1   **Drosophila Synaptotagmin 7 negatively regulates synaptic vesicle fusion and replenishment**

2   **in a dosage-dependent manner**

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1

14 **Abstract**

15 Synchronous neurotransmitter release is triggered by Ca^{2+} binding to the synaptic vesicle protein

16 Synaptotagmin 1, while asynchronous fusion and short-term facilitation is hypothesized to be

17 mediated by plasma membrane-localized Synaptotagmin 7 (SYT7). We generated mutations in

18 Drosophila *Syt7* to determine if it plays a conserved role as the Ca^{2+} sensor for these processes.

19 Electrophysiology and quantal imaging revealed evoked release was elevated 2-fold. *Syt7* mutants

20 also had a larger pool of readily-releasable vesicles, faster recovery following stimulation, and

21 robust facilitation. *Syt1/Syt7* double mutants displayed more release than *Syt1* mutants alone,

22 indicating SYT7 does not mediate the residual asynchronous release remaining in the absence of

23 SYT1. SYT7 localizes to an internal membrane tubular network within the peri-active zone, but

24 does not enrich at release sites. These findings indicate the two Ca^{2+} sensor model of SYT1 and

25 SYT7 mediating all phases of neurotransmitter release and facilitation is not applicable at

26 Drosophila synapses.

27

28   **Introduction**

29   Neurotransmitter release from presynaptic terminals is the primary mechanism of synaptic

30   communication and is mediated by fusion of synaptic vesicles (SVs) with the plasma membrane

31   at specific sites known as active zones (AZs) (Katz, 1969; Südhof, 2013; Zhai and Bellen, 2004).

32   A highly conserved protein machinery composed of the SNARE complex drives fusion between

33   the SV and AZ lipid bilayers (Littleton et al., 1998; Söllner et al., 1993; Sutton et al., 1998; Tucker

34   et al., 2004). Ca^{2+} influx through voltage-gated Ca^{2+} channels functions as the trigger to activate

35   the fusion process (Borst and Sakmann, 1996; Katz and Miledi, 1970, 1967; Schneggenburger and

36   Rosenmund, 2015; Südhof, 2012). The majority of SVs fuse during a synchronous phase that

37   occurs within a few milliseconds of Ca^{2+} entry (Borst and Sakmann, 1996; Goda and Stevens,

38   1994; Llinás et al., 1981; Sabatini and Regehr, 1996; Yoshihara and Littleton, 2002). Many

39   synapses also have an asynchronous component that results in SV release over hundreds of

40   milliseconds (Goda and Stevens, 1994; Hefft and Jonas, 2005; Kaeser and Regehr, 2014;

41   Yoshihara and Littleton, 2002). Asynchronous release normally accounts for less than 5% of SV

42   fusion following single action potentials at Drosophila neuromuscular junctions (NMJs) (Jorquera

43   et al., 2012). This slower phase of release becomes more prominent during high rates of stimulation

44   (Atluri and Regehr, 1998; Lu and Trussell, 2000; Rozov et al., 2019; Zucker and Regehr, 2002)

45   and mediates all SV fusion at some neuronal connections (Best and Regehr, 2009; Peters et al.,

46   2010). Changes in the kinetics and amount of SV fusion also occur during high frequency

47   stimulation, resulting in facilitation or depression depending on the synapse (Zucker and Regehr,

48   2002). Defining the molecular machinery and Ca^{2+} sensors that regulate the distinct modes and

49   kinetics of SV release is essential for understanding synaptic transmission.

50   The Synaptotagmin (SYT) family of Ca^{2+} binding proteins contain key regulators that control

51   the timing of SV release. SYT proteins contain a transmembrane domain and two Ca^{2+} binding C2

52   domains termed C2A and C2B (Adolfsen et al., 2004; Adolfsen and Littleton, 2001; Perin et al.,

53   1990; Sugita et al., 2002; Ullrich and Südhof, 1995). Mammals have three SYT family members

54   that localize to SVs (SYT1, SYT2 and SYT9), while *Drosophila* contains a single member of the

55   SV subfamily (SYT1) (Littleton et al., 1993a; Pang et al., 2006; Xu et al., 2007). These SYT

56   isoforms bind Ca^{2+} and activate synchronous fusion of SVs via interactions with membranes and

57   the SNARE complex (Chang et al., 2018; Chapman and Jahn, 1994; Fernández-Chacón et al.,

58   2001; Geppert et al., 1994; Guan et al., 2017; Lee et al., 2013; Lee and Littleton, 2015; Littleton

59   et al., 1994, 1993b; Mackler et al., 2002; Nishiki and Augustine, 2004; Tucker et al., 2004; Xu et

60   al., 2007; Yoshihara and Littleton, 2002; Young and Neher, 2009). Beyond SV localized SYTs,

61   SYT7 is the only other family member implicated in Ca^{2+}-dependent SV trafficking, although

62   additional SYT isoforms participate in Ca^{2+}-dependent fusion of other secretory organelles and

63   dense core vesicles (DCVs) (Adolfsen et al., 2004; Cao et al., 2011; Dean et al., 2012; Li et al.,

64   1995; Moghadam and Jackson, 2013; Park et al., 2014; Shin et al., 2002; Yoshihara et al., 2005).

65   Multiple mechanisms have been proposed to mediate the asynchronous component of

66   neurotransmitter release, including distinct Ca^{2+} sensors, heterogeneity in SV protein content, SV

67   distance from Ca^{2+} channels, distinct Ca^{2+} entry pathways, or regulation of Ca^{2+} extrusion and

68   buffering (Chanaday and Kavalali, 2018; Fesce, 1999; Kaeser and Regehr, 2014; Pang and Südhof,

69   2010; Rozov et al., 2019; Zucker and Regehr, 2002). Although several mechanisms may

70   contribute, the observation that *Syt1* mutants have enhanced asynchronous release indicates

71   another Ca^{2+} sensor(s) activates the remaining slower Ca^{2+}-dependent component of exocytosis

72   (Huson et al., 2019; Kochubey and Schneggenburger, 2011; Nishiki and Augustine, 2004; Turecek

73   and Regehr, 2019; Yang et al., 2010; Yoshihara et al., 2010; Yoshihara and Littleton, 2002).

74   Though controversial, SYT7 has emerged as a popular candidate for the asynchronous Ca^{2+} sensor

75   (Bacaj et al., 2013; Chen et al., 2017; Maximov et al., 2008; Saraswati et al., 2007; Turecek and

76   Regehr, 2019, 2018; Weber et al., 2014; Wen et al., 2010). SYT7 has also been postulated to

77   function as the Ca^{2+} sensor for short-term synaptic facilitation (Chen et al., 2017; Jackman et al.,

78   2016; Turecek and Regehr, 2018). SYT7 has higher Ca^{2+} sensitivity, tighter membrane binding

79   affinity and longer Ca^{2+}-lipid disassembly kinetics than SYT1 (Hui et al., 2005; Sugita et al., 2002,

80   2001; Voleti et al., 2017). These properties suggest SYT7 may regulate SV dynamics farther away

81   from the AZ Ca^{2+} nanodomains that are required for SYT1 activation, or during temporal windows

82   following the decay of the initial peak of Ca^{2+} influx. Together, these data have led to a two Ca^{2+}

83   sensor model for evoked SV exocytosis, with SYT1 triggering the rapid synchronous phase of

84   neurotransmitter release and SYT7 mediating asynchronous fusion and facilitation.

85   Although SYT7 manipulations can alter asynchronous release and facilitation at some

86   synapses, several studies have suggested alternative explanations for the reduced fusion or

87   identified unrelated defects in SV trafficking (Figure 1A). The recent observation that

88   asynchronous release at mammalian synapses is anti-correlated with the levels of the synchronous

89   Ca^{2+} sensors SYT1 and SYT2, but does not correlate with SYT7, prompted re-interpretation of

90   earlier data on the protein's function (Turecek and Regehr, 2019). Besides asynchronous release,

91   mammalian SYT7 has been implicated in SV endocytosis, SV replenishment, SV pool mobility,

92   and DCV fusion and replenishment (Bacaj et al., 2015; Dolai et al., 2016; Durán et al., 2018;

93   Fukuda et al., 2004; Gustavsson et al., 2011; Li et al., 2017; Liu et al., 2014; Schonn et al., 2008;

94   Tsuboi and Fukuda, 2007; Virmani et al., 2003; Wu et al., 2015). SYT7 has also been shown to

95   regulate cell migration, lysosomal fusion and membrane repair in non-neuronal cells (Barzilai-

96   Tutsch et al., 2018; Chakrabarti et al., 2003; Colvin et al., 2010; Czibener et al., 2006; Flannery et

97   al., 2010; Jaiswal et al., 2004; Martinez et al., 2000; Reddy et al., 2001; Zhao et al., 2008).

98   Similar to the uncertainty surrounding SYT7 function, its subcellular localization is also

99   unclear, with different studies localizing the protein to the plasma membrane, DCVs, lysosomes,

100   endosomes or other internal compartments (Adolfsen et al., 2004; Czibener et al., 2006; Flannery

101   et al., 2010; Martinez et al., 2000; Mendez et al., 2011; Monterrat et al., 2007; Schonn et al., 2008;

102   Shin et al., 2002; Sugita et al., 2001; Zhao et al., 2008). A key supporting argument for SYT7 as

103   the asynchronous Ca^{2+} sensor is its reported localization to the AZ plasma membrane, positioning

104   it at sites of SV fusion (Sugita et al., 2001). If SYT7 were present on endosomes or other internal

105   membrane compartments, it would be more compatible with a role in SV trafficking rather than

106   the fusion process itself. In summary, conflicting studies have generated confusion over how SYT7

107   contributes to neurotransmission and if the protein plays distinct roles across different neuronal

108   subpopulations or species.

109   To examine the function of SYT7 in Drosophila, we generated and characterized *Syt7* null

110   mutants. The Drosophila NMJ exhibits similar asynchronous release and facilitation properties to

111   those of mammals (Jan and Jan, 1976; Jorquera et al., 2012; Yoshihara and Littleton, 2002),

112   making it a useful system to examine evolutionary conserved functions of SYT7 in

113   neurotransmitter release. *Syt7* mutants and *Syt1; Syt7* double mutants display increased evoked

114   neurotransmitter release, indicating SYT7 negatively regulates SV fusion independent of SYT1.

115   In addition, CRISPR-mediated tagging of the endogenous *Syt7* locus indicates SYT7 localizes to

116   a tubular network inside the presynaptic terminal that resides within the peri-active zone (peri-AZ)

117   region, but is not enriched at sites of SV fusion. These data define a role for SYT7 in restricting

118   SV availability and fusion, and indicate SYT7 is not a major Ca^{2+} sensor for asynchronous release

119   and facilitation in Drosophila.

120 **Results**

121 **Evolutionary conservation and structural comparison of SYT1 and SYT7**

122 Synaptotagmins form one of the largest protein families involved in membrane tracking, with

123 17 *Syt* genes encoded in mammals and 7 *Syt* genes found in Drosophila (Adolfsen and Littleton,

124 2001; Craxton, 2010; Sugita et al., 2002). Unlike the SV subfamily of SYTs, only a single *Syt7*

125 gene is present in vertebrate and invertebrate genomes, making phenotypic comparisons easier. To

126 examine the evolutionary relationship between SYT1, SYT7 and the more distantly related

127 extended-Synaptotagmin (E-SYT) proteins, a phylogenetic tree was generated using the

128 BLOSUM62 matrix and neighbor joining clustering analysis with protein sequences from placozoa

129 (*Trichoplax adhaerens*), invertebrates (*Caenorhabditis elegans*, *Drosophila melanogaster, Ciona*

130 *intestinalis*) and vertebrates (*Danio rerio*, *Rattus norvegicus*, *Homo sapiens*, Figure 1B). Although

131 Trichoplax lacks neurons, it is the earliest metazoan that encodes *Syt* genes and contains both a

132 SYT1 and SYT7 homolog (Barber et al., 2009). The phylogenetic tree contains independent

133 clusters that correspond to the SYT1, SYT7 and E-SYT2 protein families. The clustering of SYT1

134 homologs across evolution correlates with nervous system complexity, with the Trichoplax

135 homolog forming the outlier member of the cluster. Within the SYT7 cluster, *C. elegans* SYT7 is

136 the most distantly related member, with the Trichoplax homolog residing closer within the cluster.

137 Drosophila SYT7 is more distant from the vertebrate subfamily clade than is Drosophila SYT1

138 within its subfamily, suggesting SYT7 sequence conservation is not as closely related to nervous

139 system complexity as SYT1. These observations are consistent with SYT7's broader expression

140 pattern and function within neuronal and non-neuronal cells (MacDougall et al., 2018).

141 To compare SYT1 and SYT7 proteins, we performed homology modeling between

142 Drosophila SYT7 and the published structure of mammalian SYT7 (*R. norvegicus* SYT7; PBD:

143 6ANK) (Voleti et al., 2017). Key structural features are highly conserved in the homology model,

144 including the eight-stranded β-barrel and the Ca^{2+} binding loops that form the core of C2 domains

145 (Figure 1C). In contrast to SYT1, both Drosophila and mammalian SYT7 lack the C2B HB helix

146 previously found to have an inhibitory role in SV fusion (Xue et al., 2010). We next performed

147 sequence alignment of SYT proteins from *H. sapiens*, *R. norvegicus* and *D. melanogaster* (Figure

148 1 – figure supplement 1). Drosophila SYT7 is 59% identical to human SYT7. Comparing the SYT1

149 and SYT7 subfamilies, the N-terminus encoding the transmembrane domain and linker region has

150 the greatest variability and shares only 21% identity. Within the C2 domains, there is 100%

6

151   conservation of the negatively charged Ca^{2+} binding residues in the C2 loops. A polybasic stretch

152   in the C2B domain that mediates Ca^{2+}-independent PI(4,5)P2-lipid interactions is also conserved.

153   These sequence conservations indicate Ca^{2+}-dependent and Ca^{2+}-independent membrane binding

154   are key properties of both SYT proteins.

155   Beyond lipid binding, SYT1's interaction with the SNARE complex is essential for its ability

156   to activate SV fusion. Five key C2B residues (S332, R334, E348, Y391, A455) form the primary

157   interaction site that docks SYT1 onto the SNARE complex (Guan et al., 2017; Zhou et al., 2015).

158   Four of the five primary SNARE binding residues are not conserved in Drosophila SYT7 (Figure

159   1C, Figure 1 – figure supplement 1). In addition, Drosophila and mammalian SYT7 contain

160   specific amino acids substitutions at two of these residues that block SNARE binding and abolish

161   SYT1 function in SV fusion (Guan et al., 2017), including C285 (corresponding to *Syt1* mutant

162   R334C) and K299 (corresponding to *Syt1* mutant E348K). A secondary SNARE complex-binding

163   interface on SYT1 is mediated by conserved basic residues at the bottom on the C2B β-barrel

164   (R451/R452 in Drosophila; R388/R389 in rodents) and is also not conserved in the SYT7

165   subfamily (Wang et al., 2016; Xue et al., 2010; Zhou et al., 2015). As such, SYT7 is unlikely to

166   engage the SNARE complex via the primary or secondary C2B interface, highlighting a key

167   difference in how the proteins regulate membrane trafficking. Beyond SNARE-binding, 20

168   nonsynonymous amino acid substitutions are conserved only in the SYT1 or SYT7 subfamilies,

169   suggesting additional interactions have likely diverged during evolution from the common

170   ancestral SYT protein. In summary, SYT1 and SYT7 are likely to regulate membrane trafficking

171   through distinct mechanisms, consistent with chimeric SYT1/SYT7 rescue experiments in

172   mammals (Xue et al., 2010).

173

174   **Generation of Drosophila *Syt7* mutations**

175   To assay SYT7 function in Drosophila, the CRISPR-Cas9 system was used to generate null

176   mutations in the *Syt7* locus. Using a guide RNA targeted near the *Syt7* start codon, several missense

177   mutations were obtained. To disrupt the coding frame of *Syt7*, a single base pair cytosine deletion

178   mutant (*Syt7^M1^*) located seven amino acids downstream of the start codon was used for most of the

179   analysis, with an unaffected Cas9 injection line as control (Figure 1D). A Minos transposon

180   insertion in the second coding exon of *Syt7* was also identified from the BDGP gene disruption

181   project (Bellen et al., 2004) that generates a premature stop codon before the C2A domain,

182   providing a second independent allele ($Syt7^{M2}$) in a distinct genetic background (Figure 1D). To

183   characterize the effects of SYT7 overexpression, a UAS-$Syt7$ transgene was crossed with the

184   neuronal $elav^{C155}$-GAL4 driver. Western analysis of adult brain extracts with anti-SYT7 antisera

185   confirmed the absence of SYT7 protein in $Syt7^{M1}$ and $Syt7^{M2}$ mutants and a 2.5-fold increase in

186   SYT7 protein levels in $elav^{C155}$-GAL4; UAS-$Syt7$ (Figure 1E). Similar to the loss of SYT7 in mice

187   (Maximov et al., 2008), Drosophila $Syt7$ null mutants are viable and fertile with no obvious

188   behavioral defects.

189

190   **Dosage-dependent regulation of neurotransmitter release by SYT7**

191   To assay SYT's role in synaptic transmission, two-electrode voltage clamp (TEVC) recordings

192   were performed at glutamatergic NMJs from 3rd instar larval motor neurons at segment A3 muscle

193   6 in 2 mM extracellular Ca^{2+}. No significant changes in spontaneous release parameters were

194   identified, as miniature excitatory junctional current (mEJC) amplitude, kinetics and frequency

195   were similar between $Syt7^{M1}$ mutants, $Syt7^{M1}$ heterozygotes ($Syt7^{M1}$/+) and controls (Figure 2A-

196   D). In contrast to spontaneous release, evoked SV fusion (excitatory evoked junctional current

197   (eEJC)) was dramatically enhanced in $Syt7^{M1}$ single mutants and elevated to an intermediate level

198   in $Syt7^{M1}$ heterozygotes (Figure 2E, F; control: 158.33 ± 19.13 nA, n=9; $Syt7^{M1}$/+: p<0.05, 233.08

199   ± 19.16 nA, n=14; $Syt7^{M1}$: p<0.005, 262.96 ± 13.01 nA, n=10). Although evoked release was

200   increased ~2-fold, there was no change in eEJC kinetics in $Syt7^{M1}$ or $Syt7^{M1}$/+ (Figure 2G, H). Both

201   eEJC rise time and eEJC half-width were unaffected (Figure 2I, J). Loss of SYT7 increased evoked

202   release regardless of whether quantal content was estimated using eEJC amplitude (which

203   primarily measures synchronous release, 98% increase, Figure 2K) or eEJC charge (which

204   measures both synchronous and asynchronous release, 128% increase, Figure 2L). We conclude

205   loss of SYT7 enhances SV fusion with no major effect on release kinetics at Drosophila NMJs.

206   The synaptic levels of SYT7 are likely to be rate-limiting for its ability to regulate synaptic

207   transmission since $Syt7^{M1}$/+ heterozygotes displayed an intermediate increase in evoked release

208   compared to $Syt7^{M1}$ null mutants. To determine if the effects of SYT7 are dosage-sensitive, SYT7

209   was overexpressed 2.5-fold by driving a UAS-$Syt7$ transgene with neuronal $elav^{C155}$-GAL4 (Figure

210   1E). Overexpression of SYT7 had no significant effect on spontaneous mEJC kinetics or amplitude

211   (Figure 3A, B), similar to the lack of effect in $Syt7$ null mutants. However, SYT7 overexpression

212   resulted in a ~2-fold decrease in mEJC frequency (Figure 2C, p<0.05), suggesting elevated levels

213 of SYT7 can reduce spontaneous fusion. Unlike the increased evoked release in *Syt7^M1* and

214 *Syt7^M1*/+ mutants, SYT7 overexpression caused a striking reduction in eEJC amplitude (Figure

215 3D, E) and eEJC charge (Figure 3F), with only mild effects on SV release kinetics (Figure 3G).

216 To determine if the inhibitory action of SYT7 on SV release is secondary to a presynaptic role,

217 SYT7 was overexpressed postsynaptically using the muscle specific *Mhc*-GAL4 driver.

218 Overexpression of SYT7 in muscles had no effect on eEJC amplitude or kinetics (Figure 3 –

219 supplemental figure 1A, B). We conclude that increased presynaptic SYT7 levels reduce both

220 spontaneous and evoked SV fusion, indicating SYT7 functions as a negative regulator of

221 neurotransmitter release.

222

223 **Analysis of synaptic structure and AZ morphology in *Syt7* mutants**

224   To determine if enhanced SV fusion in the absence of SYT7 results from an increase in AZ

225 number or SV docking, synaptic morphology and ultrastructure at the NMJ was analyzed in *Syt7^M1*

226 mutants. Motor neurons form en passant synaptic boutons along the axon that contain hundreds of

227 individual AZs marked by a central filamentous T-bar composed of the ELKS/CAST homolog

228 Bruchpilot (BRP) (Ehmann et al., 2014; Wagh et al., 2006). Immunostaining for BRP, the SV-

229 associated protein Complexin (CPX) and a general marker for neuronal membranes (anti-HRP)

230 was performed at muscle 6/7 and muscle 4, the two NMJs analyzed in this study (Figure 4A-H).

231 There was no change in the total number of synaptic boutons (Figure 4C, F), AZ number defined

232 by BRP puncta (Figure 4D, G), or AZ number per muscle surface area (Figure 4E, H). To examine

233 the AZ T-bar where SVs cluster, high-resolution structured illumination microscopy (SIM) was

234 performed on larval muscle 4 NMJs following anti-BRP immunostaining. *Syt7^M1* mutants

235 displayed the normal BRP ring architecture and showed no major difference in morphology

236 compared to controls (Figure 4I). Individual T-bar size and intra-terminal T-bar spacing was

237 quantified in controls and *Syt7^M1* mutants on a Zeiss Airyscan confocal. Although BRP ring

238 structure was intact, *Syt7^M1* mutants displayed a 25% decrease in the average volume of individual

239 BRP-labeled T-bars (Figure 4J), but no change in the spacing of T-bars relative to each other

240 (Figure 4K). We conclude that loss of SYT7 does not disrupt overall AZ morphology or AZ

241 number, though *Syt7^M1* mutants display a mild decrease in T-bar volume.

242   To determine if enhanced SV docking could increase the number of SVs available for release

243 in *Syt7* mutants, SV distribution was quantified at larval muscle 6/7 NMJs in control and *Syt7^M1*

9

244   using transmission electron microscopy (TEM, Figure 5A). No change in overall SV density was

245   observed within $Syt7^{M1}$ boutons, indicating SV recycling is largely unperturbed (Figure 5B). In

246   contrast to the mild decrease in T-bar area (Figure 4J), there was no change in the length of

247   individual AZs defined by the electron dense synaptic cleft (Figure 5C, p=0.93; control: 404 ±

248   34.5 nm, n=21 AZs from 5 larvae; $Syt7^{M1}$: 409 ± 28.9 nm, n=29 AZs from 5 larvae). To examine

249   docking, SVs in contact with the plasma membrane under the T-bar (within 100 nm, Figure 5D)

250   or just outside the T-bar (100 to 400 nm, Figure 5E) were quantified. No significant change in the

251   number of SVs docked at the AZ plasma membrane was detected (Fig 5D-F), indicating

252   morphological docking defined by EM is not altered in $Syt7^{M1}$ mutants. To quantify SV distribution

253   in the cytoplasm around AZs, SV number was binned into four concentric hemi-circles from 100

254   to 400 nm radius centered on the T-bar. No significant difference in SV distribution was observed

255   in any bin (Figure 5G, H), indicating the morphological distribution of SVs around T-bars is intact

256   in the absence of SYT7. We conclude the enhanced release in $Syt7^{M1}$ mutants in not due to

257   increased AZ number or docked SVs.

258

259   **Optical quantal mapping in *Syt7* mutants**

260   Given quantal size (Figure 2B), AZ number (Figure 4D, G) and SV docking (Figure 5H) are

261   unchanged in *Syt7* mutants, increased release probability (P_r) at individual AZs is a candidate

262   mechanism to mediate the elevated quantal content during single stimuli. We previously developed

263   a quantal imaging approach to map AZ P_r at Drosophila NMJs by expressing myristoylated

264   GCaMP6s in muscles (Akbergenova et al., 2018; Melom et al., 2013). Using this approach, P_r

265   maps for evoked release were generated for all AZs from 1b boutons at muscle 4 NMJs in control

266   and $Syt7^{M1}$ mutants. Similar to controls, AZs formed by single motor neurons in $Syt7^{M1}$ displayed

267   heterogeneous P_r (Figure 6A). However, P_r distribution was strikingly different between the

268   genotypes, with a greater number of high P_r and fewer low P_r AZs at $Syt7^{M1}$ NMJs (Figure 6B).

269    $Syt7^{M1}$ NMJs also had fewer silent AZs that showed no release (control: 19.9%; $Syt7^{M1}$: 4.6%).

270   Overall, mean P_r was increased 2-fold (Figure 6C, p<0.01; control: 0.063 ± 0.002, n=1158 AZs;

271    $Syt7^{M1}$: 0.12 ± 0.004, n=768 AZs). In contrast, the maximum AZ P_r in the two genotypes was

272   unchanged (Figure 6C, control: 0.61; $Syt7^{M1}$: 0.63), indicating an upper limit on release strength

273   for single AZs that is similar between controls and $Syt7^{M1}$. We conclude that the enhanced release

274   in the absence of SYT7 results from an increase in average P_r across the AZ population.

275

Loss of SYT7 enhances SV release in *Syt1* null mutants

Drosophila *Syt1* null mutants have dramatically reduced synchronous SV fusion and enhanced asynchronous and spontaneous release (Jorquera et al., 2012; Lee et al., 2013; Yoshihara et al., 2010; Yoshihara and Littleton, 2002). We generated *Syt1; Syt7* double mutants to determine if SYT7 mediates the residual asynchronous release present in *Syt1* nulls. A complete loss of asynchronous release in *Syt1; Syt7* double mutants should occur if SYT7 functions as the sole asynchronous Ca^{2+} sensor, while a reduction in release is expected if it is one of several sensors mediating the residual synaptic transmission in *Syt1*. Animals lacking SYT1 were obtained by crossing an intragenic *Syt1* deletion (*Syt1^{N13}*) with a point mutant containing an early stop codon (*Syt1^{AD4}*), an allelic combination referred to as *Syt1^{Null}*. Loss of SYT1 results in lethality throughout development, although some *Syt1^{Null}* mutants survive to adulthood when cultured under special conditions (Loewen et al., 2001). Surviving *Syt1^{Null}* adults are severely uncoordinated and die within several days. Quantification of survival rates demonstrated 45.3% of *Syt1^{Null}* mutants survived from the 1st instar to the pupal stage, with 44.1% of mutant pupae surviving to adulthood (n=5 groups with >40 starting animals each). In contrast, 5.6% of *Syt1^{Null}*; *Syt7^{M2}* double mutants (referred to as *Double^{Null}*) survived from the 1st instar to the pupal stage, and 6.6% of mutant pupae survived to adulthood (n=6 groups with >80 animals each). Western analysis confirmed loss of both proteins in *Double^{Null}* mutants and demonstrated no change in expression of SYT1 or SYT7 in the absence of the other family member in individual null mutant backgrounds (Figure 7A). Although loss of both SYTs caused synergistic defects in survival, residual synaptic transmission must exist given some *Double^{Null}* mutants survive.

To assay synaptic transmission, recordings were performed from 3rd instar larval muscle 6 in 2 mM extracellular Ca^{2+} in *Syt1^{Null}* and *Double^{Null}* mutants. No change in spontaneous mEJC amplitude or kinetics was found between the two genotypes (Figure 7B), indicating postsynaptic sensitivity, neurotransmitter loading, and fusion pore dynamics were not disrupted by loss of SYT7. However, a ~2-fold increase in mEJC frequency was observed in the *Double^{Null}* compared to *Syt1^{Null}* (Figure 7C, p<0.001; *Syt1^{null}*: 2.99 ± 0.23 Hz, n=16; *Double^{Null}*: 5.33 ± 0.42 Hz, n=14), demonstrating loss of both SYTs enhances the already elevated spontaneous release rate found in *Syt1^{Null}* mutants alone. Measurements of evoked release revealed both amplitude and charge transfer were increased ~2-fold in *Double^{Null}* compared to *Syt1^{Null}* mutants (Figure 7D-F; eEJC

306   amplitude: p<0.001; $Syt1^{Null}$: 3.18 ± 0.4 nA, n=15; $Double^{Null}$: 6.12 ± 0.62 nA, n=13; eEJC charge:

307   p<0.05; $Syt1^{Null}$: 33.2 ± 4.4 nA*ms, n=15; $Double^{Null}$: 52.6 ± 5.8 nA*ms, n=13). In addition, more

308   SVs fused in the first 15 ms following stimulation (Figure 7G, H), with less SVs available for

309   release later in the response. $Double^{Null}$ mutants also had a reduced rate of evoked failures

310   following nerve stimulation compared to $Syt1^{Null}$, consistent with an increased probability of SV

311   fusion (Figure 7I, p<0.01; $Syt1^{Null}$: 21.1 ± 3.5% failure rate, n=17; $Double^{Null}$: 7.4 ± 3.4% failure

312   rate, n=14). These results indicate SYT7 does not mediate the residual release found in the absence

313   of SYT1. We conclude SYT7 negatively regulates SV fusion with or without SYT1 present at the

314   synapse.

315

316   **Short-term facilitation does not require SYT7**

317   Although these results indicate SYT7 is a not a key asynchronous Ca^{2+} sensor in Drosophila,

318   the protein has also been implicated as the Ca^{2+} sensor for facilitation (Chen et al., 2017; Jackman

319   et al., 2016; Turecek and Regehr, 2018), a short-term form of presynaptic plasticity that results in

320   enhanced SV fusion during closely-spaced stimuli. To examine facilitation, $[Ca^{2+}]$ was lowered

321   from 2 mM to 0.175 mM or 0.2 mM to identify conditions where the initial P_r was matched

322   between control and $Syt7$ mutants. In 0.175 mM Ca^2, controls displayed an 11% failure ratio in

323   response to single action potentials, while $Syt7^{M1}$ had no failures (Figure 8A). In 0.2 mM Ca^{2+},

324   neither genotype had failures (Figure 8A), although evoked release was increased 3-fold in $Syt7^{M1}$

325   (Figure 7B, C, p<0.01, control: 7.73 ± 1.5 nA, n=9; $Syt7^{M1}$: 23.72 ± 6.2 nA, n=9). In contrast, EJC

326   amplitude was not statistically different between control in 0.2 mM Ca^{2+} (7.73 ± 1.5 nA, n=9) and

327    $Syt7^{M1}$ in 0.175 mM Ca^{2+} (8.70 ± 1.6 nA, n=9). Facilitation was assayed in these conditions where

328   initial P_r was comparable. Control and $Syt7^{M1}$ mutants displayed robust facilitation to paired-pulses

329   separated by 10 or 50 ms at both Ca^{2+} concentrations (Figure 8D). A modest reduction in paired-

330   pulse ratio was observed in $Syt7^{M1}$ at 0.175 Ca^{2+} compared to control at 0.2 mM Ca^{2+} (Figure 8E,

331   F, p<0.05; 10 ms interval: 31% decrease; 50 ms interval: 22% decrease). These data indicate SYT7

332   is not the sole effector of facilitation. The mild defect in $Syt7$ mutants could be due to a partially

333   redundant role for SYT7 in facilitation or secondary to differences in Ca^{2+} available to activate the

334   true facilitation sensor. Given $[Ca^{2+}]$ was lowered in $Syt7^{M1}$ to match initial P_r between the

335   genotypes, the latter explanation is more likely.

336   To determine if short-term facilitation could be elicited in the absence of both SYT1 and SYT7,
337   a 10 Hz stimulation train in 2.0 mM Ca^{2+} was given to *Double^{Null}* mutants and eEJC responses
338   were compared to *Syt1^{Null}* mutants alone. Similar to the increased quantal content to single action
339   potentials, *Double^{Null}* mutants displayed larger facilitating responses during the early phase of
340   stimulation (Figure 8G-I; cumulative average release for 10 stimuli: *Syt1^{Null}* (n=12): 87 ± 7.0
341   quanta; *Double^{Null}* (n=13): 109 ± 9.9 quanta; 20 stimuli: *Syt1^{Null}*: 209 ± 13.8 quanta; *Double^{Null}*:
342   261 ± 22.6 quanta; 50 stimuli: *Syt1^{Null}*: 594 ± 34.5 quanta; *Double^{Null}*: 745 ± 56.2 quanta, p<0.03).
343   These results indicate short-term facilitation can occur in the absence of both SYT1 and SYT7,
344   and is enhanced during the early phases of stimulation, consistent with SYT7 negatively regulating
345   SV fusion with or without SYT1.
346

347   **Syt7 mutants have access to a larger pool of fusogenic SVs but maintain a normal SV**
348   **endocytosis rate at steady-state**

349   Enhanced SV release in *Syt7* mutants could reflect increased fusogenicity of the entire SV
350   population or conversion of a non-fusogenic SV pool into one capable of fusion in the absence of
351   SYT7. To test whether SYT7 normally renders a pool of SVs non-fusogenic, 1000 stimuli at 10
352   Hz were applied in 2 mM Ca^{2+} at 3rd instar muscle 6 NMJs to deplete the readily releasable pool
353   (RRP) and drive SV cycling to steady-state. The total number of released SVs and the SV recycling
354   rate was then measured. Both control and *Syt7^{M1}* eEJCs depressed during the stimulation train.
355   However, SV release in *Syt7^{M1}* mutants remained elevated over much of the initial stimulation
356   (Figure 9A) and the integral of release during the train was greater than controls (Figure 9B),
357   indicating *Syt7* nulls have access to more fusogenic SVs. SV release rate in *Syt7^{M1}* eventually
358   reached the same level as control following depletion of the RRP (Figure 9C, control quantal
359   content: 131.5 ± 10.7, n=7; *Syt7^{M1}* quantal content: 123.1 ± 10.5, n=8). We conclude that SV
360   endocytosis and recycling rate is SYT7-independent at steady-state, although *Syt7^{M1}* mutants
361   contain a larger RRP available for fusion.
362   To further examine SV recycling, FM1-43 dye uptake and release assays were performed in
363   control and *Syt7^{M1}* mutants at 3rd instar muscle 6/7 NMJs. At low stimulation rates (0.5 Hz), *Syt7^{M1}*
364   mutants took up significantly more FM1-43 dye than controls (Figure 9D, F), consistent with the
365   increased SV fusion observed by physiology. In contrast, no significant difference in FM1-43
366   uptake was found following high frequency 10 Hz stimulation for 500 stimuli (Figure 9E, G).

367   These data suggest previously exocytosed SVs re-enter the RRP more often in the absence of SYT7

368   given the normal recycling rate (Figure 9C). Consistent with this hypothesis, no change in FM1-

369   43 release was detected with high [K$^+$] stimulation following 10 Hz loading (Figure 9H). Together

370   with the electrophysiology data, we conclude *Syt7* mutants have a larger RRP, but no changes in

371   SV endocytosis.

372

373   **Syt7 mutants have enhanced refilling of the readily-releasable SV pool independent of**

374   **endocytosis**

375   To probe how SYT7 regulates SV cycling and the transition between distinct SV pools, eEJC

376   recovery kinetics following high frequency stimulation were characterized. A paradigm consisting

377   of 30 stimuli at 0.5 Hz, 500 stimuli at 10 Hz and a final 50 stimuli at 0.5 Hz was given to *Syt7^{M1}*

378   mutants, *Syt7^{M1}*/+ heterozygotes and controls in 2 mM Ca^{2+} (Figure 10A). During 0.5 Hz

379   stimulation, *Syt7^{M1}* and *Syt7^{M1}*/+ displayed elevated levels of release. Following the onset of high

380   frequency stimulation, *Syt7^{M1}* and *Syt7^{M1}*/+ synapses depressed while controls displayed a mild

381   facilitation before quickly transitioning to depression (Figure 10B). Remarkably, *Syt7^{M1}* and

382   *Syt7^{M1}*/+ displayed an extremely rapid recovery of eEJC amplitude and quantal content during the

383   2 second interval following termination of the 10 Hz train compared to controls (Figure 10C). A

384   similar rapid recovery was observed in *Syt7^{M1}* after 2000 stimuli were given at 10 Hz to fully

385   deplete the RRP and normalize release rates to control levels (Figure 10 – figure supplement 1A-

386   C). These observations suggest SYT7 functions to reduce SV entry into the RRP, while also

387   negatively regulating fusion of newly regenerated SVs. The enhanced refilling of the RRP did not

388   require SYT1 function, as *Double^{Null}* mutants also displayed larger eEJCs than *Syt1^{Null}* alone during

389   the recovery from a 10 Hz stimulation train (Fig. 8G).

390   The partial elevation in RRP refilling rate at *Syt7^{M1}*/+ synapses indicates the amount of SYT7

391   in the presynaptic terminal regulates SV entry into the releasable pool. To determine if RRP

392   refilling is dosage-sensitive, the stimulation paradigm above (0.5 Hz/10 Hz/0.5 Hz) was applied

393   to SYT7 overexpression larvae (*elav^{C155}*-GAL4; UAS-*Syt7*) in 2 mM Ca^{2+}. Presynaptic

394   overexpression of SYT7 had the opposite effect of *Syt7* mutants and *Syt7*/+ heterozygotes, not only

395   reducing eEJC amplitude at 0.5 Hz, but greatly limiting the ability of SVs to re-enter the RRP

396   following termination of the 10 Hz stimulation train (Figure 10 – supplemental figure 2A-C). We

14

397   conclude that SYT7 limits release in a dosage-sensitive manner by negatively regulating the

398   number of SVs available for fusion and slowing recovery of the RRP following stimulation.

399   To determine if increased RRP refilling in $Syt7^{M1}$ requires an enhanced rate of SV endocytosis

400   or is mediated through refilling from a pre-existing SV pool, recordings were repeated in the

401   presence of the proton pump inhibitor bafilomycin. Bafilomycin blocks neurotransmitter reloading

402   of newly endocytosed SVs and should eliminate the enhanced refilling of the RRP if recycling is

403   essential. Alternatively, if SVs are recruited more rapidly from pre-existing pools, bafilomycin

404   would not abolish the enhanced recovery. The same 0.5 Hz/10 Hz/0.5 Hz paradigm was applied

405   in three successive epochs in the presence of 4 uM bafilomycin or DMSO (control) in the bath

406   solution. As expected, bafilomycin progressively reduced eEJC amplitude throughout the

407   experiment and eliminated most evoked responses during the 3rd stimulation epoch (Figure 10D).

408    $Syt7^{M1}$ mutants displayed a similar fold-enhancement in the recovery of the RRP in the presence

409   of bafilomycin, though the absolute numbers of SVs re-entering the pool decreased following the

410   2nd 10 Hz stimulation as the number of neurotransmitter-containing SVs declined (Figure 10E, F).

411   We conclude that the rapid refilling of the RRP can occur from pre-existing SV pools. In addition

412   to reducing fusogenicity of SVs already docked at the AZ, these data indicate SYT7 regulates

413   transition kinetics between vesicle pools by reducing the number of SVs moving from the reserve

414   pool to the RRP.

415

416   SYT7 localizes to an internal membrane network within the peri-AZ that resides in

417   proximity to multiple presynaptic compartments

418   Defining the subcellular localization of SYT7 could help elucidate how it modulates SV

419   dynamics. SYT7 could be a resident protein of the SV pool it regulates or reside on an alternative

420   compartment that exerts control over a subset of SVs. To examine the subcellular localization of

421   SYT7, an RFP tag was introduced at the 3'-end of the endogenous $Syt7$ locus using CRISPR

422   (Figure 11A). This approach generated a $SYT7^{RFP}$ C-terminal fusion protein expressed under its

423   endogenous enhancers to avoid any overexpression that might trigger changes in its normal

424   localization. A sfGFP version ($SYT7^{GFP}$) was also generated with CRISPR that showed the same

425   intra-terminal expression pattern as $SYT7^{RFP}$ (Figure 11 – supplemental figure 1A). Western

426   analysis with anti-RFP identified a single band at the predicted molecular weight (73kD) of the

427   fusion protein in $SYT7^{RFP}$ animals (Figure 11B), indicating a single SYT7 isoform is expressed in

428   Drosophila. Immunostaining of 3rd instar larvae with anti-RFP antisera revealed SYT7^{RFP} was

429   enriched in presynaptic terminals and formed an expansive tubular network near the plasma

430   membrane that extended into the center of the bouton (Figure 11C, D). Neuronal knockdown of

431   *Syt7* with two independent RNAi lines (*elav^{C155}*-GAL4; UAS-*Syt7* RNAi) dramatically reduced

432   SYT7^{RFP} on Westerns (Figure 11B) and eliminated expression of SYT7^{RFP} at the NMJ (Figure 11

433   – supplemental figure 2), indicating the signal is specific to SYT7 and localizes predominantly to

434   the presynaptic compartment.

435   To further characterize the subsynaptic localization of SYT7, fluorescently-tagged

436   compartmental markers or compartment-specific antisera were used for labeling in the Syt7^{RFP}

437   background. Images were collected on a Zeiss Airyscan and analyzed in FIJI and Matlab to

438   generate cytofluorogram co-localization plots to calculate the Pearson correlation (r) between

439   SYT7^{RFP} and labeled compartments from individual synaptic boutons at muscle 6/7 NMJs (Figure

440   12, n=3 animals each). Co-labeling of the SV proteins nSYB and SYT1 served as a positive control

441   (Figure 12A, r=0.71). SYT7^{RFP} and the Golgi marker, Golgin84, served as a negative control since

442   Golgi is absent from presynaptic terminals (Figure 12L, r=-0.43). Co-localization analysis

443   indicates SYT7 resides on a membrane compartment that does not completely overlap with any

444   protein tested (Figure 12B-L). The largest overlap was with Dynamin (Figure 12B, r=0.22), a

445   GTPase involved in endocytosis that localizes to the peri-AZ. The t-SNARE SYX1 also

446   overlapped with a subset of SYT7 immunolabeling near the plasma membrane (Figure 12C,

447   r=0.15). Although SYT7's pattern of inter-connectivity within the bouton appeared similar to

448   peripheral ER, it did not co-localize with Reticulon-like 1 (RTLN1, Figure 12D, r=0.01), a

449   peripheral ER marker. In addition, SYT7 did not co-localize with SVs (r=-0.07), DCVs labeled

450   with ANF-GFP (r=-0.19), exosomes (r=-0.29), late endosomes (r=-0.29), lysosomes (r=-0.01) or

451   the plasma membrane (anti-HRP, r=-0.06). Neither SYT7^{RFP} (Figure 12G, r=-0.11) or SYT7^{GFP}

452   (Figure 11 – supplemental figure 1B) was enriched at AZs, but instead surrounded BRP as

453   previously described for other peri-AZ proteins. These data are in agreement with anti-SYT7

454   antibody labeling of sucrose gradient-separated subcellular fractions from wildtype Drosophila

455   head extracts that localized SYT7 to a distinct membrane compartment separate from SVs and the

456   plasma membrane (Adolfsen et al., 2004). In conclusion, SYT7 surrounds AZs marked by BRP

457   (Figure 11 – supplemental figure 1B, Figure 12G), indicating the protein localizes in part to the

458   previously described peri-AZ domain. Peri-AZs are enriched in proteins regulating SV endocytosis

459   and endosomal trafficking (Coyle et al., 2004; Koh et al., 2004; Marie et al., 2004; Rodal et al.,

460   2008; Sone et al., 2000), indicating SYT7 may modulate SV re-entry into the RRP by interfacing

461   with sorting machinery within this domain.

462   SYT7 localization was widespread within the peri-AZ region, with SYT7^{RFP} tubules in close

463   proximity to other labeled membrane compartments, including endosomes, lysosomes, and the

464   plasma membrane (Figure 12 – figure supplement 1). To determine if the SYT7 compartment

465   required endosomal trafficking for its assembly or maintenance, a panel of dominant-negative,

466   constitutively-active or wildtype endosomal UAS-RAB proteins (Zhang et al., 2007) were

467   expressed with $elav^{C155}$-GAL4 in the SYT7^{RFP} background. Manipulations of RAB5 (early

468   endosomes), RAB7 (late endosomes) or RAB4 and RAB11 (recycling endosomes) did not disrupt

469   the abundance or morphology of the SYT7 tubular network (Figure 12 – supplemental figure 2).

470   Similarly, no change in the distribution of several compartment markers were found in $Syt7^{M1}$

471   mutants, including the early endosomal marker RAB5, the late endosomal/peri-AZ marker RAB11

472   and the peri-AZ protein Nervous Wreck (NWK) (Figure 12 – supplemental figure 3). In addition,

473   no defect was observed in trans-synaptic transfer of the exosomal protein SYT4 to the postsynaptic

474   compartment, indicating SYT7 does not regulate exosome trafficking as described for several other

475   peri-AZ proteins (Walsh et al., 2019). Although no sub-compartment overlapped completely with

476   SYT7, the protein is positioned within the peri-AZ to interact with SVs, endosomes and the

477   recycling machinery to negatively regulate the size of releasable SV pools (Figure 12 –

478   supplemental figure 4). We conclude that SYT7 does not localize to SVs and is not enriched at

479   AZs, consistent with SYT7 negatively regulating SV release through an indirect mechanism that

480   does not require its presence at sites of SV fusion.

Discussion

To characterize the location and function of SYT7 in Drosophila, we used the CRISPR-Cas9 system to endogenously label the protein and generate null mutations in the *Syt7* locus. Our findings indicate SYT7 acts as a negative regulator of SV release, AZ P_r, RRP size, and RRP refilling. The elevated P_r across the AZ population in *Syt7* mutants provides a robust explanation for why minor defects in asynchronous release and facilitation are present in Drosophila. SYT7's presence on an internal tubular membrane network within the peri-AZ positions the protein to interface with the SV cycle at multiple points to regulate membrane trafficking. In addition, the increased SV fusion in animals lacking both SYT1 and SYT7 indicate the full complement of Ca^{2+} sensors that mediate the distinct phases of SV fusion remain unknown.

Syt7 mutants have increased P_r at Drosophila NMJs

Using a combination of synaptic physiology and imaging approaches, our findings indicate SYT7 acts to reduce SV recruitment and fusion. Minor defects in asynchronous release and facilitation were identified in Drosophila *Syt7* mutants, as observed in mouse and zebrafish models (Bacaj et al., 2013; Chen et al., 2017; Jackman et al., 2016; Turecek and Regehr, 2019, 2018; Weber et al., 2014; Wen et al., 2010). However, we attribute these mild defects to reduced SV availability as a result of increased P_r in *Syt7* mutants. Indeed, a key feature of facilitation is its critical dependence on initial P_r (Neher and Brose, 2018; Zucker and Regehr, 2002). Low P_r synapses increase SV fusogenicity as Ca^{2+} levels rise during paired-pulses or stimulation trains, resulting in short-term increases in P_r for SVs not recruited during the initial stimuli. In contrast, depression occurs at high P_r synapses due to the rapid depletion of fusion-capable SVs during the initial response. Prior quantal imaging at Drosophila NMJs demonstrated facilitation and depression can occur across different AZs within the same neuron, with high P_r AZs depressing and low P_r AZs facilitating (Peled and Isacoff, 2011). Given the elevated P_r in *Syt7* mutants, the facilitation defects are likely related to differences in initial P_r and depletion of fusion-competent SVs available for release during the 2nd stimuli.

A similar loss of SVs due to elevated P_r in *Syt7* mutants would reduce fusogenic SVs that are available during the delayed phase of the asynchronous response. *Syt1; Syt7* double mutants continue to show asynchronous fusion and facilitation, conclusively demonstrating there must be other Ca^{2+} sensors that mediate these components of SV release. The predominant localization of

512   endogenous SYT7 to an internal tubular membrane compartment at the peri-AZ also places the

513   majority of the protein away from release sites where it would need to reside to directly activate

514   SV fusion. As such, our data indicate SYT7 regulates SV release through a distinct mechanism

515   from SYT1.

516   We can also conclude that the remaining components of asynchronous fusion and facilitation

517   must be mediated by an entirely different family of Ca^{2+}-binding proteins than Synaptotagmins (or

518   through Ca^{2+}-lipid interactions). Of the seven *Syt* genes in the Drosophila genome, only 3 SYT

519   proteins are expressed at the motor neuron synapses assayed in our study – SYT1, SYT4 and SYT7

520   (Adolfsen et al., 2004). For the remaining SYTs in the genome, SYT-α and SYT-β are expressed

521   in neurosecretory neurons and function in DCV fusion (Adolfsen et al., 2004; Park et al., 2014).

522   SYT12 and SYT14 lack Ca^{2+} binding residues in their C2 domains and are not expressed in motor

523   neurons (Adolfsen et al., 2004). In addition, SYT4 is found on exosomes and transferred to

524   postsynaptic cells, where it participates in retrograde signaling (Adolfsen et al., 2004; Harris et al.,

525   2016; Korkut et al., 2013; Walsh et al., 2019; Yoshihara et al., 2005). *Syt1; Syt4* double mutants

526   display the same SV fusion defects found in *Syt1* mutants alone, indicating SYT4 cannot

527   compensate for SYT1 function in SV release (Barber et al., 2009; Saraswati et al., 2007). As such,

528   SYT1 and SYT7 are the only remaining SYT isoforms that could contribute to SV trafficking

529   within Drosophila motor neuron terminals.

530   A prior study from our lab using *Syt7* RNAi also found no change in facilitation or

531   asynchronous release following knockdown of SYT7 (Saraswati et al., 2007). However, the RNAi

532   knockdown did not result in enhanced release. Although a reduction in ectopic expression of SYT7

533   in muscles could be seen with *Mhc*-GAL4 driving UAS-*Syt7* RNAi, our anti-SYT7 antisera could

534   not recognize the endogenous protein in neurons by immunocytochemistry, preventing a

535   determination of presynaptic SYT7 levels following neuronal RNAi. Based on the current data,

536   the *Syt7* UAS-RNAi line was likely ineffective in knocking down endogenous SYT7 in neurons.

537   Both $Syt7^{M1}$ and $Syt7^{M2}$ alleles result in early stop codons and lack SYT7 expression by Western

538   analysis. Given both null mutants show elevated release, and SYT7 overexpression reduces

539   release, our data indicate SYT7 normally acts to suppress SV fusogenicity as demonstrated by both

540   electrophysiology and optical P_r imaging.

541

542   *SYT7 regulates the recruitment and fusion of SVs in a dosage-dependent manner*

19

543   Although our data indicate SYT7 is not the primary asynchronous or facilitation Ca^{2+} sensor
544   in Drosophila, we found it inhibits SV fusion in a dosage-sensitive manner. This is not due to
545   altered endocytosis, as Syt7 mutants have a normal steady-state rate of SV release following
546   depletion of the RRP. Instead, SYT7 regulates SV fusogenicity at a stage between SV endocytosis
547   and fusion. Given the rapid enhanced refilling of the RRP observed in *Syt7* mutants, and the
548   suppression of RRP refilling following SYT7 overexpression, our data indicate SYT7 regulates
549   releasable SVs in part through changes in SV mobilization to the RRP. Ca^{2+} is well known to
550   control the replenishment rate of releasable SVs, with Calmodulin-UNC13 identified as one of
551   several molecular pathways that accelerate RRP refilling in a Ca^{2+}-dependent manner (Dittman et
552   al., 2000; Dittman and Regehr, 1998; Junge et al., 2004; Lipstein et al., 2013; Ritzau-Jost et al.,
553   2018). Our findings indicate SYT7 acts in an opposite fashion and slows RRP refilling, providing
554   a Ca^{2+}-dependent counter-balance for SV recruitment into the RRP. Although such an effect has
555   not been described for mammalian SYT7, defects in RRP replenishment have been observed when
556   both SYT1 and SYT7 are deleted or following high frequency stimulation trains (Bacaj et al.,
557   2015; Durán et al., 2018; Liu et al., 2014).

558   SYT7's role in restricting SV fusion and RRP size also affects spontaneous release. Although
559   *Syt7* mutants alone do not show elevated mini frequency, *Double^Null* mutants have a 2-fold increase
560   in spontaneous release. Similar increases in spontaneous release were observed at mammalian
561   synapses lacking both SYT7 and SYT1 (or SYT2), with the effect being attributed to a dual role
562   in clamping fusion in the absence of Ca^{2+} (Luo and Südhof, 2017; Turecek and Regehr, 2019). Our
563   results indicate the elevation in spontaneous release at Drosophila synapses is a result of an
564   increase in releasable SVs rather than a clamping function for SYT7. Following overexpression of
565   SYT7, there is a reduction in the number of fusogenic SVs available for both evoked and
566   spontaneous release. The dosage-sensitivity of the various phenotypes indicate SYT7 abundance
567   is a critical node in controlling SV release rate. Indeed, mammalian SYT7 levels are post-
568   transcriptionally modulated by γ-secretase proteolytic activity and APP, linking it to SV trafficking
569   defects in Alzheimer's disease (Barthet et al., 2018).

570

571   *How does SYT7 negatively regulate recruitment and fusion of SVs?*

572   The precise mechanism by which SYT7 reduces release and slows refilling of the RRP is
573   uncertain given it is not enriched at sites of SV fusion. Although we cannot rule out the possibility

574   that a small fraction of the protein is found at AZs, SYT7 is predominantly localized to an internal

575   membrane compartment at the peri-AZ where SV endocytosis and endosomal sorting occurs

576   (Coyle et al., 2004; Koh et al., 2004; Marie et al., 2004; Rodal et al., 2008; Sone et al., 2000).

577   SYT7 membrane tubules are in close proximity and could potentially interact with peri-AZs

578   proteins, endosomes, lysosomes and the plasma membrane. Given its primary biochemical activity

579   is to bind membranes in a Ca^{2+}-dependent manner, SYT7 could mediate cargo or lipid movement

580   across multiple compartments within peri-AZs. In addition, it is possible SYT7 tubules could form

581   part of the poorly defined SV recycling endosome compartment. However, we observed no change

582   in SV density or SV localization around AZs, making it unlikely SYT7 would be essential for

583   endosomal trafficking of SVs. The best characterized regulator of the SV endosome compartment

584   in Drosophila is the RAB35 GAP Skywalker (SKY) (Uytterhoeven et al., 2011). Although *Sky*

585   mutations display some similarities to *Syt7*, including increased neurotransmitter release and larger

586   RRP size, *Syt7* lacks most of the well-described *Sky* phenotypes such as behavioral paralysis, FM1-

587   43 uptake into discrete punctated compartments, cisternal accumulation within terminals and

588   reduced SV density. In addition, we found no co-localization between SKY-GFP and SYT7[RFP]

589   within presynaptic terminals.

590   By blocking SV refilling with bafilomycin, our findings indicate the fast recovery of the RRP

591   can occur via enhanced recruitment from the reserve pool and does not require changes in

592   endocytosis rate. The phosphoprotein Synapsin has been found to maintain the reserve SV pool by

593   tethering vesicles to actin filaments at rest (Akbergenova and Bykhovskaia, 2007; Bykhovskaia,

594   2011; Hilfiker et al., 1999; Milovanovic and De Camilli, 2017; Shupliakov et al., 2011). Synapsin

595   interacts with the peri-AZ protein Dap160/Intersectin to form a protein network within the peri-

596   AZ that regulates clustering and release of SVs (Gerth et al., 2017; Marie et al., 2004; Winther et

597   al., 2015). Synapsin-mediated phase separation is also implicated in clustering SVs near release

598   sites (Milovanovic et al., 2018; Milovanovic and De Camilli, 2017). SYT7 could similarly

599   maintain a subset of SVs in a non-releasable pool and provide a dual mechanism for modulating

600   SV mobilization. Phosphorylation of Synapsin and Ca^{2+} activation of SYT7 would allow multiple

601   activity-dependent signals to regulate SV entry into the RRP. As such, SYT7 could play a key role

602   in organizing membrane trafficking and protein interactions within the peri-AZ network by adding

603   a Ca^{2+}-dependent regulator of SV recruitment and fusogenicity.

604   Additional support for a role for SYT7 in regulating SV availability through differential SV
605   sorting comes from recent studies on the SNARE complex binding protein CPX. Analysis of
606   Drosophila *Cpx* mutants, which have a dramatic increase in minis (Buhl et al., 2013; Huntwork
607   and Littleton, 2007; Jorquera et al., 2012), revealed a segregation of recycling pathways for SVs
608   undergoing spontaneous versus evoked fusion (Sabeva et al., 2017). Under conditions where
609   intracellular Ca^{2+} is low and SYT7 is not activated, spontaneously-released SVs do not transit to
610   the reserve pool and rapidly return to the AZ for re-release. In contrast, SVs released during high
611   frequency evoked stimulation when Ca^{2+} is elevated and SYT7 is engaged, re-enter the RRP at a
612   much slower rate. This mechanism slows re-entry of SVs back into the releasable pool when
613   stimulation rates are high and large numbers of SV proteins are deposited onto the plasma
614   membrane at the same time, allowing time for endosomal sorting that might be required in these
615   conditions. In contrast, SVs released during spontaneous fusion or at low stimulation rates would
616   likely have less need for endosomal re-sorting. Given SYT7 restricts SV transit into the RRP, it
617   provides an activity-regulated Ca^{2+}-triggered switch for redirecting and retaining SVs in a non-
618   fusogenic pool that could facilitate sorting mechanisms.

619   Beyond SV fusion, presynaptic membrane trafficking is required for multiple signaling
620   pathways important for developmental maturation of NMJs (Harris and Littleton, 2015; McCabe
621   et al., 2003; Packard et al., 2002; Piccioli and Littleton, 2014; Rodal et al., 2011). In addition,
622   alterations in neuronal activity or SV endocytosis can result in synaptic undergrowth or overgrowth
623   (Akbergenova et al., 2018; Budnik et al., 1990; Dickman et al., 2006; Guan et al., 2005; Koh et
624   al., 2004). We did not find any defect in synaptic bouton or AZ number, indicating SYT7 does not
625   participate in membrane trafficking pathways that regulate synaptic growth and maturation.
626   However, a decrease in T-bar area in *Syt7* mutants was found. Although it is unclear how this
627   phenotype arises, it may represent a form of homeostatic plasticity downstream of elevated
628   synaptic transmission (Frank et al., 2020). There is also ample evidence that SV distance to Ca^{2+}
629   channels plays a key role in defining the kinetics of SV release and the size of the RRP (Böhme et
630   al., 2016; Chen et al., 2015; Neher, 2015; Neher and Brose, 2018; Wadel et al., 2007), suggesting
631   a change in such coupling in *Syt7* mutants might contribute to elevations in P_r and RRP refilling.
632   Further studies will be required to examine the role of this morphological alteration in release sites
633   in *Syt7* mutants.

634

635 **Materials and Methods**

636

637 *Drosophila stocks*

638 *Drosophila melanogaster* were cultured on standard medium at 22-25°C. Genotypes used in the

639 study include: *elav^{C155}*-GAL4 (Bloomington Drosophila Stock Center (BDSC)#8765), UAS-ANF-

640 Emerald (BDSC#7001), SYT4^{GFP-2M} (Harris et al., 2016), *Syt1^{AD4}* (DiAntonio and Schwarz, 1994),

641 *Syt1^{N13}* (Littleton et al., 1993b), UAS-*Syt7* (Saraswati et al., 2007), *Mhc*-GAL4 (BDSC#55132),

642 UAS-Syt7 RNAi#1 (Vienna#24989) and UAS-Syt7 RNAi#2 (BDSC#27279). Lines used for

643 testing co-localization with SYT7^{RFP} or mis-localization in Syt7^{M1} include: endogenous nSYB^{GFP}

644 (this study), UAS-NHE-GFP (this study), UAS-ANF-Emerald (BDSC#7001), SYT4^{GFP-2M} (Harris

645 et al., 2016), UAS-RTNL1-GFP (BDSC#77908), RAB5-YFP (BDSC#62543) and RAB11-YFP

646 (BDSC#62549). Lines used for assaying SYT7^{RFP} localization after overexpressing RABs: UAS-

647 RAB4-YFP (BDSC#9767), UAS-RAB4(Q67L)-YFP (BDSC#9770), UAS-RAB5-YFP

648 (BDSC#24616), UAS-RAB5(S43N)-YFP (BDSC#9772), UAS-RAB5(T22N)-YFP

649 (BDSC#9778), UAS-RAB7-YFP (BDSC#23641), UAS-RAB7(Q67L)-YFP (BDSC#9779), UAS-

650 RAB11-YFP (BDSC#50782) and UAS-RAB11(S25N)-YFP (BDSC#9792) (Zhang et al., 2007).

651

652 *Genome engineering of Syt7^{M1} mutant and SYT7^{RFP} knock-in*

653 Guide RNAs were selected using the CRISPR Optimal Target Finder resource (Gratz et al., 2014)

654 and cloned into the plasmid pCFD4-U6:1_U6:3tandemgRNAs (Addgene #49411) (Port et al.,

655 2014). To generate *Syt7^{M1}*, guide RNA containing pCFD4 plasmid was inject into *vasa*-Cas9

656 embryos (BDSC #56552) by Best Gene Inc (Chino Hills, CA, USA). *Syt7^{M1}* and an unaffected

657 injection line (control) were brought into the *white* background and the *vasa*-Cas9 chromosome

658 was removed. To generate SYT7^{RFP}, a donor plasmid that flanked RFP and a DsRed cassette was

659 generated from the pScarless plasmid (courtesy of Kate O'Connor-Giles) with 1 Kb homology

660 arms from the 3' end of the *Syt7* gene. The left homology arm was generated by PCR and the right

661 homology arm was synthesized by Epoch Life Science (Sugarland, TX, USA). The donor plasmid

662 and guide RNA containing pCFD4 plasmid was co-injected into Act5C-Cas9, Lig4 (BDSC

663 #58492) by Best Gene Inc. *Syt7^{M1}* and SYT7^{RFP} transformants were confirmed by DNA

664 sequencing.

665

666 *Sequence alignment, phylogenetic tree construction and molecular modeling*

667 NCBI BLAST was used to identify homologs of SYT1, SYT7 and ESYT-2 in the genomes of *C.*

668 *elegans*, *C. intestinalis*, *D. rerio*, *M. musculus*, *H. sapiens*, *R. norvegicus* and *T. adherens*. Jalview

669 was used to align SYT1 and SYT7 protein sequences from *D. melanogaster*, *M. Musculus* and *H.*

670 *sapiens* with the T-coffee multiple sequence alignment algorithm. Jalview and Matlab were used

671 to generate a phylogenetic tree using BLOSUM62 matrix and neighbor joining clustering. The

672 SWISS model server (https://swissmodel.expasy.org) was used for homology modeling of

673 Drosophila SYT7 from *R. norvegicus* SYT7 (PBD: 6ANK) (Waterhouse et al., 2018). The PyMOL

674 Molecular Graphics System (Version 2.0 Schrödinger, LLC) was used to visualize SYT1 and

675 SYT7 protein structures.

676

Sequences used for sequence alignment and phylogenetic tree		
Protein	Species	NCBI Accession number
ESYT2	*C. elegans*	NP_741181.1
	C. intestinalis	XP_018671537.1
	D. melanogaster	NP_733011.2
	D. rerio	XP_005171456.1
	H. sapiens	XP_024302614.1
	R. norvegicus	NP_001258098.1
	T. adhaerens	EDV19885.1
SYT1	*C. elegans*	NP_495394.3
	C. intestinalis	NP_001107602.1
	D. melanogaster	NP_523460.2
	D. rerio	NP_001314758.1
	H. sapiens	NP_001129277.1

24

	R. norvegicus	NP_001028852.2
	T. adhaerens	XP_002117742.1
SYT7	*C. elegans*	NP_001254022.1
	C. intestinalis	XP_026696415.1
	D. melanogaster	NP_726560.5
	D. rerio	XP_021326273.1
	H. sapiens	NP_004191.2
	R. norvegicus	NP_067691.1
	T. adhaerens	XP_002117784.1

677

678 *Western analysis and immunocytochemistry*

679 Western blotting of adult head lysates (1 head/lane) was performed using standard laboratory

680 procedures with anti-SYT7 (1:500) (Adolfsen et al., 2004), anti-SYX1 (8C3, 1:1000,

681 Developmental Studies Hybridoma Bank (DSHB, Iowa City, IA) and anti-RFP (600-401-379;

682 Rockland, 1:5000). Visualization and quantification were performed with a LI-COR Odyssey

683 Imaging System (LI-COR Biosciences, Lincoln, MA, USA). Secondary antibodies for Westerns

684 included Alexa Fluor 680-conjugated goat anti-rabbit IgG (1:5000, Invitrogen; A21109) and IR

685 Dye 800-conjugated goat anti-mouse IgG (1:5000, LICOR; 926-32211).

686   Immunostaining for AZ and bouton counting was performed on wandering stage 3rd instar

687 larvae dissected in Ca^{2+}-free HL3.1 and fixed for 17 min in Ca^{2+}-free HL3.1 containing 4% PFA.

688 Larvae were blocked and permeabilized for 1 hr in PBS containing 0.1% Triton X-100, 2.5% NGS,

689 2.5% BSA and 0.1% sodium azide. Larvae were incubated overnight with primary antibody at 4°C

690 and 2 hrs in secondary antibody at room temperature. Samples were mounted on slides with

691 Vectashield (Vector Laboratories, Burlingame, CA). Immunostaining for SYT7[RFP] and STY7[GFP]

692 co-localization analysis was similar, except larvae were blocked and permeabilized overnight in

693 PBS containing 0.25% Saponin, 2.5% normal goat serum (NGS), 2.5% bovine serum albumin

694 (BSA) and 0.1% sodium azide. Fixed larvae were incubated with primary antibody at 4°C for 24

695 hrs and with secondary antibodies for 1.5 hrs at room temperature. Fixed larvae were mounted in

25

696   ProLong® Diamond Antifade Mountant (#P36970; Thermo Fisher Scientific, Waltham, MA,

697   USA).

698   Antibodies used for immunolabeling were: mouse anti-BRP at 1:500 (Nc82; DSHB), mouse

699   anti-DYN at 1:1000 (Clone 41, Dynamin (RUO); BD Transduction Laboratories, San Jose, CA,

700   USA), mouse anti-Golgin84 at 1:50 (Golgin84 12-1; DSHB), mouse anti-RAB7 at 1:10 (Rab7;

701   DSHB), mouse anti-RFP at 1:1000 (200-301-379; Rockland, Limerick, PA, USA) mouse anti-

702   SYX1 at 1:100 (8C3; DSHB), rabbit anti-CPX at 1:5000 (Huntwork and Littleton, 2007), rabbit

703   anti-NWK at 1:1000 (gift from Avital Rodal), rabbit anti-SYT1 1:500, mouse anti-GFP at 1:1000

704   (#A-11120; Thermo Fisher Scientific, Waltham, MA, USA), rabbit anti-GFP at 1:1000 (#G10362;

705   Thermo Fisher Scientific, Waltham, MA, USA), mouse anti-RFP at 1:1000 (200-301-379;

706   Rockland), rabbit anti-RFP at 1:1000 (600-401-379; Rockland) and DyLight 649 conjugated anti-

707   HRP at 1:1000 (#123-605-021; Jackson Immuno Research, West Grove, PA, USA). Secondary

708   antibodies used for AZ and bouton counting were used at 1:1000: goat anti-rabbit Alexa Flour

709   488-conjugated antibody (A-11008; Thermofisher) and goat anti-mouse Alexa Fluor 546-

710   conjugated antibody (A-11030; ThermoFisher). Secondary antibodies used for co-localization

711   were used at 1:1000: goat anti-mouse Alexa Fluor Plus 555 (A32727; Thermofisher), goat anti-

712   mouse Alexa Fluor Plus 488 (A32723; ThermoFisher), goat anti-rabbit Alexa Fluor Plus 555

713   (A32732; ThermoFisher) and goat anti-rabbit Alexa Fluor Plus 488 (A32731; ThermoFisher).

714   Immunoreactive proteins were imaged on either a Zeiss Pascal Confocal (Carl Zeiss

715   Microscopy, Jena, GERMANY) using a 40x or 63X NA 1.3 Plan Neofluar oil immersion objective

716   or a ZEISS LSM 800 microscope with Airyscan using a 63X oil immersion objective. For AZ

717   volume and AZ proximity measurements, samples were imaged on a Zeiss Airyscan microscope

718   and BRP labeling was analyzed in Volocity 6.3.1 software (Quorum Technologies Inc., Puslinch,

719   Ontario, CAN). AZs clusters larger than 0.2 μm^3 were rarely found, but could not be resolved into

720   single objects by the software. To ensure such clusters did not affect AZ size analysis, all AZs

721   larger than 0.2 μm^3 were excluded from the analysis.

722

723   *Electrophysiology*

724   Postsynaptic currents from the indicated genotypes were recorded from 3rd instar muscle fiber 6 at

725   segment A3 using two-electrode voltage clamp with a -80 mV holding potential in HL3.1 saline

726   solution (in mM, 70 NaCl, 5 KCl, 10 NaHCO3, 4 MgCl2, 5 trehalose, 115 sucrose, 5 HEPES, pH

727   7.2) as previously described (Jorquera et al., 2012). Final [Ca^{2+}] was adjusted to the level indicated

728   in the text. For experiments using bafilomycin, 4 μm bafilomycin (LC Laboratories, Woburn, MA,

729   USA) was dissolved in dimethyl sulphoxide (DMSO, Sigma, St. Louis, MO, USA) in HL3.1 and

730   bath applied to dissected larvae. DMSO containing HL3.1 was used for control. Data acquisition

731   and analysis was performed using Axoscope 9.0 and Clampfit 9.0 software (Molecular Devices,

732   Sunnyvale, CA, USA). mEJCs were analyzed with Mini Analysis software 6.0.3 (Synaptosoft,

733   Decatur, GA, USA). Motor nerves innervating the musculature were severed and placed into a

734   suction electrode. Action potential stimulation was applied at the indicated frequencies using a

735   programmable stimulator (Master8, AMPI; Jerusalem, Israel).

736

737   *Optical quantal imaging and P_r mapping*

738   P_r mapping was performed on a Zeiss Axio Imager 2 equipped with a spinning-disk confocal head

739   (CSU-X1; Yokagawa, JAPAN) and ImagEM X2 EM-CCD camera (Hamamatsu, Hamamatsu City

740   JAPAN) as previously described (Akbergenova et al., 2018). Myristoylated-GCaMP6s was

741   expressed with 44H10-LexAp65 (provided by Gerald Rubin). Postsynaptic densities were

742   visualized by expression of GluRIIA-RFP under its endogenous promoter (provided by Stephan

743   Sigrist). An Olympus LUMFL N 60X objective with a 1.10 NA was used to acquire GCaMP6s

744   imaging data at 8 Hz. 3rd instar larvae were dissected in Ca^{2+}-free HL3 containing 20 mM $MgCl_2$.

745   After dissection, preparations were maintained in HL3 with 20 mM $MgCl_2$ and 1.0 mM Ca^{2+} for 5

746   minutes. Motor nerves were stimulated every three seconds for GCaMP6s mapping. The time and

747   location of Ca^{2+} events were imported into Excel or Matlab for further analysis. The number of

748   observed GCaMP events per AZ was divided by the number of stimuli to calculate AZ P_r.

749

750   *FM1-43 uptake and release assays*

751   3rd instar wandering larvae were dissected in Ca^{2+}-free HL3.1 and axons were severed from the

752   CNS. Axon bundles were stimulated with a suction electrode in 1.5 mM $CaCl_2$ HL3.1 solution

753   containing 2 μM of the lipophilic dye FM 1-43FX (F35355; Thermo Fisher Scientific, Waltham,

754   MA, USA). Dye loading was performed at 10 Hz for 50 seconds (500 events) or at 0.5 Hz for 300

755   seconds (150 events), 600 seconds (300 events) and 900 seconds (600 events) as indicated. After

756   stimulation, samples were washed for 2 min in Ca^{2+} free HL3.1 containing 100 μM Advacep-7

757   (Sigma; A3723) to help remove non-internalized FM 1-43 dye. Image stacks from muscle 6/7 at

758   segment A3 were obtained using a spinning disk confocal microscope. FM1-43 unloading was

759   done with a high K^+ (90 mM) HL3.1 solution for 1 min, followed by washing in a Ca^{2+} free HL3.1

760   solution for 1 min. An image stack at segment A3 muscle 6-7 was obtained on a Zeiss Axio Imager

761   2 equipped with a spinning-disk confocal head with a 63X water immersion objective. Mean FM1-

762   43 intensity at the NMJ was quantified using the Volocity 3D Image Analysis software (Quorum

763   Technologies Inc., Puslinch, Ontario, CAN).

764

765   *Electron microscopy*

766   *Syt1^{M1}* and control 3rd instar larvae were dissected in Ca^{2+}-free HL3.1 solution and fixed in 1%

767   glutaraldehyde, 4% formaldehyde, and 0.1 m sodium cacodylate for 10 min at room temperature

768   as previously described (Akbergenova and Bykhovskaia, 2009). Fresh fixative was added and

769   samples were microwaved in a BioWave Pro Pelco (Ted Pella, Inc., Redding, CA, USA) with the

770   following protocol: (1) 100W 1 min, (2) 1 min off, (3) 100W 1 min, (4) 300W 20 secs, (5) 20 secs

771   off, (6) 300W 20 secs. Steps 4- 6 were repeated twice more. Samples were then incubated for 30

772   min at room temperature with fixative. After washing in 0.1 M sodium cacodylate and 0.1 M

773   sucrose, samples were stained for 30 min in 1% osmium tetroxide and 1.5% potassium

774   ferrocyanide in 0.1 M sodium cacodylate solution. After washing with 0.1 M sodium cacodylate,

775   samples were stained for 30 mins in 2% uranyl acetate and dehydrated through a graded series of

776   ethanol and acetone, before embedding in epoxy resin (Embed 812; Electron Microscopy

777   Sciences). Thin sections (50–60 nm) were collected on Formvar/carbon-coated copper slot grids

778   and contrasted with lead citrate. Sections were imaged at 49,000× magnification at 80 kV with a

779   Tecnai G2 electron microscope (FEI, Hillsboro, OR, USA) equipped with a charge-coupled device

780   camera (Advanced Microscopy Techniques, Woburn, MA, USA). Type 1b boutons at muscle 6/7

781   were analyzed. All data analysis was done blinded.

782   For SV counting, T-bars at Ib boutons were identified and a FIJI macro was used to draw four

783   concentric circles with 100 nm, 200 nm, 300 nm or 400 nm radius. The concentric circles were

784   drawn with the T-bar at the center. To quantify vesicle density, FIJI was used to measure the area

785   of the bouton and quantify the total number of vesicles within it. Final analysis was performed in

786   Matlab and Excel.

787

788   *Co-localization analysis and 3D reconstruction*

789 The JaCOP FIJI algorithm (Bolte and Cordelières, 2006) was used to obtain cytofluorogram plots

790 of bouton image stacks that were probed for RFP and a 2nd labeled compartment in SYT7RFP 3rd

791 instar larvae. Automatic thresholding was used to identify pixels above background for both

792 channels. To obtain an average Pearson correlation, cytofluorograms from boutons obtained from

793 3 animals were analyzed in Matlab. All data analysis was done blinded. 3D reconstruction was

794 performed using the 3D Viewer plugin in FIJI (Schmid et al., 2010). The bouton stack was

795 displayed as a surface and labeled with SYT7RFP in magenta and HRP in black.

796

797 *Statistical analysis*

798 Statistical analysis and graphing was performed with either Origin Software (OriginLab

799 Corporation, Northampton, MA, USA) or GraphPad Prism (San Diego, CA, USA). Statistical

800 significance was determined using specific tests as indicated in the text. Appropriate sample size

801 was determined using GraphPad Statmate. Asterisks denote p-values of: *, $P \leq 0.05$; **, $P \leq 0.01$;

802 and ***, $P \leq 0.001$. All histograms and measurements are shown as mean ± SEM.

803

812

813 **Competing Interests**

814 The authors declare no competing interests.

815

816

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1276

1277   **Figure Legends**

1278

1279   **Figure 1.** SYT1 and SYT7 comparison and generation of *Syt7* mutants. **(A)** Proposed roles for

1280   SYT7 in Ca^{2+}-regulated membrane trafficking. **(B)** Phylogenetic tree of SYT1, SYT7 and E-SYT2

1281   from the indicated species generated using the BLOSUM62 matrix with neighbor joining

1282   clustering. **(C)** Comparison of the structure of the C2A and C2B domains of *R. norvegicus* SYT1

1283   (magenta) with a homology model of *D. melanogaster* SYT7 (blue). The C2B residues that form

1284   the SYT1-SNARE complex primary binding site are highlighted in yellow, with the counterpart

1285   changes noted in SYT7. The C2B HB helix in SYT1 is highlighted in green and missing from

1286   SYT7. **(D)** Diagram of the *Syt7* genomic locus on chromosome 4 with coding exons indicated with

1287   boxes. Exon 1 (teal) encodes the intravesicular and transmembrane (TM) domains; exons 2 and 3

1288   (white) encode the linker region; exons 4 and 5 encode the C2A domain (dark blue); and exons 6

1289   and 7 encode the C2B domain (light blue). The location of the *Syt7^M2* Minos transposon insertion

1290   in exon 2 is indicated in red. Sequence of the *Syt7^M1* CRISPR mutant versus control is shown

1291   below with the start codon in green. The guide RNA sequence used to target *Syt7* is bolded, with

1292   the cleavage site noted by the red arrowhead and the deleted cytosine with a red dash. **(E)** Western

1293   of SYT7 protein levels in head extracts of *white*, CRISPR control, *Syt7^M1*, *Syt7^M2* and *elav^C155*-

1294   GAL4; UAS-*Syt7* (OE SYT7) with anti-SYT7 antisera (top panel). Syntaxin 1 (SYX1) antisera

1295   was used as a loading control (bottom panel). SYT7 is overexpressed 2.48 ± 0.4-fold compared to

1296   controls ($p<0.05$, Mann-Whitney unpaired t-test, n=4).

1297

1298   **Figure 1 – figure supplement 1**. SYT1 and SYT7 sequence comparisons. Annotated sequence

1299   alignment of the C2A Ca^{2+} binding loops and the C2B domain of SYT1 and SYT7 from the

1300   indicated species. Conserved resides are shaded dark blue, with conservative amino acid

1301   substitutions in light blue. Grey shading denotes subfamily-specific reside conservation in only

1302   SYT1 or SYT7. C2A and C2B Ca^{2+} binding residues are shaded red. Yellow circles denote

1303   residues that form the primary SYT1-SNARE complex binding interface. Four of the five residues

1304   are not conserved in Drosophila SYT7, with two containing identical substitutions previously

1305   found to abolish SYT1 function (R334H and E348K).

1306

1307 **Figure 2.** *Syt7* mutants and *Syt7*/+ heterozygotes display enhanced neurotransmitter release. **(A)**

1308 Average mEJC traces in control (black), *Syt7^{M1}*/+ (green) and *Syt7^{M1}* mutants (blue). **(B)**

1309 Quantification of mean mEJC amplitude for the indicated genotypes (control: 0.62 ± 0.020 nA,

1310 n=17; *Syt7^{M1}*/+: 0.61 ± 0.021 nA, n=21; *Syt7^{M1}*: 0.57 ± 0.013 nA, n=20). **(C)** Normalized

1311 cumulative mEJC charge for each genotype. **(D)** Quantification of mean mEJC frequency for the

1312 indicated genotypes (control: 1.30 ± 0.10 Hz, n=17; *Syt7^{M1}*/+: 1.66 ± 0.13 Hz, n=19; *Syt7^{M1}*: 1.36

1313 ± 0.12 Hz, n=19). **(E)** Average eEJC traces in control (black), *Syt7^{M1}*/+ (green) and *Syt7^{M1}* (blue).

1314 **(F)** Quantification of mean eEJC amplitude for the indicated genotypes. **(G)** Average normalized

1315 responses for each genotype plotted on a semi-logarithmic graph to display release components.

1316 **(H)** Cumulative release normalized to the maximum response in 2 mM Ca^{2+} for each genotype. **(I)**

1317 Quantification of mean eEJC rise time in the indicated genotypes (control: 1.09 ± 0.08 ms, n=9;

1318 *Syt7^{M1}*/+: 1.06 ± 0.09 ms, n=14; *Syt7^{M1}*: 1.03 ± 0.08 ms, n=10). **(J)** Quantification of mean eEJC

1319 half-width in the indicated genotypes (control: 7.81 ± 0.47 ms, n=9; *Syt7^{M1}*/+: 7.77 ± 0.26 ms,

1320 n=14; *Syt7^{M1}*: 7.15 ± 0.34 ms, n=10). **(K)** Quantification of evoked quantal content with mEJC

1321 amplitude for the indicated genotypes (control: 250.1 ± 30.58 SVs, n=9; *Syt7^{M1}*/+: 377.9 ± 31.13,

1322 n=14; *Syt7^{M1}*: 495.3 ± 36.75, n=10). **(L)** Quantification of evoked quantal content with mEJC

1323 charge for the indicated genotypes (control: 221.3 ± 20.54 SVs, n=9; *Syt7^{M1}*/+: 371.6 ± 43.56,

1324 n=14; *Syt7^{M1}*: 503.6 ± 31.99, n=10). Recordings were performed from 3rd instar segment A3

1325 muscle 6 in 2 mM Ca^{2+}. Statistical significance for all comparisons was determined using one-way

1326 ANOVA (nonparametric) with post hoc Tukey's multiple comparisons test. N.S. = no significant

1327 change. Error bars represent SEM.

1328

1329 **Figure 3.** Neuronal overexpression of SYT7 reduces spontaneous and evoked SV release. **(A)**

1330 Average mEJC traces in control (black) and *elav^{C155}*-GAL4; UAS-*Syt7* (OE SYT7, magenta). **(B)**

1331 Quantification of mean mEJC amplitudes in the indicated genotypes (control: 0.66 ± 0.03 nA, n=9;

1332 OE SYT7: 0.73 ± 0.03 nA, n=8). **(C)** Quantification of mean mEJC frequency in the indicated

1333 genotypes (control: 2.81 ± 0.42 Hz, n=9; OE SYT7: 1.45 ± 0.18 Hz, n=8). **(D)** Average eEJC traces

1334 in control (black) and *elav^{C155}*-GAL4; UAS-*Syt7* (OE SYT7, magenta). **(E)** Quantification of mean

1335 eEJC amplitudes in the indicated genotypes (control: 256.24 ± 22.38 nA, n=10; OE SYT7: 166.66

1336 ± 10.74 nA, n=7). **(F)** Quantification of mean eEJC charge in the indicated genotypes (control:

1337 2.5x10^3 ± 0.25 x10^3 nA*ms, n=10; OE SYT7: 1.4x10^3 ± 0.12x10^3 nA*ms, n=7). **(G)** Average

1338 normalized responses for each genotype plotted on a semi-logarithmic graph to display release

1339 components. Recordings were performed from 3rd instar segment A3 muscle 6 in 2 mM Ca^{2+}.

1340 Statistical significance was determined with a Mann-Whitney unpaired t-test.

1341

1342 **Figure 3 – figure supplement 1.** Overexpression of SYT7 in postsynaptic muscles does not

1343 disrupt synaptic transmission. **(A)** Average eEJC traces in control (black) and *Mhc*-GAL4; UAS-

1344 *Syt7* (OE SYT7, orange). **(B)** Quantification of mean eEJC amplitudes in the indicated genotypes

1345 (control: 252.82 ± 10.98 nA, n=12; *Mhc*-GAL4; UAS-*Syt7*: 243.91 ± 7.46 nA, n=16). Recordings

1346 were performed from 3rd instar segment A3 muscle 6 in 2 mM Ca^{2+}. Statistical significance was

1347 determined with the Mann-Whitney unpaired test.

1348

1349 **Figure 4.** Analysis of synaptic morphology in *Syt7* mutants. **(A, B)** Immunocytochemistry of 3rd

1350 instar muscle 6/7 NMJs with anti-HRP (blue), anti-CPX (magenta) and anti-BRP (green) in control

1351 and *Syt7^{M1}*. The boxed region is magnified below with channels showing BRP, CPX and the merge.

1352 Scale bar = 20 μm for large panels and 2 μm for boxed regions. Synaptic morphology was

1353 quantified for 3rd instar muscle 6/7 **(C-E)** and muscle 4 **(F-H)** in controls and *Syt7^{M1}* mutants. No

1354 significant differences were detected in synaptic bouton number **(C, F**; muscle 6/7: p = 0.78;

1355 control: 81.87 ± 5.301, n=15; *Syt7^{M1}*: 79.60 ± 5.824, n=15; muscle 4: p = 0.24; control: 55.86 ±

1356 3.141, n=14; *Syt7^{M1}*: 62.50 ± 4.575, n=14), BRP puncta **(D, G**, muscle 6/7: p = 0.94; control: 621.1

1357 ± 26.28, n=15; *Syt7^{M1}*: 618.1 ± 25.73, n=15; muscle 4: p = 0.83; control: 450.5 ± 23.25, n=14;

1358 *Syt7^{M1}*: 443.5 ± 21.47, n=14) or BRP puncta per muscle surface area **(E, H**, muscle 6/7: p = 0.13;

1359 control: 0.0088 ± 0.0004, n=15; *Syt7^{M1}*: 0.0098 ± 0.0005, n=15; muscle 4: p = 0.88; control:

1360 0.0105± 0.0008, n=14; *Syt7^{M1}*: 0.0107± 0.0007, n=14). **(I)** Anti-BRP staining at 3rd instar muscle

1361 4 in control and *Syt7^{M1}* imaged with SIM microscopy. Scale bar = 1 μm. **(J)** Relative cumulative

1362 frequency of AZ T-bar volume defined with anti-BRP staining at 3rd instar muscle 6/7 NMJs (p =

1363 0.026; control: 0.055 ± 0.004 μm^2, n = 19 NMJs from 5 larvae; *Syt7^{M1}*: 0.044 ± 0.003 μm^2, n=15

1364 NMJs from 4 larvae). **(K)** Relative cumulative frequency of T-bar spacing defined by distance

1365 between nearest BRP puncta at 3rd instar muscle 6/7 NMJs (p = 0.48; control: 0.28 ± 0.016 μm,

1366 n=20 NMJs from 5 larvae; *Syt7^{M1}*: 0.27 ± 0.014 μm, n=15 NMJs from 4 larvae). Statistical

1367 significance was determined with Student's t-test.

1368

42

1369   **Figure 5.** Ultrastructural analysis of SV distribution in *Syt7* mutants. **(A)** Representative EM

1370   micrographs of muscle 6/7 synaptic boutons in control and *Syt7^{M1}* 3rd instar larvae. An AZ with its

1371   associated electron dense T-bar is denoted with an arrowhead in each micrograph. **(B)**

1372   Quantification of SV density (p = 0.41; control = 0.34 ± 0.033 SVs/μm^2, n = 20; *Syt7^{M1}* = 0.30 ±

1373   0.031 SVs/μm^2, n = 20). **(C)** Quantification of AZ length defined by the electron dense synaptic

1374   cleft (p=0.93; control: 404 ± 34.5 nm, n=21 AZs from 5 larvae; *Syt7^{M1}*: 409 ± 28.9 nm, n=29 AZs

1375   from 5 larvae). **(D)** Quantification of SVs docked within 100 nm of the T-bar (p = 0.41; control =

1376   1.69 ± 0.15 SVs n = 84; *Syt7^{M1}* = 1.43 ± 0.15 SVs, n = 58). **(E)** Quantification of SVs docked

1377   within 100 - 400 nm of the T-bar (p = 0.68; control = 2.46 ± 0.17 SVs n = 84; *Syt7^{M1}* = 2.35 ± 0.25

1378   SVs, n = 58). **(F)** Quantification of all docked SVs at 0-400 nm from the T-bar (p = 0.31; control

1379   = 4.16± 0.23 SVs n = 84; *Syt7^{M1}* = 3.78 ± 0.29 SVs, n = 58). **(G)** Quantification of all SVs within

1380   a 400 nm radius from the T-bar (p = 0.38; control = 71.98 ± 4.05 SVs n = 84; *Syt7^{M1}* = 78.12 ±

1381   5.89 SVs, n = 58). **(H)** Quantification of SV distribution at AZs in control and *Syt7^{M1}* mutants.

1382   Statistical significance was determined with Student's t-test.

1383

1384   **Figure 6.** Quantal imaging reveals elevated release probability across the AZ population in *Syt7*

1385   mutants. **(A)** P_r heatmaps for muscle 4 NMJs generated following 0.3 Hz stimulation for 5 minutes

1386   in control and *Syt7^{M1}* mutants. The P_r color map is displayed in the upper right. **(B)** Frequency

1387   distribution of AZ P_r after a 0.3 Hz 5-minute stimulation for control (black dashed line) and *Syt7^{M1}*

1388   (blue line). **(C)** Quantification of mean AZ P_r for the two genotypes (p≤0.01, Student's t-test;

1389   control: 0.063 ± 0.002, n=1158; *Syt7^{M2}*: 0.12 ± 0.004, n=768).

1390

1391   **Figure 7.** Loss of SYT7 enhances the residual release observed in *Syt1* null mutants. **(A)** Western

1392   of head extracts from control, *Syt7^{M2}*, *Syt1^{Null}* and *Syt1^{null}*; *Syt7^{M2}* (*Double^{Null}*) probed with anti-

1393   SYT7, anti-SYT1 and anti-SYX1 (loading control). SYT1 migrates as a doublet at 55 and 70 kD

1394   (Littleton et al., 1993a). **(B)** Average mEJC traces in *Syt1^{Null}* (black trace) and *Double^{Null}* (red trace)

1395   mutants obtained by summing all mEPSC events under the first peak distribution. **(C)**

1396   Quantification of mean mEJC frequency for the indicated genotypes. **(D)** Average eEJC traces in

1397   *Syt1^{Null}* (black trace) and *Double^{Null}* (red trace). **(E)** Quantification of mean eEJC amplitude for the

1398   indicated genotypes. **(F)** Quantification of mean eEJC charge for the indicated genotypes obtained

1399   by measuring total release over time. **(G)** Average normalized responses for each genotype plotted

1400   on a semi-logarithmic graph to display release components. (**H**) Cumulative release normalized to

1401   the maximum response in 2 mM Ca^{2+} for each genotype. Each trace was adjusted to a double

1402   exponential fit. (**I**) Quantification of eEJC failure ratio (%) in the indicated genotypes. Recordings

1403   were performed from 3rd instar segment A3 muscle 6 in 2 mM extracellular Ca^{2+}. Statistical

1404   significance was determined with the Mann-Whitney unpaired t-test.

1405

1406   **Figure 8.** Short-term synaptic facilitation can occur without SYT7 or SYT1. (**A**) Quantification of

1407   eEJC failure ratio (%) in the indicated genotypes. (**B**) Average eEJC traces recorded in 0.175 mM

1408 Ca^{2+} (control, grey; *Syt7^{M1}*, light blue) or 0.2 mM Ca^{2+} (control, black; *Syt7^{M1}*, dark blue). (**C**)

1409   Quantification of mean eEJC amplitude for the indicated genotypes (0.175 mM Ca^{2+}: control, 5.42

1410   ± 2.0 nA, n=7; *Syt7^{M1}*, 8.70 ± 1.6 nA, n=14; 0.2 mM Ca^{2+}: control, 7.73 ± 1.5 nA, n=9; *Syt7^{M1}*,

1411   23.72 ± 6.2 nA, n=9). (**D**) Representative eEJC traces to 10 ms or 50 ms paired-pulse stimuli

1412   recorded in 0.2 mM Ca^{2+} (control, black; *Syt7^{M1}*, dark blue) or 0.175 mM Ca^{2+} (*Syt7^{M1}*, light blue).

1413   (**E**) Quantification of facilitation (P2/P1) at 10 ms interval for the indicated genotypes (0.2 mM

1414 Ca^{2+}: 1.93 ± 0.095, n=9; *Syt7^{M1}*, 1.28 ± 0.12, n=9; 0.175 mM Ca^{2+}: *Syt7^{M1}*, 1.47 ± 0.11, n=12). (**F**)

1415   Quantification of facilitation (P2/P1) at 50 ms interval for the indicated genotypes (0.2 mM Ca^{2+}:

1416   control, 1.64 ± 0.043, n=9; *Syt7^{M1}*, 1.23 ± 0.056, n=9; 0.175 mM Ca^{2+}: *Syt7^{M1}*, 1.34 ± 0.054, n=12).

1417   Statistical significance was determined using one-way ANOVA (nonparametric) with post hoc

1418   Tukey's multiple comparisons test for panels A-F. (**G**) Average eEJC quantal content determined

1419   from mEJC charge in 2 mM Ca^{2+} during a 10 Hz stimulation paradigm (30 stimuli at 0.5 Hz, 500

1420   stimuli at 10 Hz, and return to 0.5 Hz) in *Syt1^{Null}* (black) and *Double^{Null}* (red). (**H**) Average quantal

1421   content for the last four responses of 0.5 Hz stimulation and the first 14 responses during 10 Hz

1422   stimulation in *Syt1^{Null}* (black) and *Double^{Null}* (red). P1 denotes the 1st response and P2 the 2nd

1423   response to 10 Hz stimulation. (**I**) Quantification of P2/P1 ratio in *Syt1^{Null}* (black, 1.15 ± 0.089,

1424   n=12) and *Double^{Null}* (red, 1.55 ± 0.22, n=13) at onset of 10 Hz stimulation. Statistical significance

1425   was determined with a Mann-Whitney unpaired t-test for panels H and I.

1426

1427   **Figure 9.** *Syt7* mutants have a larger releasable pool of SVs and normal endocytosis. (**A**)

1428   Representative mean eEJC quantal content determined by mEJC charge during 1000 stimuli at 10

1429   Hz in 2 mM Ca^{2+} in control (black) and *Syt7^{M1}* (blue). The inset shows representative eEJC traces

1430   in control (black) and *Syt7^{M1}* (blue). (**B**) Quantification of average cumulative quanta released

1431   during the 1000 stimuli at 10 Hz tetanic stimulation in control (black, 19.21K ± 2.88K, n=7) and

1432   *Syt7^{M1}* (blue, 36.18K ± 5.67K, n=8). (**C**) Quantification of average quantal content at steady-state

1433   release at the end of the 10 Hz stimulation in control (black, 131.54 ± 10.71, n=7) and *Syt7^{M1}* (blue,

1434   123.05 ± 10.47, n=8). Statistical significance for B and C was determined with a Mann-Whitney

1435   unpaired t-test. (**D**) FM1-43 loading in control and *Syt7^{M1}* larvae at muscle 6/7 NMJs in 2 mM

1436 Ca^{2+} following 150, 300 or 600 stimuli delivered at 0.5 Hz. (**E**) FM1-43 loading with 500 stimuli

1437   at 10 Hz in 2 mM Ca^{2+} and FM1-43 unloading with high K^+ (90 mM) in control and *Syt7^{M1}* larvae

1438   at muscle 6/7 NMJs. (**F**) Quantification of FM1-43 loading following 150, 300 or 600 stimuli

1439   delivered at 0.5 Hz. (**G**) Quantification of FM1-43 loading after 500 stimulati at 10 Hz. (**H**)

1440   Quantification of FM1-43 unloading with high K^+ (90 mM). Statistical significance was

1441   determined with Student's t-test for F-H. Scale bar = 5µm.

1442

1443   **Figure 10.** *Syt7* mutants have enhanced refilling of the RRP that does not require endocytosis. (**A**)

1444   Average eEJC quantal content during the indicated stimulation protocol in 2 mM external Ca^{2+} for

1445   control (black), *Syt7$^{M1/+}$* (green) and *Syt7^{M1}* (blue). (**B**) Quantification of P2/P1 ratio (P1 = 1st

1446   response to 10 Hz, P2 = 2nd response to 10 Hz) in control (black, 1.13 ± 0.03, n=8), *Syt7$^{M1/+}$* (green,

1447   0.95 ± 0.009, n=14) and *Syt7^{M1}* (blue, 0.82 ± 0.01, n=8). Representative eEJC traces of P1 and P2

1448   for control (black) and *Syt7^{M1}* (blue) are shown on the right. (**C**) Quantification of P531/P530 ratio

1449   (P530 is the last response to 10 Hz and P531 is the 1st response to 0.5 Hz stimulation delivered 2

1450   seconds after P530) in control (black, 0.93 ± 0.06, n=8), *Syt7$^{M1/+}$* (green, 1.33 ± 0.04, n=12) and

1451   *Syt7^{M1}* (blue, 1.91 ± 0.09, n=8). Representative eEJC traces of P530 and P531 for control (black)

1452   and *Syt7^{M1}* (blue) are shown on the right. (**D**) Representative eEJC traces for control with DMSO

1453   (black) or 4 µM bafilomycin (gray) and *Syt7^{M1}* with DMSO (dark blue) or 4 µM bafilomycin (light

1454   blue) in 2 mM external Ca^{2+} with the indicated stimulation protocol repeated three times. (**E**)

1455   Quantification of P531/P530 for the indicated genotypes (1st stimulation protocol: Control +

1456   DMSO, 0.98 ± 0.056, n=17; Control + bafilomycin , 1.53 ± 0.12, n=17; *Syt7^{M1}* + DMSO, 1.83 ±

1457   0.058, n=17; *Syt7^{M1}* + bafilomycin, 2.10 ± 0.11, n=17; 2nd stimulation protocol: Control + DMSO,

1458   0.97 ± 0.045, n=17; Control + bafilomycin , 1.25 ± 0.064, n=17; *Syt7^{M1}* + DMSO, 1.95 ± 0.10,

1459   n=17; *Syt7^{M1}* + bafilomycin, 2.09 ± 0.19, n=17). Statistical significance was determined with a

1460   one-way Anova with Sidak's multiple comparisons test. (**F**) Quantification of mean eEJC

1461   amplitudes for P530 and P531 for the indicated genotypes (1st stimulation protocol: P530 in

1462   Control + DMSO, 87.39 ± 3.85, n=17; P531 in Control + DMSO, 80.22 ± 5.25, n=17; P530 in

1463   Control + bafilomycin, 44.68 ± 2.80, n=17; P531 in Control + bafilomycin, 66.26 ± 5.03, n=17;

1464   P530 in $Syt7^{M1}$ + DMSO, 97.62 ± 4.04, n=17; P531 in $Syt7^{M1}$ + DMSO, 177.34 ± 7.80, n=17; P530

1465   in $Syt7^{M1}$ + bafilomycin, 52.44 ± 3.83, n=17; P531 in $Syt7^{M1}$ + bafilomycin, 102.50 ± 8.07, n=17;

1466   2nd stimulation protocol: P530 in Control + DMSO, 68.21 ± 3.97, n=17; P531 in Control + DMSO,

1467   70.05 ± 5.95, n=17; P530 in Control + bafilomycin, 15.09 ± 1.26, n=17; P531 in Control +

1468   bafilomycin, 18.15 ± 1.34, n=17; P531 in $Syt7^{M1}$ + DMSO, 82.89 ± 4.64, n=17; P531 in $Syt7^{M1}$ +

1469   DMSO, 163.52 ± 9.74, n=17; P530 in $Syt7^{M1}$ + bafilomycin, 11.98 ± 1.26, n=17; P531 in $Syt7^{M1}$ +

1470   bafilomycin, 24.71 ± 3.00, n=17). Statistical significance was determined with a Student's paired

1471   t-test.

1472

1473   **Figure 10 – figure supplement 1.** Enhanced recovery after termination of 10 Hz stimulation in

1474   *Syt7* mutants. (**A**) Average eEJC quantal content to 2000 stimuli at 10 Hz stimulation at 3rd instar

1475   segment A3 muscle 6 in 2 mM external Ca^{2+} for control (black) and $Syt7^{M1}$ (blue). (**B**)

1476   Representative average quantal content of the last 3 responses to 10 Hz and the 1st 12 responses to

1477   0.5 Hz stimulation. P2000 = last response to 10 Hz stimulation, P2001 = 1st response to 0.5 Hz

1478   stimulation 2 seconds after P2000. (**C**) Quantification of P2001/P2000 ratio for control (black,

1479   0.90 ± 0.03, n=9) and $Syt7^{M1}$ (blue, 1.84 ± 0.10, n=8). Statistical significance was determined with

1480   a Mann-Whitney unpaired t-test.

1481

1482   **Figure 10 – figure supplement 2.** SYT7 overexpression reduces RRP refilling following 10 Hz

1483   stimulation. (**A**) Representative average eEJC quantal content for the indicated stimulation in 2

1484   mM external Ca^{2+} in control (black) and $elav^{C155}$-GAL4; UAS-*Syt7* (OE SYT7, magenta). (**B**)

1485   Representative average quantal content for the last 4 responses during 10 Hz and the 1st 14

1486   responses during 0.5 Hz in control (black) and OE SYT7 (magenta). (**C**) Quantification of

1487   P531/P530 ratio for control (black, 1.43 ± 0.052, n=9) and OE SYT7 (magenta, 0.85 ± 0.068, n=7).

1488   Statistical significance was determined with a Mann-Whitney unpaired t-test.

1489

1490   **Figure 11.** Tagging and location of endogenous SYT7. (**A**) CRISPR strategy used to insert RFP

1491   in frame at the *Syt7* 3'end to generate $SYT7^{RFP}$. Exon coloring is the same as Figure 1D. The guide

1492   RNA cleavage site is displayed in yellow. (**B**) Two *Syt7* UAS-RNAi lines (#1 and #2) were used

1493   to pan-neuronally knockdown SYT7^{RFP}. Western analysis of head extracts probed with anti-RFP

1494   (top panel) from SYT7^{RFP} adults following pan-neuronal knockdown of SYT7: lane 1: UAS-*Syt7*

1495   RNAi#1; SYT7^{RFP}: lane 2: *elav*^{C155}-GAL4, UAS-Dicer2; UAS-*Syt7* RNAi#1; SYT7^{RFP}: lane 3:

1496   UAS-*Syt7* RNAi#2; SYT7^{RFP}: lane 4: *elav*^{C155}-GAL4, UAS-Dicer2; UAS-*Syt7* RNAi line#2;

1497   SYT7^{RFP}. SYX1 antisera was used as a loading control (bottom panel). (**C**) Immunocytochemistry

1498   with anti-HRP (top) and anti-RFP (bottom) in SYT7^{RFP} 3rd instar larvae at muscle 6/7 NMJs.

1499   SYT7^{RFP} staining is abundant in the presynaptic terminal, with a few postsynaptic membrane

1500   compartments also labeled. (**D**) 3D rendering of the terminal bouton (left) from above. The

1501   SYT7^{RFP} intra-terminal compartment is labeled in magenta, with HRP-labeled plasma membrane

1502   indicated with a grey mesh. Scale bar = 2 μm.

1503

1504   **Figure 11 – figure supplement 1.** Location of SYT7^{GFP} within synaptic boutons. (**A**)

1505   Immunocytochemistry with anti-HRP (top) and anti-GFP (bottom) in SYT7^{GFP} 3rd instar larvae at

1506   muscle 6/7 NMJs. SYT7^{GFP} staining, like SYT7^{RFP}, localizes throughout the presynaptic terminal.

1507   Scale bar = 2 μm. (**B**) Synaptic bouton immunolabeled with anti-BRP and anti-GFP in SYT7^{GFP}

1508   larvae. SYT7 surrounds but does not co-localize with BRP-labeled AZs, similar to other previously

1509   described peri-AZ proteins. Scale bar = 1 μm.

1510

1511   **Figure 11 – figure supplement 2.** Knockdown of SYT7^{RFP} with *Syt7* RNAi eliminates RFP

1512   immunostaining. (**A**) Immunocytochemistry with anti-HRP (cyan) and anti-RFP (magenta) in

1513   SYT7^{RFP} 3rd instar larvae without (left) or with *elav*^{C155}-GAL4, UAS-Dicer2; UAS-*Syt7* RNAi#1.

1514   (**B**) Immunocytochemistry with anti-HRP and anti-RFP in SYT7^{RFP} without (left) or with *elav*^{C155}-

1515   GAL4, UAS-Dicer2; UAS-*Syt7* RNAi#2 (right). Neuronal knockdown of SYT7^{RFP} eliminates

1516   presynaptic and most postsynaptic SYT7 staining, suggesting a small fraction of SYT7^{RFP} may

1517   undergo exosome-mediated transfer, as shown for SYT4. The majority of SYT7 protein resides in

1518   the presynaptic terminal. Scale bar = 2 μm.

1519

1520   **Figure 12.** Localization of SYT7 in presynaptic terminals. Immunostaining for the indicated

1521   proteins in each panel was performed at 3rd instar larval muscle 6/7 NMJs. Staining for all panels

1522   except A were done in the SYT7^{RFP} endogenously tagged background using anti-RFP to label the

1523   SYT7 compartment, with the merged image shown on the right. The Pearson correlation

1524   coefficient (r) calculated from the cytofluorogram co-localization plots is shown on the upper right.

1525   All images are from single confocal planes. **(A)** Co-localization of the SV proteins SYT1 (left,

1526   magenta, anti-SYT1 antisera) and nSYB (middle, green, endogenous nSYB^{GFP}) as a positive

1527   control. The remaining panels show boutons co-stained for SYT7^{RFP} (left, magenta, anti-RFP

1528   antisera) and the indicated compartment marker (middle, green): **(B)** Dynamin (anti-DYN

1529   antisera); **(C)** SYX1 (anti-SYX1 antisera); **(D)** Reticulin like-1 (*elav^{C155}*-GAL4; UAS-RTNL1-

1530   GFP); **(E)** lysosomal Na$^+$/H$^+$ exchanger 1 (*elav^{C155}*-GAL4; UAS-NHE-GFP); **(F)** HRP (anti-HRP

1531   antisera); **(G)** BRP (anti-BRP Nc82 antisera); **(H)** nSYB (nSYB^{GFP}); **(I)** Atrial natriuretic peptide

1532   (*elav^{C155}*-GAL4; UAS-ANF-GFP); **(J)** SYT4 (endogenously tagged SYT4^{GFP-2M}); **(K)** RAB7

1533   (anti-RAB7 antisera); and **(L)** Golgin84 (anti-GOLGIN84 antisera). Co-localization plots were

1534   generated with normalized pixel intensity of stacked images of boutons from 3 animals, with the

1535   color representing the frequency of data points as shown in the right scale bar. The vertical line on

1536   the X-axis indicates the threshold used to identify pixels above background for the compartment

1537   stain. The horizontal line on the Y-axis represents the threshold used to identify pixels above

1538   background for SYT7. Scale bar = 1 μm.

1539

1540   **Figure 12 – figure supplement 1.** SYT7 tubules reside in proximity to multiple presynaptic

1541   compartments. Immunostaining for endogenously-tagged SYT7^{RFP} (label) and a lysosomal marker

1542   (NHE), a late endosomal marker (RAB7) and a peri-AZ endosomal protein (RAB11). The merged

1543   image is shown on the right. Scale bar = 0.5 μm.

1544

1545   **Figure 12 – figure supplement 2.** SYT7 localization is not altered by specific RAB protein

1546   manipulations. Dominant-negative (D.N), constitutively-active (C.A.) or wildtype (WT) RAB4,

1547   RAB5, RAB7 and RAB11 were expressed from UAS constructs with *elav^{C155}*-GAL4 in the

1548   SYT7^{RFP} background. Immunostaining with anti-HRP and anti-RFP to label the SYT7

1549   compartment is shown for each manipulation. Several manipulations resulted in extremely reduced

1550   larval viability and could not be analyzed. Scale bar = 1 μm.

1551

1552   **Figure 12 – figure supplement 3.** Localization of compartment-specific markers in *Syt7* mutants.

1553   Immunocytochemistry with anti-HRP (cyan) and anti-GFP (green) in control and *Syt7^{M1}* 3rd instar

1554   larvae to label: **(A)** endogenously-tagged RAB5; **(B)** endogenously-tagged SYT4 (SYT4^{GFP-2M});

1555   **(C)** RAB7 (anti-RAB7 antisera); **(D)** NWK (anti-NWK antisera); and **(E)** endogenously tagged

1556   RAB11. No changes were observed in $Syt7^{M1}$ mutants. Scale bar = 1 μm.

1557

1558   **Figure 12 – figure supplement 4.** Model for SYT7 localization and function. SYT7 functions to

1559   decrease the fusogenicity of SVs in the RRP and slow refilling of the RRP following stimulation.

1560   SYT7 localizes to internal tubular membranes within the peri-AZ network. This location places

1561   SYT7 at a key node to modulate SV re-entry into the RRP in a Ca^{2+}-dependent manner by

1562   interfacing with other membrane compartments and the SV sorting machinery at peri-AZs.

1563

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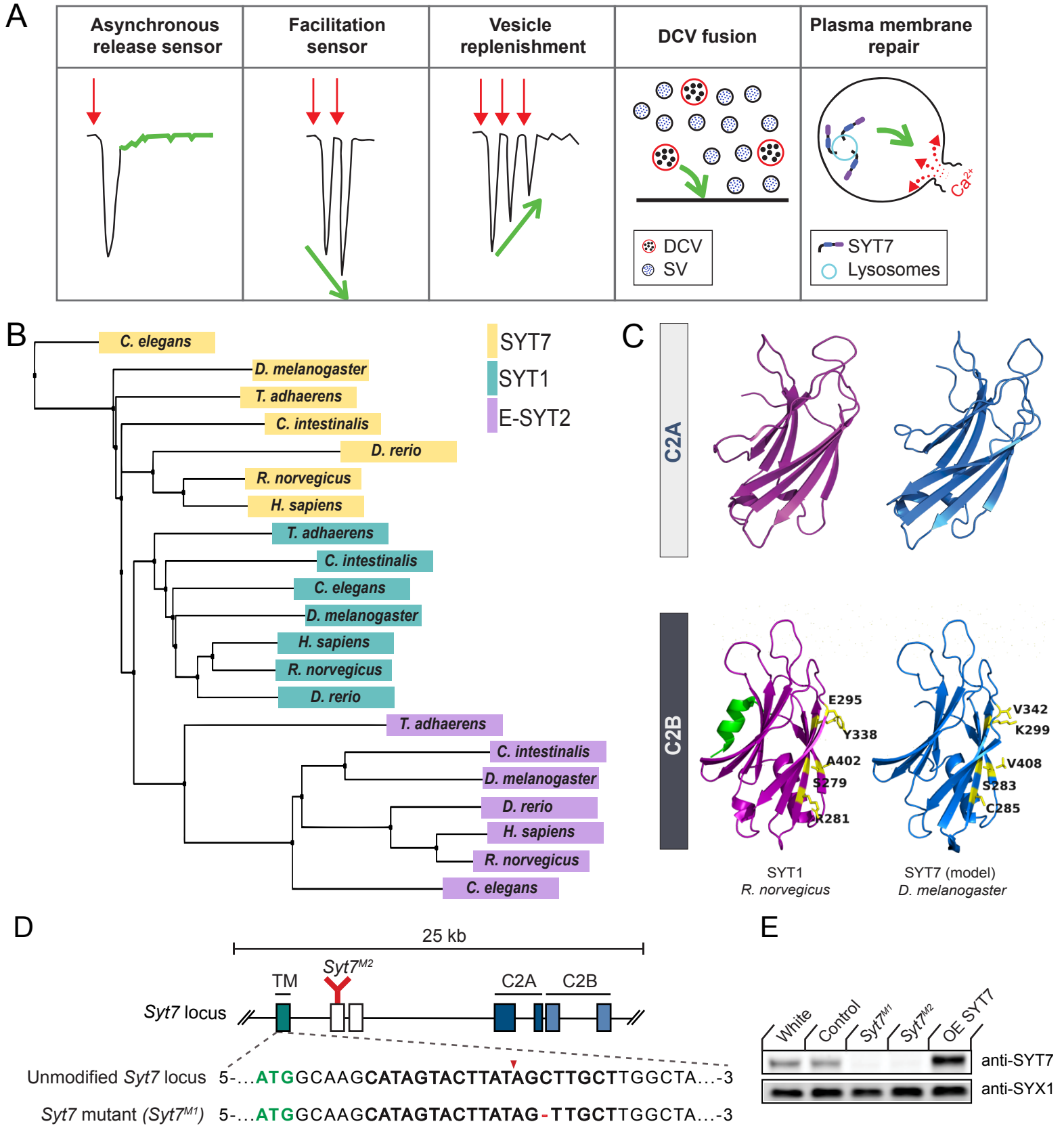
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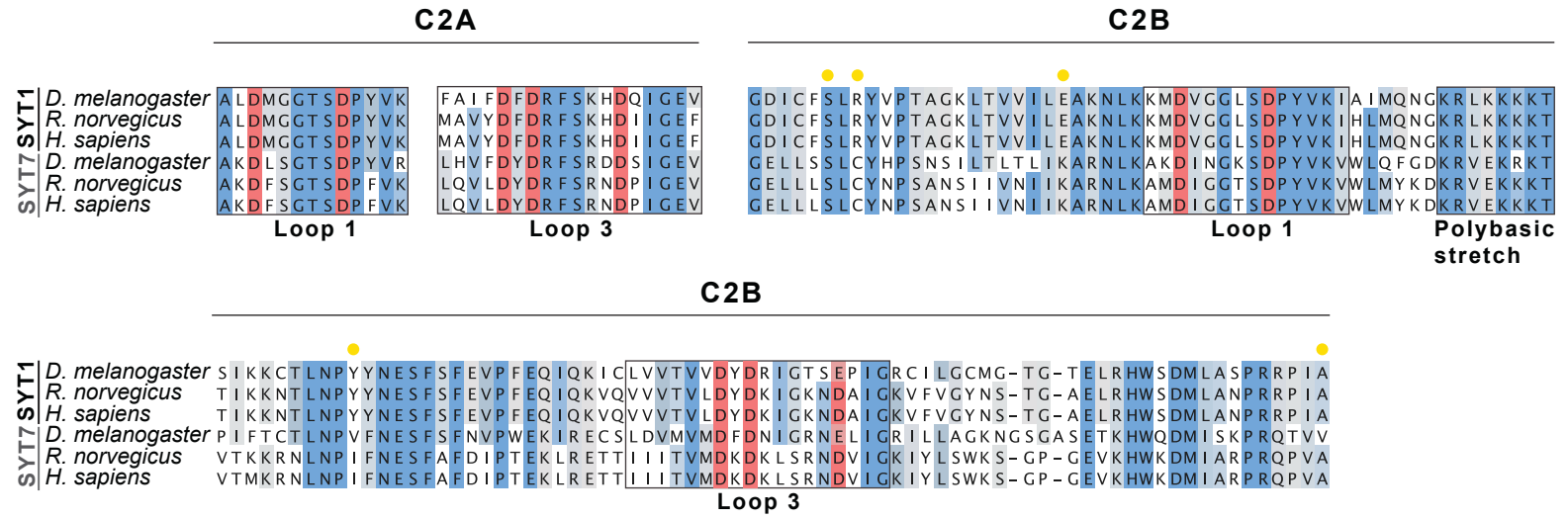
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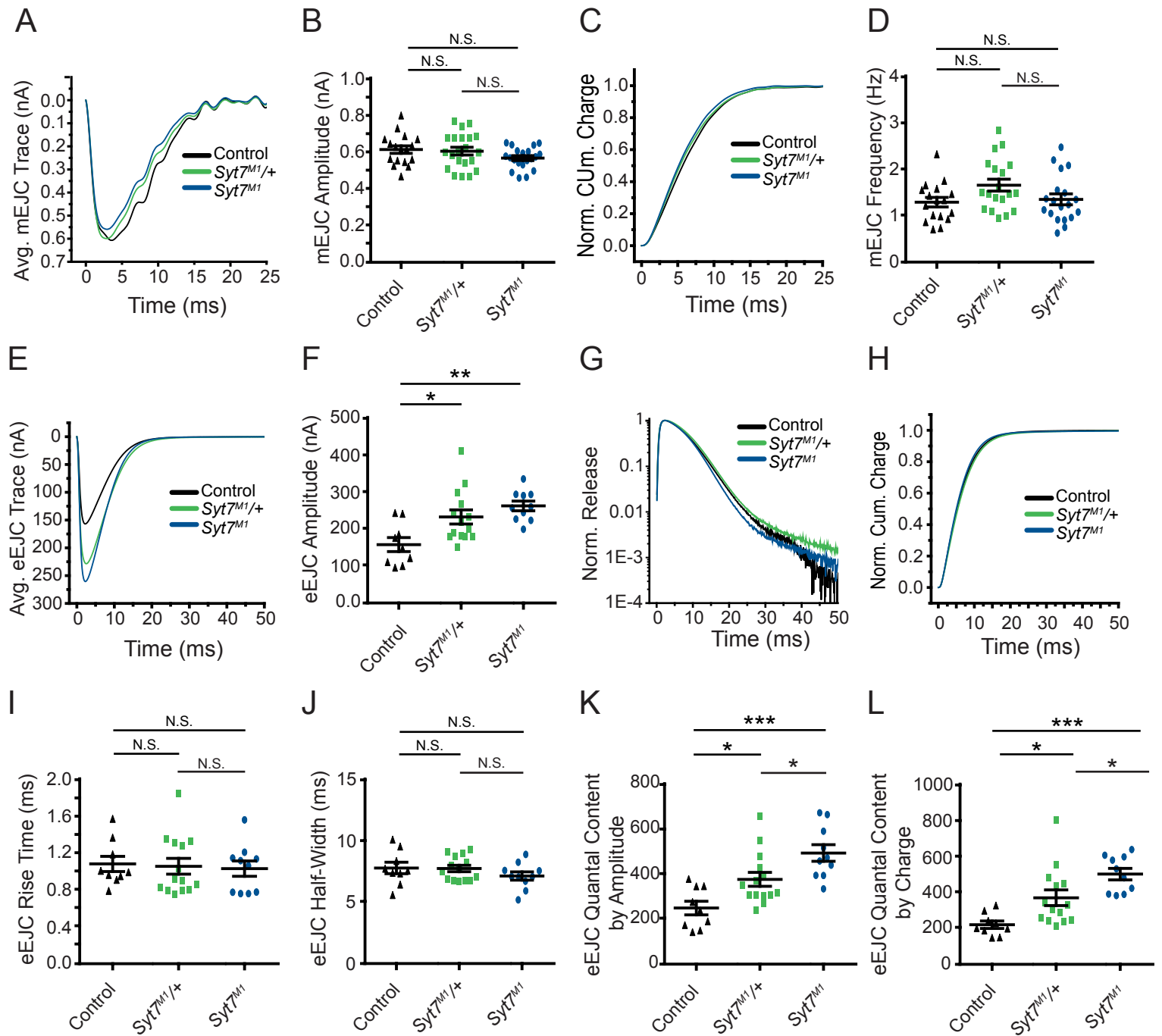
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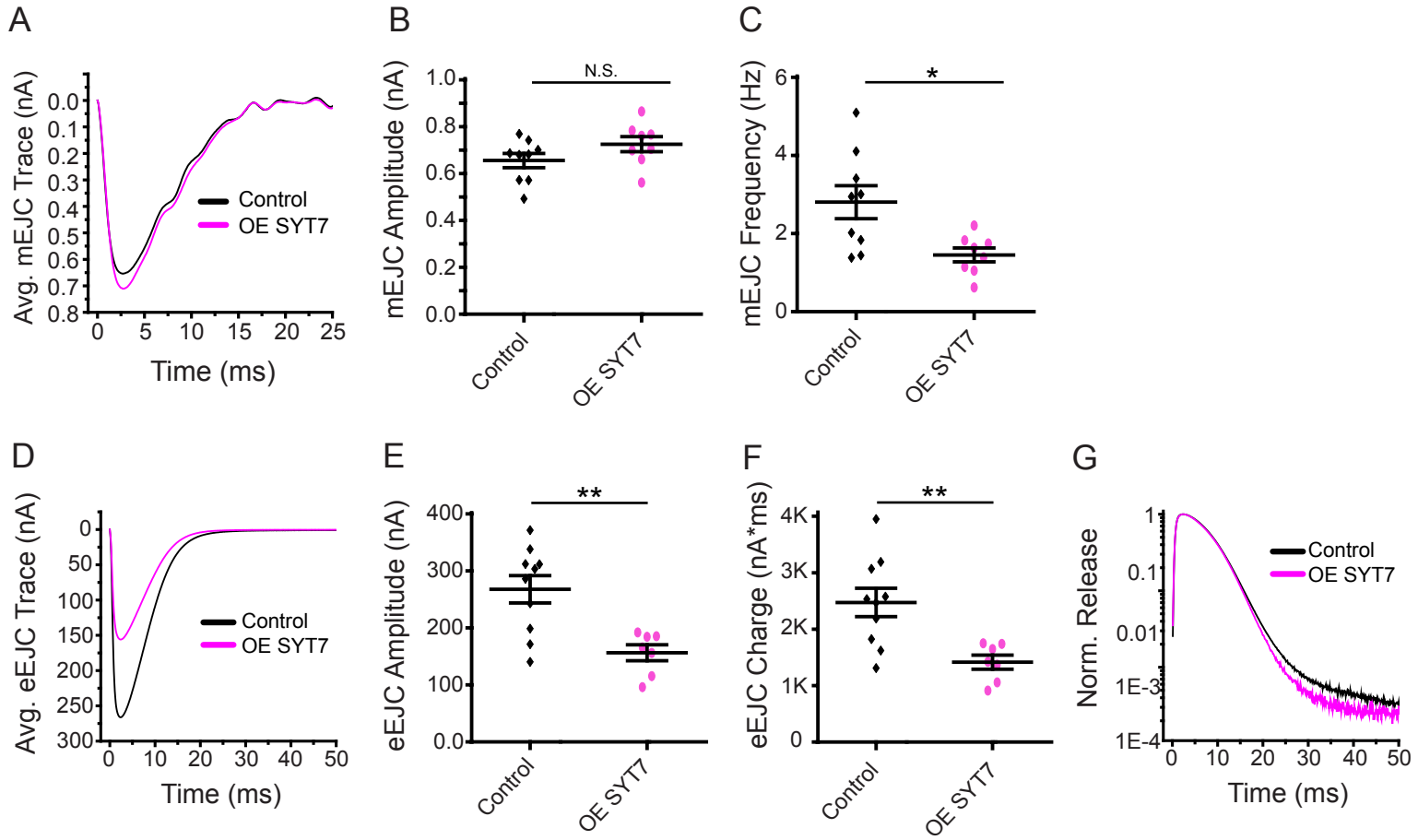
Figure 3 - supplemental figure 1

A

B

Figure 4

Figure 5

Figure 6

Figure 7

Figure 8

Figure 9
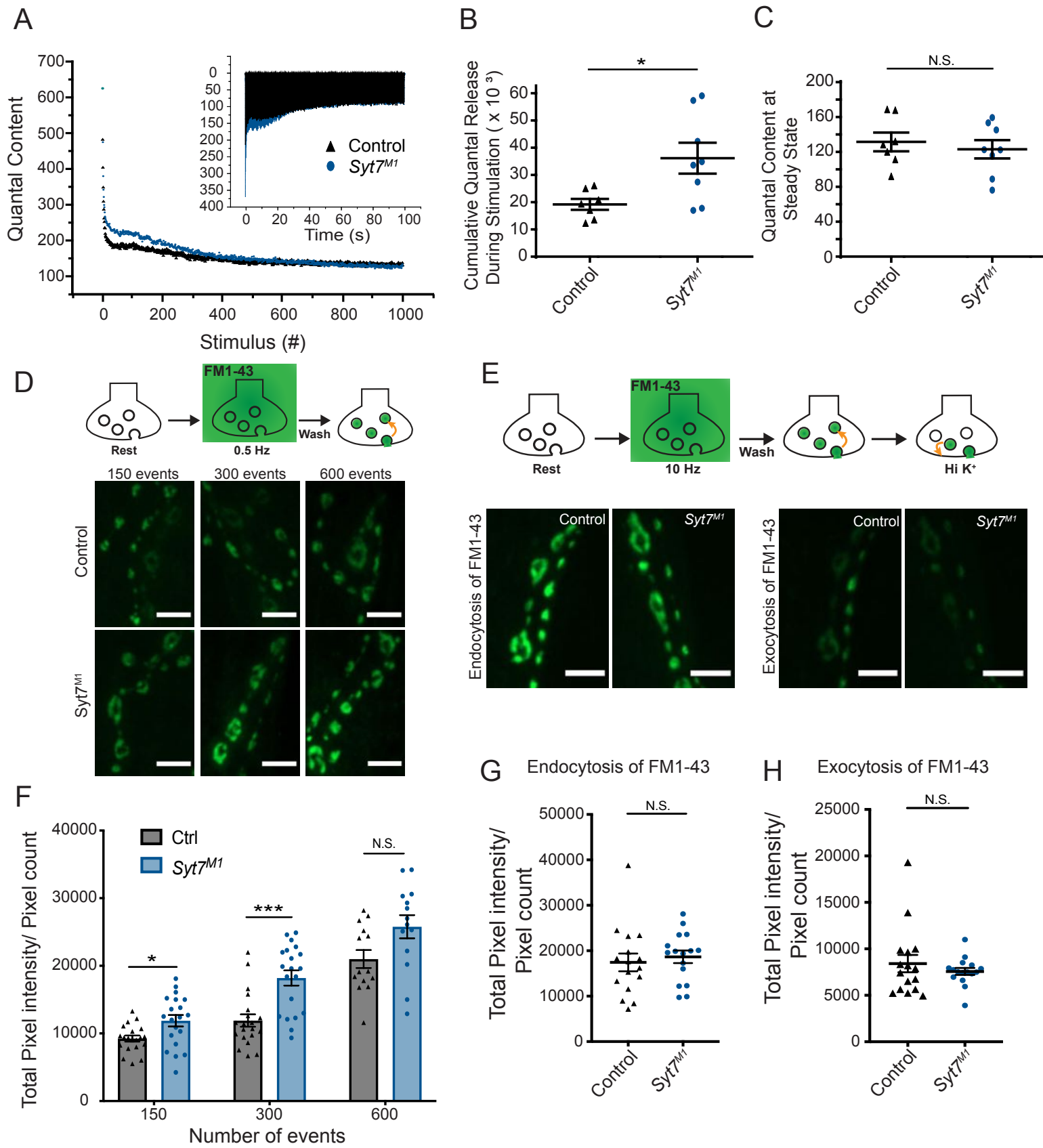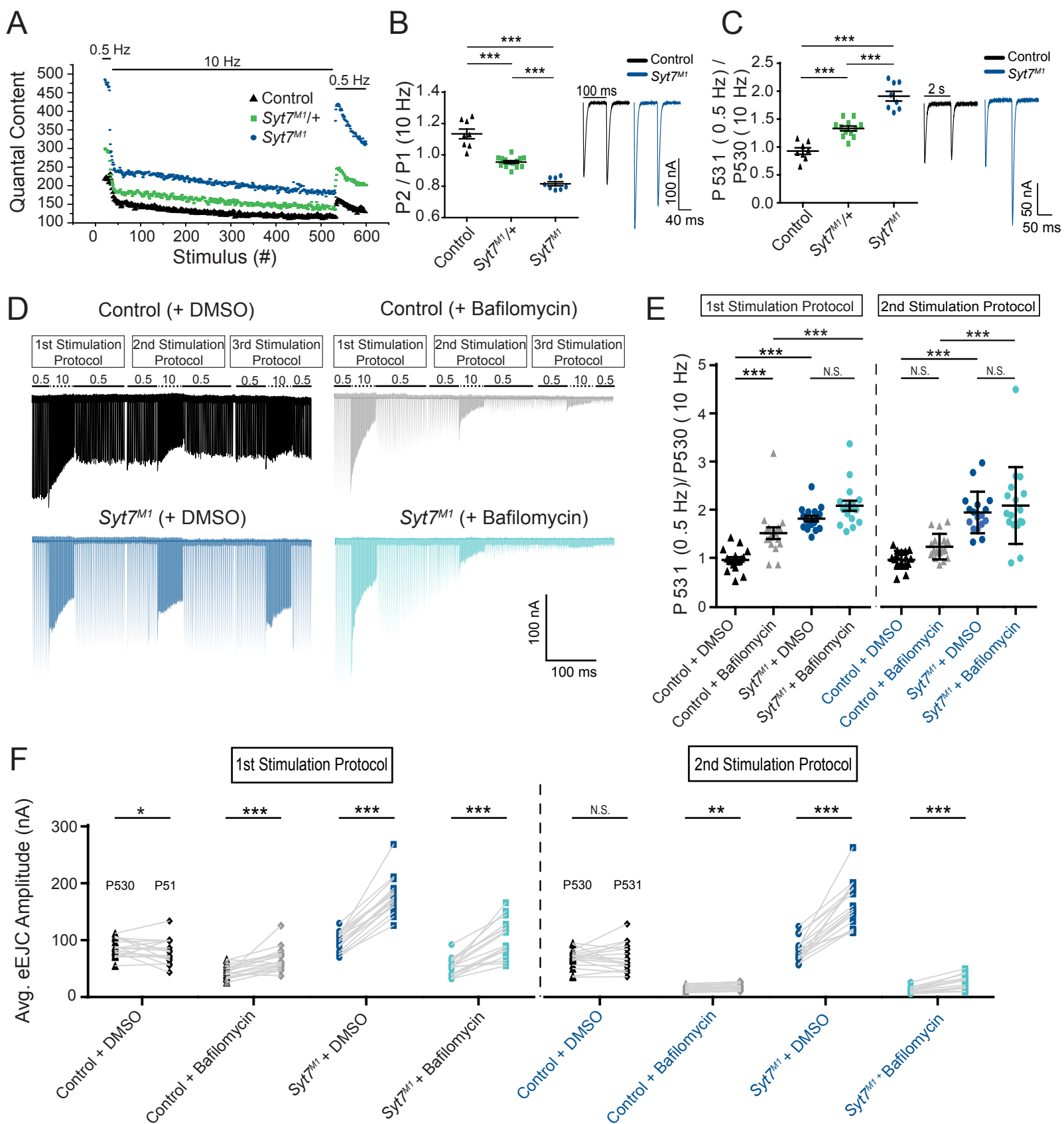
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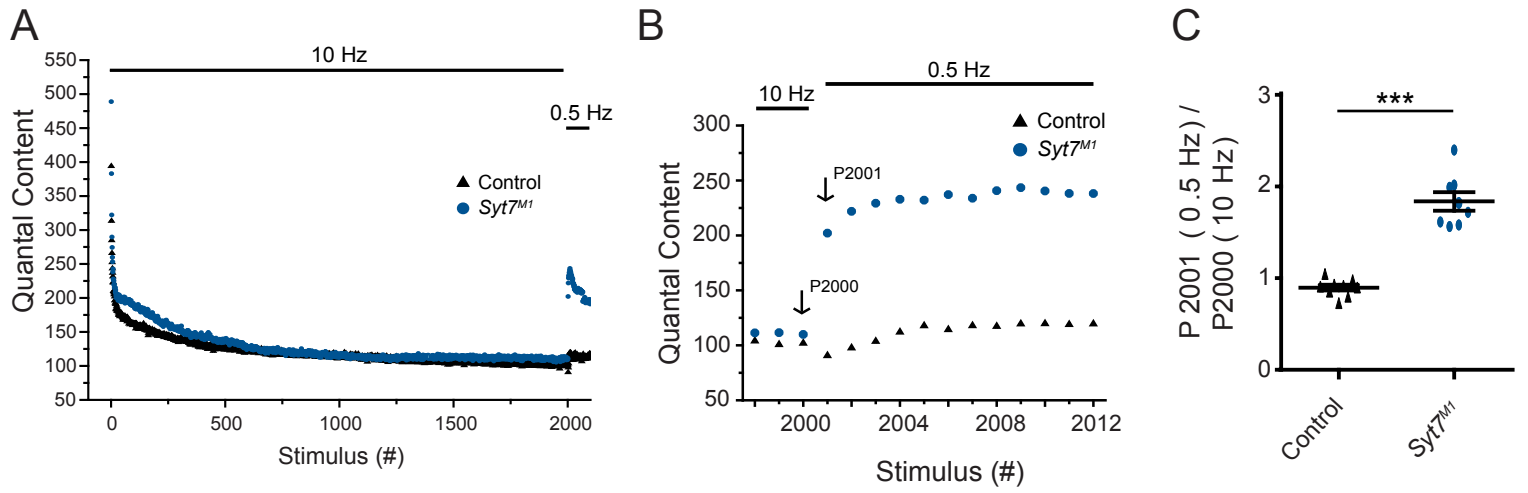
Figure 10 - supplemental figure 2

Figure 11

A

B

anti-RFP				
anti- SYX1				

Genotype

elav^{C155}-GAL4
UAS-Dicer2
UAS-Syt7 RNAi #1
UAS-Syt7 RNAi #2

C

HRP

2 µm

Syt7^{RFP}

D
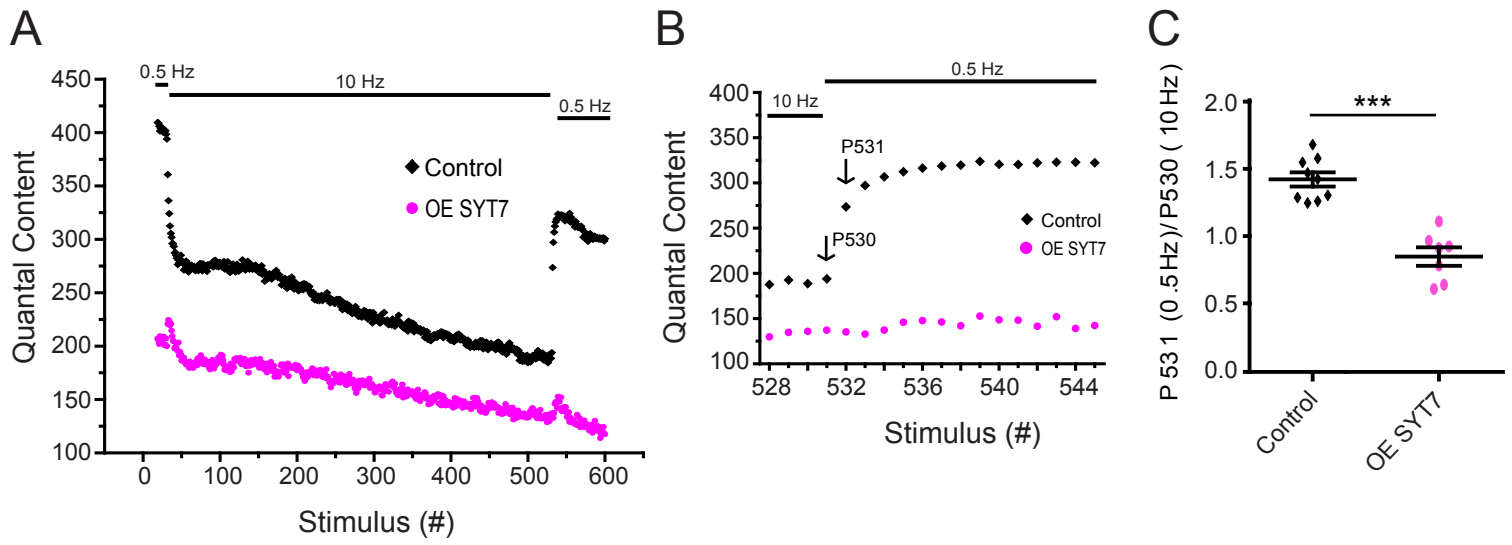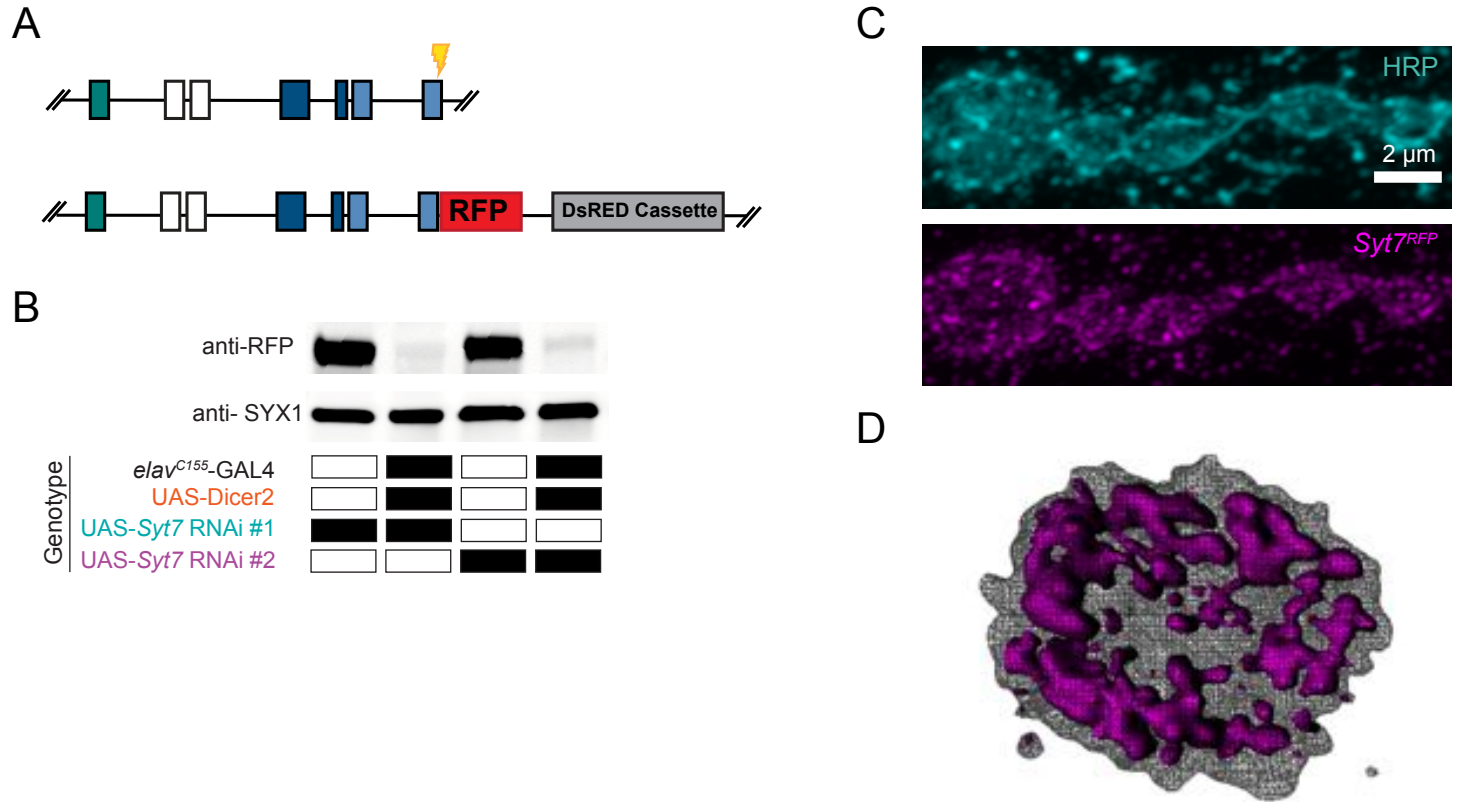
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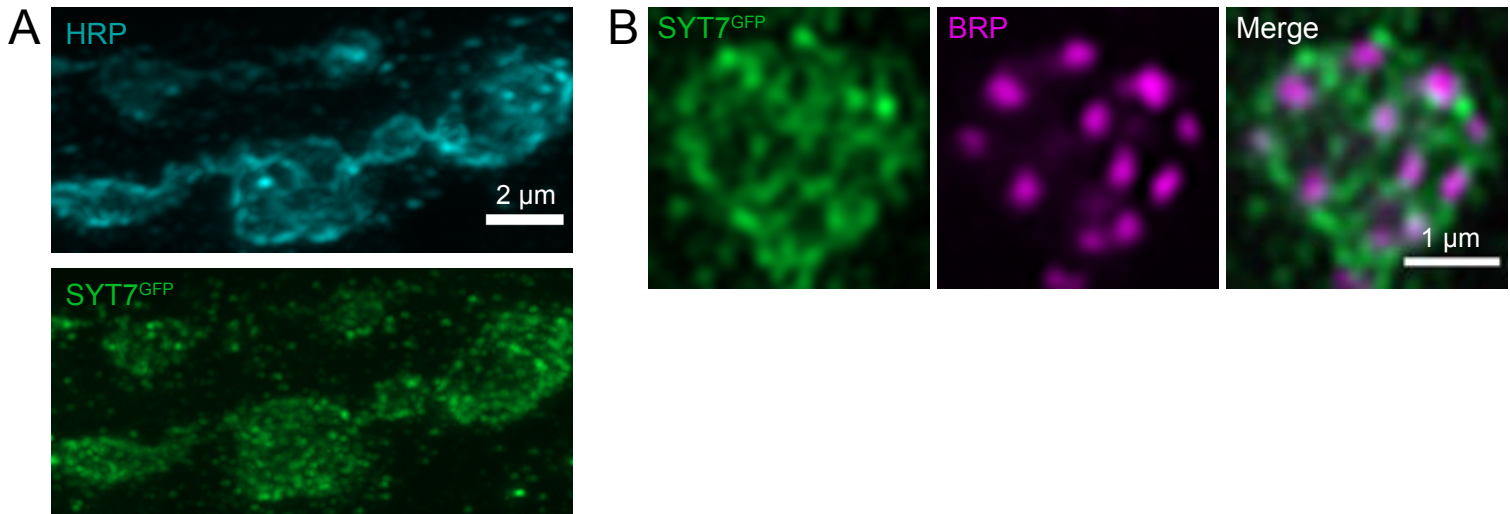
Figure 11 - supplemental figure 2

A

Syt7 RNAi 1

B

UAS-*Syt7* RNAi 2

UAS-*Syt7* RNAi 1
+ SYT7^{RFP}

elav^{C155}-GAL4>UAS-*Syt7* RNAi 1
+ SYT7^{RFP}

UAS-*Syt7* RNAi 2
+ SYT7^{RFP}

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+ SYT7^{RFP}
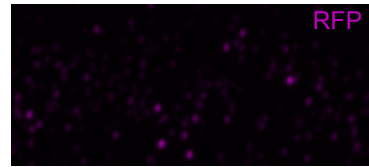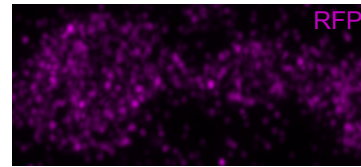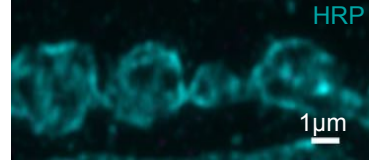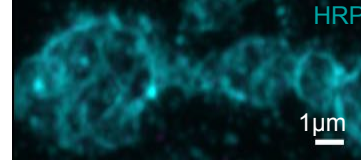
Figure 12

Figure 12 - supplemental figure 1

Figure 12 - supplemental figure 2
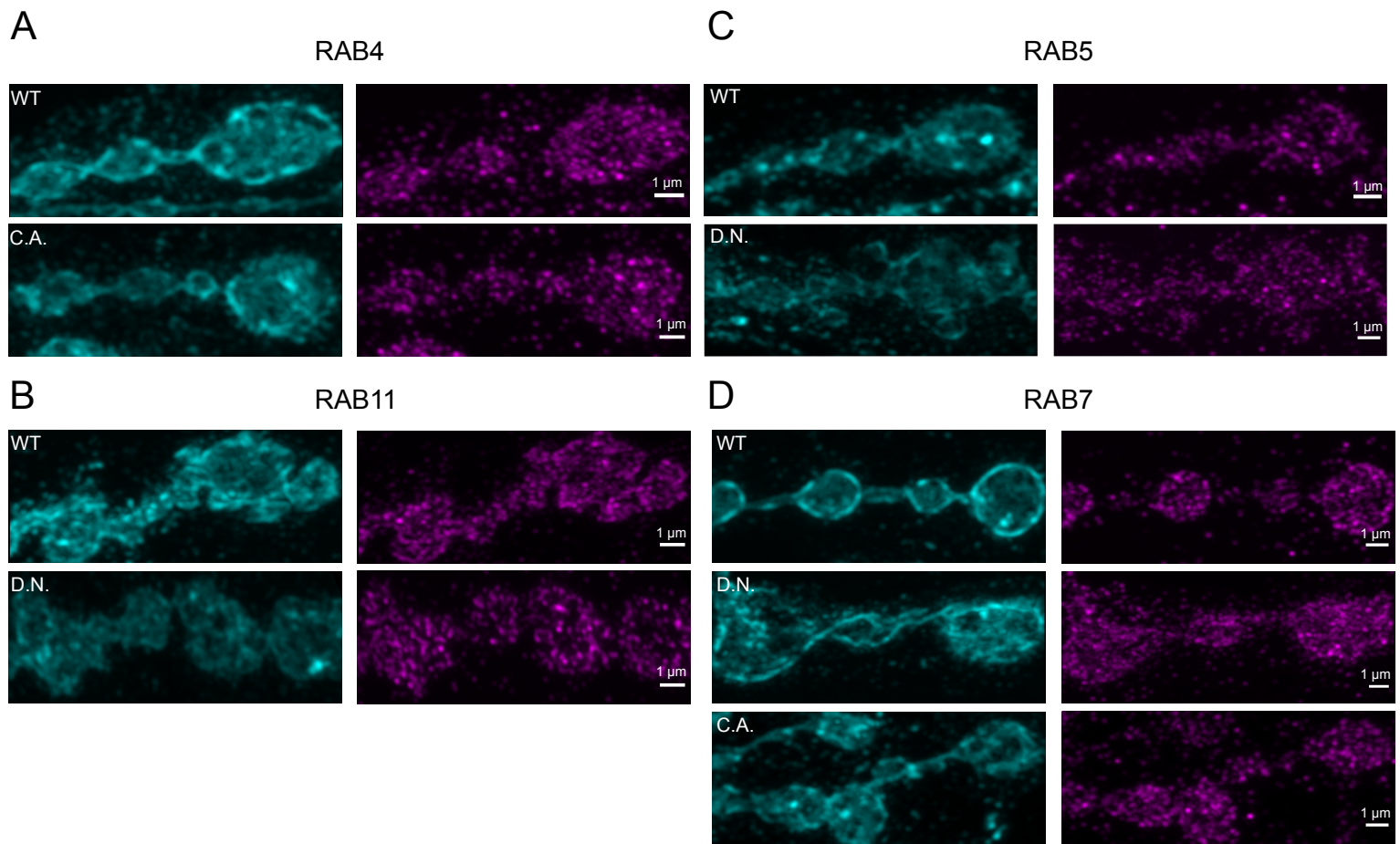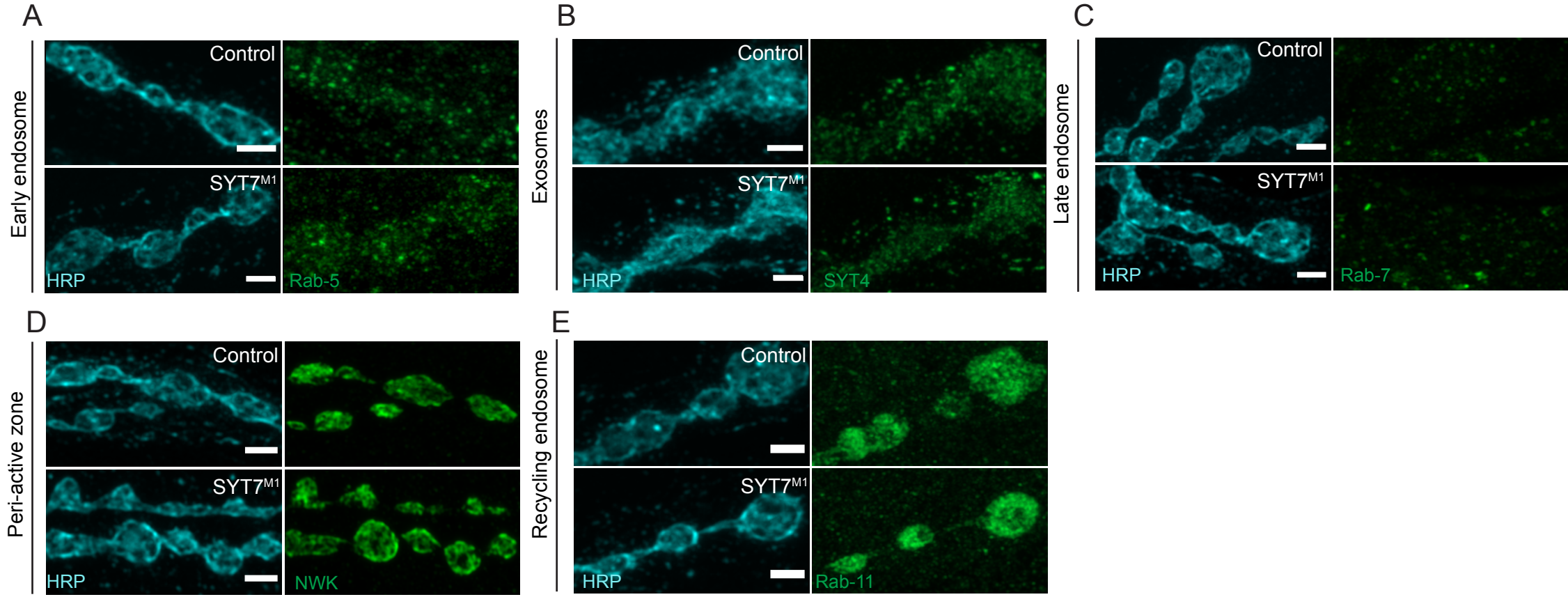
A

RAB4

C

RAB5

B

RAB11

D

RAB7
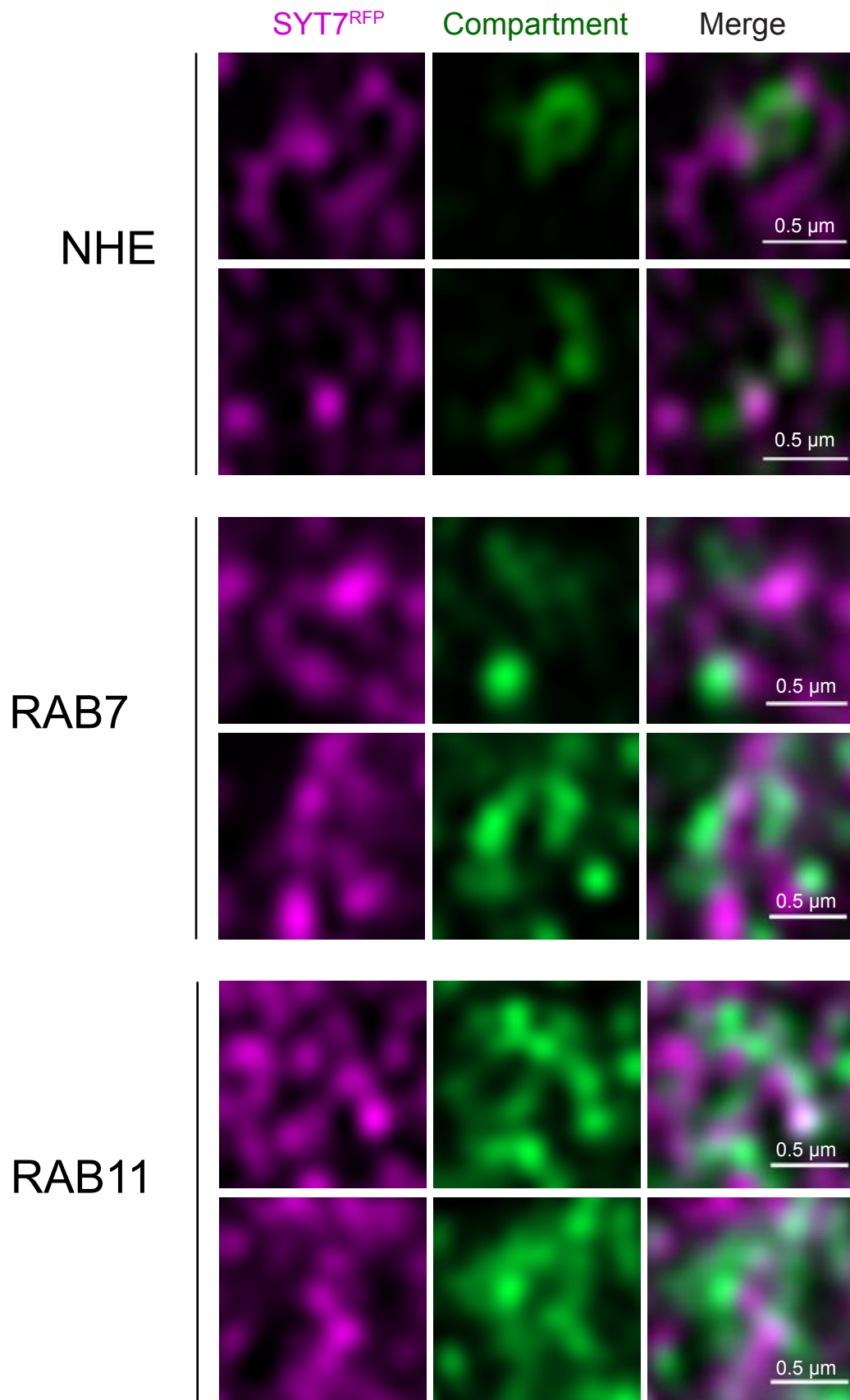
Figure 12 - supplemental figure 3

Figure 12 - supplemental figure 4

Legend:
- **BRP**
- **Calcium channels**
- **RRP vesicles**
- **RP vesicles**
- **SYT7 positive compartment**