SNAP25 disease mutations change the energy landscape for synaptic exocytosis due to aberrant SNARE interactions

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Running title: SNAP25 disease mutations changing priming and energy barriers

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Abstract

SNAP25 is one of three neuronal SNAREs driving synaptic vesicle exocytosis. We studied three mutations in SNAP25 that cause epileptic encephalopathy: V48F, and D166Y in the Synaptotagmin-1 (Syt1) binding interface, and I67N, which destabilizes the SNARE-complex. All three mutations reduced Syt1-dependent vesicle docking to SNARE-carrying liposomes and Ca^{2+}-stimulated membrane fusion *in vitro* and in neurons. The V48F and D166Y mutants (with potency D166Y > V48F) led to reduced Readily Releasable Pool (RRP) size, due to increased spontaneous (mEPSC) release and decreased priming rates. These mutations lowered the energy barrier for fusion and increased the release probability, which are gain-of-function features not found in *Syt1* knockout (KO) neurons; normalized mEPSC release rates were higher (potency D166Y>V48F) than in the *Syt1* KO. These mutations (potency D166Y > V48F) increased spontaneous association to partner SNAREs, resulting in unregulated membrane fusion. In contrast, the I67N mutant decreased mEPSC frequency and evoked EPSC amplitudes due to an increase in the apparent height of the energy barrier for fusion, whereas the RRP size was unaffected. This could be partly compensated by positive charges lowering the energy barrier. Overall, pathogenic mutations in SNAP25 cause complex changes in the energy landscape for priming and fusion.

42 ## Introduction

43 The fusion machinery responsible for chemical synaptic transmission is well known: it consists of
44 the SNARE-complex, a ternary complex formed by the proteins VAMP2, syntaxin-1 and SNAP25
45 (Sutton et al., 1998). This complex is under tight control by upstream partner protein Munc18-1,
46 which acts as a template for SNARE-complex formation, and Munc13s, which assist in the transitions
47 required along the pathway of assembly (Rizo, 2022). SNARE-complexes assemble in a zipper-like
48 manner; partially assembled SNAREpins bind synaptotagmin-1 (Syt1) and complexin within two
49 separate interaction sites: the primary interface formed between a synaptotagmin 1 molecule and
50 syntaxin-1 and SNAP25 (Zhou et al., 2015), and the tripartite interface formed by syntaxin-1 and
51 VAMP2 with another Syt1 molecule and complexin (Zhou et al., 2017). Upon arrival of an action
52 potential, Ca^{2+} binds to the two C2-domains of Syt1, which results in rapid vesicle-plasma membrane
53 fusion and release of neurotransmitter within a fraction of a millisecond (Sudhof, 2013).

54 The strong functional integration and specialization of the neuronal SNARE for speed has rendered
55 the release machinery exquisitely susceptible to insults. *De novo* mutations in SNAREs and
56 associated proteins lead to complex neurological disease, characterized by drug-resistant epilepsy,
57 intellectual disability, movement disorders and often autism; a syndrome which has been denoted
58 "SNAREopathy" (Verhage and Sorensen, 2020). Although rare, these are devastating conditions for
59 the patients and their families. Consequently, there is considerable interest in revealing the
60 molecular/cellular mechanisms for these conditions, which is seen as key to the development of
61 treatment. Disease-causing mutations in the SNARE-machinery fall into distinct categories according
62 to the nature of the defect (Verhage and Sorensen, 2020). These include different forms of haplo-
63 insufficiency, where the mutated protein is either lost altogether or has lost its functionality, and
64 dominant-negative or recessive variants, as well as variants with new or changed protein
65 interactions, referred to as neomorphs.

66 Most SNAREopathy mutations have been described in *STXBP1* (Abramov et al., 2021a; Verhage and
67 Sorensen, 2020; Xian et al., 2022), the gene encoding Munc18-1. These mutations are generally
68 found to cause Munc18-1 hypo-expression, probably due to protein instability (Guiberson et al.,
69 2018; Kovacevic et al., 2018; Martin et al., 2014; Saitsu et al., 2008), and thus the mutations belong
70 in the haploinsufficiency category. Synaptic phenotypes of Munc18-1 hypoexpression have been

71 identified in the *Drosophila* neuromuscular junction (Wu et al., 1998), in cultured mouse neurons
72 (Toonen et al., 2006) and in brain slices of *STXBP1* heterozygous mice (Chen et al., 2020; dos Santos
73 et al., 2023), as well as in human neurons (Patzke et al., 2015), whereas expression of human
74 missense mutations in *STXBP1* null mouse neurons led to synaptic phenotypes for some mutations,
75 but not for others (Kovacevic et al., 2018). Attempts to rescue the disease phenotypes have focused
76 on mechanisms for increasing expression levels, for instance chemical chaperones that might
77 prevent Munc18-1 misfolding and degradation (Abramov et al., 2021b; Guiberson et al., 2018).

78 The situation is different for disease mutations in Syt1 and SNAP25. In these cases, mutation
79 generally does not cause protein instability, but instead changes function in different ways. For Syt1,
80 disease mutations were found to cluster in the C2B-domain around the top loops that coordinate
81 Ca^{2+} (Baker et al., 2018). Expression of these mutants in neurons demonstrated reduced evoked
82 release, in some cases an increase in spontaneous release, and a dominant-negative phenotype
83 when co-expressed with wildtype protein (Bradberry et al., 2020). The molecular mechanism was
84 identified as a decrease in Ca^{2+}-dependent lipid binding (Bradberry et al., 2020). In SNAP25, disease
85 causing mutations are found within the SNARE-domains (Hamdan et al., 2017; Klockner et al., 2021;
86 Rohena et al., 2013; Shen et al., 2014). Alten et al. studied a selection of SNAP25 mutants, and found
87 no changes in expression levels, but changes in both spontaneous and evoked release (Alten et al.,
88 2021). Specifically, mutations in the primary Syt1:SNARE interface (Zhou et al., 2015) caused an
89 increase in spontaneous release rates, and a decrease in evoked release amplitudes. Conversely, C-
90 terminal mutations in the so-called 'layer residues', whose side chains point to the center of the
91 SNARE complex and are involved in SNAREpin zippering (Sutton et al., 1998), led to a decrease in
92 both evoked and spontaneous release (Alten et al., 2021). This is expected because the C-terminal
93 end of the SNARE-complex is required for both types of release (Weber et al., 2010).

94 Alten et al. (2021) described striking phenotypes for most SNAP25 mutations tested, but the
95 molecular reason for these phenotypes remains incompletely understood, and a few findings were
96 surprising. For instance, Alten et al. reported that the V48F and D166Y mutants supported an
97 unchanged Readily Releasable Pool (RRP) of vesicles. The RRP is the pool of vesicles available for
98 immediate release upon invasion of the presynapse by the action potential, and it is often assessed
99 by applying a hyperosmotic solution (often 0.5 M sucrose), which causes primed vesicles to fuse
100 (Rosenmund and Stevens, 1996). Previously, we concluded that the primary Syt1:SNARE-interface

101 is involved in vesicle priming (Schupp et al., 2016), which agrees with the suggestion that Syt1 binds
102 to the SNAREs before Ca^{2+} arrival (Zhou et al., 2015). Conversely, Alten et al. reported a smaller RRP
103 for the I67N mutant, but the I67 residue is present in the internal of layer +4, which we expected to
104 affect final SNARE complex zippering causing membrane fusion rather than priming (Sorensen et al.,
105 2006; Weber et al., 2010). These discrepancies might be explained by the fact that Alten et al. used
106 mixed cultures and mainly studied GABAergic transmission. Glutamatergic transmission is hard to
107 investigate in mixed cultures due to reverberating activity, but it can be studied using autaptic
108 neurons, which was our approach (Schupp et al., 2016; Weber et al., 2010). Thus, there is a need to
109 further study these disease mutations in glutamatergic neurons. Another open question is whether
110 the phenotype of the mutants in the primary interface (V48F, D166Y) are explainable solely by the
111 loss of Syt1 binding, or whether other features of these mutations add to, or detract from, the
112 phenotype.

113 Here, we reexamined three SNAP25 disease mutations using glutamatergic autaptic neurons: I67N,
114 V48F and D166Y. We confirmed the increase in spontaneous release rate and decrease in evoked
115 release by V48F and D166Y reported previously (Alten et al., 2021). Through *in vitro* analysis we find
116 that both mutations lower Syt1 association to SNARE-protein liposomes. Additional experiments
117 both *in vitro* and in cells demonstrate that V48F and especially D166Y represent partial gain-of-
118 function mutations that increase association to partner SNAREs and lower the energy barrier for
119 fusion, bypassing Syt1-dependent control. Thus, these mutants do not phenocopy the loss of Syt1,
120 but combine loss of Syt1 binding with a gain-of-function phenotype. At the same time, the mutants
121 act as loss-of-function in upstream reactions, through effects on priming. The I67N is a classical
122 dominant-negative mutation, which increases the energy barrier for fusion, but does not change
123 the size of the RRP if probed by a sufficiently high concentration of sucrose, nor does the I67N
124 change the electrostatics of triggering itself. Thus, for V48F and D166Y, loss-of-function and gain-of-
125 function features combine to change the energy landscape for vesicle priming and fusion in a
126 complex way. These findings have consequences for our understanding of the simultaneous role of
127 the primary SNARE:Syt1 interface in vesicle priming and release clamping. It further demonstrates
128 the challenge faced by finding mechanism-based treatments of these disorders in the presence of
129 multiple effects caused by single point mutations.

130 **Results**

131 We first investigated two SNAP25 disease-causing mutations within the primary Syt1:SNARE-
132 interface (V48F, D166Y; Fig. 1A-C). The mutations occurred *de novo* and were identified in single
133 heterozygous subjects. The V48F mutant was identified in a 15-year-old female with
134 encephalopathy, intellectual disability and generalized epilepsy with seizures started at 5 months of
135 age; MRI was normal except for delayed myelination (Rohena et al., 2013). The D166Y mutants was
136 found in a 23-year-old male with global developmental delay, nocturnal tonic-clonic seizures, and
137 moderate intellectual disability; the MRI showed mild diffuse cortical atrophy (Hamdan et al., 2017).
138 We aimed to achieve a detailed understanding of the reason for synaptic dysfunction.

139 *The V48F and D166Y mutants disinhibit spontaneous release and desynchronize evoked release*

140 We constructed lentiviral vectors, which expressed wildtype (WT) or mutant SNAP25b fused N-
141 terminally to EGFP as an expression marker (Delgado-Martinez et al., 2007). In the absence of
142 SNAP25, neuronal viability is compromised (Delgado-Martinez et al., 2007; Peng et al., 2013; Santos
143 et al., 2017; Weber et al., 2010) with low-density autaptic glutamatergic neurons dying within a few
144 days, whereas *Snap25* KO neurons growing at higher densities, or in the intact brain, can survive
145 longer, but also eventually degenerate (Bronk et al., 2007; Hoerder-Suabedissen et al., 2019). We
146 therefore first examined the morphology and survival of autaptic hippocampal neurons from
147 *Snap25* KO after expressing mutated or WT EGFP-SNAP25b (henceforth denoted 'WT' in rescue
148 experiments; not to be confused with '*Syt1* WT', which refers to wildtype littermates of *Syt1* KO
149 mice).

150 As expected, *Snap25* KO autaptic neurons did not survive in the absence of expression of
151 exogeneous SNAP25 (Fig. 2G), whereas expression of WT EGFP-SNAP25b restored survival (Delgado-
152 Martinez et al., 2007; Ruiter et al., 2019; Weber et al., 2010). Both mutations (V48F and D166Y)
153 caused rescue of survival; however, the number of neurons per islet was lower than in WT-
154 expressing neurons prepared in parallel. This difference was statistically significant for D166Y, but
155 not for V48F (Fig. 2G). Since patients harbor one mutated and one WT allele, we coexpressed WT
156 EGFP-SNAP25b with mutated EGFP-SNAP25b, by combining infection with separate viruses
157 encoding WT and mutant protein in a 1:1 ratio, as done previously by others for Syt1 (Bradberry et
158 al., 2020). As a prerequisite for this, we demonstrated by Western blot analysis that both mutant

159 viruses expressed similar amounts of protein as WT viruses (Fig. 2A-C). In co-expressing neurons, we
160 added half the volume of WT and mutant virus as compared to the WT condition, thus keeping the
161 total amount of virus constant. We preferred to combine two single-cistronic viruses, rather than to
162 construct bicistronic viruses, since the larger insert would be expected to lower the titer of viruses,
163 which might compromise survival rescue of *Snap25* KO neurons. Using co-expression of WT and
164 mutant SNAP25b, neuronal survival was mildly and significantly reduced in the V48F + WT condition
165 from WT (Fig. 2G). Overall, mutations seemed to mildly reduce survivability of neurons. Staining of
166 neurons against VGlut1 (a marker for glutamatergic synapses) and MAP2 (a dendritic marker)
167 revealed no significant difference in the dendritic length or the number of synapses in neurons
168 expressing V48F, and D166Y alone (Fig. 2D-F).

169 Patch-clamp was performed on days 10-14 on autaptic neurons expressing either the WT EGFP-
170 SNAP25b, the mutants, or both the mutant and WT in a 1:1 ratio. Spontaneous miniature events
171 were recorded at a holding voltage of -70 mV. Both primary interface mutations (V48F, D166Y)
172 strongly increased the frequency of mEPSCs (Fig. 3A-B, D-E); the mEPSC amplitude was significantly
173 increased in the V48F, and insignificantly (p=0.14) increased for the D166Y (Fig. 3C, F). The mEPSC
174 frequency was at least as high as in *Syt1* KO neurons measured in separate experiments (Fig. 3G-I).
175 V48F and D166Y co-expressed with WT resulted in mEPSC frequencies close to the arithmetic mean
176 between frequencies in WT and the mutant alone (Fig. 3B, E), indicating that the mutations are
177 incompletely dominant, or co-dominant.

178 Brief depolarization elicits an unclamped action potential in the axon, which makes it possible to
179 study evoked release, which is essentially absent in *Snap25* KO neurons (Bronk et al., 2007; Delgado-
180 Martinez et al., 2007). The V48F and D166Y mutants both supported evoked release, but the evoked
181 EPSC (eEPSC) amplitude was significantly reduced in the mutant condition (Fig. 4A-B, E-F), whereas
182 when co-expressed with the WT, only the D166Y mutant had a significantly reduced eEPSC (Fig.
183 4B,F). Integration of individual eEPSCs allowed determination of the total charge and the
184 assessment of synchronous and asynchronous release components (Fig. 4 – Figure supplement 1).
185 The eEPSC charge was significantly reduced in the D166Y, but not in the V48F (Fig. 4C,G), whereas
186 in both cases the kinetics was significantly shifted in the direction of more asynchronous release,
187 and the fast time constant was prolonged (Fig. 4D,H; Fig. 4 - Figure supplement 2). We compared
188 the data obtained from V48F and D166Y SNAP25 to *Syt1* KO neurons recorded in separate

experiments. *Syt1* WT neurons in this set of experiments had larger eEPSC amplitudes, and a larger charge than WT-rescued *Snap25* KO neurons (Fig. 4I-K), which might be caused by differences between cell cultures, or animal lines (*SNAP25* vs *Syt1* lines). Note that our experiments using WT, mutant and WT + mutant SNAP25 were always carried out in neurons prepared and recorded in parallel from the same *SNAP25* KO embryos (Materials and Methods). In the *Syt1* KO, the kinetic change was similar to the V48F and D166Y (Fig. 4L), but the total charge was also strongly reduced (Fig. 4K), unlike the two mutations that displayed at most a mild reduction (Fig. 4C,G).

Overall, D166Y and V48F caused a strong disinhibition of spontaneous release and a desynchronization of evoked release, as demonstrated before (Alten et al., 2021), and consistent with previous mutational studies of the primary interface, both in SNAP25 (Schupp et al., 2016) and Syt1 (Zhou et al., 2015). Accordingly, these phenotypes are similar to *Syt1* KO, but the effect of V48F and D166Y on total evoked charge was milder than in the *Syt1* KO. The preserved overall charge of evoked release in V48F and mild reduction in D166Y might point to a compensatory gain-of-function aspect to these mutations, in addition to the impaired Syt1 interaction.

V48F and D166Y mutations lower the energy barrier for release and reduce the readily releasable pool size

The role of Syt1 and Syt1:SNARE interactions in vesicle priming has been controversially discussed. The RRP is often assessed by applying a pulse of hypertonic solution to the neurons, usually 0.5 M sucrose (Rosenmund and Stevens, 1996; Schotten et al., 2015). However, in some experiments this did not lead to a change in RRP size in the absence of Syt1 (Bacaj et al., 2015; Xu et al., 2009), whereas experiments both from our laboratories and others showed a decrease in RRP in the absence of Syt1 (Bouazza-Arostegui et al., 2022; Chang et al., 2018; Courtney et al., 2019; Huson et al., 2020; Liu et al., 2009; Ruiter et al., 2019). The reason for this discrepancy is likely partly technical and has to do with how fast sucrose can be applied to the dendritic tree (see also Discussion). Using neurons growing on small autaptic islands makes it possible to apply sucrose to the entire dendritic tree (which is confined to the 30-50 µm island) within tens of milliseconds, which is fast enough to dissect the RRP with a short (few s) sucrose application. We set out to understand whether the V48F and D166Y mutants changed the size of the RRP.

217 Application of 0.5M sucrose to neurons with the V48F or D166Y mutation resulted in estimates of
218 the RRP (denoted $RRP_{0.5}$) that were significantly reduced compared to the WT condition (Fig. 5
219 A,C,F,H). Also the co-expressed condition displayed significantly reduced $RRP_{0.5}$. Application of two
220 different sucrose concentrations is used to probe the size of the energy barrier for release (Basu et
221 al., 2007; Schotten et al., 2015), because the use of a lower sucrose concentration (typically 0.25 M)
222 will only release a fraction of the RRP, and this fraction depends sensitively on the energy barrier
223 for release; the size of the RRP at 0.5M sucrose ($RRP_{0.5}$) is used for normalization. Application of 0.25
224 M sucrose to V48F and D166Y expressing neurons strikingly led to unchanged pool release ($RRP_{0.25}$)
225 (Fig. 5B,G). Consequently, the ratio of pools ($RRP_{0.25}/RRP_{0.5}$) was significantly increased for both the
226 V48F and the D166Y mutations (Fig. 5D,I), indicating that these two mutations decrease the
227 apparent energy barrier for fusion (Schotten et al., 2015). The co-expressed conditions were in-
228 between WT and mutant and did not reach statistical significance. Consistent with these results,
229 both mutations on average increased the release probability, calculated as the ratio between the
230 eEPSC charge and the $RRP_{0.5}$ charge measured in the same cell. The increase was statistically
231 significant for the D166Y mutation (Fig. 5J), but not for V48F (Fig. 5E). This is different from the
232 situation in the *Syt1* KO, where the $RRP_{0.25}/RRP_{0.5}$ and the release probability were both significantly
233 decreased (Fig. 5K-O), although in this data set the reduction in $RRP_{0.5}$ size by removing Syt1 did not
234 reach statistical significance (P=0.0548, unpaired t-test). Thus, the increased mEPSC release rate
235 from the *Syt1* KO does not correlate with a reduced energy barrier as assayed by sucrose, which was
236 reported before (Huson et al., 2020), and the V48F and D166Y have specific gain-of-function
237 features that lower the energy barrier for vesicle fusion.

238 To investigate the reasons for the change in RRP-size by D166Y and V48F, we considered a one-pool
239 model for the RRP (Fig. 6A), where the RRP is filled by priming (k_1) from an upstream pool and
240 depleted by de-priming (k_{-1}) or spontaneous fusion (k_f). Dividing the miniature release rates (r_{mini})
241 with the size of the RRP yields the spontaneous fusion rate k_f. The current plateau during 0.5 M
242 sucrose application (Fig. 6B) essentially reports on the priming rate (k_1), providing that the fusion
243 rate is sufficiently increased by sucrose (Materials and Methods). However, high sucrose
244 concentrations can change the baseline current level, which will cause an error in estimation of k_1.
245 We therefore corrected the plateau level using a plot of the variance versus mean during the sucrose
246 application (Fig. 6C); this plot is linear for the type of noise generated by synaptic transmission (shot

247 noise). Back-extrapolation of a regression line allows a determination of the baseline current level
248 in the presence of sucrose (Fig. 6B-C; see Materials and Methods). Combining RRP size with the
249 estimates of k_1 and k_f allows determining the depriming rate, k_{-1}.

250 These calculations showed that k_f, the spontaneous fusion rate was strongly increased in the V48F
251 and D166Y, and this increase was much larger for the mutations than for the *Syt1* KO (Fig. 6D)
252 fulfilling D166Y>V48F>*Syt1* KO. Further analysis showed that in both mutations, the forward priming
253 rate, k_1, and the depriming rate, k_{-1}, were both decreased – the latter effect was only significant for
254 D166Y (Table 1). Summing up the effects of changes in the three parameters on the RRP-size for the
255 V48F, D166Y and *Syt1* KO (Fig. 6E), we can conclude that for the V48F and even more for the D166Y,
256 spontaneous release contributes to the reduction in RRP size, whereas this effect is minimal in the
257 *Syt1* KO. However, the major reason for the smaller RRP size is a reduction in priming rate, k_1, which
258 is partly counteracted by the decrease in k_{-1} (which would increase RRP size). Overall, changes in
259 priming, depriming and spontaneous fusion rates combine to change RRP size.

260 Repetitive stimulation to determine the RRPs often results in lower estimates than sucrose
261 application, because action potentials draw on a sub-pool of the RRP (Moulder and Mennerick,
262 2005), whereas sucrose releases the entire RRP (Rosenmund and Stevens, 1996). To determine the
263 RRP sub-pool that evoked release draws on (denoted RRP_{ev}) we applied repetitive stimulation (50
264 APs @ 40 Hz) and used back-extrapolation to determine the RRP (Neher, 2015). Performing this in
265 our standard 2 mM Ca^{2+}-containing extracellular solution resulted in overall smaller estimates for
266 RRP_{ev} for V48F and D166Y compared to WT (Fig. 7 - Figure supplement 1); however, the differences
267 were not statistically significant. The back-extrapolation method works best with high release
268 probabilities (Neher, 2015); therefore, we repeated these experiments in the presence of 4 mM
269 extracellular Ca^{2+}. Under these conditions, the RRP_{ev} was significantly reduced for both the V48F
270 and D166Y mutation (Fig. 7C,G; Fig. 7 - Figure supplement 2). Strikingly, the release probabilities
271 (calculated as the charge of the first eEPSC of the train divided by the RRP_{ev}) were decreased for
272 both mutations (Fig. 7D,H), which correlated with an increased paired-pulse ratio (Fig. 7A,E, *inserts)*
273 (Zucker and Regehr, 2002). Thus, although the release probability calculated by normalizing evoked
274 charge to the sucrose-determined RRP ($RRP_{0.5}$) was increased (see above), the release probability
275 when normalizing to RRP_{ev} was decreased. This points to a difference in the organization of the
276 RRP_{ev} and $RRP_{0.5}$ (see Discussion). The forward priming rate is determined as the slope of the linear

277 fit used for the back extrapolation; this parameter was reduced in both mutations (Fig. 7B, F; Fig. 7
278 - Figure supplement 2). Thus, the difference in priming rate extends to the RRP_{ev}.

279 Overall, these data demonstrate a rather complex phenotype of the D166Y and V48F mutations,
280 which combine a lowering of the energy barrier – a gain-of-function feature – with a loss of vesicle
281 priming – a loss-of-function feature. The D166Y and V48F phenotypes can be summarized as 1)
282 desynchronized eEPSCs with at most mildly reduced total charge, 2) lowered energy barrier for
283 fusion, 3) increased release probability when normalized to the sucrose pools, 4) decreased RRP-
284 size due to unclamped spontaneous release and lowered forward priming rates, 5) short-term
285 facilitation. These phenotypes are distinctive from the Syt1 KO, which does not have a preserved
286 charge of the eEPSC, or lowered energy barrier when probed by sucrose, or an increased release
287 probability. Thus, the D166Y and V48F cannot be understood solely in terms of a lack of Syt1
288 coupling; instead, gain-of-function features are present in the mutants which are absent upon
289 deletion of Syt1.

290 *V48F and D166Y mutants show increased partner SNARE interactions and cause unregulated fusion*
291 *in vitro*

292 We next tried to identify the biochemical properties of V48F and D166Y, which could support a gain-
293 of-function phenotype during exocytosis. These data are displayed in Fig. 8-9 - data on the I67N
294 mutant were obtained in parallel and will be presented later. To test to which degree the mutants
295 may change the stability of SNARE complexes, full-length t-SNAREs (syntaxin-1 and SNAP25) were
296 incubated with the cytosolic domain of VAMP2 (VAMP2cd) overnight. Cis-SNARE complex stability
297 was tested in the presence of SDS at the indicated temperatures (Fig. 8A), and the release of
298 syntaxin-1 as a single protein band was used as a measure of the complex dissociation. The wildtype
299 (WT), V48F and D166Y v-/t-SNARE complexes showed a similar stability with half-maximal
300 dissociation occurring at approximately 71°C.

301 Next, we asked whether the V48F and D166Y mutants would change interaction with Syt1. To this
302 end, Atto647 labelled Giant Unilamellar Vesicles (GUVs) filled with isosmotic sucrose and containing
303 preassembled t-SNARE complexes were preincubated with Atto488/Atto550 labeled Small
304 Unilamellar Vesicles (SUVs) containing Syt1 as well as VAMP2 for 10 minutes on ice, followed by
305 centrifugation to reisolate GUVs with attached SUVs. Fusion was blocked by performing the assay

306 on ice. Attachment of SUVs was determined by measuring the Atto550 fluorescence of SUVs. In the
307 absence of PI(4,5)P_2, vesicle attachment occurs by Syt1:SNARE interactions (Kim et al., 2012;
308 Parisotto et al., 2012), probably involving the primary interface (Zhou et al., 2015). In the cell,
309 PI(4,5)P_2-binding by Syt1 appears to happen first (Honigmann et al., 2013), whereas subsequent
310 Syt1:SNARE-binding leads to a tightly docked state (Chen et al., 2021). Under our conditions, both
311 SNAP25 mutants (V48F and D166Y) showed significantly impaired attachment of Syt1/VAMP2 SUVs
312 to t-SNARE GUVs (Fig. 8B). Both V48 and D166 directly interact with Syt1 (Fig. 1B-C, (Zhou et al.,
313 2015)), so that changing these two amino acids to more bulky or hydrophobic amino acids reduced
314 the docking from 42.7 ± 2.6 % wildtype (WT) docking efficiency to 29.6 ± 4.1 % and 20.9 ± 3.7 %,
315 respectively. In the presence of 1% PI(4,5)P_2, the vesicle attachment increased from 42.7 ± 2.6 % to
316 66.0 ± 0.5 % for WT t-SNARE. Although the Syt1:PI(4,5)P_2 interaction predominated SUV-GUV
317 docking, both mutants still showed significantly reduced vesicle attachment by approximately 6%
318 compared to WT (Fig. 8C) (V48F: 59.1 ± 1.4 %, and D166Y: 60.0 ± 2.0 %).

319 To understand how the mutations affect fusion in a well-defined reconstituted membrane fusion
320 system, we performed *in vitro* lipid mixing assays using GUVs containing both t-SNAREs (syntaxin-1
321 and SNAP25) with 1% PI(4,5)P_2, and 0.5% Atto488/0.5% Atto550 labeled SUVs containing Syt1 and
322 VAMP2 in the presence or absence of complexin-II (6 µM) (Kedar et al., 2015; Malsam et al., 2012).
323 Fusion was measured at 37°C by Atto488 fluorescence dequenching, which occurs upon lipid mixing
324 with GUVs. Calcium (100 µM free Ca^{2+} final) was added after 2 minutes to the t-SNARE GUV assay.
325 Measurements were continued for another 5 minutes. SUVs treated with botulinum toxin D, which
326 cleaves VAMP2 and abolishes membrane fusion, served as negative control and the corresponding
327 background fluorescence was subtracted. Measurements were normalized to total fluorescence
328 after detergent lysis.

329 Complexin-II clamps spontaneous Ca^{2+}-independent membrane fusion in the reconstituted assay
330 (i.e. fusion before addition of Ca^{2+}), via laterally binding the membrane-proximal C-terminal ends of
331 SNAP25 and VAMP2 (Malsam et al., 2020). Remarkably, V48F and D166Y showed impaired clamping
332 by complexin, as apparent by increased fusion before Ca^{2+} addition (Fig. 8D). The decreased
333 clamping is likely caused by the reduced interaction of V48F and D166Y with Syt1. As a note, the
334 clamping function of Syt1 becomes only obvious in the presence of complexin, whereas the
335 clamping function of complexin depends on the presence of Syt1 (Malsam et al., 2012); thus, the

336 clamping function of the two proteins cannot be separated. After Ca^{2+}-triggering, WT SNAP25
337 supported the largest amount of fusion, followed by V48F, whereas D166Y Ca^{2+}-dependent fusion
338 was clearly reduced (Fig. 8D). Notably, this is the same sequence as found for evoked release in
339 synapses, considering either the amplitude or the charge of the eEPSC (Fig. 4B-C, F-G). Thus, the *in*
340 *vitro* assay reproduces both the increased spontaneous release and the reduced Ca^{2+}-dependent
341 release found in neurons, indicating that these features are present within the minimal set of fusion
342 proteins included in this assay (i.e. the SNAREs, Syt1 and complexin).

343 There is evidence that SNAP25 might enter the SNARE-complex last, after syntaxin-1 and VAMP2
344 are joined by Munc18-1 (Baker et al., 2015; Jiao et al., 2018; Sitarska et al., 2017), although another
345 view is that a syntaxin/SNAP25 dimer bound to Munc18-1 acts as an intermediary (Jakhanwal et al.,
346 2017). In the former case, mutations changing association of SNAP25 to the SNAREs might change
347 exocytosis efficiency. To test this, we used a syntaxin-1 GUV assay, where the incorporation of
348 soluble SNAP25 into the SNARE complex becomes a rate-limiting step. In this assay, fusion kinetics
349 are much slower when compared with fusion reactions containing GUVs with pre-assembled t-
350 SNAREs. Accordingly, we allowed 30 min for pre-stimulation fusion to take place, and after addition
351 of Ca^{2+} (100 µM free Ca^{2+}), fusion was followed for another 30 min (Fig. 9A). The assay was
352 performed in the presence and absence of complexin-II. D166Y and to a lesser degree V48F revealed
353 enhanced stimulation of fusion in comparison to WT before calcium was added, regardless of the
354 presence or absence of complexin (Fig. 9B). Comparing data with and without complexin established
355 that complexin barely suppressed spontaneous fusion in reactions containing the V48F mutant. The
356 presence of complexin partially reduced Ca^{2+}-independent fusion, but D166Y still stimulated pre-
357 Ca^{2+} fusion compared to wildtype. Overall, these data demonstrate that D166Y and V48F are gain-
358 of-function mutants under conditions where SNAP25 association to the other SNAREs is rate
359 limiting.

360 To test directly whether V48F and especially D166Y enhance SNARE interactions, co-flotation assays
361 were performed. SUVs containing either syntaxin-1 (Stx-1), or VAMP2, or Syt1, or the Syt1/VAMP2
362 combination were incubated with SNAP25 and re-isolated by flotation using a Nycodenz density
363 gradient. An additional reaction, reflecting the fusion assay, contained SNAP25 in combination with
364 both Syt1/VAMP2 SUVs and Syntaxin-1 SUVs (Fig. 9D). SNAP25 recruitment for each condition was
365 determined by SDS PAGE followed by Coomassie blue and silver staining. Silver stained SNAP25

366 bands were quantified, and the mutants were plotted relatively to the wildtype SNAP25 (Fig. 9D and
367 Fig. 9 – Figure supplement 1).

368 SNAP25 WT did not show any binding to Syt1 SUVs (Fig. 9 – Figure supplement 1) , although direct
369 interactions with Syt1 would be predicted based on the primary interface, indicating that such
370 interactions are not stable under the employed conditions, which is expected because SNAP25 is
371 unstructured until it binds its SNARE partners (Fasshauer et al., 1997). D166Y showed profoundly
372 increased interactions with SUVs containing either syntaxin-1, or VAMP2, or Syt1/VAMP2, and the
373 combination used in the fusion assay (Fig. 9D; Fig. 9 – Figure supplement 1). V48F displayed mildly
374 increased binding to syntaxin-1 and Syt1/VAMP1 SUVs, and a tendency to increased association to
375 VAMP2 SUVs (p=0.0629, one-sample t-test). These data show that loss of Syt1 interaction upon
376 mutation in the primary interface can be accompanied by a gain-of-function phenotype stimulating
377 interactions with the other SNARE-partners. This association of SNAP25 to the other SNAREs might
378 happen as one of the last steps towards fusion; consequently, when D166Y and V48F join the
379 complex prematurely, it will bypass layers of control and result in uncontrolled fusion.

380 *The I67N mutation supports an intact RRP, but an increased energy barrier for fusion*

381 We next addressed the phenotype of the I67N disease mutation, which was found in an 11-year-old
382 female, who suffered from myasthenia, cortical hyperexcitability, ataxia and intellectual disability,
383 but with normal brain MRI (Shen et al., 2014). The I67 is found within the interaction layer +4
384 (Fasshauer et al., 1998), which helps in assembly of the C-terminal of the SNARE-complex (Gao et
385 al., 2012), and it might therefore have a different synaptic phenotype than V48F and D166Y.

386 In *in vitro* experiments, SNARE-complexes formed with the I67N mutant displayed a lower stability,
387 with the melting temperature reduced from 71°C to approximately 56°C (Fig. 8A), as expected for a
388 mutation that destabilizes the SNARE-complex. I67N also showed a strong decrease in SUV docking
389 (Fig. 8B-C), which is likely caused by the destabilization of the t-SNARE complex, which indirectly
390 perturbs the Syt1 binding interface(s). In lipid mixing assays, I67N strongly reduced both Ca^{2+}-
391 independent and Ca^{2+}-dependent fusion, whether the t-SNAREs were preassembled (Fig. 8D-E) or
392 not (Fig. 9B-C). The co-flotation assay did not display any binding of I67N to t-SNAREs or Syt1 (Fig.
393 9D). The binding to Syt1/VAMP2 SUVs was reduced compared to SNAP25 WT, but since binding of

394 the WT protein is already very low, the biological significance of this result is unclear. Overall, these
395 data indicate that I67N is inferior in membrane fusion.

396 Lentiviruses encoding the I67N mutant N-terminally fused to EGFP expressed similar amounts as WT
397 EGFP-SNAP25 (Fig. 10A-C). Expression in SNAP25 KO neurons resulted in reduced rescue of survival
398 in neurons expressing I67N alone, whereas neurons co-expressing WT and I67N had intermediate
399 survival, not significantly different from WT (Fig. 10D). Staining against MAP2 (dendritic marker) and
400 VGlut1 (synapse marker) showed that the number of synapses on average was reduced in the I67N
401 (Fig. 10E-F), and the dendritic length was on average reduced (Fig. 10G), but the changes did not
402 reach statistical significance. Patch-clamp measurements demonstrated strongly reduced
403 spontaneous release frequencies (Fig. 10H-J) and evoked release amplitude (Fig 10K-L) with the I67N
404 mutation, see also (Alten et al., 2021). mEPSCs were absent in most I67N expressing neurons,
405 whereas WT and I67N co-expressing neurons had a very low mEPSC rate, much closer to the I67N
406 than the WT phenotype; similar for eEPSC amplitudes and charges (Fig. 10I, L-M). The I67N therefore
407 is dominant-negative for both types of release, in contrast to the incompletely dominant
408 phenotypes of the V48F and D166Y mutants (see above). The fraction of synchronous release was
409 unchanged in WT and I67N coexpressing neuron (Fig. 10N); this number could not be estimated for
410 I67N-expressing neurons due to the low amount of release.

411 Reduced spontaneous and evoked release could result from a decrease in priming, or fusion, or
412 both. To distinguish between these possibilities, we turned to sucrose applications. Application of
413 0.25M sucrose did not lead to any measurable release in I67N expressing cells, and only minimal
414 release in cells co-expressing WT and I67N (Fig. 11B). This indicates that the energy barrier is
415 increased in amplitude, and therefore the RRP might be underestimated when probed by 0.5M
416 sucrose (Schotten et al., 2015). Indeed, 0.5M sucrose displayed reduced release in the I67N and
417 WT+I67N condition (Fig. 11C), but this could be due to defects in priming or fusion. To investigate
418 this, we applied a stronger stimulus, 0.75M sucrose, to these cells (Schotten et al., 2015). Strikingly,
419 the RRP as assessed by 0.75M sucrose ($RRP_{0.75}$) was unchanged between WT, I67N and WT+I67N co-
420 expressed cells (Fig. 11E,G). Application of 0.375M sucrose led to small amounts of release in the
421 I67N, but more in the WT+I67N co-expressed situation (Fig. 11F). Forming the ratio $RRP_{0.375}/RRP_{0.75}$
422 revealed a statistically significant reduction in I67N compared to WT (Fig. 11H). Therefore, the RRP
423 *per se* appears intact in the I67N (when probed by sufficiently high concentrations of sucrose), but

424 the vesicles face a higher apparent fusion barrier, which makes the RRP appear smaller if assessed
425 by 0.5M sucrose. The higher apparent fusion barrier explains the lower frequency of mEPSC in the
426 I67N mutation, the lower degree of spontaneous fusion in *in vitro* assays, as well as the lower
427 amount of Ca^{2+}-dependent release in vitro and in the cell.

428 The I67N mutation profoundly affected trains in 2 mM extracellular Ca^{2+} (Fig. 11I) leading to strong
429 facilitation, which is expected due to the strong phenotype of this mutation, which radically lowers
430 release probability. Even when co-expressed with WT protein, the train facilitated over the first
431 several stimulations, attesting to the strong dominant-negative feature of the I67N mutation (Fig.
432 11I-J). Consequently, the paired-pulse ratio was increased in the I67N and intermediate in the
433 WT+I67N condition (Fig. 11K). Back-extrapolation of these trains was not reliable, because the low
434 release probability in the I67N mutation made it impossible to achieve sufficient depletion of the
435 RRP.

436 The energy barrier for fusion is exquisitely sensitive to the charges on the surface of the SNARE-
437 complex, with positive charges decreasing and negative charges increasing the fusion barrier
438 amplitude (Ruiter et al., 2019). To investigate whether the same electrostatic mechanism applies to
439 the I67N, we combined the I67N mutation with a mutation of four amino acids ("4K"=SNAP25
440 E183K/S187K/T190K/E194K) in the second SNARE-domain of SNAP25, constructing the quintuple
441 mutation ("I67N/4K"=SNAP25 I67N/E183K/S187K/T190K/E194K). The 4K mutation lowers the
442 energy barrier for fusion by increasing the charge of the SNARE-complex surface by +6 via charge
443 introduction and charge reversal (Ruiter et al., 2019). The 4K-mutation increased the mEPSC release
444 rate compared to WT (Ruiter et al., 2019), whereas in the combined I67N/4K mutation the
445 spontaneous release rate was indistinguishable from WT (Fig. 12A-C), showing that increased
446 positive charges rescued the defect of spontaneous release in the I67N mutant. Evoked release was
447 also increased in the 4K mutation compared to WT (Ruiter et al., 2019), but in the I67N/4K mutation,
448 evoked release was still strongly depressed compared to WT (Fig. 12D-E). Nevertheless, evoked
449 release was noticeable in the combined mutation, whereas it was almost absent in I67N-expressing
450 cells (comp. Fig. 10K-L), indicating a positive effect of the 4K mutation, which amounted to an
451 increase by a factor ~5 (eEPSC amplitude, I67N: 0.0475 ± 0.0087; I67N/4K: 0.2668 ± 0.12 nA; Mann-
452 Whitney test, P=0.035).

We previously created a simple mathematical model that links the release rate to the number of charges added to the SNARE-complex (Ruiter et al., 2019). This model includes both spontaneous and evoked release, which are separated by the addition of 35 positive charges in the latter case (Fig. 12F, black points are WT spontaneous and evoked release rates; blue line is the model fitted to WT data). Placing the spontaneous release rates for I67N and I67N/4K on this curve (by dividing the spontaneous release rate with RRP size and finding a corresponding charge-value using the model) resulted in two points (red) separated by 5.6 charges, which is close to the nominal 6 charges added by the 4K mutation (Fig. 12F). Similarly, evoked release in the I67N and the I67N/4K were separated by 5.9 charges (Fig. 12F). The fact that these numbers are close to the nominal 6 charges introduced shows that even for the I67N mutation, the same basal electrostatic model still applies, but the deleterious effect of the I67N on evoked release rates is larger than on spontaneous rates and therefore the rescue of evoked release rate by positive charges is insufficient to reach WT values. Moreover, because of the saturating form of the curve (i.e. the model) adding positive charges is an effective way of rescuing spontaneous, but not evoked release.

Overall, the I67N disease mutation increases the amplitude of the energy barrier for fusion, and it does so more for evoked than for spontaneous release, but the electrostatic mechanism, which we assume is part of release triggering (Ruiter et al., 2019), appears to be intact.

470 ## Discussion

471 We have shown that two SNAP25 mutations (V48F, D166Y) that compromise interaction with Syt1
472 lead to complex phenotypes characterized by a combination of loss-of-function and gain-of-function
473 features. Thereby, the mutations fall into the 'neomorph' category, where the mutated protein has
474 novel or changed interactions or functions (Verhage and Sorensen, 2020). In contrast, the I67N
475 substitution within the SNARE-bundle is a dominant-negative mutation.

476 *SNAP25 disease mutations change protein-protein interactions and the energy landscape of fusion*

477 Both the V48F and the D166Y resulted in a decrease in the amplitude of the apparent energy barrier
478 for fusion, whereas the I67N increased the amplitude of the apparent fusion barrier. A vesicle's
479 release willingness can only be assessed by fusing it; therefore, it is not possible to distinguish
480 between effects on the fusion barrier *per se*, and effects on the fusion machinery. In recognition of
481 this fact, we here refer to the "apparent energy barrier". Using Arrhenius' equation to convert
482 fusion, priming and depriming rates to their respective energy barrier heights (see Materials and
483 Methods for the assumptions behind this procedure), we can derive the energy landscape for fusion
484 of the three mutants (Fig. 13A-D). This shows the multiple changes in the V48F and D166Y, which
485 affect at least two different barriers (priming and fusion, Fig 13A-B), leading to a complex
486 phenotype, whereas for I67N the fusion barrier is primarily (or solely) affected (Fig. 13D).

487 When combining the I67N with the 4K-mutation, which introduces 6 extra positive fixed charges,
488 we could place our data within the framework of our model for electrostatic triggering (Ruiter et al.,
489 2019) and show that the effect of charge *per se* is approximately the same in the I67N mutant as in
490 the WT. Note that there are endogenous positively charged amino acids towards the C-terminal end
491 of SNAP25 that are important for release rates (Fang et al., 2015). Rescue of spontaneous release
492 was completed by adding 6 positive charges, which is consistent with the idea that the assembly of
493 the C-terminal end of the SNARE-complex, which is compromised by I67N (Rebane et al., 2018),
494 works against the electrostatic energy barrier, which is affected by the SNARE surface charge (Ruiter
495 et al., 2019). In contrast, rescue of evoked release by charges were incomplete, due to the larger
496 effect of I67N on evoked release, combined with the shallow effect of charges on evoked release
497 (Ruiter et al., 2019). The larger susceptibility of evoked release to C-terminal mutation of the

498 SNAREs might be partly due to the higher number of SNARE-complexes involved in evoked than in
499 sustained/spontaneous release (Mohrmann et al., 2010).

500 The effect of the I67N mutation in the energy domain at rest can be calculated from the
501 spontaneous release rate, which was reduced by a factor 22.4 (from 1.31 ± 0.36 to 0.0583 ± 0.0274
502 Hz). This corresponds to an effect in the energy domain of 3.1 k_BT (where k_B is Boltzmann's constant;
503 assuming unchanged RRP size, pool normalization is not required). Work with single-molecular
504 optical tweezers showed that the I67N mutation destabilizes the overall SNARE C-terminal and
505 linker domain, which are supposed to deliver the power stroke for membrane fusion, by 14 k_BT
506 (Rebane et al., 2018), which is substantially more. The reduction in spontaneous release rate is more
507 comparable to the reduction in the transition rate of folding by the C-terminal and linker domain by
508 a factor of ~10 (Rebane et al., 2018). Since at least three SNARE-complexes, possibly more,
509 contribute to vesicle fusion (Bao et al., 2018; Manca et al., 2019; Mohrmann et al., 2010; Shi et al.,
510 2012), folding kinetics correlates better to spontaneous fusion rates than overall SNARE-complex
511 stability.

512 The D166Y and V48F mutations lead to increases in spontaneous release, and more asynchronous
513 eEPSCs, consistent with their localization in the primary SNARE:Syt1 interface (Schupp et al., 2016;
514 Zhou et al., 2015; Zhou et al., 2017), and the demonstrated impaired Syt1 binding (Fig. 8B-C), see
515 also (Alten et al., 2021). These phenotypes are at first glance similar to Syt1 knockout/knockdown
516 (Bouazza-Arostegui et al., 2022; Chang et al., 2018; Huson et al., 2020; Ruiter et al., 2019). However,
517 when normalized to the RRP size, mEPSC frequencies were much higher in the V48F and the D166Y
518 than in the *Syt1* KO (Fig. 6D), and dual sucrose applications indicated a decrease in the amplitude of
519 the sucrose-probed apparent energy barrier and increased release probability, features not found
520 in the *Syt1* KO (Bouazza-Arostegui et al., 2022; Huson et al., 2020). This indicates a gain-of-function
521 feature of these mutations, which fulfills D166Y>V48F; such a feature was identified as an increased
522 interaction of V48F and D166Y with VAMP2- and syntaxin-1-containing SUVs (Fig. 9D); the
523 interaction was stronger for D166Y than for V48F. Molecular Dynamics (MD) simulations of the
524 SNAP25 mutants did not reveal major structural changes in the SNAP25 backbone compared to WT
525 (Fig. 9 - Figure supplement 2A-C). Nevertheless, the calculation of electrostatic (Coulomb) and van
526 der Waals (Lennard-Jonson, LJ) interactions for residues 48-52 (Fig. 9 - Figure supplement 2D) and
527 residues 162-166 (Fig. 9 - Figure supplement 2E) shows that for D166Y the interaction energy is

528 substantially more negative. This suggests that the interaction between D166Y and nearby residue
529 H162 is stronger than in WT. Overall, this may result in stabilization of a structure consistent with
530 SNARE-complex formation and explain why D166Y is a stronger gain-of-function mutation than
531 V48F. However, note that the AlphaFold prediction (Jumper et al., 2021; Mirdita et al., 2022) used
532 as a starting point for these simulations is identical to the structure of SNAP25 in the assembled
533 SNARE-complex (Zhou et al., 2015), whereas unassembled SNAP25 is likely less structured or
534 unstructured (Fasshauer et al., 1997). Overall, these disease mutants do not only fail in their
535 interaction with Syt1, they bypass fusion control, resulting in premature SNARE-complex assembly.
536 Although the increased spontaneous release rate might cause patient symptoms, Alten et al. (2021)
537 suggested that the resulting postsynaptic depolarization might compensate for the smaller eEPSC
538 amplitude to normalize the overall firing rate.

539 In a SUV:GUV fusion assay, where folding of SNAP25 onto syntaxin-1/VAMP2 is rate-limiting, D166Y
540 and V48F caused an increase in pre-stimulation fusion rates. This is consistent with recent data
541 showing that folding of SNAP25 onto a template formed by VAMP2 and syntaxin-1 held in place by
542 Munc18-1 might be a late, rate-limiting, step in exocytosis (Jiao et al., 2018). This process is
543 regulated and sped up by Munc13-1 (Kalyana Sundaram et al., 2021; Shu et al., 2020; Wang et al.,
544 2019). The fact that a similar effect on spontaneous fusion was seen in the assay with preformed t-
545 SNAREs in the presence of complexin indicates that there is an assembly step, even in that assay,
546 which can be sped up by the mutations. This aligns with the demonstration by single molecule FRET
547 of a further assembled ('tighter') state of the trans-SNARE-complex induced by Ca^{2+}-unbound Syt1,
548 which becomes committed for fusion once Ca^{2+} binds (Das et al., 2020).

549 *V48F and D166Y change the size of the RRP via effects on priming, depriming and fusion*

550 We found that V48F and D166Y cause a decrease in RRP size, whether measured by sucrose
551 application or by train stimulation. The reduced RRP appears to be at variance with the publication
552 by Alten et al., who used sucrose application to larger mixed cultures (Alten et al., 2021). There
553 might be several reasons for this discrepancy. First, Alten et al. used longer duration of sucrose
554 application (~1 min) and applied sucrose to a large mixed culture, which might result in a variable
555 delay such that all synapses are not stimulated simultaneously. This will result in temporally
556 overlapping release of RRP and upstream vesicle pools, which then cannot be distinguished from

557 each other. When grown on 50-µm micro-islands, sucrose can be applied acutely (within ~0.05 s) to
558 the entire dendritic tree and all synapses using a local perfusion system, which allows distinguishing
559 the RRP from upstream pools. Second, Alten assessed the RRP for GABAergic synaptic transmission
560 in mixed culture, whereas we measured the RRP for glutamatergic neurons in autaptic culture. The
561 RRP has a different substructure in the two types of neurons (Moulder and Mennerick, 2005), which
562 might lead to different findings. Third, it was recently shown that sufficient neuronal maturation is
563 necessary to detect the decrease in RRP upon Syt1 elimination (Bouazza-Arostegui et al., 2022),
564 which is likely modulated by the presence of other neurons (Chang et al., 2018; Liu et al., 2009;
565 Wierda and Sorensen, 2014). This adds an additional layer of complexity since neuronal maturation
566 likely varies between laboratories.

567 Dissection of the three rates that determine RRP size (priming, depriming and fusion rates) showed
568 that all three are changed in the V48F and D166Y mutations. Especially for D166Y, but also for V48F,
569 spontaneous release contributed to RRP depletion by triggering premature fusion. Significant RRP
570 depletion has not been expected for moderate increases in mEPSC frequency (Rhee et al., 2005;
571 Ruiter et al., 2019), based on the argument that RRP refilling should be fast enough to counteract
572 depletion. However, to properly make this argument, priming, depriming and spontaneous release
573 rates must be compared in the steady-state situation. Similarly, we recently showed by cryo-
574 electron tomography that the 4K-mutation, which increased the mEPSC frequency to ~30 Hz and
575 had a reduced RRP (Ruiter et al., 2019) caused a loss of synaptic vesicles tethered to the membrane
576 with three tethers (Radecke et al., 2023), which is the structural correlate of the RRP (Fernandez-
577 Busnadiego et al., 2010).

578 The major effect on RRP size is caused by a reduction in forward priming rate by D166Y and V48F.
579 Comparison to the Syt1 KO showed qualitatively similar changes, but of a smaller magnitude, with
580 spontaneous release playing a negligible role for RRP size. Notably, in all three cases a reduction in
581 depriming rate partly counteracted the lowered priming rate (Table 1) – this was only significant for
582 D166Y. Indeed, part of the role of Syt1:SNARE interaction might be catalytic, lowering the energy
583 level of a transition state along the path to priming, which will affect both rates (Walter et al., 2013).
584 This might happen because transient binding to Syt1 might structure SNAP25 and assist in formation
585 of the SNARE-complex, whereas SNAP25 mutants might prestructure the protein, bypassing the
586 need for Syt1. In an energy diagram (Fig. 13) it becomes clear that vesicle priming and regulation of

587 spontaneous release are interdependent. Stabilization of the RRP state will both increase RRP size
588 at rest and reduce spontaneous release, since lowering the energy level of (i.e. stabilizing) RRP
589 vesicles will increase the size of the energy barrier that the RRP vesicles face (Fig. 13). Consistently,
590 downregulation (clamping) of spontaneous release and upregulation of evoked release are often
591 interdependent under conditions where Syt1 expression level, or Syt1 interaction with the SNAREs,
592 are up or down regulated (Courtney et al., 2021; Courtney et al., 2019; Vevea and Chapman, 2020;
593 Zhou et al., 2015; Zhou et al., 2017). This does not rule out the existence of mutations that can affect
594 one mode of release more than the other (e.g. the I67N/4K mutation). By inference, assembly of
595 the primary Syt1:SNARE interface (Zhou et al., 2017) is most likely involved in both clamping release
596 and setting up a RRP. In further support of this, the minimal *in vitro* assay with preassembled t-
597 SNARE dimers displayed a qualitatively similar reduction in calcium-dependent release, with D166Y
598 being more impaired than V48F (Fig. 8D). Overall, the energetic contribution of Syt1:SNARE
599 interaction to Ca^{2+}-triggered release is a stabilization of upstream steps, and the increase of the
600 fusion barrier downstream of the RRP (Fig. 13). The electrostatic nature of the fusion barrier (Ruiter
601 et al., 2019) ensures its rapid dissolution by Ca^{2+}, possibly by unbinding of Syt1 from the SNARE
602 complex (Voleti et al., 2020).

603 For the I67N-mutation, the sucrose-RRP was also reduced in size when using 0.5 M sucrose, which
604 is consistent with previous observations (Alten et al., 2021). However, when the energy barrier for
605 release is increased, 0.5 M sucrose is insufficient to deplete the RRP (Schotten et al., 2015).
606 Accordingly, 0.75 M sucrose released an RRP of similar size in WT, I67N and I67N+WT co-expressing
607 cells, showing that the vesicle priming reaction is intact, but the vesicles face a larger fusion barrier
608 (Fig. 11). In this mutant, train stimulations in the I67N or co-expressed WT + I67N case resulted in a
609 phenotype quite distinct from the WT, with strong facilitation throughout the train. Thus, the I67N
610 is a strongly dominant-negative mutant, whether considering mEPSC frequency, eEPSC amplitude
611 or train stimulations, whereas V48F and D166Y are incompletely dominant. This can be explained
612 by the different function of SNAP25 domains during fusion, where V48 and D166 help set up an
613 arrested primed vesicle state by interacting with Syt1 (Schupp et al., 2016; Zhou et al., 2015; Zhou
614 et al., 2017), whereas I67 participates in the final conformational change, the 'stroke' that leads to
615 assembly of the C-terminal end of the complex and the linker domain. Due to their defect in priming,
616 V48F and D166Y might not enter the super-complex (the complex of SNARE-complexes driving

617 fusion) as often as WT protein. In contrast, I67N will readily enter the super-complex and
618 compromise its function, which will lead to a dominant-negative phenotype due to the multiple
619 SNARE-complexes involved in fusion (Bao et al., 2018; Mohrmann et al., 2010; Shi et al., 2012).

620 Back-extrapolation of 40 Hz trains (@4 mM Ca^{2+}) also led to the conclusion that V48F and D166Y
621 have a reduced RRP_{ev}, the RRP-subpool that action potentials draw on, and a reduced forward
622 priming rate. Interestingly, the release probability when normalized to the sucrose pool ($RRP_{0.5}$) was
623 increased non-significantly for the V48F and significantly for the D166Y mutation, but when
624 considering 40Hz trains, the release probabilities of both mutations were decreased, consistent with
625 a shift towards facilitation. The latter finding is likely caused by the defective interaction with Syt1,
626 which leads to suboptimal priming and/or defective super-priming, which is an additional priming
627 step after entry of the vesicle into the RRP (Lee et al., 2013; Taschenberger et al., 2016). This will
628 lead to reduction of the first eEPSC of a train and thereby a lowered nominal release probability.
629 However, when comparing the eEPSC charge to the $RRP_{0.5}$, the strong reduction in the $RRP_{0.5}$ pool
630 (especially in the D166Y mutation) accounts for the increase in the overall release probability. This
631 can be explained if spontaneous release causes a disproportional depletion of vesicles, which are in
632 the $RRP_{0.5}$, but not in the RRP_{ev}.

633 *Conclusion*

634 SNAP25 disease missense mutations change the function of the protein without compromising its
635 expression, leading to dominant negative or neomorphic mutations. Missense mutations in the
636 primary SNARE:Syt1 interface (V48F, D166Y) result in a complex phenotype characterized by loss-
637 of-function in the priming step and gain-of-function in the fusion step. Missense mutation in the
638 SNARE-bundle (I67N) leads to an increased amplitude of the energy barrier for fusion. In addition,
639 disease mutations display inefficient rescue of neuronal survival. Overall, SNAP25 encephalopathy
640 caused by single missense point mutations presents with interdependent functional deficits, which
641 must be overcome for successful treatment.

642

643 ## Materials and Methods

644 *Animals*

645 SNAP25 KO C57/Bl6-mice: Heterozygous animals were routinely backcrossed to Bl6 to generate new
646 heterozygotes. The strain was kept in the heterozygous condition and timed pregnancies were used
647 to recover knockout embryos by caesarean section at embryonic day 18 (E18). Pregnant females
648 were killed by cervical dislocation; E18 embryos of either sex were collected and killed by
649 decapitation. Permission to keep and breed *Snap25* and *Syt1* mice was obtained from the Danish
650 Animal Experiments Inspectorate (2018-15-0202-00157) and followed institutional guidelines as
651 overseen by the Institutional Animal Care and Use Committee (IACUC). CD1 outbred mice were used
652 to create astrocytic cultures and mass cultures for Western blotting. Newborns (P0-P2) of either sex
653 were used. Pups were killed by decapitation.

654 *Cell lines*

655 HEK293-FT cells for production of lentiviruses were obtained from the Max-Planck-Institute for
656 biophysical chemistry. The cells were passaged once a week, and they were used between passage
657 11 and 25 for generation of lentiviral particles. The cells were kept in DMEM + Glutamax (Gibco, cat.
658 31966047) supplemented with Fetal Bovine Serum (Gibco, cat. 10500064), Pen/Strep (Gibco, cat.
659 15140122) and Geneticin G418 (Gibco, cat. 11811064) at 37°C in 5% CO_2.

660 *Preparation of neuronal culture*

661 Self-innervating ("autaptic") hippocampal cultures were used (Bekkers and Stevens, 1991).
662 Astrocytes were isolated from CD1 outbred mice (P0-P2). The cortices were isolated from the brains
663 in HBSS-HEPES medium (HBSS supplemented with 1 M HEPES) and the meninges were removed.
664 The cortical tissue was chopped into smaller fragments, transferred to 0.25% trypsin dissolved in
665 DMEM solution (450 ml Dulbecco's MEM with 10% Foetal calf serum, 20000 IU Penicillin, 20 mg
666 Streptomycin, 1% MEM non-essential Amino Acids) and incubated for 15 min at 37 °C. Subsequently,
667 inactivation medium (12,5 mg Albumin + 12,5 mg Trypsin-Inhibitor in 5 ml 10% DMEM) was added,
668 the tissue was washed with HBSS-HEPES, triturated and the cells were plated in 75 cm^2 flasks with
669 pre-warmed DMEM solution (one hemisphere per flask) and stored at 37 °C with 5% CO_2. Glial cells
670 were used after 10 days.

671 Glass coverslips were washed overnight in 1 M HCl; for an hour in 1 M NaOH and washed with water
672 before storage in 96% ethanol. Coverslips were first coated by 0.15 % agarose and islands were
673 made by stamping the coating mixture (3 parts acetic acid (17 mM), 1 part collagen (4 mg/ml) and
674 1 part poly-D-lysine (0.5 mg/ml)) onto the glass coverslips using a custom rubber stamp. Glial cells
675 were washed with pre-warmed HBSS-HEPES. Trypsin was added and the flasks were incubated at
676 37 °C for 10 min. Cells were triturated and counted in a Bürker chamber before plating onto the
677 glass coverslip with DMEM solution. After 2-5 days, neurons were plated on the islands.

678 Neurons for autaptic culture were isolated from E18 *Snap25* KO mice. Pups were selected based on
679 the absence of motion after tactile stimulation and bloated neck (Washbourne et al., 2002); the
680 genotype was confirmed by PCR in all cases. The cortices were isolated from the brains in the HBSS-
681 HEPES medium. The meninges were removed and the hippocampi were cut from the cortices before
682 being transferred to 0.25% trypsin dissolved in HBSS-HEPES solution. The hippocampi were
683 incubated for 20 min at 37 °C, subsequently washed with HBSS-HEPES, triturated and the cell count
684 was determined with a Bürker chamber before plating on the islands (7000-8000 neurons per well).
685 Cells were incubated in the NB medium (Neurobasal with 2% B-27, 1 M HEPES, 0,26% Glutamax,
686 14,3 mM β-mercaptoethanol, 20000 IU Penicillin, 20 mg Streptomycin) and used for measurements
687 between DIV10-14.

688 Hippocampal neurons for high-density cell culture for Western blotting were obtained from P0-P1
689 CD1 outbred mice. The dissection, tissue digestion and cell counting were performed the same way
690 as the neurons for the autaptic culture, the high-density culture (600,000 neurons per well) was
691 then kept in NB-A medium (Neurobasal-A with 2% B-27, 1 M HEPES, 0,26% Glutamax, 20000 IU
692 Penicillin, 20 mg Streptomycin) and half the volume of the media was replaced every 2-3 days before
693 harvesting the cells at DIV14.

694 *Constructs for rescue experiments*

695 SNAP25B was N-terminally fused to GFP and cloned into a pLenti construct with a CMV promoter
696 (Delgado-Martinez et al., 2007). Mutations were made using the QuikChange II XL kit (Agilent).
697 Primers were ordered from TAGC Copenhagen. All mutations were verified by sequencing before
698 virus production. Viruses were prepared as previously described using transfection of HEK293FT-

699 cells (Naldini et al., 1996). Neurons were infected with lentiviruses on DIV 0-1, 30 µl total virus per
700 well (WT or mutant; 15 µl + 15 µl WT + mutant virus for co-expressed condition).

701 *Immunostaining and confocal microscopy*

702 Autaptic hippocampal neurons were fixed at DIV14 in 2% PFA in culture medium for 10 min and
703 subsequently in 4% PFA for 10 min. Cells were then washed with PBS, permeabilized by 0.5% Triton
704 X-100 for 5 min and blocked with 4% normal goat serum in 0.1% Triton X-100 (blocking solution) for
705 30 min. Cells were incubated with primary antibodies diluted in blocking solution (anti-MAP2, 1:500,
706 chicken, ab5392, Abcam; and anti-vGlut1 1:1000, guinea pig, AB5905, Merck Millipore) for 2 h at RT.
707 After washing with PBS, the cells incubated with secondary antibodies in blocking solution for 1h at
708 RT in the dark (anti-chicken Alexa 568, 1:1000, A11041, Thermo Fisher Scientific; and anti-guinea
709 pig Alexa 647, 1:1000, A-21450, Thermo Fisher Scientific) and washed again. Coverslips were
710 mounted with FluorSave and imaged on Zeiss CellObserver spinning disc confocal microscope (40x
711 water immersion objective; NA 1.2) with Zeiss Zen Blue 2012 software. Images were acquired as Z-
712 stack and 9 images per plane to capture the whole island in the field of view. The images were post-
713 processed with Zeiss Zen Black software and neuronal morphology was analyzed using SynD
714 automated image analysis (Schmitz et al., 2011).

715 *Western Blotting*

716 Harvesting the high-density hippocampal culture, BCA assay and transferring the protein samples
717 on a PVDF membrane were performed as described before (Ruiter et al., 2019). Incubation in
718 primary antibodies (a-SNAP25: mouse, 1:10000, SYSY 111011, Synaptic Systems; a-VCP: mouse,
719 1:2000, ab11433, Abcam) was performed overnight with 70 rpm shaking at 4 °C, followed by
720 washing in TBST (0.1%) and a 1 h incubation in secondary antibody (goat a-mouse-HRP: 1:10000,
721 P0447, Dako). After washing, Pierce ECL Western blotting substrate was added and
722 chemiluminescence was visualized with FluorChem E (Proteinsimple). The western blots were
723 quantified using ImageJ (1.52a) to measure the signal intensity of the protein bands relative to the
724 loading control (VCP). All relative levels of the target protein were normalized to the average relative
725 level of the same target protein in the WT samples.

726 *Electrophysiology*

727 Autaptic cultures were used from DIV10 until DIV14. The intracellular pipette medium contained:
728 KCl 136 mM, HEPES 17.8 mM, Creatine Phosphate 15 mM, Na-ATP 4 mM, Creatine Phosphokinase
729 50 U, $MgCl_2$ 4.6 mM, EGTA 1 mM (pH 7.4, osmolarity ~300 mOsm). The standard extracellular
730 recording medium contained: NaCl 140 mM, KCl 2.4 mM, HEPES 10 mM, Glucose 14 mM, $CaCl_2$ 2
731 mM, $MgCl_2$ 2 mM. The extracellular recording medium for de-priming experiments contained: NaCl
732 140 mM, KCl 2.4 mM, HEPES 10 mM, Glucose 14 mM, $CaCl_2$ 4 mM, $MgCl_2$ 4 mM (pH 7.4, osmolarity
733 ~300 mOsm). An Axio Observer A1 inverted microscope (Zeiss) was used to visualize the cells. The
734 recordings were performed at room temperature. An EPC10 amplifier (HEKA) was used with the
735 program Patchmaster v2.73 (HEKA). Traces were filtered with a 3kHz Bessel low-pass filter and data
736 were acquired at 20 kHz. The series resistance was compensated to 70%. Glass pipettes were freshly
737 pulled on a P1000 pipette puller (Sutter Instruments) from borosilicate glass capillaries. Pipets
738 ranging from 2.5 to 5 MΩ were selected for recordings. Cells with starting access resistance above
739 10 MΩ were rejected. Recordings were performed in voltage clamp, with the holding potential kept
740 at -70 mV. Evoked excitatory postsynaptic currents (eEPSC) were induced by raising the holding
741 voltage to 0 mV for 2 ms. Sucrose was dissolved into the standard extracellular recording medium.
742 Application of the extracellular media was done using a custom-made barrel system, controlled by
743 SF-77B perfusion fast step (Warner Instruments) controlled via digital output switches from the
744 EPC10. Electrophysiological data was analyzed in IGOR Pro (v6.21 and v6.37, WaveMetrics) using a
745 custom-written script. mEPSCs were analyzed with MiniAnalysis (v6.0.7, Synaptosoft).

746 *Electrophysiological data: Calculations*

747 Deconvolution was calculated and the electrostatic model for triggering was fitted to 4K and
748 I67N/4K data as described before (Ruiter et al., 2019). Since the evoked release for I67N was so
749 small that deconvolution became unreliable, we downscaled the peak release rate of the I67N/4K
750 mutation with a factor of 5.616, corresponding to the reduction in eEPSC amplitude in the I67N
751 compared to I67N/4K.

752 In order to determine the reasons for the reduced RRP size for V48F and D166Y, we considered a
753 single pool model for the RRP, with priming rate k_1, depriming rate k_{-1}, and fusion rate k_f (Fig. 6A).
754 The equation describing the evolution in RRP size is:

(Eq. 1) $$\frac{d\mathrm{RPP}(t)}{dt} = k_1 - \left(k_{-1} + k_f\right) \cdot \mathrm{RRP}(t)$$

where k_1 has the unit vesicles/s, whereas k_{-1}, and k_f have the unit 1/s. The solution of this differential equation is

(Eq. 2) $$\mathrm{RPP}(t) = \frac{k_1}{(k_{-1}+k_f)}\left(1 - e^{-(k_{-1}+k_f)t}\right)$$

The steady-state RPP size is

(Eq. 3) $$RRP = \frac{k_1}{(k_{-1}+k_f)}$$

The miniature release rate at steady state is

(Eq. 4) $$r_{mini} = \frac{k_f k_1}{(k_{-1}+k_f)}$$

When stimulated by sucrose, k_f increases, and if $k_f >> k_{-1}$ (for justification, see below) the current plateau will report on the forward priming rate alone:

(Eq. 5a) $$r_{prime} \approx k_1$$

Thus, the current plateau during sucrose application can be used for estimating the forward priming rate. However, application of 0.5 M sucrose causes cell shrinkage and changes in solution viscosity, which in turn can change the leak current. This might cause the plateau current to change, which might be invisible during the experiment due to the synaptic events. To correct for this, we implemented variance-mean analysis to identify the true baseline current (the current corresponding to the lack of synaptic release). Synaptic release is essentially a source of shot noise, for which the variance is proportional to the mean. We therefore calculated the variance (after subtraction of a running average) and mean of the current in 50 ms intervals during the sucrose application, and performed linear regression in a variance-mean plot. The corrected baseline was identified by backextrapolation to the variance level found in the absence of synaptic activity (using a stretch of current before sucrose application), as illustrated in Fig. 6C.

If sucrose does not sufficiently increase k_f, the equation 5a above would be replaced by

(Eq. 5b) $$r_{mini} = \frac{k_f k_1 N_{suc}}{\left(k_{-1} + k_f N_{suc}\right)}$$

where N_{suc} is the fold-increase in k_f induced by sucrose application. Plotting the solutions to Eqs 3, 4, and 5b at different values of N_{suc}, the dependency of the estimated k_{-1} and k_1 upon N_{suc} can be investigated (Figure 6 – Figure supplement 1). Notably, in wildtype cells N_{suc} >5,000 (Schotten et al., 2015), indicating that the estimated values of k_{-1} and k_1 using Eq. 5a (Table 1) is quite accurate for the WT case, and the estimation in the case of the D166Y and V48F is even less dependent on N_{suc}, because the value of k_f is higher at rest (Figure 6 – Figure supplement 1). Importantly, for no realistic value of N_{suc} would the conclusion of decreased k_1 and k_{-1} in the two mutants be in jeopardy.

For calculating the energy profiles of WT and mutants, we used Arrhenius' Equation:

(Eq. 6) $$k = Ae^{(\frac{-E_A}{RT})}$$

where E_A is the activation energy, R and T are the gas constants and the absolute temperature, respectively, and A is an empirical constant that depends on collision rates (Schotten et al., 2015). Solving for the activation energy, we get:

(Eq. 7) $$E_a = RT(\ln(A) - \ln(k))$$

Since A is unknown, we cannot use this equation to calculate the absolute values of the transition energies; however, when we compare a mutation to the WT condition, and under the assumption that A is unchanged by mutation, we can calculate the difference in energy level of the transition states:

(Eq. 8) $$\Delta E_a^{Mut-WT} = RT(\ln(k^{WT}) - \ln(k^{Mut}))$$

Using this equation sequentially, for the priming rate, the depriming rate and the fusion rate, we can derive the entire energy diagram, under the additional assumption that the energy in the pre-primed state is identical between WT and mutation, and using that at room temperature, RT=2.479 kJ/mol.

The assumption that the empiric factor, *A*, is unchanged by mutation is likely to hold for the fusion reaction, which depends on conformational changes in a preformed complex. In contrast, collision rates might be involved in priming; in that case, the effect of the V48F and D166Y mutations on priming, which we here attribute to an increase in the energy level of the priming transition state

805 might reflect a lower collision rate between vesicles and plasma membrane fusion machinery,
806 and/or a lower energy level of the pre-primed state.

807 *Constructs for in vitro protein expression*

808 The following constructs were used: Glutathione S-Transferase (GST) - full length VAMP2 is encoded
809 by plasmid pSK28 (Kedar et al., 2015), GST-cytosolic domain VAMP2 (amino acids 1-94) (pSK74,
810 (Ruiter et al., 2019)), synaptotagmin 1-His6 lacking the luminal domain (amino acids 57-421) (pLM6,
811 (Mahal et al., 2002), His6-complexin II (CpxII) (pMDL80, (Malsam et al., 2012)), His6-syntaxin-1A
812 (pSK270, (Schollmeier et al., 2011)), His6-SNAP25B (pFP247, (Parlati et al., 1999)), t-SNARE
813 consisting of syntaxin-1A and His6-SNAP25B (pTW34, (Parlati et al., 1999)). Point mutants in soluble
814 His6-SNAP25B and in the t-SNARE complex were generated by using the DNA templates pFP247 and
815 pTW34, respectively, and the Quikchange DNA mutagenesis kit (Qiagen) (Ruiter et al., 2019).
816 Thereby, the following SNAP25 constructs were established: His6-SNAP25B mutant I67N (pUG1),
817 V48F (pUG2), D166Y (pUG3), and t-SNAREs containing the corresponding SNAP25B mutants I67N
818 (pUG7), V48F (pUG8), and D166Y (pUG9). The identity of all constructs was validated by DNA
819 sequencing.

820 *Protein expression and purification*

821 In general, the expression vectors, encoding the desired protein constructs were transfected into
822 *Escheria coli* BL21 (DE3) (Stratagene). At an OD_{660} of 0.8, protein expression was induced by the
823 addition of 0.3 mM IPTG. Alternatively, proteins were expressed by autoinduction using buffered
824 media containing lactose (Studier, 2005). Cells were harvested by centrifugation (3500 rpm, 15
825 minutes, H-12000 rotor, Sorvall) and lysed using the high-pressure pneumatic processor 110L
826 (Microfluidics). Cell fragments were removed by centrifugation at 60.000 rpm (70Ti rotor, Beckman)
827 for 1 hour and the clarified supernatant was snap-frozen in liquid nitrogen.

828 The purification of full length VAMP2 was performed as described previously (Kedar et al., 2015)
829 with the following modifications. Cells were grown in ZYM media (Studier, 2005) and protein
830 expression was induced with IPTG for 3 hours at 25°C. The purification and expression of the GST-
831 tagged cytosolic domain of VAMP2 was described previously (Ruiter et al., 2019). Synaptotagmin 1-
832 His6 lacking the luminal domain was purified as described previously (Malsam et al., 2012) with the
833 following modification. After dilution to 50 mM salt, the protein was further purified on a MonoS

834 Sepharose column (GE healthcare) applying a gradient of 50 to 500 mM KCl in 25 mM Hepes-KOH
835 (pH 7.4).

836 His6-syntaxin-1A purification was performed as outlined by (Schollmeier et al., 2011) with the
837 following modifications. Briefly, cells were grown in ZYM media (Studier, 2005) and autoinduction
838 was used for the protein expression at 22°C overnight. Syntaxin-1A was eluted from Ni-NTA-beads
839 (Qiagen) by over-night cleavage with Prescission protease (GE healthcare) at 4°C, removing the His_6
840 tag. After dilution to 80 mM salt, the protein was further purified on a MonoQ Sepharose column
841 (GE healthcare) applying a gradient of 50 to 500 mM KCl in 25 mM HEPES-KOH (pH 7.4).

842 His6-SNAP25 was expressed as depicted for syntaxin-1A and purified via Ni-NTA beads, followed by
843 MonoQ Sepharose column chromatography (Ruiter et al., 2019). Preassembled full-length t-SNARE
844 complexes were expressed and purified as described previously (Weber et al., 1998). His_6-Complexin
845 II expression and purification was performed according to (Malsam et al., 2012) with the following
846 modifications. His6-CpxII was expressed in BL21-DE3 codon+ bacteria for 2 hours at 27°C.

847 The concentrations of purified proteins were determined by SDS-PAGE and Coomassie Blue staining
848 using BSA as a standard and the Fiji software for quantification. Furthermore, mutant and wildtype
849 protein concentrations were directly compared on a single gel.

850 *Protein reconstitution into liposomes*

851 All lipids were from Avanti Polar Lipids, except of Atto488-DPPE and Atto550-DPPE, which were
852 purchased from ATTO-TEC. For VAMP2 and Syt1 reconstitution into small unilamellar vesicles
853 (SUVs), lipid mixes (3 µmol total lipid) with the following composition were prepared: 25 mol% POPE
854 (1-hexadecanoyl-2-octadecenoyl-SN-glycero-3-phosphoethanolamine), 15 mol% DOPS (1,2-
855 dioleoyl-SN-glycero-3-phosphoserine), 29 mol% POPC (1-palmitoyl-2-oleoyl-SN-glycero-3-
856 phosphocholine), 25 mol% cholesterol (from ovine wool), 5 mol% PI (L-α-phosphatidylinositol), 0.5
857 mol% Atto488-DPPE (1,2-dipalmitoyl-SN-glycero-3-phosphoethanolamine) and 0.5 mol% Atto550-
858 DPPE. For docking assays, the t-SNARE liposome lipid mix (5 µmol total lipid) had the following
859 composition: 35 mol% POPC, 15 mol% DOPS, 20 mol% POPE, 25 mol% cholesterol, 4 mol% PI, 0,05
860 mol% Atto647-DPPE and 0,5 mol% tocopherol. For the preparation of PI(4,5)P2-containing t-SNARE
861 and syntaxin-1A liposomes, the t-SNARE liposome lipid mix was used, but 1 mol% PI(4,5)P2 (L-α-

862 phosphatidylinositol-4,5-bisphosphate) was added and the POPC concentration lowered by 1%
863 accordingly.

864 Proteins were reconstituted as described previously (Malsam et al., 2012). For the docking and
865 fusion assays, t-SNARE wildtype and mutants were reconstituted at a protein to lipid ratio of 1:900.
866 For the syntaxin-1A membrane fusion assay, syntaxin-1A was reconstituted at a protein to lipid ratio
867 of 1:1000. Briefly, 5 µmol dried lipids were dissolved in 0.7 ml reconstitution buffer (25 mM HEPES-
868 KOH, pH 7.4, 550 mM KCl, 1 mM EDTA-NaOH, 1 mM DTT) containing final 1.4 weight% octyl-ß-D-
869 glucopyranoside (ß-OG) and either 6.5 nmol (390 µg) t-SNARE complex or 5 nmol (165 µg) syntaxin-
870 1A. SUVs containing either t-SNARE or syntaxin-1A were formed by rapid ß-OG dilution below the
871 critical micelle concentration by adding 1.4 ml reconstitution buffer. For the quantification of lipid
872 recovery, a 20 µl aliquot (GUV input) was removed and stored at -20°C. The liposome suspension
873 was desalted using a PD10 column (GE Healthcare) equilibrated with desalting buffer 1 (1 mM
874 HEPES-KOH, pH 7.5, 1 w/v% glycerol, 10 µM EGTA-KOH, 1 mM DTT) and snap frozen in four aliquots
875 in liquid nitrogen and stored in -80°C. These syntaxin-1A and t-SNARE SUVs were later used to
876 prepare giant unilamellar vesicles (GUVs). The final protein to lipid ratios were determined by SDS-
877 PAGE and Coomassie Blue staining of the proteins and Atto647 fluorescence intensity
878 measurements of the lipids.

879 VAMP2/Synaptotagmin 1-SUVs were prepared as described previously (Malsam et al., 2012; Weber
880 et al., 1998) with the following modification: VAMP2 and Synaptotagmin 1 were reconstituted at a
881 protein to lipid ratio of 1:350 and 1:800, respectively. SUVs, which were used for the SNAP25
882 recruitment assay, were not dialyzed twice, but directly harvested after the density gradient
883 flotation and lipid recovery and protein-to-lipid-ratio were determined (Stx1-SUVs: 1:1900; Syt1/V2-
884 SUVs: Syt1 1:700, VAMP2 1:300; V2-SUVs: VAMP2 1:200; Syt1-SUVs: Syt1 1:700).

885 *GUV preparation*

886 GUVs were prepared as described previously (Kedar et al., 2015). Briefly, t-SNARE or syntaxin-1A
887 liposomes (1.25 µmol lipid) were loaded onto a midi column (GE Healthcare) equilibrated with
888 desalting buffer 2 containing trehalose (1 mM HEPES-KOH, pH 7.5, 0.5 w/v% glycerol, 10 µM EGTA-
889 KOH, pH 7.4, 50 µM MgCl2, 1 mM DTT, 10 mM trehalose). 1.4 ml eluate were collected and
890 liposomes sedimented in a TLA-55 rotor (Beckman) at 35.000 rpm for 2 h at 4°C. The pellet was

891 resuspended by rigorous vortexing and, while vortexing, was diluted with 10 µl of pellet
892 resuspension buffer (1 mM HEPES-KOH, pH 7.4, 10 µM EGTA-KOH, 50 µM $MgCl_2$) to lower the
893 osmotic strength. The total volume (20-25 µl) was spread as a uniform layer (14 mm diameter) on
894 the surface of a platinum foil (Alfa Aesar; 25 x 25 mm, 0.025 mm thick) attached to a glass slide as
895 support. After drying the liposome suspension for 50 min at 50 mbar, the incubation chamber was
896 assembled using an O-ring (2 mm x 18 mm), filled with 620 µl of swelling buffer (1mM EPPS-KOH,
897 pH 8.0, 240 mM sucrose (Ca^{2+} free), 1 mM DTT) and closed using a second platinum plate.
898 Conductive copper tape (3M) was attached to the platinum foil to connect the assembly with a
899 function generator (Voltcraft 8202). GUVs were generated by electro-formation at 10 Hz and 1 V at
900 0°C overnight.

901 *Lipid mixing assay*

902 The membrane fusion assay was performed as described previously (Malsam et al., 2012), except
903 that in the fusion buffer HEPES was replaced with 20 mM MOPS pH 7.4. Briefly, t-SNARE-GUVs (14
904 nmol lipid, 15 $\pm$ 0,7 pmol t-SNARE) were preincubated with or without 6 µM (0,6 nmol) CpxII for 5
905 min on ice in fusion buffer containing 0,1 mM EGTA-KOH and 0.5 mM $MgCl_2$. When using syntaxin-
906 1A-GUVs (14 nmol lipid, 14 pmol syntaxin-1A), these preincubations contained 2 µM (0.2 nmol) of
907 soluble SNAP25 in addition. Subsequently, VAMP2/Syt1-SUVs (2.5 nmol lipid, 4.5 pmol VAMP2, 2
908 pmol Syt1) were added to the GUV reaction mix resulting in 104 µl sample volume. After 10 minutes
909 on ice, 100 µl of the GUV-SUV mixes were transferred into a prewarmed 96-well plate (37°C) and
910 fluorescence emitted by Atto488 (λ_{ex} = 485 nm, λ_{em} = 538 nm) was measured in a Synergy 4 plate
911 reader (BioTek Instruments GmbH) at intervals of 10 seconds. Ca^{2+} was added to a final free
912 concentration of 100 µM
913 (https://somapp.ucdmc.ucdavis.edu/pharmacology/bers/maxchelator/CaEGTA-TS.htm) after 2
914 minutes or 30 minutes for t-SNARE-GUVs or syntaxin-1A-GUVs, respectively. The fusion reactions
915 were stopped after 4 minutes for t-SNARE-GUVs or after 1 h for syntaxin-1A-GUVs by the addition
916 of 0.7% SDS and 0.7% n-Dodecyl-ß-D-Maltosid (DDM). The resulting “maximum” fluorescent signal
917 was used to normalize the fusion-dependent fluorescence. As a negative control, SUVs were treated
918 with Botulinum NeuroToxin type D (BoNT-D) and their fluorescence signals were subtracted from
919 individual measurement sets. Three independent fusion experiments were performed for each
920 mutant.

921 *SUV-GUV binding assay*

922 All SUV-GUV binding studies were performed in an ice bath and all pipetting steps were carried out
923 in the cold room to avoid membrane fusion (Parisotto et al, 2012; Malsam et al, 2012; Weber et al,
924 1998). Before starting the incubation, potential SUV aggregates were removed by centrifugation. T-
925 SNARE-GUVs (28 nmol lipid, 30 $\pm$ 1,4 pmol t-SNARE) were preincubated with VAMP2/Syt1-SUVs (5
926 nmol lipid, 9 pmol VAMP2, 4 pmol Syt1) on ice in 100 µL fusion buffer (20 mM MOPS-KOH, pH 7.4,
927 135 mM KCl, 1mM DTT) with 0.1 mM EGTA and 1 mM $MgCl_2$. After 10 min incubation, the reactions
928 were underlaid with 20 µL of a sucrose cushion (1 mM MOPS-KOH, pH 7.4, 60 mM sucrose, 1 mM
929 DTT) and the GUVs with attached SUVs were re-isolated by centrifugation for 10 min. After removing
930 the supernatant, the pellets (in 10 µL remaining volume) were resuspended, transferred into new
931 tubes, treated with 100 µL of 1% SDS/1% DDM and the SUV recovery was determined by measuring
932 the Atto488 fluorescence.

933 To determine the respective inputs, 28 nmol GUV lipids or 5 nmol SUV lipids were treated with 1%
934 SDS/1% DDM (final) and the corresponding Atto647 and Atto488 fluorescence was measured at λ_{ex}
935 = 620/40 nm, λ_{em} = 680/30 nm and λ_{ex} = 485/20 nm, λ_{em} = 528/20 nm, respectively. A sample lacking
936 SUVs was used to determine the GUV recovery (usually 80-95%). GUV recovery of each sample was
937 used to normalize the respective SUV docking. A sample without GUVs was used to determine the
938 absolute background (usually <15%), which was subtracted from all samples.

939 *SNAP25 recruitment assay / SUV flotation assay*

940 Small unilamellar liposomes (SUVs) containing 35 pmol syntaxin-1A or 210 pmol VAMP2 and/or 100
941 pmol Synaptotagmin-1 (66 nmol lipid for Stx1-, V2/Syt1- or Syt1-SUVs, 40 nmol lipid for V2-SUVs)
942 were mixed with 180 pmol SNAP25 in a final assay volume of 50 µl in fusion buffer (20 mM MOPS-
943 KOH, pH 7.4, 135 mM KCl, 1 mM DTT). After two hours on ice, allowing the SNARE complex
944 formation, the samples were diluted five times with fusion buffer and mixed with the equivalent
945 volume of 80% nycodenz solution. After overlaying the sample with 100 µl 35% nycodenz, 25 µl 20%
946 nycodenz solution and finally with 5 µl fusion buffer in an ultra-clear tube (5 x 41 mm), the liposomes
947 were isolated by centrifugation for 3 h 40 mins at 55,000 rpm at 4°C in a SW 60 rotor (SW 60 Ti,
948 Beckman). 20 µl were harvested from the top of the gradient and mixed with 8 µl of 4x Laemmli
949 buffer (final 62.5 mM Tris-HCl, pH 6.8, 10% glycerol, 2% SDS, 50 mM ß-mercaptoethanol, 0.1%

950 bromphenol blue). 18 µl of this mixture were used to quantify the amount of recruited SNAP25 by
951 SDS PAGE followed by Coomassie Staining and Silver Staining. Using the Fiji software (Image J based)
952 the Coomassie stained band intensity of syntaxin-1A or VAMP2 or Syt1, respectively, were
953 determined and normalized to the respective mean. Subsequently, Silver Staining was used to
954 quantify the band intensities of SNAP25, and these values were normalized to the intensities of the
955 relative protein (e.g. Syntaxin-1A) based on the Coomassie Staining. From this, the ratios between
956 the SNAP25 mutants and wild type were determined.

957 *Temperature-dependent dissociation of v-/t-SNARE complexes in SDS*

958 SNARE complex stability was determined as described previously (Schupp et al., 2016). Briefly,
959 preassembled full-length t-SNARE complexes (WT and mutants, 10 µM) were incubated with the
960 cytoplasmic domain of VAMP2 (30 µM) in 25 mM MOPS (3-(Nmorpholino) propanesulfonic acid)-
961 KOH, pH 7.4, 135 mM KCl, 1% Octyl ß-D-glucopyranoside, 1 mM Dithiothreitol, 10 mM TECEP (Tris(2-
962 carboxyethyl)phosphine hydrochloride)-KOH, pH 7.4, 1 mM EDTA-NaOH, pH 7.4 overnight at 0°C
963 and subsequently for 1 h at 25°C. Subsequently, 37 µl of reaction mixture (36 µg of total protein)
964 was diluted with 213 µl of 1x Laemmli buffer. 7.5 µl aliquots were incubated at the indicated
965 temperatures for 5 min. Samples were analyzed by SDS-PAGE (15% gels) and proteins were
966 visualized by Coomassie brilliant blue staining. Temperature-dependent dissociation of the SNARE
967 complex was quantified by the appearance of free syntaxin-1A (35 kDa protein band released from
968 the high MW SNARE complex) using the Fiji (Image J based) software. Data were normalized to the
969 maximum value of a measurement set.

970 *Silver Staining*

971 Coomassie prestained gels from SDS PAGE were destained overnight in destain solution (30 %
972 methanol, 10 % acetic acid). Gels were gently washed 30 minutes with 10% ethanol and one minute
973 with 0.02% sodium thiosulfate. After short washing with deionized water, gels were stained (0.03%
974 paraformaldehyde, 0.002% silver nitrate) for 15 minutes and again quickly washed with water.
975 Developing solution (0.06% sodium carbonate, 0.018% paraformaldehyde, 0.0002% sodium
976 thiosulfate) was applied to the gels until protein band intensities were satisfactorily stained. The
977 reactions were stopped by replacing the staining solution with 0.07% acetic acid. Gels were scanned
978 and quantified by using the Fiji (Image J based) software.

979 *Atomistic molecular dynamics simulations*

980 Simulations were carried out at the atomic level using classical molecular dynamics (MD). We used
981 the CHARMM36m force field for protein, CHARMM TIP3P for water, and the standard CHARMM36
982 for ions (Huang et al., 2017). GROMACS 2022 software package was used for performing these
983 simulations (Abraham et al., 2015). The 3D structure of SNAP25 wild-type protein was generated
984 through AlphaFold2, utilizing the first ranked structure obtained using the default settings of
985 ColabFold v1.5.2 (Mirdita et al., 2022). Both wild-type and the V48F and D166Y mutant variants'
986 topology files and initial 3D structures, inclusive of water and ions, were produced via the CHARMM-
987 GUI web interface (Lee et al., 2016). All systems were hydrated, neutralized with counter ions, and
988 supplemented with 150 mM potassium chloride to replicate experimental conditions. Following the
989 CHARMM-GUI recommended protocol, systems were energy-minimized, equilibrated under NpT
990 conditions, temperature-stabilized at 310 K by the Nose-Hoover thermostat with a 1.0 ps time
991 constant (Evans and Holian, 1985), and maintained a constant 1 atm pressure via the Parrinello–
992 Rahman barostat, setting the time constant at 5.0 ps and an isothermal compressibility at 4.5×10^{-5}
993 bar^{-1} (Parrinello and Rahman, 1981). Isotropic pressure coupling was utilized in the simulations. The
994 Verlet scheme determined neighbor searches, updating once every 20 steps (Verlet, 1967).
995 Electrostatics were computed using the Particle Mesh Ewald method (Darden et al., 1993) with
996 parameters set to a 0.12 nm spacing, a tolerance 10^{-5}, and a 1.2 nm cut-off. Periodic boundary
997 conditions were applied in all three dimensions. Simulations ran with a 2 fs timestep until 800 ns
998 was achieved. As shown in Figure 9 – Figure Supplement 2 panel A, the structures used for protein
999 alignment represent the predominant structure from the most populated cluster. Clustering was
1000 based on the RMSD value using the GROMACS "gmx cluster" tool and the Gromos algorithm (Daura
1001 et al., 1999), setting an RMSD cut-off for neighbor structures at 0.6 nm (Legrand et al., 2020). The
1002 RMSD analysis was conducted using the average structure of the wild-type as a reference.
1003 Interaction energies, including both short-range Coulomb and Lennard-Jones forces, were
1004 computed throughout the 800 ns trajectory. The autocorrelation function of the data indicated that
1005 correlations diminished substantially after approximately 6 ns. Given this reduced correlation, we
1006 adopted a block size of 200 ns to ensure statistical independence between blocks.

1007 All structures generated using AlphaFold2, as well as the initial structures and topology files for
1008 atomistic molecular dynamics simulations, were deposited to ZENODO (DOI:
1009 10.5281/zenodo.10051665)

1010 *Statistical analysis*

1011 Graphs (bar and line) display mean ± SEM with all points displayed, except when otherwise noted;
1012 for electrophysiological experiments *n* denotes the number of cells recorded and is given in the
1013 legends. For *in vitro* experiments the number of biological replicates was 3 unless stated otherwise
1014 in the legends. Statistics were performed using GraphPad Prism 9. Unless otherwise noted,
1015 statistical differences between several groups were determined by one-way ANOVA; post-test was
1016 Dunnett's test comparing to the WT condition as a reference, unless otherwise noted. Equal
1017 variance of groups was tested by the Brown-Forsythe test; in case of a significant test, the Brown-
1018 Forsythe ANOVA test, which does not assume equal variances, was used instead. Kruskal-Wallis test
1019 was used in cases, where the data structure contained many identical values (zeros). Pairwise testing
1020 was carried out using unpaired t-test, or Welch's t-test in case of significantly different variances as
1021 determined by an F-test. The test is mentioned in the Figure legend; if no test is mentioned, the
1022 difference was not significantly different. Significance was assumed when $p<0.05$ and the level of
1023 significance is indicated by asterisk: * $p < 0.05$, ** $p < 0.01$, *** $p < 0.001$, **** $p < 0.0001$.

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1033

1034 **Table 1.**

Mean±SEM	**WT**	**V48F**	**WT**	**D166Y**	***Syt1* WT**	***Syt1* KO**
$\mathbf{k_1}$ [vesicles/s]	385.6 ±52.2	79.87 *** ±12.5	457.4 ±77.4	37.68 **** ±7.33	1227 ±189	646 * ±114
$\mathbf{k_{-1}}$ [1/s]	0.0903 ±0.0091	0.0605 # ±0.011	0.1114 ±0.012	0.0294 **** ±0.0122	0.140 ±0.016	0.114 ¤ ±0.013
$\mathbf{k_f}$ [1/s]	0.000844 ±0.000178	0.0164**** ±0.00230	0.000398 ±0.000077	0.03522**** ±0.00352	0.000235 ±0.000043	0.00286*** ±0.00062

1035

1036 **Estimated parameters affecting the size of the RRP.** Displayed is mean ±SEM. Two-sample t-test or
1037 Welch's t-test comparing mutant to WT: *p<0.05; ***p<0.001; ****p<0.0001, # non-significant
1038 (p=0.125), ¤ non-significant (p=0.210).

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1331 **Figure Legends**

1332 **Figure 1 – Localization of three pathogenic mutations in SNAP25**
1333 **A** Schematic of the neuronal SNARE complex interacting with C2B domain of synaptotagmin-
1334 1 (Syt1; not to scale) via the primary interface. Position of the I67N mutation in the first SNARE
1335 domain of SNAP25 is depicted by an asterisk.
1336 **B** Interaction site of the C2B domain of Syt1 and SNAP25. Syt1 interacts with SNAP25 both
1337 electrostatically (region I and II) and within the hydrophobic patch (HP patch)(Zhou et al., 2015).
1338 **C** Position of the disease-linked mutations V48F (hydrophobic patch) and D166Y (region I) in
1339 the SNARE complex.
1340
1341 **Figure 2** – **Pathogenic SNAP25 mutations compromise neuronal viability, but not synaptogenesis**
1342 **A** SNAP25 V48F and D166Y mutations are similarly expressed as the WT SNAP25 protein.
1343 EGFP-SNAP25 was overexpressed in neurons from CD1 (wildtype) mice; both endogenous and
1344 overexpressed SNAP25 are shown. Valosin-containing protein (VCP) was used as loading control.
1345 **B, C** Quantification of EGFP-SNAP25 (B) and endogenous SNAP25 (C) from Western Blots (as in
1346 A). Displayed are the intensity of EGFP-SNAP25 or endogenous SNAP25 bands, divided by the
1347 intensity of VCP bands, normalized to the WT situation (n = 3 independent experiments). The
1348 expression level of mutants was indistinguishable from expressed WT protein (ANOVA).
1349 **D** Representative images of control (WT) and mutant (V48F, D166Y) hippocampal neurons
1350 stained by dendritic (MAP2) and synaptic (vGlut1) markers. Displayed is MAP2 staining, representing
1351 the cell morphology, in inserts MAP2 staining is depicted in red and vGlut staining in cyan. The scale
1352 bar represents 50 µm.
1353 **E** Number of synapses per neuron in WT and mutant cells.
1354 **F** Total dendritic length of WT and mutant neurons.
1355 **G** Cell viability represented as the number of neurons per glia island. ****p <0.0001, **p
1356 <0.01, *p<0.05, Brown-Forsythe ANOVA test with Dunnett's multiple comparisons test.
1357
1358 **Figure 3 –V48F and D166Y mutations increase miniature EPSC frequency**
1359 **A,D,G** Example traces of mEPSC release for WT, mutant and 1:1 co-expression of WT and mutant
1360 SNAP25, or (**G**) Syt1 WT and KO.
1361 **B, E** The mEPSC frequencies were increased in both V48F and D166Y mutants and co-expressed
1362 conditions (V48F: n = 49, 47, 48 for WT, coexpressed and mutant conditions, respectively; D166Y: n
1363 = 54, 43, 50). ****p <0.0001, ***p <0.001, Brown-Forsythe ANOVA test with Dunnett's multiple
1364 comparisons test.
1365 **C, F** mEPSC amplitudes were on average increased by the V48F and D166Y mutations; this was
1366 significant for the V48F. *p <0.05, ANOVA with Dunnett's multiple comparison test.
1367 **H, I** Syt1 WT and KO data (Syt1: n = 28, 26 for the WT and KO condition). The mEPSC frequencies
1368 and amplitudes were increased and decreased in the KO, respectively. ****p <0.0001, Welch's t-
1369 test, *p<0.05, unpaired t-test.
1370
1371 **Figure 4 – V48F and D166Y mutations reduce the amplitude of the eEPSC**

1372 **A, E, I** Example evoked excitatory post-synaptic currents (eEPSC) for WT, SNAP25 mutants and co-
1373 expressed WT/mutants, or (**I**) Syt1 WT and KO.
1374 **B, F, J** eEPSC amplitude was decreased by both SNAP25 mutations (V48F: n = 50, 50, 45 for WT,
1375 co-expressed and mutant conditions, respectively; D166Y: n = 56, 35, 44) and by Syt1 KO (Syt1: n =
1376 19, 26 for the WT and KO condition). SNAP25 mutations: ****p <0.0001, **p <0.01, Brown-Forsythe
1377 ANOVA test with Dunnett's multiple comparisons test; Syt1: ****p<0.0001, Welch's t-test.
1378 **C, G, K** Overall evoked charge after a single depolarization (V48F: n = 50, 45, 50 for WT, mutant
1379 and co-expressed conditions, respectively; D166Y: 56, 44, 35; Syt1: 19, 20 for WT and KO). SNAP25:
1380 *p <0.05, Brown-Forsythe ANOVA with Dunnett's multiple comparison test; Syt1: ****p<0.0001,
1381 Welch's t-test.
1382 **D, H, L** Fractional contribution of the synchronous release component to the overall charge (V48F:
1383 n = 50, 50, 45 for WT, co-expressed and mutant conditions, respectively; D166Y: 56, 35, 44; Syt1:
1384 19, 20 for WT and KO). SNAP25: ****p <0.0001, ***p <0.001, Brown-Forsythe ANOVA (V48F) or
1385 standard ANOVA (D166Y) with Dunnett's multiple comparisons test; Syt1: ****p<0.0001, Welch's t-
1386 test.
1387
1388 **Figure 5 – The apparent energy barrier for vesicle fusion is lowered by V48F and D166Y, but not**
1389 **by removing Syt1.**
1390 **A, F, K** Example traces for the WT, mutant and co-expressed condition. Each cell was stimulated
1391 by 0.25 M (in grey) and 0.5 M sucrose (in black or color).
1392 **B, G, L** The charge released by 0.25 M sucrose (V48F: n = 28, 30, 29 for WT, co-expressed and
1393 mutant conditions, respectively; D166Y: n = 33, 30, 35; Syt1: n = 23, 18 for WT and KO). Syt1: p<0.05,
1394 Welch's t-test.
1395 **C, H, M** The charge released by 0.5 M sucrose (V48F: n = 28, 30, 29 for WT, co-expressed and
1396 mutant conditions, respectively; D166Y: n = 33, 30, 35; Syt1: n = 23, 26 for WT and KO). SNAP25:
1397 ****p <0.0001, **p <0.01, *p<0.05, Brown-Forsythe ANOVA with Dunnett's multiple comparisons
1398 test; Syt1: p=0.0548, unpaired t-test.
1399 **D, I, N** The ratio of 0.25 and 0.5 M sucrose pool (V48F: n = 28, 30, 29 for WT, co-expressed and
1400 mutant conditions, respectively; D166Y: n = 33, 30, 35; Syt1: n = 23, 18 for WT and KO). SNAP25:
1401 ****p <0.0001, **p <0.01, ANOVA with Dunnett's multiple comparisons test; Syt1: *p < 0.05,
1402 unpaired t-test.
1403 **E, J, O** Release probability calculated by dividing the charge of an eEPSC with the 0.5 M sucrose
1404 pool (V48F: n = 24, 25, 22 for WT, co-expressed and mutant conditions, respectively; D166Y: n = 33,
1405 24, 30; Syt1, n = 16, 21 for WT and KO). SNAP25: ***p <0.001, ANOVA with Dunnett's multiple
1406 comparisons test; Syt1: ****p<0.0001, unpaired t-test.
1407
1408 **Figure 6 – Dissection of the RRP reduction in V48F and D166Y mutations.**
1409 **A** One-pool model of the Readily Releasable Pool (RRP). k_1 is the rate of priming (units
1410 vesicles/s), k_{-1} is the rate of depriming (s^{-1}), k_f is the rate of fusion (s^{-1}).
1411 **B** Estimation of the three parameters from the response to 0.5 M sucrose and a
1412 measurement of the spontaneous release rate.

1413 **C** Variance-mean analysis in 50 ms intervals during the sucrose application allows
1414 determination of the corrected baseline by back-extrapolation of a regression line to the variance
1415 of the baseline.
1416 **D** Normalized mEPSC frequency (k_f) for V48F, D166Y and Syt1 KO. (V48F: n = 23, 24 for WT
1417 and mutant conditions, respectively; D166Y: n = 19, 19; Syt1: n = 23, 26). Brown-Forsythe ANOVA
1418 test with Dunnett's multiple comparison test, testing the three mutant conditions against each
1419 other. ****p <0.0001, ***p <0.001.
1420 **E** Normalized RRP size for WT and mutant conditions, with indications of the effect of the
1421 mutant-induced changes in k_1, k_{-1}, and k_f on the RRP size.
1422
1423 **Figure 7 – SNAP25 V48F and D166Y mutations change short-term plasticity towards facilitation.**
1424 **A, E** eEPSCs in response to 50 APs at 40 Hz recorded in 4 mM extracellular Ca^{2+} (V48F: 27, 17, 15
1425 for WT, co-expressed and mutant conditions, respectively; D166Y: 27, 18, 16). Inserts: Normalized
1426 eEPSC amplitudes demonstrating facilitation of mutant conditions. ****p<0.0001; **p<0.01, Brown-
1427 Forsythe ANOVA with Dunnett's multiple comparison test.
1428 **B, F** Priming rate calculated as the slope of a linear fit to the cumulative evoked charges during
1429 the last part of stimulation (V48F: 27, 17, 15 for WT, co-expressed and mutant conditions,
1430 respectively; D166Y: 27, 18, 16). *p <0.05, ANOVA (V48F) or Brown-Forsythe ANOVA (D166Y) with
1431 Dunnett's multiple comparisons test.
1432 **C, G** RRP calculated by back-extrapolation of a linear fit to the cumulative evoked charges during
1433 the last part of stimulation (V48F: 27, 17, 15 for WT, co-expressed and mutant conditions,
1434 respectively; D166Y: 27, 18, 16). **p <0.01, *p <0.05, ANOVA (V48F) or Brown-Forsythe ANOVA
1435 (D166Y) with Dunnett's multiple comparisons test.
1436 **D, H** Release probability calculated as the charge of the first evoked response divided by the RRP
1437 obtained by back-extrapolation (V48F: 27, 17, 15 for WT, co-expressed and mutant conditions,
1438 respectively; D166Y: 27, 18, 16). ***p<0.001; **p <0.01, ANOVA (V48F) or Brown-Forsythe ANOVA
1439 (D166Y) with Dunnett's multiple comparisons test.
1440
1441 **Figure 8 – Pathogenic SNAP25 mutations affect synaptotagmin-1 interaction and fusion rates *in*
1442 *vitro*.**
1443 **A** In the presence of SDS, SNAP25 I67N containing v-/t-SNARE complexes were more sensitive
1444 to temperature-dependent dissociation. Shown are mean ± SEM (n = 3) for SNARE-complexes
1445 including SNAP25 WT and the I67N, V48F and D166Y mutations.
1446 **B-C** *In vitro* Syt1/VAMP2 SUVs docking to t-SNARE GUVs was significantly reduced by SNAP25
1447 V48F, I67N and D166Y mutants either in absence (**B**) or presence (**C**) of $PI(4,5)P_2$. Fusion was blocked
1448 by performing the assay on ice. **** p<0.0001; *** p<0.001, ANOVA with Dunnett's multiple
1449 comparison test.
1450 **D-E** *In vitro* lipid mixing assays of VAMP/Syt1 SUVs with t-SNARE GUVs containing SNAP25 V48F,
1451 I67N, or D166Y mutants showed impaired membrane fusion in the absence (left) or presence (right)
1452 of complexin-II. Fusion clamping in the presence of complexin was selectively reduced by V48F and
1453 D166Y. Bar diagrams show lipid mixing just before (pre) and after (post) Ca^{2+} addition and at the end

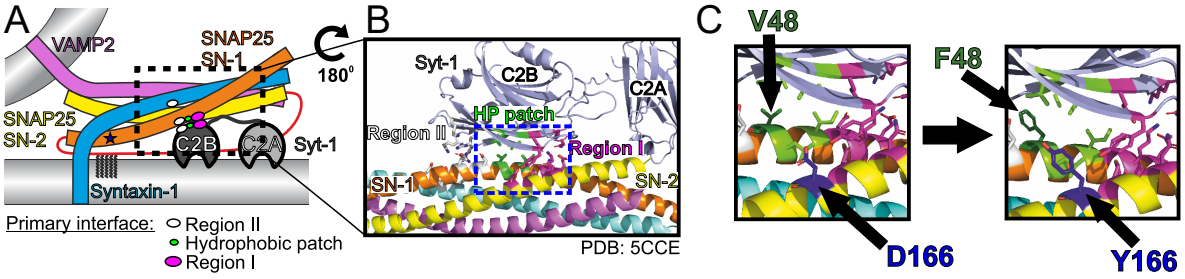
1454 of the reaction. Shown is mean ± SEM (n = 3). ****p<0.0001; **p <0.01, ANOVA with Dunnett's
1455 multiple comparisons test, comparing each mutation to the corresponding WT condition.
1456
1457 **Figure 9 – The D166Y mutation increases binding to its SNARE partners.**
1458 **A-C** *In vitro* lipid mixing assays of VAMP/Syt1 SUVs with syntaxin-1A GUVs in the presence of
1459 soluble SNAP25. V48F, and D166Y mutants showed impaired fusion clamping in the absence (left)
1460 or presence (right) of complexin-II; I67N (red) showed impaired Ca^{2+}-independent and Ca^{2+}-
1461 triggered fusion. Bar diagrams show lipid mixing just before (pre) and after (post) Ca^{2+} addition and
1462 at the end of the reaction. Mean ± SEM (n = 3). ****p<0.0001; ***p<0.001; **p <0.01; *p <0.05,
1463 ANOVA with Dunnett's multiple comparisons test, comparing each mutation to the corresponding
1464 WT condition.
1465 **D** SNAP25 D166Y showed enhanced interactions with SUVs carrying reconstituted syntaxin-
1466 1A (Stx-1), VAMP2, Syt1/VAMP2 or a SUV-mixture containing Syntaxin-1A and VAMP2/Syt1 in co-
1467 flotation assays, whereas V48F displayed weaker increases in interactions with SUVs containing
1468 syntaxin-1A, or Syt1/VAMP2. Shown is mean ± SEM on a logarithmic scale. *** p<0.001, ** p<0.01,
1469 * p<0.05, two-tailed one-sample t-test comparing to 1.
1470
1471 **Figure 10 – The I67N mutation inhibits spontaneous and evoked release.**
1472 **A** SNAP25 I67N is similarly expressed as the WT SNAP25 protein. EGFP-SNAP25 was
1473 overexpressed in neurons from CD1 (wildtype) mice; both endogenous and overexpressed SNAP25
1474 are shown. Valosin-containing protein (VCP) was used as the loading control.
1475 **B, C** Quantification of EGFP-SNAP25 (B) and endogenous SNAP25 (C) from Western Blots (as in
1476 A). Displayed are the intensity of EGFP-SNAP25 or endogenous SNAP25 bands, divided by the
1477 intensity of VCP bands, normalized to the WT situation (n = 3 independent experiments). The
1478 expression level of the I67N mutant was indistinguishable from WT protein (ANOVA).
1479 **D** Cell viability represented as the number of neurons per glial islet. ****p <0.0001, Brown-
1480 Forsythe ANOVA test with Dunnett's multiple comparisons test.
1481 **E** Representative image of mutant (I67N) hippocampal neurons stained for the dendritic
1482 marker MAP2 and the synaptic markers vGlut1. Displayed is MAP2 staining, representing the cell
1483 morphology, in inserts MAP2 staining is depicted in red and vGlut staining in cyan. The scale bar
1484 represents 50 µm.
1485 **F** Number of synapses per neuron in WT and mutant cells. The WT data are the same as in
1486 Fig. 2D-E because these experiments were carried out in parallel. The difference was tested using
1487 ANOVA between all conditions, which was non-significant.
1488 **G** Total dendritic length of WT and mutant neurons.
1489 **H** Example traces of mEPSC release for WT, mutant (I67N) and 1:1 co-expression of WT and
1490 SNAP25 mutant.
1491 **I** The mini frequency was decreased in both I67N mutant and the WT+I67N combination
1492 (I67N: n = 39, 36, 30 for WT, coexpressed and mutant). ****p <0.0001, ***p <0.001, Kruskal-Wallis
1493 with Dunn's multiple comparisons.
1494 **J** mEPSC amplitudes were unchanged by the I67N mutation.

1495 **K** Example evoked excitatory post-synaptic currents (eEPSC) for WT, mutant (I67N) and co-
1496 expressed WT and mutant.
1497 **L** eEPSC amplitude was decreased by the I67N mutations (I67N: n = 39, 37, 30 for WT, co-
1498 expressed and mutant conditions, respectively). SNAP25 mutations: ****p <0.0001, **p <0.01,
1499 Brown-Forsythe ANOVA test with Dunnett's multiple comparisons test.
1500 **M** Overall evoked charge after a single depolarization (I67N: 24, 10, 0 for WT, co-expressed
1501 and mutant conditions, respectively).
1502 **N** Fractional contribution of the synchronous release component to the overall charge (I67N:
1503 24, 10, 0 for WT, co-expressed and mutant conditions, respectively).
1504
1505 **Figure 11 – The I67N mutation has normal RRP size, but increased energy barrier for fusion.**
1506 **A, E** Example traces for the WT, mutant and co-expressed condition. Each cell was stimulated
1507 by 0.25 M (**A**, in grey) and 0.5 M sucrose (**A**, in color) or 0.375 M sucrose (**E**, in grey) and 0.75 M (**E**,
1508 in color).
1509 **B, F** The charge released by 0.25 M sucrose (**B**, I67N: n = 21, 15, 8 for WT, co-expressed and
1510 mutant conditions, respectively) or 0.375 M sucrose (**F**, I67N: n = 12, 16, 18; a few cells were
1511 stimulated with 0.35 M sucrose – shown with open symbols). **B**, **p<0.01; *p<0.05, Kruskal-Wallis
1512 test with Dunn's multiple comparison test; **F**, p=0.0339 Brown Forsythe ANOVA test; Dunnett's
1513 multiple comparison test, p=0.0571.
1514 **C, G** The charge released by 0.5 M sucrose (**C**, I67N: n = 21, 15, 8 for WT, co-expressed and
1515 mutant conditions, respectively), or 0.75 M sucrose (**G**, I67N: n = 13, 16, 18). **C**, **p<0.01, *p<0.05,
1516 Brown-Forsythe ANOVA with Dunnett's multiple comparisons test.
1517 **D, H** The ratio of the 0.25 M and 0.5 M sucrose pool (**D**, I67N: n = 21, 15, 8 for WT, coexpressed
1518 and mutant conditions, respectively), or the ratio of 0.375 and 0.75 M sucrose pool (**H**, n = 13, 16,
1519 18). **D**, * p<0.05, Kruskal-Wallis test with Dunn's multiple comparisons test. **H**, **** p<0.0001; ***
1520 p<0.001, ANOVA with Dunnett's multiple comparisons test.
1521 **I** eEPSCs in response to 50 APs at 40 Hz recorded in 2 mM extracellular Ca^{2+} (I67N: n = 23, 16,
1522 20 for WT, co-expressed and mutant conditions, respectively). Inserts: Normalized eEPSC
1523 amplitudes demonstrating facilitation of mutant conditions.
1524 **J** Normalized eEPSC amplitudes in response to 50 APs at 40 Hz recorded in 2 mM extracellular
1525 Ca^{2+}.
1526 **K** Paired pulse ratio at interstimulus interval 25 ms (I67N: n = 24, 14, 17 for WT, co-expressed
1527 and mutant conditions, respectively). *p<0.05, ANOVA with Dunnett's multiple comparison test.
1528
1529 **Figure 12 - Adding positive surface charges to the SNARE complex partly compensate for the I67N**
1530 **mutation.**
1531 **A** Example traces of mEPSC release for WT, I67N/E183K/S187K/T190K/E194K ('I67N/4K') and
1532 E183K/S187K/T190K/E194K ('4K') SNAP25. Data from the 4K mutation was obtained in a separate
1533 experiment and is shown for comparison, but statistical tests with 4K mutation data were not
1534 carried out.
1535 **B** The mini frequencies for the WT and I67N/4K are not significantly different; data from the
1536 4K mutation is shown for comparison (n = 19, 25, 13 for WT, I67N/4K and 4K, respectively).
1537 **C** Mini amplitudes remain unaffected by I67N/4K mutation.

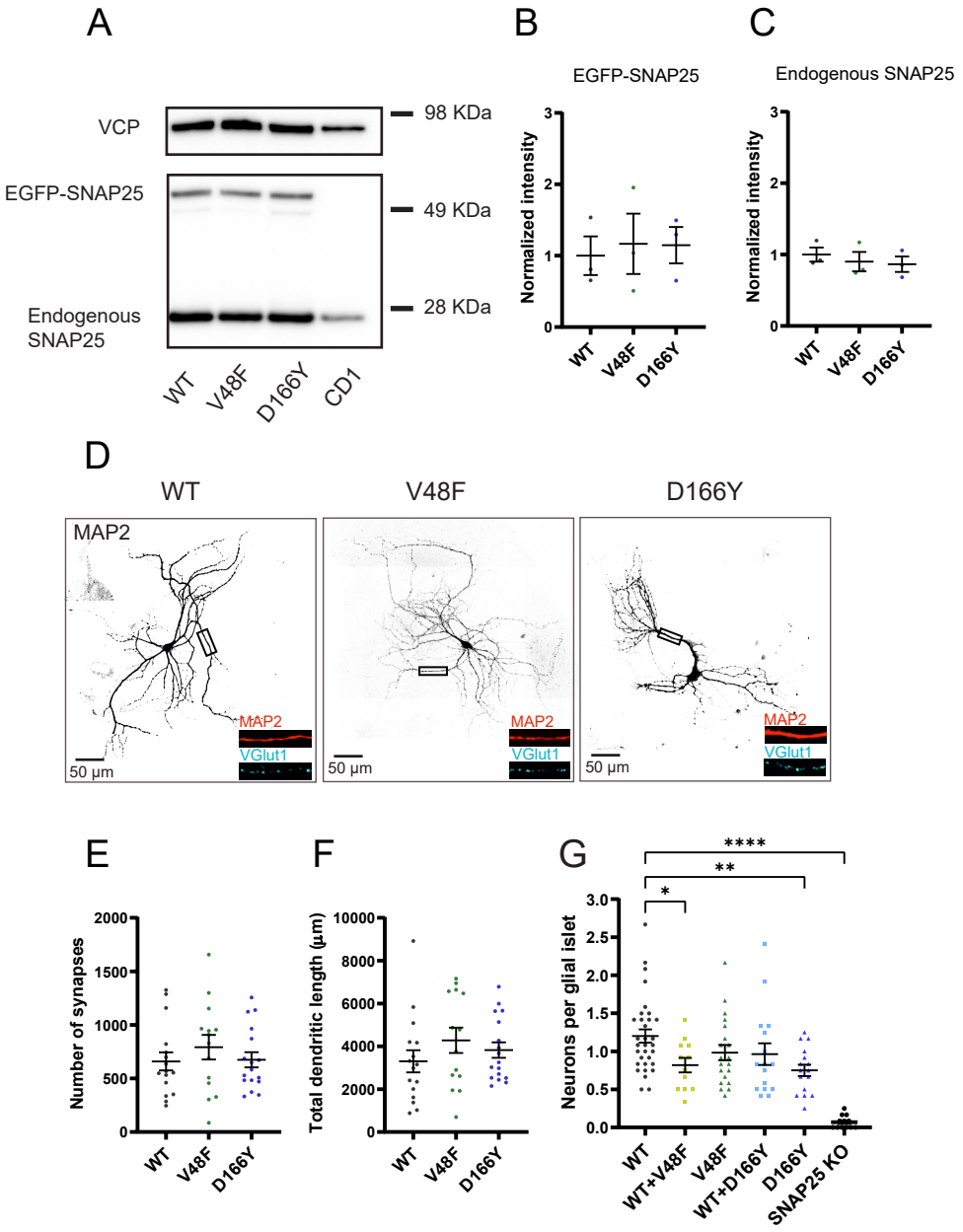
1538 **D-E** eEPSC examples (**D**) and amplitudes (**E**) for WT and I67N/4K; 4K is shown for comparison (n
1539 = 19, 25, 13 for WT, I67N/4K and 4K, respectively). ****p<0.0001 Mann-Whitney test.
1540 **F** Electrostatic triggering model (blue line; Ruiter et al., 2019) refitted to WT spontaneous
1541 and evoked data points (black points). Fitted parameters: rate 0.00029 s^{-1} at zero (0) charge (Z);
1542 fraction f=0.030; the maximum rate was fixed at 6000 s^{-1}. WT (black points), I67N, I67N/4K (red
1543 points): means of log-transformed data. The charge-values (Z, horizontal axis) for I67N and I67N/4K
1544 were found by interpolation in the model; the two spontaneous points (I67N, I67N/4K) are
1545 separated by 5.6 charges. For evoked release, rates were found by deconvolution and normalizing
1546 to $RRP_{0.5}$ (Ruiter et al., 2019). The Z-values for evoked release were found by interpolation in the
1547 model; the two mutants (I67N, I67N/4K) are separated by 5.9 charges.
1548
1549 **Figure 13 – Energy landscapes.**
1550 The energy landscapes of WT and mutants were calculated as explained in Materials and Methods
1551 and displayed to scale. Energy landscapes for D166Y (**A**), V48F (**B**) and *Syt1* KO (**C**) are shown at rest
1552 and are characterized by a higher priming barrier ("loss-of-function" phenotype), a destabilized RRP
1553 and a lower fusion barrier ("gain-of-function" phenotype). The I67N (**D**) is characterized by a higher
1554 fusion barrier ("loss-of-function" phenotype). The relative increase in the fusion barrier by the I67N
1555 mutation is higher during stimulation than at rest. Dotted lines represent energy levels for which
1556 less is known.
1557
1558 **Figure 4 – Figure Supplement 1. Kinetic parameters of evoked EPSCs.**
1559 **A** eEPSC (black trace), and integrated eEPSC (after multiplication with -1, red trace) with
1560 double exponential fit (green trace).
1561 **B** Zoom-in of eEPSC (black trace), and integrated eEPSC (after multiplication with -1, red trace)
1562 with double exponential fit (green trace). Equations for integration and double exponential function
1563 used for fit is given.
1564
1565 **Figure 4 – Figure Supplement 2. Kinetic parameters of eEPSCs.**
1566 **A, E, I** Synchronous release components (**A**, V48F: n = 50, 50, 45 for WT, co-expressed and mutant
1567 conditions, respectively; **E**, D166Y: n = 56, 35, 44; **I**, *Syt1*: 19, 20 for WT and KO). **A**: * p < 0.05, Welch's
1568 ANOVA with Dunnett's multiple comparison test, **E**: *** p <0.001, Brown-Forsythe ANOVA with
1569 Dunnett's multiple comparison test, **I**: **** p < 0.0001, Welch's unpaired t-test.
1570 **B, F, J** Asynchronous release components. **B**: ** p< 0.01; * p<0.05, Brown-Forsythe ANOVA with
1571 Dunnett's multiple comparison test.
1572 **C, G, K** Fast time constants. **C**: **** p< 0.0001; ** p<0.01, Brown-Forsythe ANOVA with Dunnett's
1573 multiple comparison test, **G**: **** p< 0.0001, Brown-Forsythe ANOVA with Dunnett's multiple
1574 comparison test, **K**: **** p < 0.0001, Welch's unpaired t-test.
1575 **D, H, L** Slow time constants.
1576
1577 **Figure 6 – Figure Supplement 1. Effect of sucrose stimulation on estimates of k_1 and k_{-1}.**
1578 The figure shows the effect of the fold-increase in fusion rate (*N*) induced by sucrose (abscissa) on
1579 the estimates of k_1 (**A**, **C**) or k_{-1} (**B, D**) using Eqs. 3, 4 and 5b and the values estimated for D166Y (**A**,
1580 **B**), V48F (**C**, **D**) and WT (Table 1). Previous data showed that 0.5 M sucrose increases the fusion rate

1581 by a factor ~5000 (Schotten et al., 2015). The plots show that the estimates in Table 1 are not strongly
1582 affected by small changes in the effect of sucrose upon k_f.
1583
1584 **Figure 7 – Figure Supplement 1. Train stimulations of V48F and D166Y in 2 mM Ca^{2+}.**
1585 **A, D** eEPSCs in response to 50 APs at 40 Hz recorded in 2 mM extracellular Ca^{2+} (V48F: 25, 18, 24
1586 for WT, co-expressed and mutant conditions, respectively; D166Y: 23, 15, 15). Inserts: Normalized
1587 eEPSC amplitudes of first five stimulations. * $p < 0.05$, *** $p < 0.001$, one-way ANOVA with Dunnett's
1588 multiple comparison test.
1589 **B, E** Priming rate calculated by as the slope of a linear fit to the cumulative evoked charges
1590 during the last part of stimulation (V48F: 24, 18, 24 for WT, co-expressed and mutant conditions,
1591 respectively; D166Y: 23, 15, 15).
1592 **C, F** RRP calculated by back-extrapolation of a linear fit to the cumulative evoked charges during
1593 the last part of stimulation (V48F: 24, 18, 24 for WT, co-expressed and mutant conditions,
1594 respectively; D166Y: 23, 15, 15).
1595
1596 **Figure 7 – Figure Supplement 2. Cumulative charges of V48F and D166Y trains in 4 mM Ca^{2+}.**
1597 **A, B** Cumulative charges obtained by integrating eEPSCs during 40 Hz trains. The slope of the
1598 linear part of the curve reports on the priming rate, which is reduced by the mutations. The back
1599 extrapolation of the linear fit to zero time reports on the RRP_{ev}, the part of the RRP which APs draw
1600 on, which is also reduced by mutation (V48F: n= 27, 17, 15 for WT, co-expressed and mutant
1601 conditions, respectively; D166Y: 27, 18, 16).

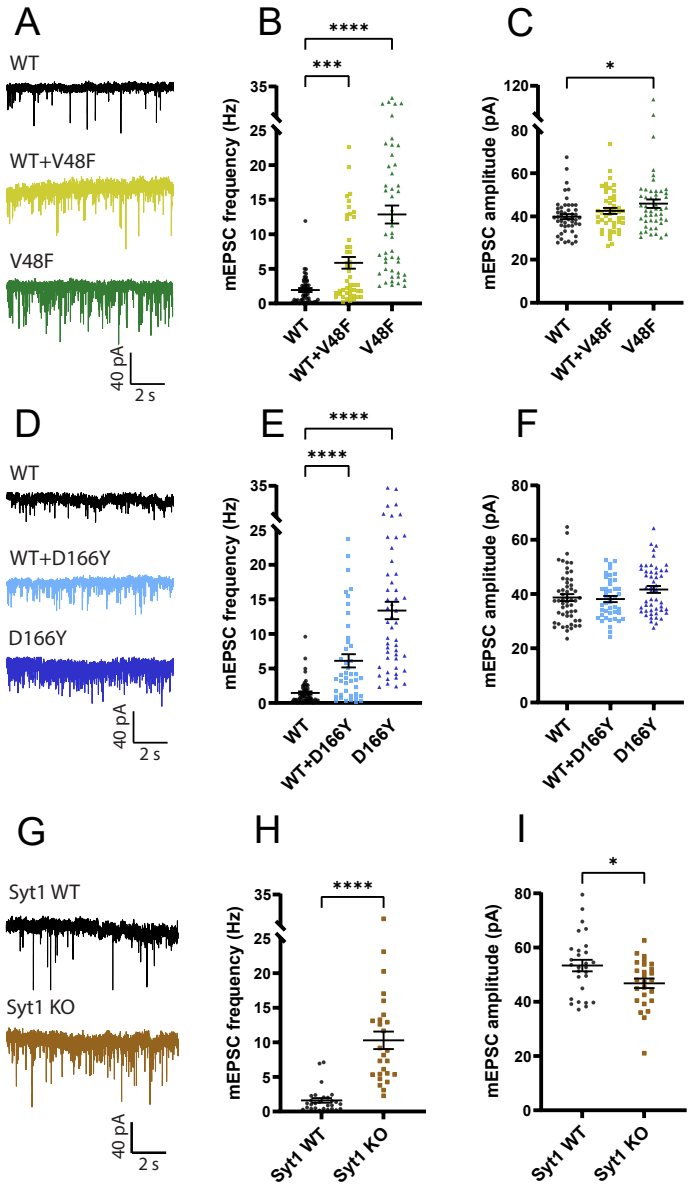
1602 **Figure 9 – Figure Supplement 1. Floatation assay.**
1603 Example Coomassie and silver stained gels demonstrating binding of SNAP25 WT and mutants to
1604 different populations of SUVs: Syntaxin-1 (Stx-1) and VAMP2/Syt1, Syntaxin-1 (Stx-1), VAMP2/Syt1,
1605 VAMP2, or Syt1 SUVs. Note increased binding of D166Y and V48F to most SUV populations, strongest
1606 for D166Y (quantified data in Fig. 9D).
1607
1608 **Figure 9 – Figure Supplement 2. Molecular dynamics simulations of mutants.**
1609 **A** Alignment of helices across the three systems (WT, V48F, and D166Y), reveals close
1610 correspondence. The structures displayed represent the most prevalent configurations from the
1611 dominant cluster observed during simulations.
1612 **B** Stability evaluation (RMSD) of the two helices across the three systems relative to WT's
1613 average structure during their simulations.
1614 **C** A detailed view of the region displaying residue pairs 48-52 and 162-166 on the structures.
1615 **D, E** Computed electrostatic (Coulomb) and van der Waals (Lennard-Jonson, LJ) interactions for
1616 residue pairs 48-52 (D) and 162-166 (E) calculated in 200 ns blocks within the 800 ns trajectory (see
1617 Materials and Methods). The bar plots represent the means calculated using the block averaging
1618 method, while each block's average is depicted as a dot alongside. The error bars capture the
1619 standard error of the mean, premised on treating each block as an independent measure (i.e., n=4).
1620 Notably, for D166Y (panel E, blue bar), the interaction energy is considerably more negative,
1621 indicating a stronger interaction compared to WT. * $p < 0.05$, ** $p < 0.01$, **** $p < 0.0001$, one-way
1622 ANOVA with Tukey HSD post-hoc tests.



Kádková et al., Fig. 1



Kádková et al., Fig. 2



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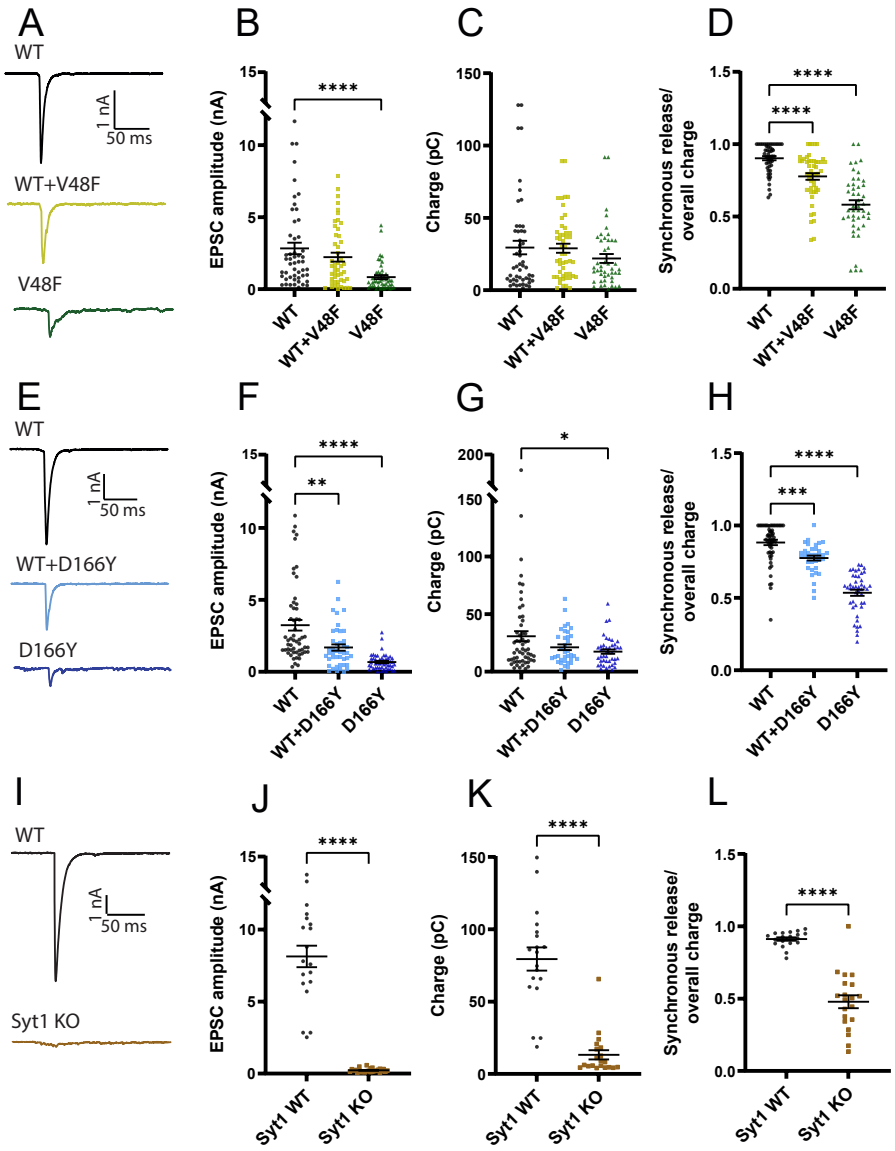

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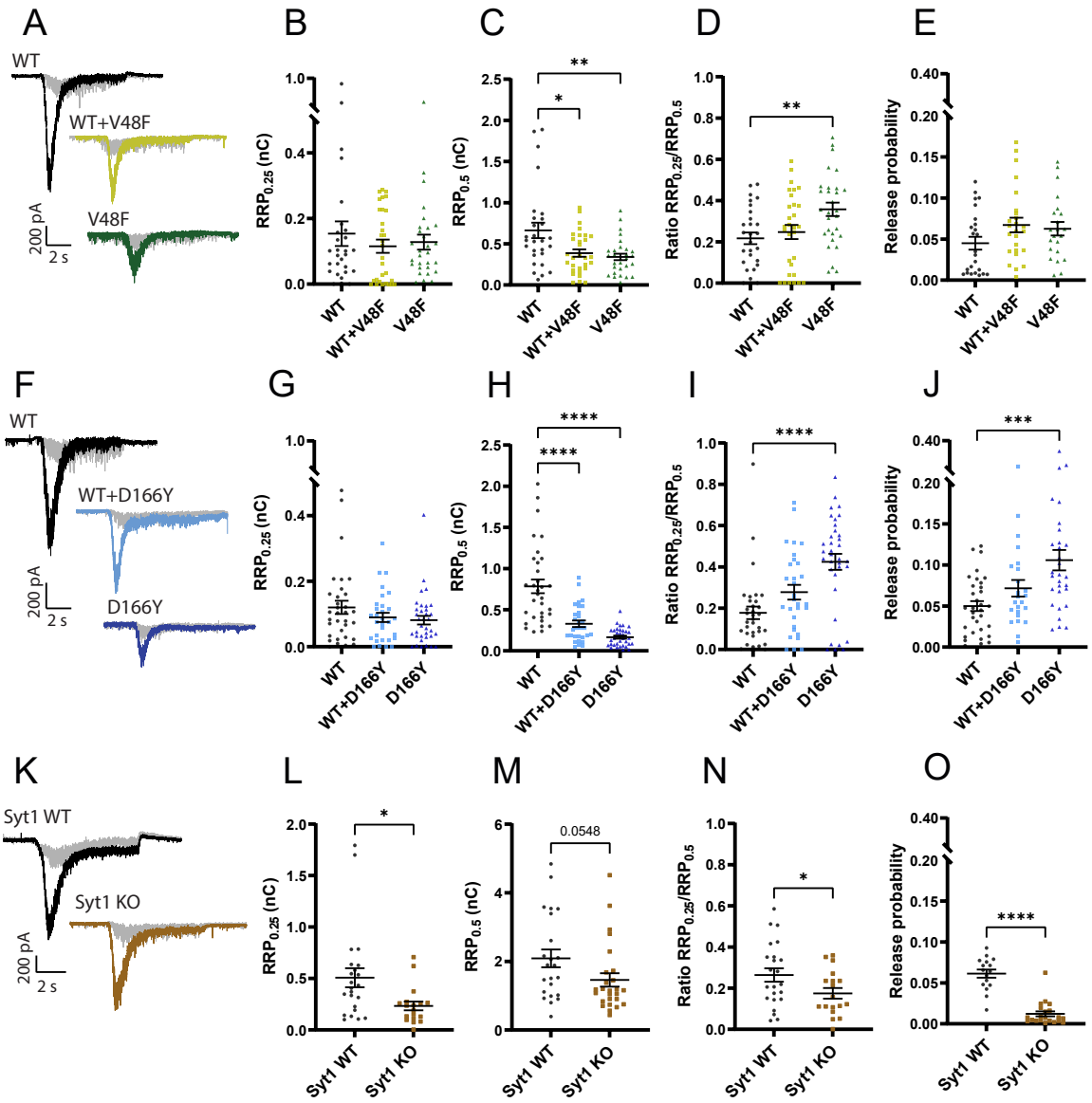

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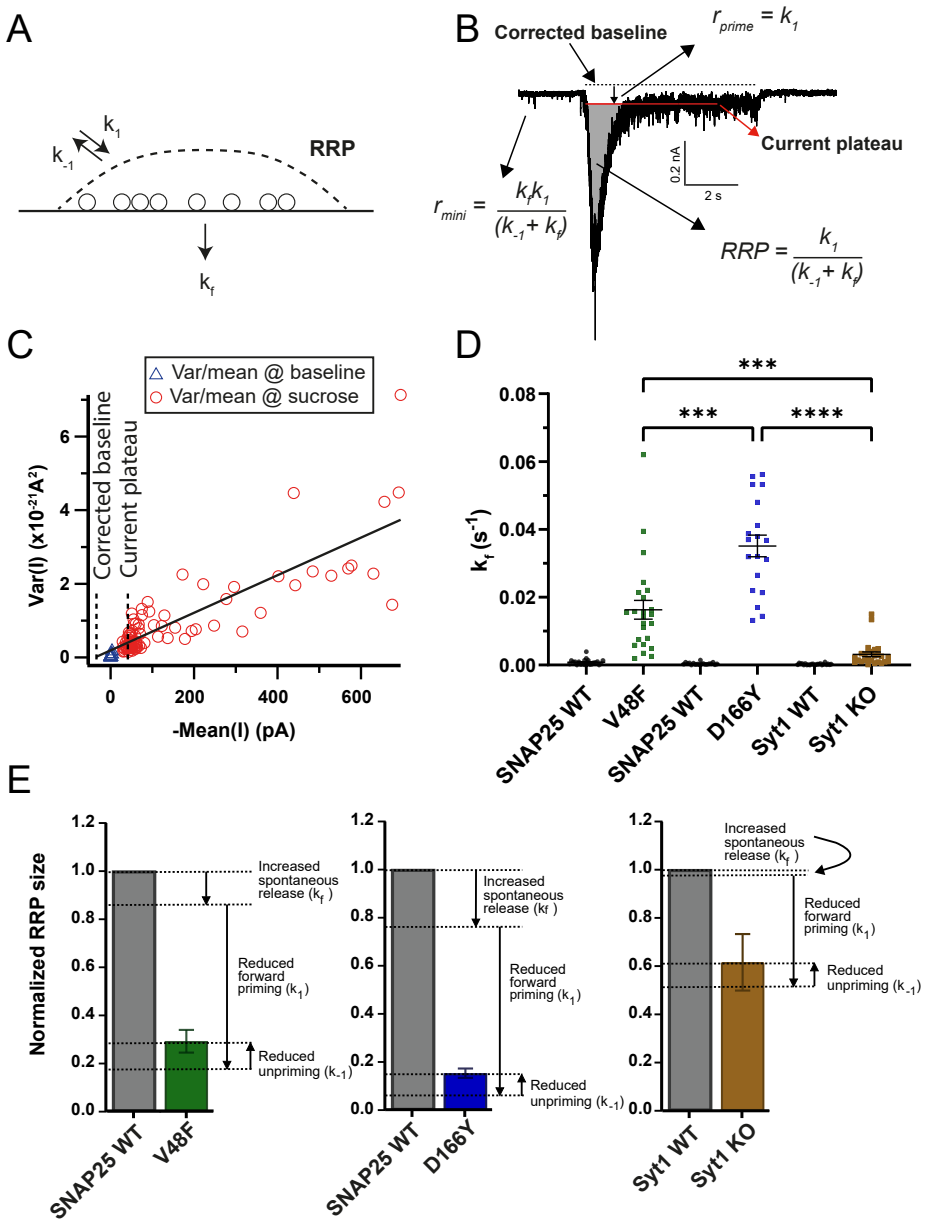

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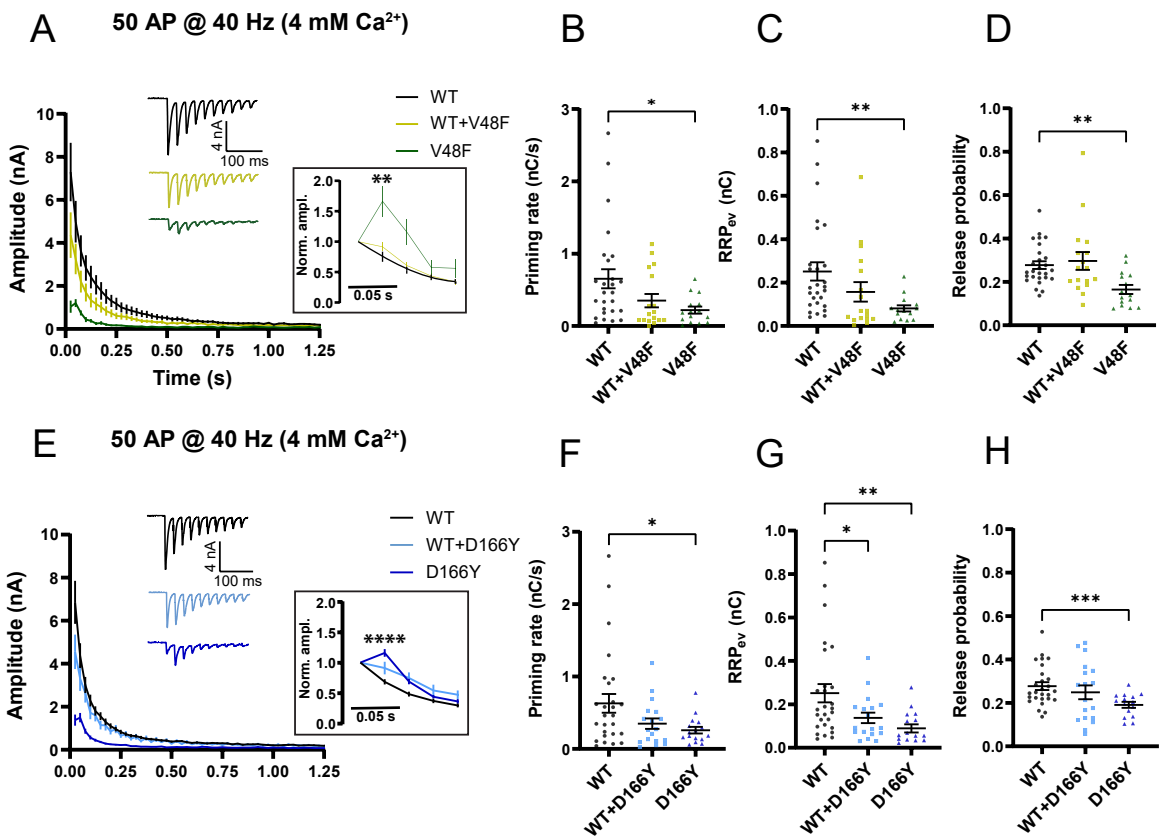

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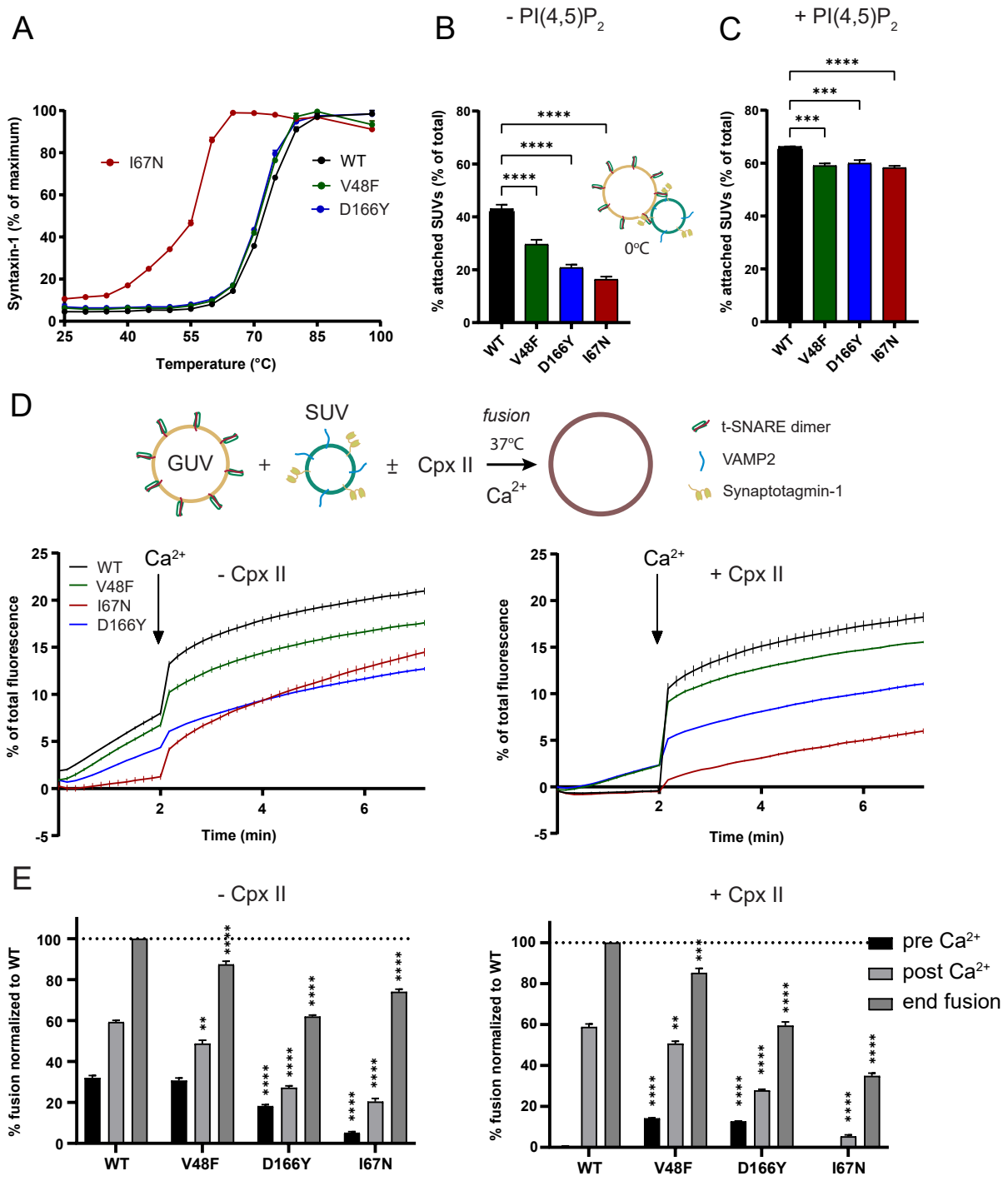

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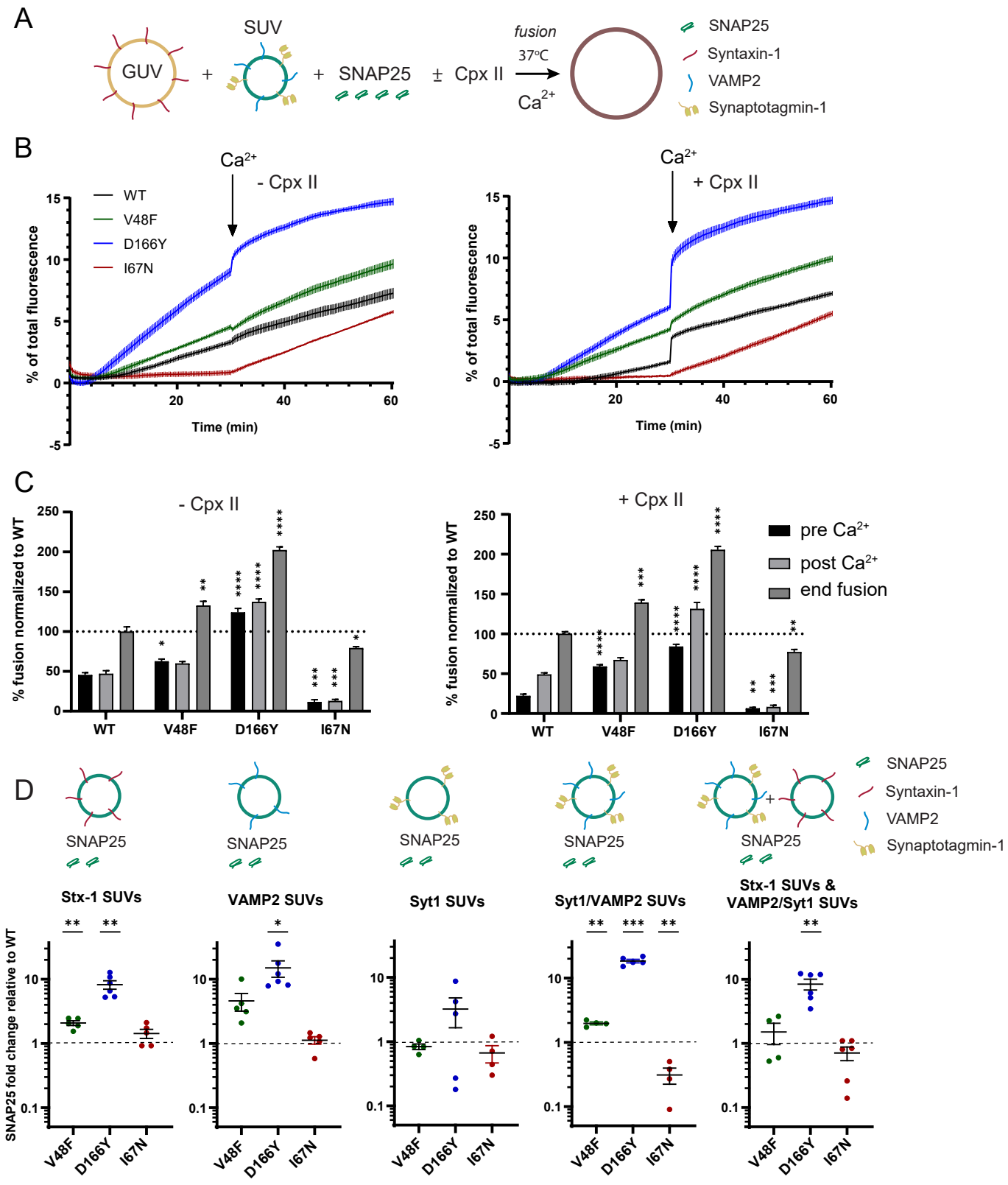

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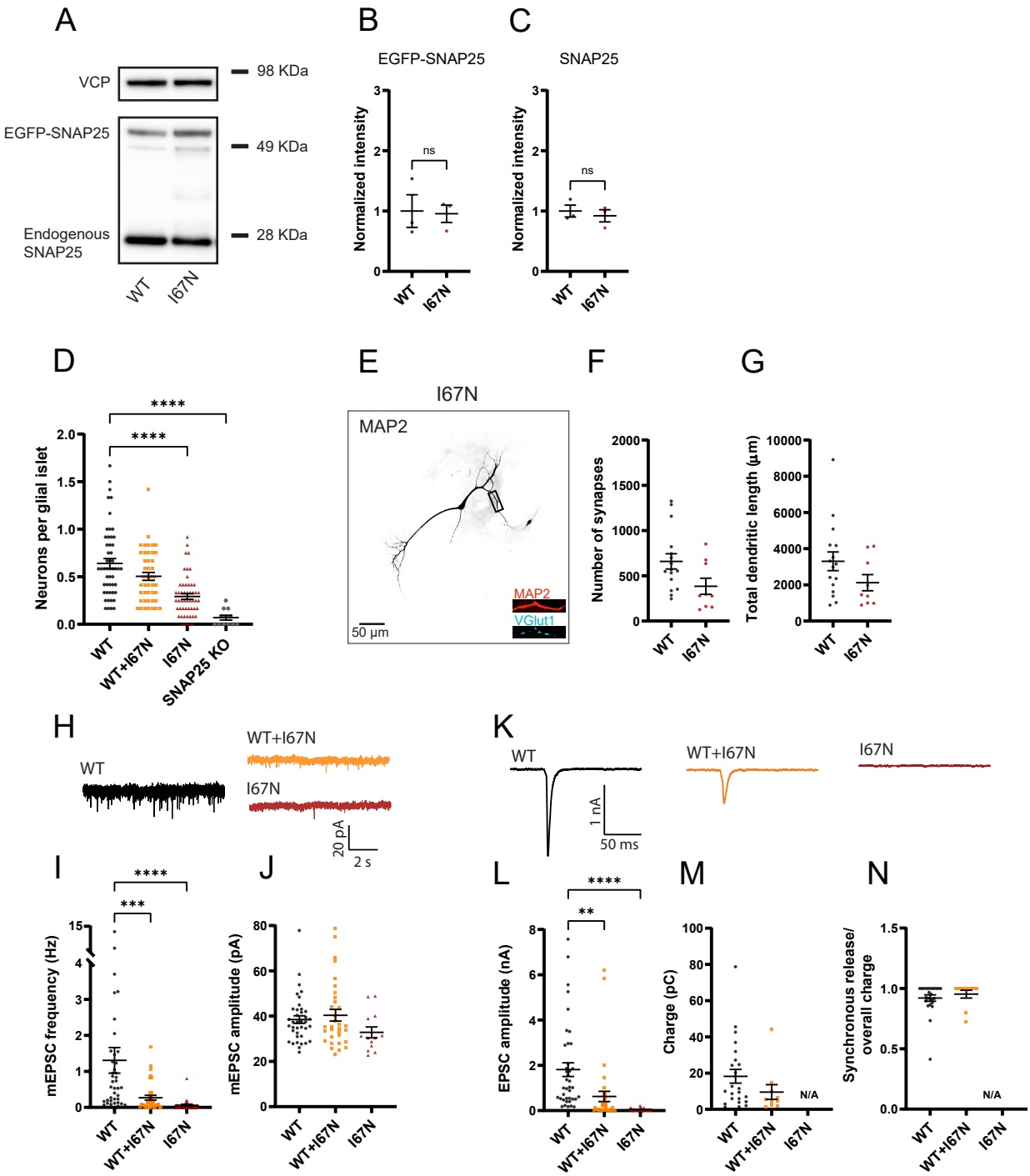

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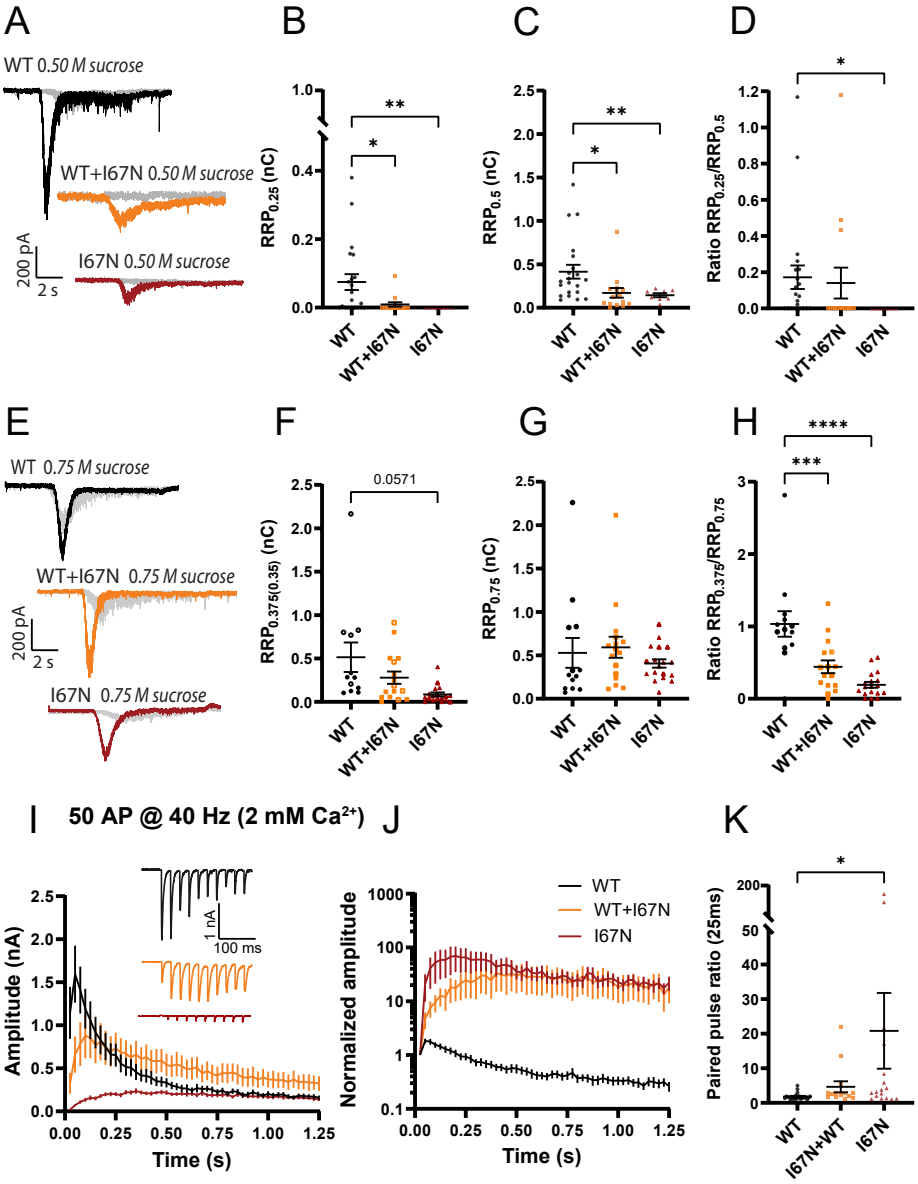

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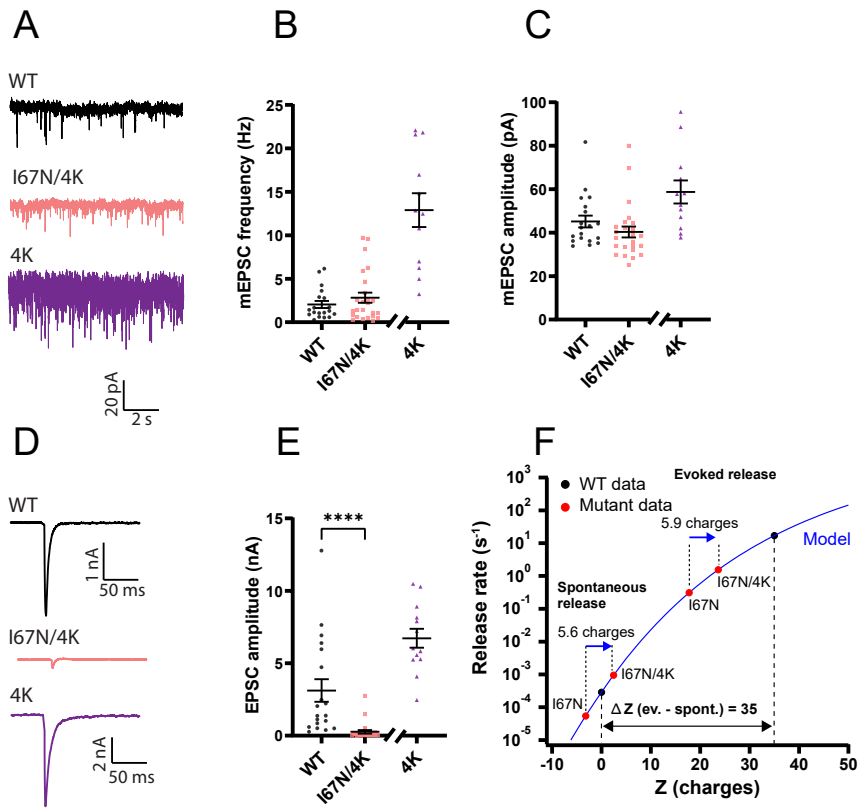

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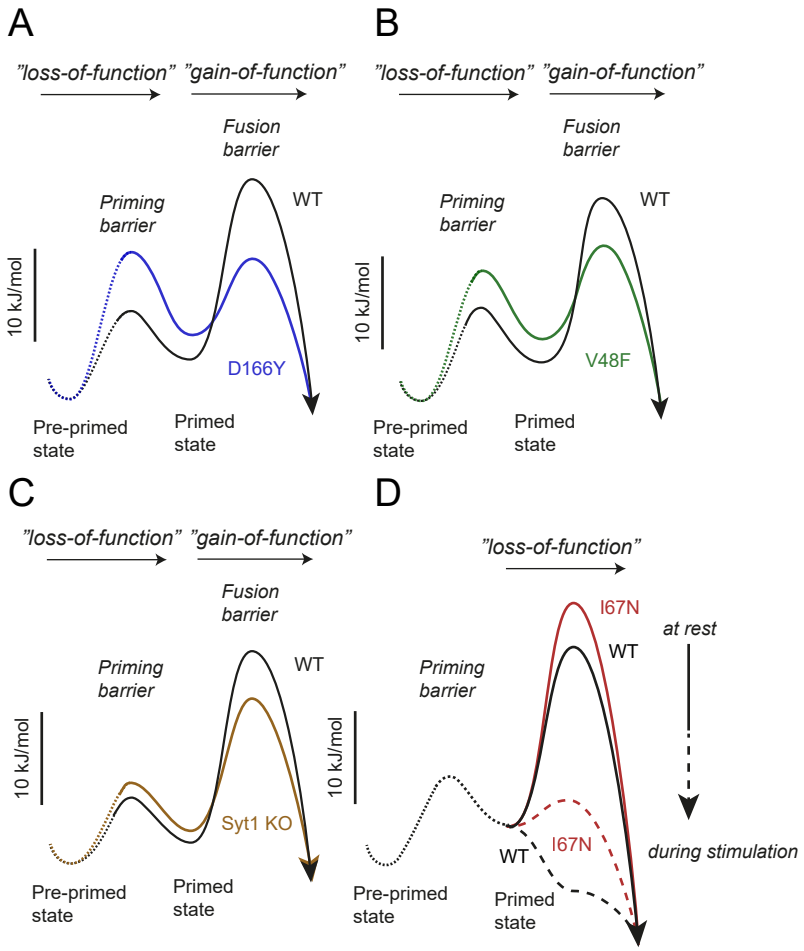

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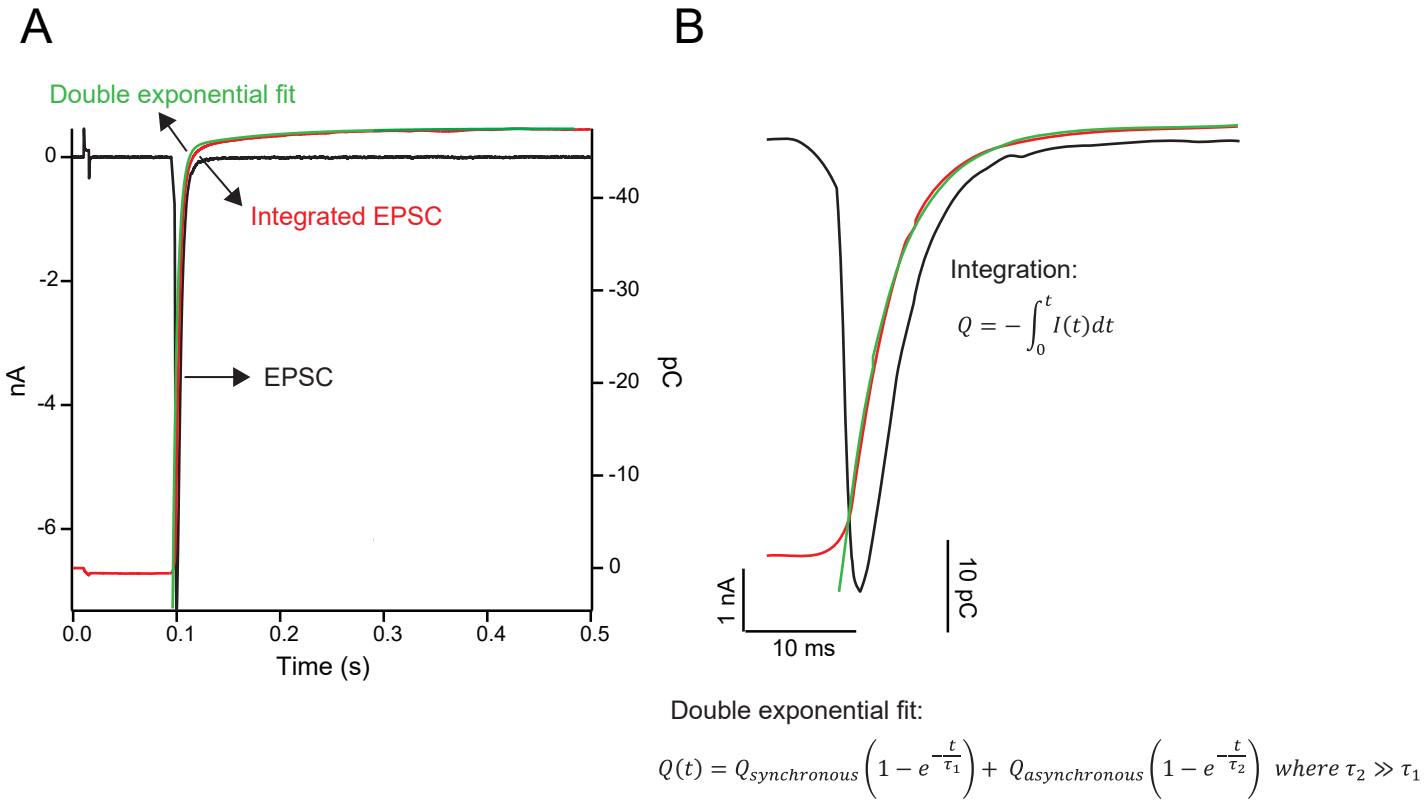

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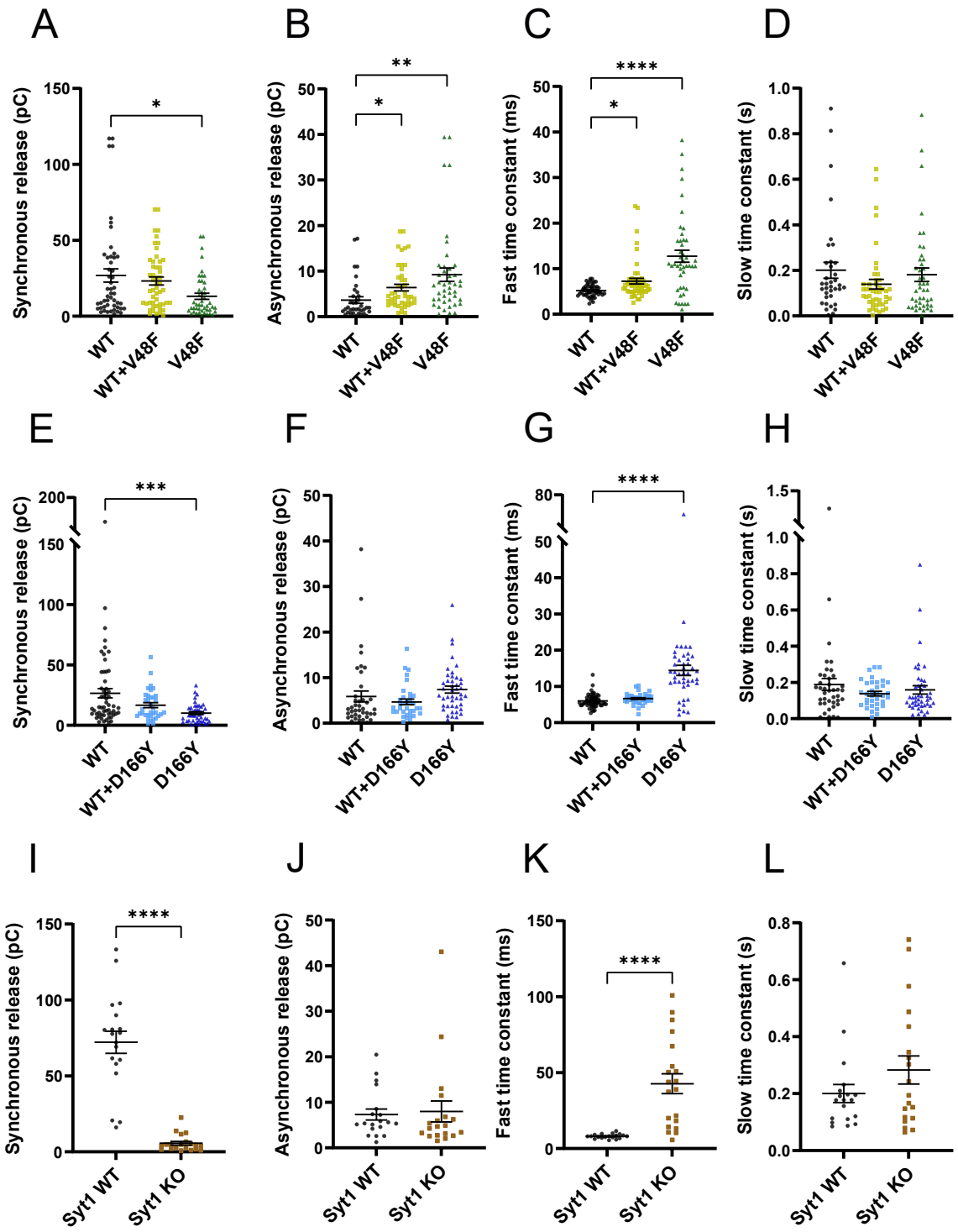

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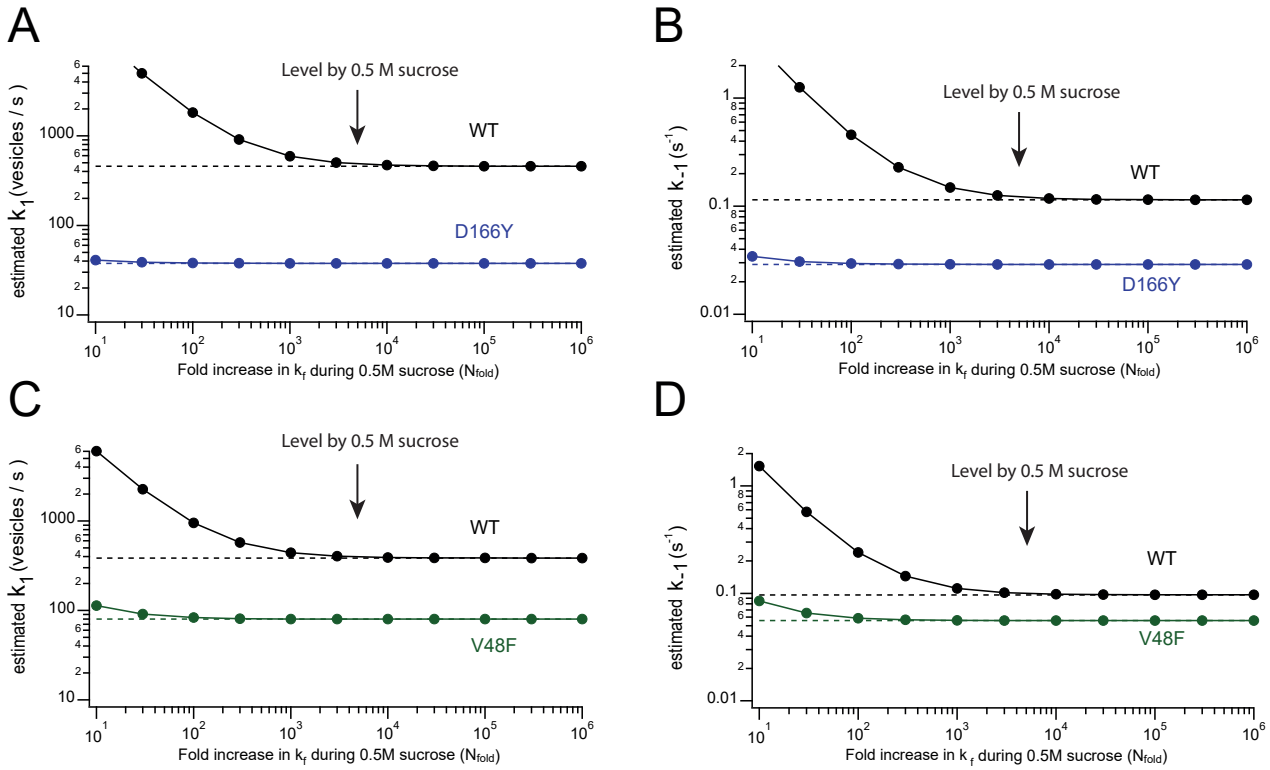

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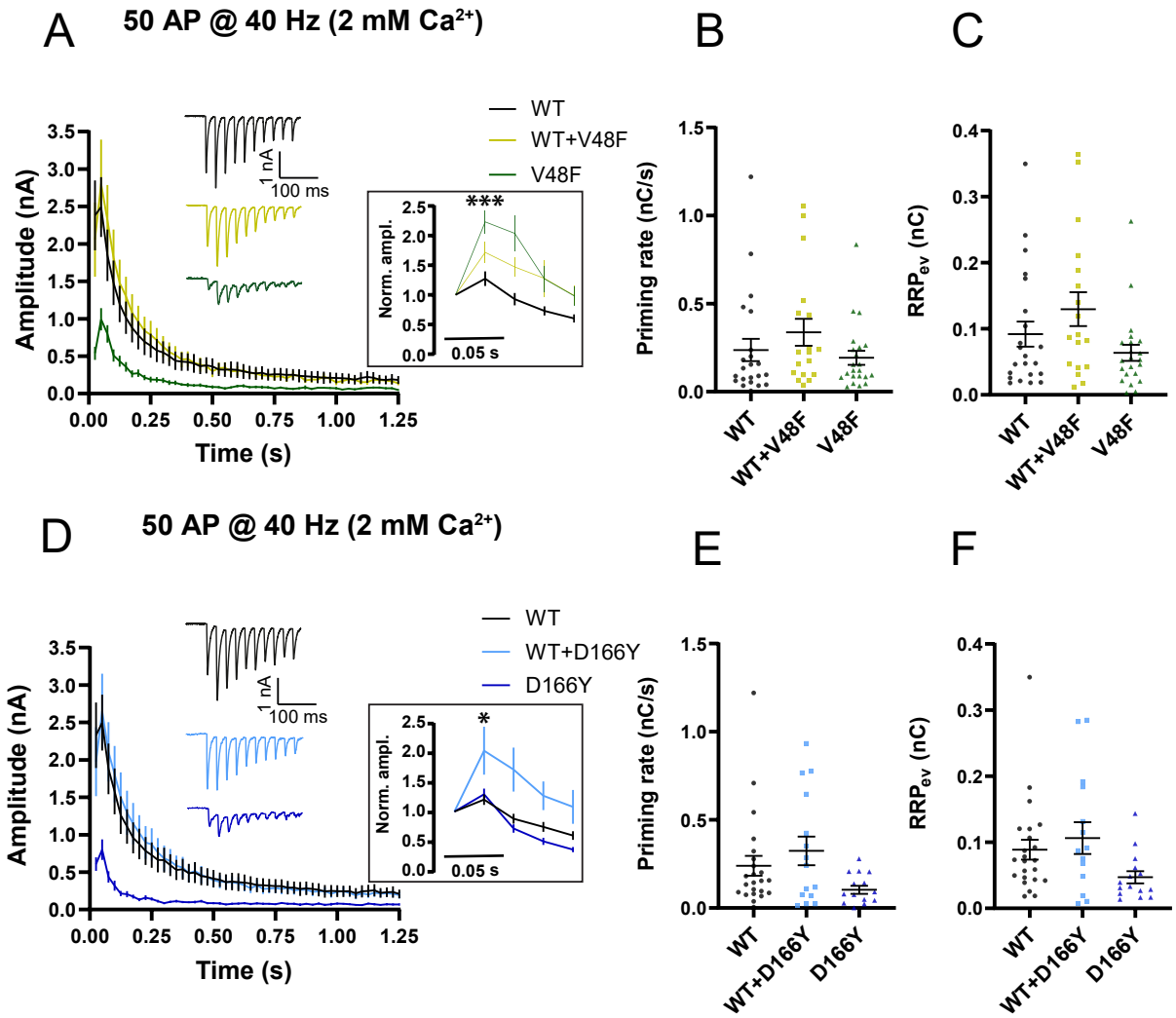

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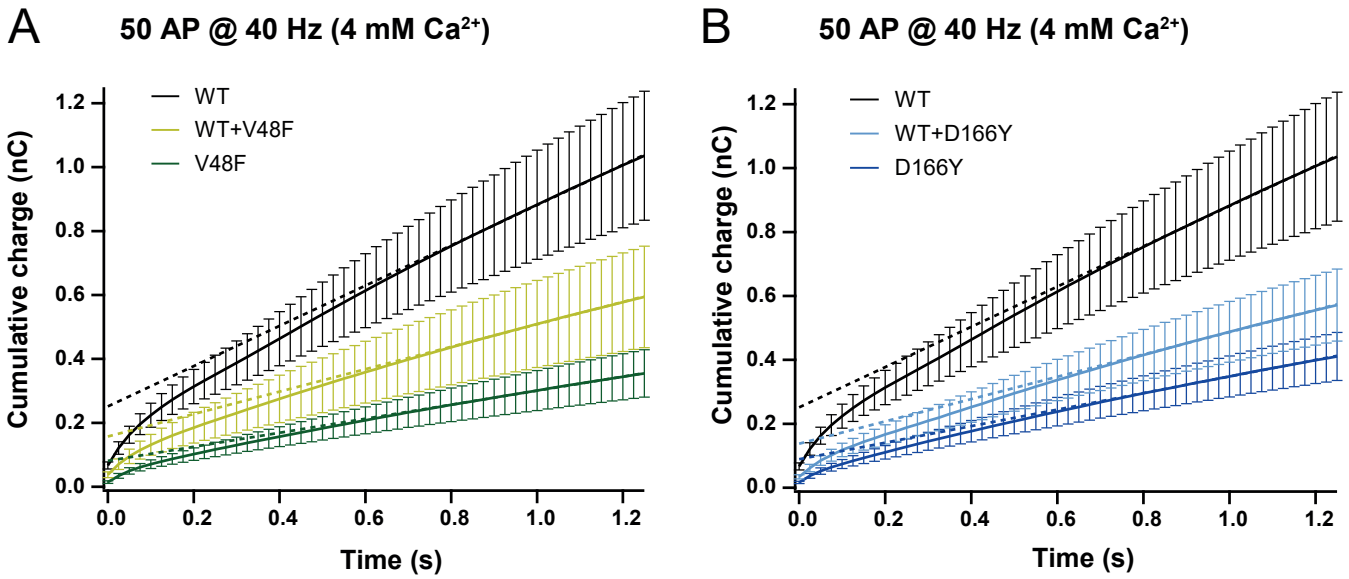

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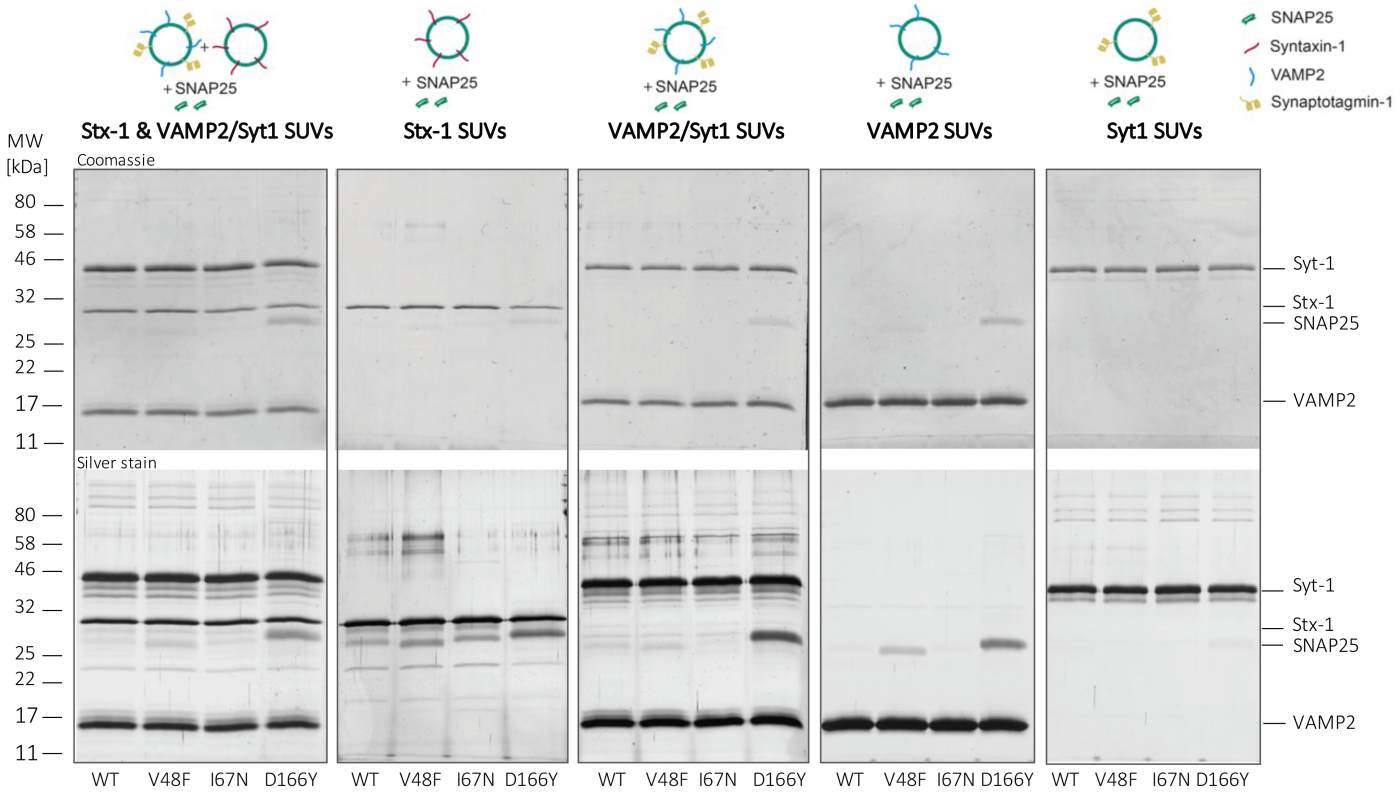

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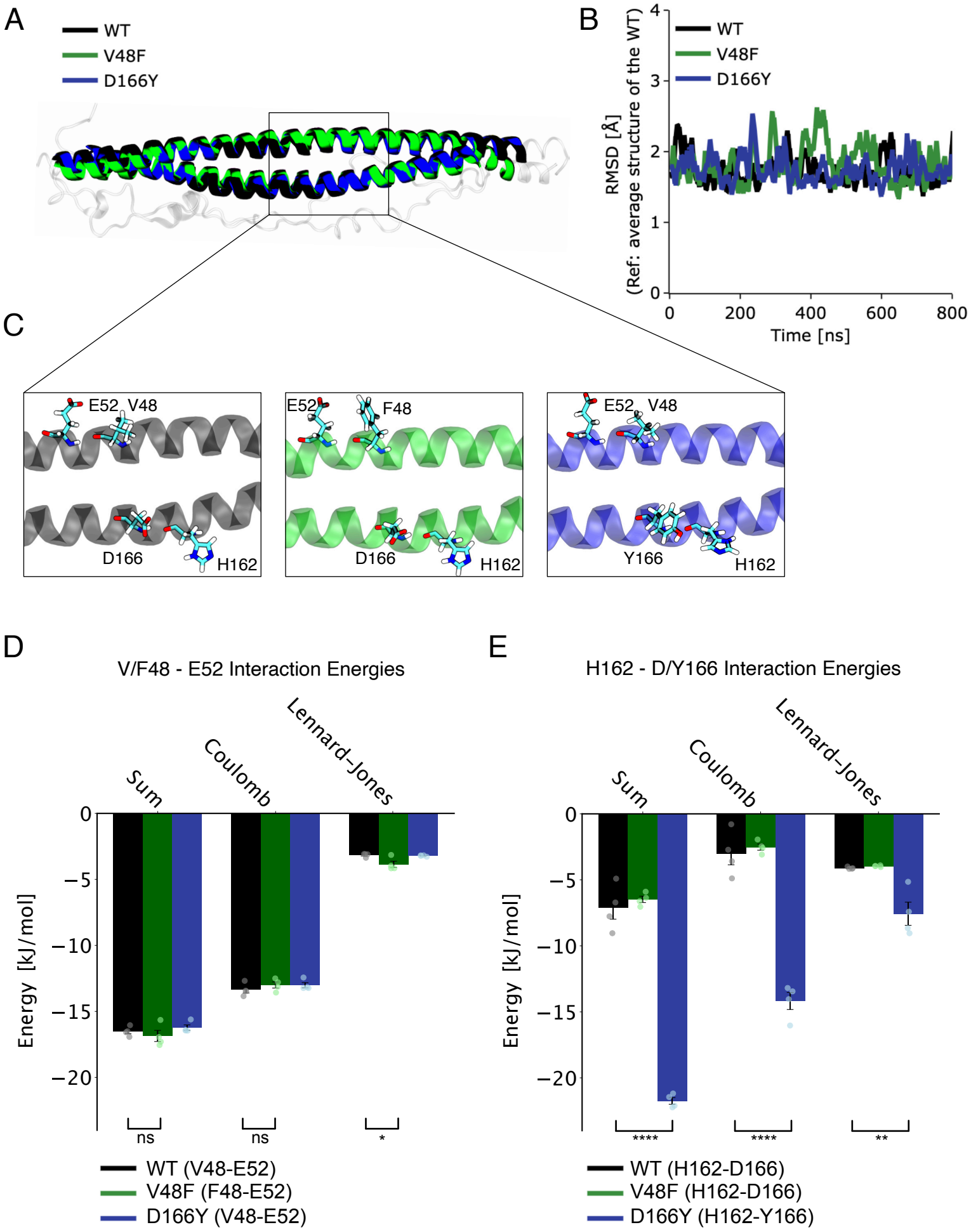

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