A.W., P.P. and B.C. performed research; K-M.M., C.I., A.W., P.P., D.S.,

936   J.R., J.S. and S.W. analyzed data; K-M.M., C.I., A.W., J.S., N.B. and S.W. wrote the

937   paper.

938
939

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1210
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Figure legends

Figure 1.

1215   **Expression of Munc13 isoforms in the mouse adrenal gland**. KO mouse lines of

1216   the respective Munc13 isoform were used as control. The antibodies used to detect

1217   individual Munc13 isoforms and loading controls are indicated on the left. (**A**)

1218   Munc13-1 (*) is barely detectable in perinatal adrenal gland. (**B**) ubMunc13-2, but not

1219   bMunc13-2, is expressed. (**C**) Munc13-3 was not detected. (**D**) Baiap3 was detected,

1220   but not (**E**) Munc13-4. *Jx* refers to mice homozygous for the *Unc13d^{Jinx}* mutation

1221   (Crozat et al., 2007). (**F**) Munc13-1 and Baiap3 are mainly located in the medulla

1222   (Med), but ubMunc13-2 is present in cortex (Cort) as well. Please note that the

1223   difference in the position of ubMunc13-2 relative to the marker in panels (**B**) and (**F**)

1224   is due to how far the respective gels were run. Loading controls were valosin-

1225   containing protein (VCP), glyceraldehyde-3-phosphate dehydrogenase (GAPDH) and

1226   CgA. Brain samples, and spleen tissue in the case of Munc13-4, were used for

1227   comparison. Please note that the *Baiap3*^{KO} (Wojcik et al., 2013) and *Unc13a*^{KO}

1228   animals express truncated protein products, whereas the truncated product present in

1229   the *Unc13b*^{KO} (Cooper et al., 2012) is not shown here. Based on previous analyses of

1230   the *Unc13a*^{KO} and *Unc13b*^{KO} mice (Augustin et al., 1999; Cooper et al., 2012;

1231   Varoqueaux et al., 2002), the truncated Munc13-1 and Munc13-2 products are neither

1232   functional, nor do they have a dominant-negative effect. The truncated Baiap3

1233   product was not detected in adrenal gland, and its effect in the brain, where it can be

1234   detected in young animals up to P21, is currently unknown (Wojcik et al., 2013). See

1235   also Figure 1–figure supplement 1.

1236

Figure 1–figure supplement 1.

1238 **Comparison of Munc13-1, Munc13-2, and Munc13-3 expression.** (**A**) Adrenal

1239 gland and brain homogenates from *Unc13a*-EYFP, *Unc13b*-EYFP, and *Unc13c*-

1240 EGFP knockin (KI) mice were probed using an antibody that recognizes the GFP-

1241 derived tags, which allowed us to compare expression levels of Munc13-1,

1242 ubMunc13-2, bMunc13-2 and Munc13-3 with the same antibody. The two bands

1243 detected in the *Unc13b*-EYFP KI brain sample represent ubMunc13-2 (lower band)

1244 and bMunc13-2 (upper band). While all isoforms were detected in brain, ubMunc13-

1245 2-EYFP was the only isoform readily detectable in the adrenal gland. Valosin-

1246 containing protein (VCP) was used as a loading control. (**B**) Adrenal gland and brain

1247 samples from *Unc13a*[KO]*Unc13b*[KO] (DKO) mice and WT controls, probed with

1248 antibodies to Munc13-1, ubMunc13-2 and VCP as a loading control. Expression of

1249 Munc13-1 (marked with *, to distinguish it from co-migrating bands of higher

1250 molecular weight) is low in E18 adrenal glands, while ubMunc13-2 can be readily

1251 detected. The expression of both isoforms was abrogated in DKO mice. Please note

1252 that, like the single KO lines, the DKO animals express truncated Munc13-1 protein

1253 products and a truncated Munc13-2 product (Cooper et al., 2012), the latter is not

1254 shown here.

1255

1256 **Figure 2.**

1257 **Flash photolysis induced LDCV exocytosis in chromaffin cells.** For each KO line,

1258 the average intracellular $[Ca^{2+}] \pm$ SEM and the average ΔCm are shown in panels (**A**-

1259 **C**, and **E**). Single gene deletions of (**A**) *Unc13a*, (**B**) *Unc13c*, or (**C**) *Baiap3* did not

1260 impair LDCV exocytosis. (**D**) Summary of burst sizes, sustained release rates, and

1261   time constants. (**E**) LDCV exocytosis is dramatically reduced in $Unc13a^{KO}Unc13b^{KO}$

1262   cells, $Unc13a^{WT}Unc13b^{KO}$ cells, and $Unc13a^{Het}Unc13b^{KO}$ cells. This reduction is

1263   primarily due to the absence of ubMunc13-2. (**F**) Fast burst, slow burst and the rate of

1264   sustained release are reduced in the absence of Munc13-1 and ubMunc13-2, as well as

1265   in the absence of ubMunc13-2 alone (ANOVA with post-hoc Tukey's test). (**G**)

1266   Compared to $Unc13a^{WT}Unc13b^{KO}$ cells, the deletion of Munc13-1 causes significant

1267   reductions in the slow burst and the rate of sustained release (Student's *t*-test, two-

1268   tailed). (**H**) Delay of the onset of exocytosis after the flash stimulus (ANOVA with

1269   post-hoc Tukey's test). (**I**) Normalized traces show identical release kinetics of the

1270   exocytotic burst. (**J**) Summary of the release components shown in panels (**E**, **F, G**).

1271   Time constants are not significantly different (ANOVA with post-hoc Tukey's test).

1272   ($p < 0.05$: *, $p < 0.01$: **, $p < 0.001$: ***). See also Figure 2–figure supplement 1.

1273

1274   **Figure 2–figure supplement 1.**

1275   **Single gene deletion or overexpression of Baiap3 does not affect LDCV**

1276   **exocytosis in chromaffin cells.** (**A**) LDCV exocytosis was elicited by a series of

1277   depolarizing steps to analyze the Immediately-Releasable Pool (IRP) of vesicles

1278   localized in the vicinity of Ca^{2+} channels and the Readily-Releasable Pool (RRP).

1279   Shown are the averaged $[Ca^{2+}] \pm$ SEM, ΔCm, and whole-cell current traces. The inset

1280   in the whole-cell current panel shows an enlargement of the first 100 ms

1281   depolarization. (**B**) The size of the IRP, estimated based on the release elicited by 6

1282   short (10 ms) depolarization pulses, was not altered. (**C**) The total ΔCm caused by the

1283   depolarization train was also not altered in $Baiap3^{KO}$ cells. Note that the data from

1284 $Baiap3^{KO}$ and $Baiap3^{WT}$ were matched for equal ionic influx according to the ionic

1285 charge of the first 100 ms depolarization to eliminate effects of differential ionic

1286 influx. (**D**) Overexpression (OE) of Baiap3 in WT chromaffin cells. EGFP expression

1287 in WT cells was used as control. Shown are the averaged $[Ca^{2+}] \pm$ SEM and ΔCm

1288 traces. (**E**) Burst sizes, rate of sustained release and the kinetics of release were not

1289 altered by the overexpression of Baiap3 (Student's *t*-test, two-tailed). (**F**) Normalized

1290 traces of *Baiap3*^WT and *Baiap3*^KO cells overlapped with each other, indicating no

1291 alterations in release kinetics in *Baiap3*^KO cells.

1292

1293 **Figure 3.**

1294 **Absence of Munc13-2 results in a significant release deficit in response to**

1295 **depolarization.** (**A**) Shown are the averaged $[Ca^{2+}] \pm$ SEM, ΔCm, and whole-cell

1296 current traces of *Unc13b*^WT and *Unc13b*^KO cells. The inset in the whole-cell current

1297 panel shows an enlargement of the first 100 ms depolarization. (**B**) ΔCm elicited by

1298 individual depolarizations was significantly different between the two groups. RRP,

1299 Readily-releasable Pool; IRP, Immediately-Releasable Pool. (**C**) The size of the RRP

1300 was measured as the ΔCm after the train of depolarization pulses and was

1301 significantly reduced in *Unc13b*^KO cells. ($p < 0.05$: *****, $p < 0.01$: ******, $p < 0.001$: *******;

1302 Student's *t*-test, two-tailed).

1303

1304 **Figure 4.**

1305 **Reduced number of fusion events of catecholamine-containing LDCVs in the**

1306 **absence of Munc13-2.** (**A**) Illustration of a single amperometric spike, corresponding

1307 to the release of catecholamines from a single LDCV, and the parameters analyzed.

1308   (**B**) Representative amperometric recordings of a $Unc13b^{WT}$ and a $Unc13b^{KO}$ cell. (**C**)

1309   Dramatic reduction in spike frequency in $Unc13b^{KO}$ chromaffin cells. (**D**) Spike

1310   duration, (**E**) width at half amplitude ($t_{1/2}$), (**F**) maximum spike amplitude, (**G**)

1311   amperometric charge, (**H**) rise time, and (**I**) decay time were unchanged in the

1312 $Unc13b^{KO}$. The stability of fusion pores was also not altered, as shown by the

1313   unchanged (**J**) foot amplitude, (**K**) duration and (**L**) charge. (**M**) The fraction of

1314   amperometric spikes with a detectable foot was reduced in $Unc13b^{KO}$ cells. ($p <$

1315 0.05:*, $p < 0.001$:***; Student's t-test, two-tailed).

1316

1317   **Figure 5.**

1318   **LDCV exocytosis in $Unc13a^{KO}Unc13b^{KO}$ chromaffin cells is rescued by**

1319   **overexpression (OE) of Munc13-1, ubMunc13-2 and Munc13-4, but not Baiap3.**

1320 $Unc13a^{KO}Unc13b^{KO}$ cells were infected with SFV-Munc13-1-EGFP, SFV-

1321   ubMunc13-2-EGFP, SFV-Munc13-4-IRES-EGFP or SFV-Baiap3-IRES-EGFP using

1322   SFV-EGFP as control. (**A**) Averaged $[Ca^{2+}] \pm$ SEM and capacitance traces ΔCm are

1323   shown in the same graph to compare the efficiency of rescue: ubMunc13-2 >

1324   Munc13-1 > Munc13-4 > Baiap3. (**B**) Burst sizes and rates of sustained release after

1325   analysis of individual traces. ($p < 0.05$: *, $p < 0.001$: ***; Student's t-test, two-tailed).

1326   See Figure 5–figure supplement 1 for a direct comparison of Munc13-1-EGFP and

1327   ubMunc13-2-EGFP expressing cell matched for fluorescence intensity and Figure 5–

1328   figure supplement 2 for Western blotting to confirm the expression of Munc13-4 and

1329   Baiap3 from the IRES-constructs.

1330

1331 **Figure 5–figure supplement 1.**

1332 **Direct comparison of Munc13-1 and ubMunc13-2 over-expressing cells with**

1333 **matching EGFP fluorescence. (A)** Averaged $[Ca^{2+}] \pm$ SEM and capacitance traces

1334 ΔCm are shown in the same graph to compare the efficiency of rescue. **(B)**

1335 Fluorescence intensity in arbitrary units (a.u.) of $Unc13a^{KO}Unc13b^{KO}$ chromaffin

1336 cells over-expressing Munc13-1-EGFP and ubMunc13-2-EGFP. **(C)** Expression of

1337 ubMunc13-2 resulted in a significantly larger enhancement of burst size and rate of

1338 sustained release than expression of Munc13-1. ($p < 0.01$: **, $p < 0.001$: ***;

1339 Student's *t*-test, two-tailed).

1340

1341 **Figure 5–figure supplement 2.**

1342 **Western blot analysis confirming expression of Munc13-4 and Baiap3 SFV**

1343 **constructs.** Neuronal cultures were infected with SFV-Baiap3-IRES-EGFP and SFV-

1344 Munc13-4-IRES-EGFP, respectively. Tissue samples from *Baiap3* and *Unc13d* (Jx)

1345 WT and KO mice were used as controls. Both Baiap3 and Munc13-4 were expressed.

1346 Please note, that we had to use infected neurons for this analysis, because our

1347 chromaffin cell cultures do not provide enough material for Western blotting.

1348

1349 **Figure 6.**

1350 **Ultrastructural analysis of LDCV docking in adrenal chromaffin cells.** 2D-EM of

1351 **(A)** $Unc13a^{Het}Unc13b^{Het}$ (CTRL) and **(B)** $Unc13a^{KO}Unc13b^{KO}$ (DKO) adrenal glands.

1352 **(C)** Frequency distribution of LDCVs within 0-2 μm of the plasma membrane (PM).

1353 **(D)** Membrane-proximal LDCVs (0-40 nm of PM) normalized to PM circumference.

1354   (**E**) Total number of LDCVs normalized to cytoplasmic area. (**F-K**) Tomographically

1355   reconstructed subvolumes from 400 nm-thick sections through (**F-H**)

1356 $Unc13a^{\text{Het}}Unc13b^{\text{Het}}$ and (**I-K**) $Unc13a^{\text{KO}}Unc13b^{\text{KO}}$ cells in which docked LDCVs

1357   (enlarged in panels **G**, **J**) and undocked LDCVs (enlarged in panels **H** and **K**, small

1358   gaps separating undocked LDCVs from the PM indicated with arrowheads) can be

1359   distinguished. (**L**) Frequency distribution of membrane-proximal LDCVs distributed

1360   within 0-40 nm of the PM. (**M**) Number of membrane-proximal LDCVs expressed as

1361   a percentage of all LDCVs within 0-100 nm of the plasma membrane. (**N**) Percentage

1362   of docked LDCVs with respect to all membrane-proximal LDCVs (within 0-40 nm of

1363   PM). Scale bars represent 1 μm in (**A**, **B**), 200 nm in (**F**, **I**), and 50 nm in (**H**, **K**). *C:*

1364   *5779 LDCVs in CTRL and 4120 LDCVs in DKO profiles. **D, E**: CTRL: N=2, n=36; DKO:*

1365   *N=2; n=27. **L**: 473 LDCVs in CTRL and 386 LDCVs in DKO tomographic subvolumes.*

1366   ***M, N**: CTRL: N=2, n=24;DKO: N=2, n=26.* Values indicate mean ± SEM. (Student's *t*-

1367   test, two-tailed). See also Figure 6–figure supplement 1.

1368

1369   **Figure 6–figure supplement 1.**

1370   **Ultrastructural analysis of LDCV size in adrenal chromaffin cells.** 2D-EM of (**A**)

1371 $Unc13a^{\text{Het}}Unc13b^{\text{Het}}$ (CTRL) and (**B**) $Unc13a^{\text{KO}}Unc13b^{\text{KO}}$ (DKO) adrenal glands. (**C**)

1372   Frequency distribution of LDCV diameters measured by 3D-ET. (**D**) Mean LDCV

1373   diameter in randomly imaged chromaffin cell areas. (**E**) LDCV diameter of docked

1374   (0-4 nm of PM) vesicles. Scale bar in B represents 5 μm. *C: 846 LDCVs in CTRL and*

1375   *831 LDCVs in DKO tomographic subvolumes. **D**: CTRL: N=2, n=24; DKO: N=2,*

1376   *n=26. **E**: CTRL: N=2, n=98; DKO: N=2, n=140.* Values indicate mean ± SEM.

1377 (Student's t-test, two-tailed; diameters of docked LDCVs were tested with Mann-

1378 Whitney U test).

1379

1380 **Figure 7.**

1381 **Munc13-1 and ubMunc13-2 accelerate upstream vesicle priming ("priming step**

1382 **1") with identical Ca^{2+} affinities, but distinct rates.** (**A**) Estimation of steady-state

1383 Ca^{2+} affinities of vesicle priming driven by Munc13-1 or ubMunc13-2. Left two

1384 panels: binned and averaged secretory responses in $Unc13a^{KO}Unc13b^{KO}$ cells

1385 overexpressing (OE) either Munc13-1 (green) or ubMunc13-2 (blue). The release

1386 fraction at 30 ms (traces normalized to their amplitude after 3 s) after the stimulus was

1387 determined as the read-out for priming (vertical broken lines). Right panel: the

1388 fraction of release plotted as a function of pre-flash [Ca^{2+}]. Both genotypes are

1389 described by the same Hill function, suggesting a similar Ca^{2+}-dependence of priming

1390 (i.e. identical cooperativity and affinity). (**B**) Fits of a secretion model (see panel **E**) to

1391 the capacitance responses observed experimentally in $Unc13a^{KO}Unc13b^{KO}$ cells

1392 expressing Munc13-1 at intermediate and low pre-flash [Ca^{2+}]. Top panel: measured

1393 Ca^{2+} values were used to drive the secretion model. The chemical equation shows a

1394 priming sensor (PS), which is active in the Ca^{2+} bound state (Ca$_n$PS). The lower panel

1395 shows the experimental capacitance data (solid lines) together with simulations with

1396 the best fit parameters (broken lines; see Table 1). Insert: magnified view with

1397 horizontal/vertical scale bars: 200 ms/200 fF. (**C**) Same as (**B**), but for

1398 $Unc13a^{KO}Unc13b^{KO}$ cells expressing ubMunc13-2. The secretion model was fitted

1399 using identical K$_{DS}$ and n for the Ca^{2+} binding to the PS (determined by the analysis

1400 shown in panel **A**). The best-fit values suggest a ~2.5-fold slower on-rate (activation

58

1401 rate), but a 4.5-fold higher maximal priming rate for ubMunc13-2 (see Table 1),

1402 resulting in a sigmoidal secretion response from low pre-flash [Ca^{2+}]. (**D**) Fitting our

1403 secretion model to the experimental data of several genotypes suggests that Munc13-1

1404 and ubMunc13-2 both primarily act by increasing the forward priming rate (k_1, see

1405 also panel **E**), while loss of ubMunc13-2 – the dominant endogenous isoform – has

1406 the opposite effect. The downstream priming step ("priming 2", k_2) changes in the

1407 opposite direction. (**E**) Secretion model: Munc13-1 (green) and ubMunc13-2 (blue)

1408 regulate the Ca^{2+} binding rates (k_{on}) to a PS, which controls the asymptotic forward

1409 priming rate k_1 (see text for details, Table 1 for fitted parameters, and Walter et al.,

1410 2013 for model development). NRP: Non-Releasable Pool; RRP: Readily-Releasable

1411 Pool; F: Fused pool. See also Figure 7–figure supplement 1.

1412

1413 **Figure 7–figure supplement 1.**

1414 **Exocytotic burst size as a function of pre-flash [Ca^{2+}]. *Unc13a*^{KO}*Unc13b*^{KO} cells**

1415 **were rescued by Munc13-1 or ubMunc13-2 overexpression (OE).** For ubMunc13-

1416 2, data from 23 cells already analyzed for Figure 5 were included here as well. The

1417 average size of the exocytotic burst is plotted as the ΔCm 0.5 s after the flash stimulus

1418 ± SEM against average pre-flash [Ca^{2+}] ± SEM. Cells were binned according to pre-

1419 flash [Ca^{2+}]. *For Munc13-1, bins are 250-350 nM (n = 17), 350-450 nM (n = 15),*

1420 *450-550 nM (n = 11), 550-800 nM (n = 11), 800-1200 nM (n = 13), 1200-1700 nM (n*

1421 *= 7). For ubMunc13-2, bins are 250-350 nM (n = 11), 350-450 nM (n = 20), 450-540*

1422 *nM (n = 10), 540-800 nM (n = 12), 800-1200 nM (n = 10) and 1200-1700 nM (n =*

1423 *4).*

1424

1425   Table 1. Parameters of the exocytosis model.

Parameter	Control	$Unc13b^{KO}$	Munc13-1 OE	ubMunc13-2 OE	Comment
V_{tot}	2350				Total number of vesicles, best fit
k_1	$p(Ca^{2+}) \cdot k_{1max}$				
$p(Ca^{2+})$	$\dfrac{(Ca^{2+})^n}{(Ca^{2+})^n + (K_{D,cat})^n}$				fraction of activated PS
k_{on}	$8.96*10^{-9}$ s^{-1} μM^{-n}	$2.25*10^{-8}$ s^{-1} μM^{-n}	$2.25*10^{-8}$ s^{-1} μM^{-n}	$8.96*10^{-9}$ s^{-1} μM^{-n}	On-rate calcium binding to PS
k_{1Max}	$1.99*10^{-2}$ s^{-1}	$6.89*10^{-3}$ s^{-1}	$7.44*10^{-2}$ s^{-1}	$3.42*10^{-1}$ s^{-1}	Maximal priming rate, best fit
$\sqrt[n]{K_D}$	0.407 μM				Experiment, Hill plot Figure 7
k_{-1}	$4.70*10^{-1}$ s^{-1}				Best fit
n	7.38				Cooperativity PS, experiment, Hill plot Figure 7
k_2	$= k_{20} + g(Ca^{2+}) \cdot k_{2cat}$				(Walter et al., 2013)
k_{-2}	$= k_{-20} + g(Ca^{2+}) \cdot k_{-2cat}$				(Walter et al., 2013)
$g(Ca^{2+})$	$\dfrac{Ca^{2+}}{Ca^{2+} + K_{D,cat}}$				(Walter et al., 2013)
k_{20}	$2.37*10^{-2}$ s^{-1}	$2.95*10^{-2}$ s^{-1}	$1.29*10^{-2}$ s^{-1}	$5.80*10^{-3}$ s^{-1}	Best fit
k_{2cat}	$3.95*10^{1}$ s^{-1}	$4.91*10^{1}$ s^{-1}	$2.14*10^{1}$ s^{-1}	$9.65*10^{0}$ s^{-1}	Best fit
k_{-20}	$2.10*10^{-2}$ s^{-1}				Best fit
k_{-2cat}	$= k_{2cat} \cdot k_{-20} / k_{20}$				(Walter et al., 2013)
$K_{D, cat}$	138 μM				Best fit
k_3	4.4 $s^{-1}\mu M^{-1}$				(Voets, 2000)
k_{-3}	56 s^{-1}				(Voets, 2000)
k_4	1450 s^{-1}				(Voets, 2000)

1426

1427

Man et al. Figure 1

A *Unc13a*

E18 Brain | E18 Adrenal
WT KO | WT KO

Munc13-1

kDa
−200
−150
−120

VCP

−85

B *Unc13b*

P0 Brain | P0 Adrenal
WT KO | WT KO

ubMunc13-2

−200

VCP

−85

bMunc13-2

−200
−100

VCP

C *Unc13c*

P0 Brain | P0 Adrenal
WT KO | WT KO

kDa

Munc13-3

−200

VCP

−85

D *Baiap3*

P0 Brain | P0 Adrenal
WT KO | WT KO

Baiap3

−120

VCP

−85

E *Unc13d*

P0 Spleen | P0 Adrenal
WT Jx | WT Jx

Munc13-4

−130

GAPDH

−35

F

Unc13a P0 Brain | WT Adult Adrenal
WT KO | Cort Med

Munc13-1

kDa
−200

CgA

−85

Unc13b Adult Brain | WT Adult Adrenal
WT KO | Cort Med

ubMunc13-2

−200

CgA

−85

Baiap3 Adult Brain | WT Adult Adrenal
WT KO | Cort Med

Baiap3

−120

CgA

−85

A

B

C

D

Genotypes	*n*	Fast burst	Slow burst	Rate of sustained release	Fast time constant	Slow time constant
*Unc13a*WT	41	130.73±12.31 fF	112.78±11.25 fF	38.86±3.35 fF/s	23.32±3.96 ms	646.14±121.99 ms
*Unc13a*KO	45	137.11±10.38 fF	130.45±11.23 fF	45.28±3.47 fF/s	21.42±2.04 ms	772.96±168.40 ms
*Unc13c*WT	51	88.37±6.37 fF	86.53±8.53 fF	34.20±2.67 fF/s	33.17±6.96 ms	533.33±155.96 ms
*Unc13c*KO	50	95.43±7.06 fF	94.95±9.07 fF	34.93±2.51 fF/s	26.01±3.68 ms	503.31±83.80 ms
*Baiap3*WT	56	102.70±7.32 fF	106.19±7.43 fF	41.47±2.83 fF/s	20.29±2.03 ms	431.50±76.57 ms
*Baiap3*KO	59	119.48±8.16 fF	126.20±10.87 fF	41.66±3.03 fF/s	22.79±2.43 ms	686.55±120.68 ms

E

F

G

H

I

Normalized at 1s

— *Unc13a*WT/Het *Unc13b*Het
— *Unc13a*KO *Unc13b*Het
— *Unc13a*WT *Unc13b*KO
— *Unc13a*Het *Unc13b*KO
— *Unc13a*KO *Unc13b*KO

J

| Genotypes	Fast burst	Slow burst	Rate of sustained release	Fast time constant	Slow time constant
*Unc13a*WT/Het *Unc13b*Het	84.02±4.32 fF	78.34±4.39 fF	22.48±1.43 fF/s	19.40±0.93 ms	504.88±65.45 ms
*Unc13a*KO *Unc13b*Het	85.82±6.19 fF	81.21±7.17 fF	24.24±1.96 fF/s	16.14±1.33 ms	441.52±69.08 ms
*Unc13a*WT *Unc13b*KO	32.52±3.33 fF	42.15±4.11 fF	5.85±0.68 fF/s	17.56±2.35 ms	454.40±78.51 ms
*Unc13a*Het *Unc13b*KO	27.23±2.34 fF	40.57±3.66 fF	4.26±0.74 fF/s	22.06±3.31 ms	580.62±123.37 ms
*Unc13a*KO *Unc13b*KO	22.75±3.55 fF	32.96±3.95 fF	2.79±0.36 fF/s	22.33±2.66 ms	461.09±66.99 ms

Man et al. Figure 3

Man et al. Figure 4
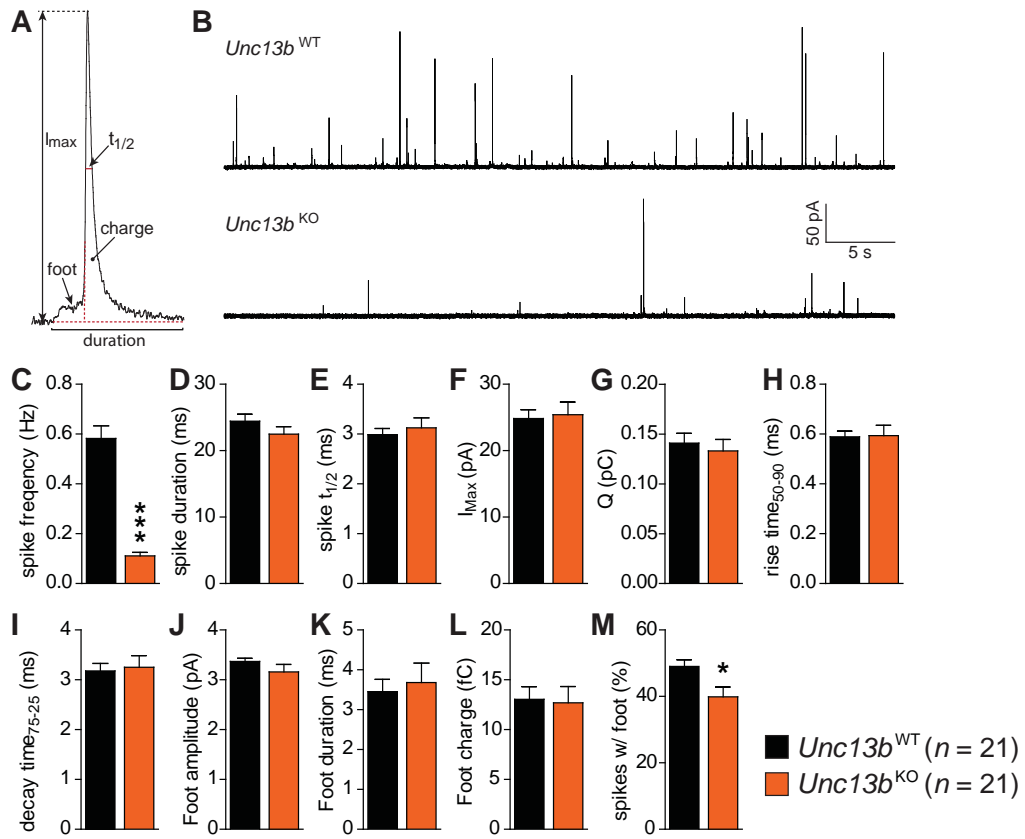
Man et al. Figure 5

Man et al. Figure 6

2D Analysis: Ultrathin Sections

3D Analysis: Tomography

Unc13a Het *Unc13b* Het

A

B

Unc13a KO *Unc13b* KO

F

G

H

I

J

K

C

Unc13a Het *Unc13b* Het (CTRL)
Unc13a KO *Unc13b* KO (DKO)

Frequency Distribution (%) vs LDCV Distance from the PM (µm)

D

ns.
P=0.146

Membrane-Proximal LDCVs per µm PM — CTRL DKO

E

ns.
P=0.129

Total LDCVs per µm² Area — CTRL DKO

L

Unc13a Het *Unc13b* Het (CTRL)
Unc13a KO *Unc13b* KO (DKO)

Frequency Distribution of Membrane-Proximal LDCVs (%) vs LDCV Distance from the PM (nm)

M

ns.
P=0.985

Membrane-Proximal LDCVs (%) — CTRL DKO

N

ns.
P=0.819

Docked LDCVs (%) — CTRL DKO