The stability of the primed pool of synaptic vesicles and the clamping of spontaneous neurotransmitter release relies on the integrity of the C-terminal half of the SNARE domain of Syntaxin-1A

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Abstract

The SNARE proteins are central in membrane fusion and, at the synapse, neurotransmitter release. However, their involvement in the dual regulation of the synchronous release while maintaining a pool of readily releasable vesicles remains unclear. Using a chimeric approach, we performed a systematic analysis of the SNARE domain of STX1A by exchanging the whole SNARE domain or its N- or C-terminus subdomains with those of STX2. We expressed these chimeric constructs in STX1-null hippocampal mouse neurons. Exchanging the C-terminal half of STX1's SNARE domain with that of STX2 resulted in a reduced RRP accompanied by an increased release rate, while inserting the C-terminal half of STX1's SNARE domain into STX2 lead to an enhanced priming and decreased release rate. Additionally, we found that the mechanisms for clamping spontaneous, but not for Ca^{2+}-evoked release, are particularly susceptible to changes in specific residues on the outer surface of the C-terminus of the SNARE domain of STX1A. Particularly, mutations of D231 and R232 affected the fusogenicity of the vesicles. We propose that the C-terminal half of the SNARE domain of STX1A plays a crucial role in the stabilization of the RRP as well as in the clamping of spontaneous synaptic vesicle fusion through the regulation of the energetic landscape for fusion. Additionally, although it may facilitate Ca^{2+}-dependent release, its role in this process is less significant.

Introduction

The molecular release machinery underlying synaptic vesicle fusion in mammalian central synapses consists of the SNARE complex at its core and several accessory proteins. The SNARE complex is formed by the interaction of plasma membrane-anchored proteins Syntaxin-1 (STX1 refers to Syntaxin-1A and Syntaxin-1B throughout this study unless stated otherwise) and SNAP-25 and vesicle-associated membrane protein Synaptobrevin-2 (VAMP/SYB2). The SNARE proteins assemble their 55 amino acid-long, highly conserved SNARE motifs into a parallel 4-helix bundle. The resulting SNARE complex that zippers up sequentially from N- to C-terminus physically brings the vesicle and plasma membranes together and provides the necessary energy to mechanically force membrane merger (Fasshauer et al., 1998; Sutton et al., 1998; Sørensen et al., 2006; Weber et al., 2010; Zhang et al., 2017, 2022). The assembly of the SNARE complex creates a highly charged outer surface (Fasshauer et al., 1998; Sutton et al., 1998; Ruiter et al., 2019) to which accessory proteins such as complexins, synaptotagmins or Sec1/Munc18(SM)-proteins can bind to and thereby modulate the efficacy and timing of neurotransmitter release (Rizo, 2022; Rizo & Rosenmund, 2008; Südhof, 2013).

Among the unique components of the SNARE complex, STX1 stands out by its complex structural characteristics. On the N-terminal extreme of STX1, the short regulatory N-peptide and the autonomously folded three-helical H_{abc}-domain are essential for the formation of fusogenic SNARE complexes and the readily releasable pool (RRP) of vesicles (Dulubova et al., 2007; Fernandez et al., 1998; Gerber et al., 2008; Khvotchev et al., 2007; Ma et al., 2011; Vardar et al., 2021; Zhou et al., 2013). On the C-terminal extreme, STX1 contains the juxtamembrane domain (JMD) and the transmembrane domain (TMD) that are actively involved in membrane fusion (Dhara et al., 2016; Gao et al., 2012; McNew et al., 1999; Sutton et al., 1998; Vardar et al., 2022). The most crucial, however, is its SNARE domain as it directly regulates the membrane merger. SNAP-25 and SYB2 mutation studies that

destabilize the inner hydrophobic pocket of the SNARE domain have determined that the SNARE motif of these proteins can be functionally compartimentalized into two subdomains: the N-terminus, necessary for priming and nucleation of the SNARE complex, and the C-terminus, essential for fusion (Gao et al., 2012; Sørensen et al., 2006; Walter et al., 2010; Weber et al., 2010). This was further confirmed by single-molecule optical tweezer studies which determined the sequential assembly of the SNARE complex through diverse staged assisted by these two subdomains (Gao et al., 2012). Additionally, it has been determined that accessory proteins, such as Munc18-1 and Complexin, chaperone the stability of the N-terminus and facilitate or clamp the assembly of the C-terminus in the partially-zippered SNARE domain, which without their regulation is fast and spontaneous (Gao et al., 2012; Hao et al., 2023; Ma et al., 2015). Although studies using STX1/STX3 chimeric constructs revealed that the SNARE motif of STX1 may carry structural features that regulate spontaneous release (Vardar et al., 2022), research on the SNARE domain of STX1 has been limited to the studies of its cognate interaction partners such as Munc18-1, Synaptotagmin-1 (SYT1) and Complexin (Hao et al., 2023; Jiao et al., 2018; Ma et al., 2015; Zhou et al., 2015, 2017).

In this study, we address the specific function of the entire SNARE domain of STX1 in regulating neurotransmitter release in intact synapses. For that purpose, we used a comprehensive structure-function approach and compared the efficacy of STX1 to the closely-related isoform STX2 (Sherry et al., 2006), which is implicated in the secretion of various peripheral secretory cells (Abonyo et al., 2004; Dolai et al., 2018; Hutt et al., 2005). When expressed in STX1-null hippocampal mouse neurons (Vardar et al., 2016), STX2 synapses showed impaired vesicle priming, unclamping of spontaneous release, as well as inefficient and slowed Ca^{2+}-evoked fusion, consistent with an impaired SNARE complex function (Hao et al., 2023; Jiao et al., 2018; Lai et al., 2017; Stepien et al., 2022; Voleti et al., 2020). Therefore, we proceeded with a structure-function chimeric analysis between STX1 and STX2, to isolate the precise involvement of the SNARE domain of STX1 in the regulatory mechanisms of release. We found that the C-terminal half of the SNARE domain of STX1 is a key regulator in the stability of the readily releasable pool and is part of the clamping mechanism for spontaneous release in central synapses, while it is minimally involved in the regulation of Ca^{2+}-evoked release. Overall, we discovered that the role of the SNARE complex goes beyond zippering and interacting with regulators of release and has evolved to participate in the finetuning of the fusion energy barrier and the precision of synaptic vesicle release alongside its accessory proteins.

Results

STX2 supports neuronal viability

Previously we have shown that comparison of non-canonical syntaxin isoforms to STX1 can offer insight into the specificity of vesicle fusion (Vardar et al., 2022). For this, Syntaxin-2 (STX2), Syntaxin-3 (STX3) and Syntaxin-4 (STX4) are the best candidates as they share the same domain structure with STX1 (Quiñones et al., 1999). For example, the isoform STX2 has been found in brain tissue and is present in the synaptosome fraction (Chen et al., 1999). STX1A and STX2 share a 63% total sequence homology and a 69% homology in their SNARE domain (Figure 1A). As expected from the promiscuity of syntaxins in particular and SNARE proteins in general, overexpression of STX2 can rescue neuronal survival in the absence of functional STX1 by binding to SNAP-25

119 (Peng et al., 2013), although its cognate SNARE partner in non-neuronal intracellular trafficking is SNAP-23
120 (Abonyo et al., 2004).

121 Having this in mind, we lentivirally expressed STX2 in STX1-null neurons using our well-established model of
122 STX1-null mouse hippocampal neurons (Vardar et al., 2016) and conducted a series of rescue experiments. STX1-
123 null neurons typically do not survive in culture longer that 8 days *in vitro* (DIV) and exhibit a complete loss of
124 neurotransmitter release (Vardar et al., 2016) (Figure 1C). Yet healthy synapses are vital to provide a solid
125 background to further analyze the electrophysiological properties. Therefore, our first objective was to compare
126 the ability of STX1A and STX2 isoforms to rescue neuronal survival. We quantified the amount of mouse
127 hippocampal neurons in high-density neuronal cultures at DIV8, DIV15, DIV22 and DIV29 for each group and
128 found that STX2 rescues neuronal survival at a comparable level to STX1A (Fig. 1B and 1D). Furthermore,
129 immunocytochemistry experiments confirmed that STX2, as well as STX1A, is strongly expressed in our model
130 neurons (Figure 1-figure supplement 1).
131
132 **STX2 does not support synchronous Ca2+-evoked release, the RRP size, not the spontaneous release clamp.**
133
134 Next, we analyzed the electrophysiological properties of autaptic neurons rescued with STX1A or STX2. First,
135 STX2 neurons showed a dramatic reduction in the excitatory postsynaptic current (EPSC) amplitude from 4.3nA
136 (±0.53) to 0.7nA (±0.16) (Figure 1E). We plotted the cumulative charge transfer of the EPSC over more than
137 550ms after the stimulation. A rapid and synchronous release of vesicles was reflected in a much more rapid
138 cumulative charge transfer in STX1A neurons (black line; Figure 1F), compared to STX2 neurons, where the
139 cumulative charge transfer of the response was much slower (purple line; Figure 1F). Additionally, the half width
140 of the EPSC was increased more than 2-fold in STX2 neurons (Figure 1G). This resonates with previous studies
141 that showed a decrease in the speed of secretion of STX2-expressing non-neuronal cells (Abonyo et al., 2004).
142 Overall, our results indicate that STX2 does not support synchronous Ca^{2+}-evoked release, but a slow
143 asynchronous response. We also evaluated synaptic vesicle priming by applying a hypertonic sucrose solution
144 (500mM) for 5s and measuring the charge of the response (Rosenmund & Stevens, 1996) and found that STX2
145 neurons exhibit a 76% reduction in the readily releasable pool (RRP) of vesicles compared to STX1A neurons
146 (Figure 1H). This suggests that STX2 does not support synaptic vesicle priming to the same extent as STX1A.
147 STX2-expressing neurons also displayed more than 2-fold increase in the frequency of spontaneous release
148 (mEPSC) relative to STX1A neurons (Figure 1I) and a 55% increase in the probability of release (PVR), calculated
149 by dividing the EPSC charge by the RRP charge (Figure 1J). When we normalized the mEPSC frequency to the
150 RRP size we found a nearly 15-fold increase in the spontaneous vesicular release rate in STX2 compared to
151 STX1A neurons (Figure 1L). Finally, no change was observed in the paired-pulse ratio (PPR) between STX1A
152 and STX2 groups (Figure 1L). The increase in the spontaneous vesicular release rate and the PVR indicates that
153 STX2-containing SNARE complexes render vesicles more fusogenic. Taken together, this data suggests that
154 STX2 neurons follow a fusion energetic landscape that differs from STX1A and resembles constitutive versus
155 regulated release (reviewed by Sørensen, 2009).
156
157 **The C-terminal half of the SNARE domain of STX1A has a regulatory effect on the RRP size and on**
158 **spontaneous release**.

Previous structure-function studies have shown the critical role of the distinct domains of STX1 in different aspects of synaptic vesicle fusion. However, what characterizes the protein is its SNARE domain. Not only does the SNARE domain of STX1A form one of the 4 helixes of the SNARE complex (Sutton et al., 1998), it also interacts with several regulatory proteins that modulate priming and fusion, such as SM protein Munc18-1, calcium sensor Synaototagmin-1 (SYT1) and accessory protein Complexin. Additionally, the SNARE domain has been compartmentalized into two functional subdomains: N-terminus, key in priming the vesicles to the plasma membrane and C-terminus, critical for the fusion process (Gao et al., 2012; Schotten et al., 2015; Sørensen et al., 2006; Walter et al, 2010; Weber et al., 2010). Our neuronal survival test shows that we can utilize STX2 for electrophysiological studies. To confine our studies to the SNARE domain, we used a chimeric approach, where we exchanged either the entire SNARE domain or the N- or C-terminal halves of the SNARE domain (N-terminal from E/D200-Q226; C-terminal from Q226-V/I255) between STX1A and STX2 (Figure 2A). Whereas the N-terminal halves of STX1A and STX2 share an 81% homology, their C-terminal halves, only share 60% homology (Figure 2A), and each half potentially contributes to a different step in vesicle fusion (Gao et al., 2012; Sørensen et al., 2006; Weber et al., 2010).

Replacement of the full-SNARE domain (STX1A-2(SNARE)) or the C-terminal half (STX1A-2(Cter)) of the SNARE domain of STX1A with the same domain from STX2 resulted in a reduction in the EPSC amplitude (Figure 2B). However, there was no change in the cumulative charge transfer (Figure 2C) or the half width of the EPSC (Figure 2D, Figure 2-figure supplement 1) in any of the groups. This suggests that the SNARE domain of STX1A may not be directly involved in the regulation of the kinetics of the release. Our analysis also showed that neurons expressing STX1A-2(SNARE) or STX1A-2(Cter) exhibited a nearly 3-fold increase in the mEPSC frequency (Figure 2E), potentially indicating an unclamping of spontaneous release. Additionally, the total charge transfer in response to a hypertonic sucrose application was significantly decreased in these two groups from 0.5nC (±0.06) to 0.18nC (±0.02) and 0.2nC (±0.02) respectively, compared to STX1A WT, indicating fewer primed synaptic vesicles and a smaller RRP (Figure 2C). Notably, the common modification of these STX1A chimera groups is the addition of the C-terminal half of the SNARE domain of STX2. These results suggest that the C-terminal half of the SNARE domain of STX1A may be directly involved in the clamping mechanism for spontaneous release of synaptic vesicles and may also play a role in the modulation of the RRP. However, it should be noted that we also observed a significant increase from 6.84Hz (±0.62) to 11.8Hz (±1.28) in the mEPSC frequency of STX1A-2(Nter)-expressing neurons (Figure 2E). Therefore, we cannot rule out the possibility that the N-terminal half of the SNARE domain may also play a role in the regulation of spontaneous release.

We next examined release efficacy in the STX1A-STX2 SNARE domain chimeras. STX1A-2(SNARE) showed a 39% increase and STX1A-2(Cter) showed a 42% increase in the PVR (Figure 2G), which indicates that the reduction in the EPSC is not proportional to the decrease in the RRP. This suggests that the changes we observed could be due to a faulty priming mechanism, but it could also mean that for these two groups there is a change in an additional mechanism that goes beyond the RRP size regulation and increases the efficacy of the Ca^{2+}-evoked release. Finally, STX1A-2(SNARE) and STX1A-2(Cter) had a 17- and 8-fold increase, respectively, in the spontaneous vesicular release rate (Figure 2H), a 40% decrease in the pair-pulse ratio (PPR) (Figure 2I) and increased depression in a 10Hz train stimulation (Figure 2J). Taken together our results suggest that the C-terminal

198 half of the SNARE domain of STX1A is involved in the regulation of the efficacy of Ca^{2+}-evoked release, the
199 formation of the RRP and in the clamping of spontaneous release.
200
201 **The insertion of the full-length SNARE domain or the C-terminal half of the SNARE domain of STX1A**
202 **into the STX2 backbone has an impact on the kinetics of evoked release, RRP size and spontaneous release.**
203
204 In the same manner as before, we wanted to test whether any of the electrophysiological properties that differ
205 between the two syntaxin isoforms are attributable to the SNARE domain itself and can be transferred to STX2.
206 For this, we generated chimeric proteins by introducing the entire SNARE domain of STX1A (STX2-
207 1A(SNARE)), its N- terminal half (STX2-1A(Nter)) or its C-terminal half (STX2-1A(Cter)) into STX2 (Figure
208 3A). Not only does this chimeric approach allow us to isolate certain functions of the SNARE domain, but it can
209 also help us determine whether the function of the SNARE domain of STX1A depends on domains or sequences
210 present in its natural structure. One of the benefits of these experiments is how significantly STX1A is regulated
211 throughout its various domains is put into perspective.

212 When recording from the STX2-STX1A SNARE domain chimera-expressing neurons we observed that we were
213 able to partially rescue the EPSC amplitude when STX2 contained the entire SNARE domain of STX1A (STX2-
214 1A(SNARE)) compared to STX2, from 0.35nA (±0.044) to 2.6nA (±0.28), respectively, although not to STX1A
215 WT levels 4.06nA (±0.31). Additionally, STX2-1A(Cter) showed a slight, albeit significant, rescue of the
216 amplitude of the Ca^{2+}-evoked response to 1.13nA (±0.28). STX2-1A(Nter) also showed a 50% increase in the
217 Ca^{2+}-evoked release to 0.68nA (±0.08) (Figure 3B), suggesting an insufficient rescue of the evoked response. To
218 analyze the kinetics of release in STX2-chimeras, we plotted the cumulative charge transfer of the EPSC over
219 time for each group and quantified kinetic parameters such as half width, rise time and decay time. STX2 and
220 STX2-1A(Nter) exhibited the slowest cumulative charge transfer as well as the highest half width (Figure 3C and
221 D: blue and light blue line respectively, Figure 3-figure supplement 1). These findings indicate that the N-terminal
222 half of the SNARE domain of STX1A is not enough to support the fast kinetics of the response. However, the
223 introduction of the entire SNARE domain or the C-terminal half rescued the speed and synchronicity of the EPSC
224 to almost STX1A WT levels (Figure 3C and D: dark and light turquois lines, respectively, Figure 3-figure
225 supplement 1). Notably, the speed of release did not change in any STX1A-chimera (Figure 2D and E), suggesting
226 that there is a dominant regulatory domain which supports the fast kinetics of the response outside of the SNARE
227 domain in STX1A, such as the juxtamembrane or transmembrane domain (Vardar et al., 2022). However, the C-
228 terminal half of STX1A alone is sufficient to evoke faster responses in STX2. These results support major
229 regulatory differences of the domains outside of the SNARE domain between the isoforms, which should not be
230 discarded in the interpretation of our results.

231 As reported previously, our STX1A chimeric analysis suggested a clamping function of the C-terminal half of the
232 SNARE domain of STX1A (Figure 2E). In line with these findings, STX2-1A(Cter) showed a significant
233 reduction in the mEPSC frequency to almost STX1A WT levels. STX2-1A(SNARE) unexpectedly showed a 47%
234 increase in the spontaneous release frequency compared to STX2, which was also observed in STX2-1A(Nter)
235 (Figure 3E). Additionally, we found that only STX2-1A(SNARE) and STX2-1A(Cter) could rescue the RRP to
236 around double of what we measured from STX2 and STX2-1A(Nter) (figure 3F), albeit not to the extent of STX1A

237 WT. This supports our hypothesis that the C-terminal half of the SNARE domain of STX1A plays a significant
238 role in the regulation in vesicle priming, also revealed by STX1A-chimeras (Figure 2F). Furthermore, STX2-
239 1A(SNARE) and STX2-1A(Nter) had an increased PVR (Figure 3G), no change in the release rate (Figure 3I)
240 and an increase in short-term depression during 10Hz train stimulation (Figure 3I), compared to STX2 WT. On
241 the other hand, STX2-1A(Cter) showed no change in the PVR compared to STX2 or STX1A WT, a 4.5-fold
242 decrease in the spontaneous release rate (STX1A WT has a 12-fold decrease compared to STX2), and an increase
243 in the short-term depression in a 10Hz train stimulation albeit the difficulty of analysis of this data given the small
244 values of the release (Figure 3J). These data indicate that the suppression of spontaneous release and the regulation
245 of the RRP relies on the C-terminal half of the SNARE domain of STX1A, while the efficacy of Ca^{2+}-evoked
246 release depends on the integrity of the entire SNARE domain.
247
248 **The insertion of the full-length SNARE domain or the C-terminal half of the SNARE domain of STX1A**
249 **into the Stx2 backbone rescues Munc18-1 levels at the synapse.**
250
251 Munc18-1 binds not only to the N-terminal regions of STX1A (Burkhardt, Hattendorf, Weis, & Fasshauer, 2008;
252 Khvotchev et al., 2007; Meijer et al., 2018) but also to the SNARE complex (Baker et al., 2015; Jiao et al., 2018;
253 Stepien et al., 2022). The expression levels of Munc18-1 and STX1A influence each other and their interaction is
254 important for priming and release (Gerber et al., 2008; Vardar et al., 2016). Thus, we analyzed the exogenous
255 expression of STX1A, STX1A-2(SNARE), STX1A-2(Nter) and STX1A-2(Cter) and the endogenous expression
256 of Munc18-1 at the synapse (Figure 4). We used VGlut1 as the synaptic marker in the co-localization assays. We
257 found that the introduction of the SNARE domain of STX2 into STX1A (STX1A-2(SNARE)) increased the
258 expression of this construct compared to STX1A WT while the rest showed similar levels (Figure 4B).
259 Additionally, the exchange of the entire SNARE domain or the C-terminus of STX1A with that of STX2 caused
260 an increase in the levels of endogenous Munc18-1 at the synapse, which may be relative to the increase in STX1A-
261 construct levels (Figure 4C). Because the loss or overexpression of Munc18-1 affect the properties of secretion
262 and release (Oh et al., 2012; Toonen et al., 2006; Verhage et al., 2000; Voets et al., 2001), we wanted to determine
263 whether changes in the levels of Munc18-1 may correlate with the changes in neurotransmitter release from the
264 neurons which carry the exogenous STX1A-chimeric constructs. We observed that higher levels of Munc18-1
265 seem to correlate with less Ca^{2+}-evoked release (Figure 4E), and a smaller RRP (Figure 4F) but higher release
266 efficacy (Figure 4G) and release rates (Figure 4H), which suggest it may be the cause of these changes in the
267 release parameters.

268 Additionally, we quantified the levels of exogenous expression of STX2, STX2-1A(SNARE), STX2-1A(Nter)
269 and STX2-1A(Cter) and the corresponding levels of endogenous Munc18-1 levels at the synapse (Figure 5). We
270 found that all STX2 constructs were expressed at similar levels at the synapse (co-localized to VGlut1 puncta)
271 (Figure 5A and B). However, STX2-1A(SNARE) and STX2-1A(Cter) showed a trend towards increase in their
272 expression (Figure 5B and C). We then quantified the endogenous levels of Munc18-1 at the synapse in these
273 neurons and found a decrease in Munc18-1 levels when expressing STX2 WT compared to STX1A WT.
274 Interestingly, Munc18-1 levels were rescued when either the entire SNARE domain (STX2-1A(SNARE) or the
275 C-terminal half (STX2-1A(Cter)) of STX1A were present (Figure 5E and 5F) compared to STX1A WT neurons.
276 The interaction between Munc18-1 and the SNARE domain, which is thought to be important for templating

SNARE complex assembly (Baker et al., 2015; Stepien et al., 2022), may be directly involved in rescuing some of the electrophysiological parameters observed in STX2 chimeras. To investigate this, we correlated the levels of Munc18-1 with electrophysiological phenotypes observed in neurons expressing the STX2-chimeras (Figure 5G-J). Our superposition of phenotypes revealed that higher levels of Munc18-1, observed in STX1A WT and in the STX2-1A(Cter) neurons, showed a trend in increasing Ca^{2+}-evoked release (Figure 5G) and the RRP (Figure 5H) while the effects on PVR were more randomly distributed (Figure 5I). Additionally, we observed that higher release rates correspond to groups which show less amounts of Munc18-1 at the synapse (Figure 5J). Taking together these results it seems like we have contradictory observations: while increased levels of Munc18-1 in the STX1A background seem to correlate with parameters that depict an increase in the fusogenicity of the vesicles (Figure 4), an increase in the levels of Munc18-1 in the STX2 background correlates to release properties that incline towards a decreased fusogenicity. In summary, although Munc18-1 levels does depend on the construct used and may be causative of changes in certain electrophysiological characteristics, we may not be able to explain these phenotypes by the levels of Munc18-1.

The residues on the outer surface of the C-terminal half of the SNARE domain are crucial in the regulation of spontaneous release and the RRP.

So far, we have highlighted the importance of the C-terminal half of the SNARE domain of STX1A in the regulation of the RRP size and for the clamping of spontaneous release. Our previous study that aligned STX1A and different STX3A and STX3B chimeric constructs also suggested the involvement of the C-terminus half in the spontaneous release clamping mechanisms, but only through elimination (Vardar et al., 2022). These data compelled us to examine whether spontaneous release regulatory mechanisms can be attributed to individual or sequentially paired amino acid residues in the C-terminal half of the SNARE domain of STX1A. To test this, we introduced single- or double-point mutations in the C-terminus of the SNARE domain of STX1A, targeting residues that differ from STX2 in their charge or polarity and that are located on the outer surface of the SNARE complex. It is important to note that electrostatic charges play a critical role in the interaction of STX1A's SNARE domain with other proteins and the membrane (Ruiter et al., 2019). Thus, we hypothesized that if the charge of the amino acid was not significantly altered, it may not play a significant role in the observed phenotypic differences between STX1A and STX2. Given this we generated single point mutations ($STX1A^{D231N}$, $STX1A^{R232N}$, $STX1A^{Y235R}$, $STX1A^{E238V}$, $STX1A^{V248K}$ and $STX1A^{S249E}$) and double point mutations ($STX1A^{D231N,R232N}$ and $STX1A^{V248K,S249E}$) if the residues were in sequential positions and analyzed their expression at the synapse and the corresponding levels of Munc18-1 at the synapse (Figure 6A, Figure 6-figure supplement 1). Notably, we excluded $STX1A^{A240S}$, which is present in STX1B, redundant in function to STX1A (Vardar et al., 2016) and $STX1A^{R246}$ and $STX1A^{D250}$ which are electrochemically similar to $Stx2^{H246}$ and $Stx2^{E250}$ (Figure 6A). For unknown reasons, the levels of some of the mutant constructs of STX1A seem to be reduced more than 50%, which has been shown to be detrimental for release (Arancillo et al., 2013; Vardar et al., 2016) while all electrophysiological parameters showed comparable levels to STX1A WT or even an enhanced spontaneous release (Figure 6). For this reason, we did not explore the expression results any further. Additionally, levels in Munc18-1 of the corresponding mutants were also decreased (Figure 6-figure supplement 1).

316 The electrophysiological analysis of our point mutations showed that none of the mutations caused a change in
317 the EPSC amplitude (Figure 6B). However, the double point mutant STX1A^{D231N,R232N} was the only mutant that
318 showed a decrease in the RRP size from 0.469nC (±0.08) to 0.19nC (±0.03) (Figure 6C), as well as a 2-fold
319 increase in the PVR (Figure 6D). This is consistent with the changes in our previous findings from the STX1A
320 chimeric analysis, which showed a decreased RRP and an increased PVR (Figure 2G). All mutants showed an
321 increase in the mEPSC frequency; however, it was not significant for STX1A^{E238V} and STX1A^{V248K} (Figure 6E).
322 This suggests that any small alteration in the outer surface of the C-terminal half of the SNARE domain of STX1A
323 could de-stabilize the clamp for spontaneous release. Therefore, the whole domain is crucial in the regulation of
324 the synaptic vesicle clamp. The mutants with increased spontaneous release frequency also exhibited an increase
325 in the release rate (Figure 6F and G), particularly those with a simultaneous increase in the mEPSC frequency and
326 a trend towards RRP reduction. This included all the double point mutants STX1A^{D231N,R232N}, STX1A^{D231D,E238V}
327 and STX1A^{V248K,S249E} and the single point mutants STX1A^{D231} and STX1A^{S249E}. These results suggest that the
328 more alterations the C-terminal half of the SNARE domain undergoes, the less effective it is in maintaining the
329 stability of the vesicles in the RRP and in clamping spontaneous release.

330 Finally, we examined the relationship between RRP size and release rate (Figure 6I) and PVR and release rate
331 (Figure 6H). Changes that de-stabilize the primed state might be seen as a decrease in the RRP and an increase
332 the release rate. Changes in the height of the energy barrier for fusion may make it harder or easier for vesicles to
333 fuse and could be indicated by a decrease or increase in the PVR, respectively, accompanied by a change in the
334 release rate. Surprisingly, STX1A-2(SNARE), STX1A-2(Cter) and STX1A^{D231N,R232N} exhibit changes in both
335 these correlations: decreased RRP and increased spontaneous release rate (Figure 6I), and increased PVR and
336 spontaneous release rate (Figure 6H). This may suggest D231, R232 may have a role on the stability of primed
337 vesicles and on the energy barrier for fusion. However, the integrity of the SNARE domain of STX1A is more
338 important than single-point changes, suggesting an additive effect of the residues of the C-terminal half of the
339 SNARE domain.
340

341 ## Discussion

342

343 In this study we investigated the role and specificity of the SNARE domain of STX1 in regulating neurotransmitter
344 release. We found that the C-terminus of the SNARE domain of STX1 is crucial in the regulation of multiple
345 aspects of synaptic transmission, such as the clamping of spontaneous release, the formation and maintenance of
346 the RRP of vesicles, while it plays a minimal role in the regulation for Ca^{2+}-evoked release.
347

348 ### STX1 is fine-tuned for synaptic vesicle release.

349

350 Current understanding of constitutive and regulated membrane fusion involves the idea of the energetically
351 favorable interaction of four cognate SNARE domains that "zipper-up" and pull the vesicle and plasma membrane
352 together (Fasshauer et al., 1998). In the physiological context, vesicle fusion can vary dramatically in terms of
353 efficacy and acceleration upon triggering, arguing for an extensive regulation machinery for the fusion process.
354 An important role is attributed to regulatory proteins, such as complexins and synaptotagmins, that regulate

various aspects of vesicle fusion including synaptic vesicle docking and priming, clamping of spontaneous release and speed and efficacy of neurotransmitter release (Rizo, 2022; Rizo & Rosenmund, 2008; Südhof, 2013). Although SNARE proteins are quite promiscuous in their assembly (Bajohrs et al., 2005; Brunger, 2005; Fasshauer et al., 1998; Peng et al., 2013; Vardar et al., 2022), variations in their SNARE motif sequence can lead to differential regulation of the fusion reaction beyond their role in zippering, e.g. by modifying the surface charge (Kádková et al., 2023; Ruiter et al., 2019) or regulating interaction with modulatory proteins (Schupp et al., 2016; Stepien et al., 2022; Zhou et al., 2015). We show that STX2 can rescue some aspects of neurotransmitter release in STX1-null neurons, supporting redundant functions among syntaxin isoforms in executing vesicle fusion. However, regulated fusion with non-cognate trans-SNARE complexes containing STX2 showed several unfavorable release properties, including slowed and inefficient Ca^{2+}-evoked release, a reduced pool of fusion competent vesicles, while spontaneous release was greatly increased (Figure 1). Given these findings, and our previous findings which compared the release parameters of the tonic release isoform STX3 with STX1 (Vardar et al., 2022), we argue that STX1 is finetuned for phasic release with a high signal-to-noise ratio for evoked over spontaneous release.

<u>The C-terminus of the STX1 SNARE domain is important for stability of the primed state of vesicles and the clamping of spontaneous fusion.</u>

As a general observation, the exchange of the whole or only the C-terminal half of the SNARE domains of STX1 and STX2 resulted in altered Ca^{2+}-dependent responses, PVR, PPR, short-term depression, as well as release rate (Figure 2 and 3). It is clear that the C-terminus of the STX1 SNARE domain is important for the stability of the primed state of the vesicles and the clamping of spontaneous fusion. While conducting a sub-saturating sucrose experiment (Basu et al., 2007; Ruiter et al., 2019; Schotten et al., 2015) could further confirm the hypothesis of reduced fusogenicity in STX1 chimeras, the small RRP obtained at 500 mM sucrose poses a limitation to our study. Therefore, we must rely on other parameters to interpret these results. Generally, changes in the molecular mechanisms for priming proportionally change the Ca^{2+}-evoked response while leaving the PVR unchanged. Changes in the PVR, PPR, release rate and depression during high-frequency train stimulation, however, may indicate alterations in the fusogenicity of the vesicles that stem from aberrations in the mechanisms underlying the regulation of the fusion energy barrier (Schotten et al., 2015). In this light, we propose a model where the C-terminal half of the SNARE domain of STX1 plays a major role in the stabilization of the primed state and in the clamping of the spontaneous release. Disrupting the C-terminus of the STX1A SNARE domain by adding that of the non-cognate STX2 (Figure 7-red dotted line) may impact vesicle fusogenicity by destabilizing the primed state and decreasing the fusion energy barrier that now becomes easier for vesicles to overcome (Schotten et al., 2015). Additionally, adding the C-terminus of the SNARE domain of STX1A to STX2 may increase the ability of the SNARE complex to stabilize the primed state by increasing the degree of the fusion energy barrier and thereby establish a clamp for spontaneous release (Figure 7-light green dotted line). This model supports our previous findings in which, by performing a similar chimeric analysis with STX3, we suggested the C-terminus of the SNARE domain of STX1 as a key regulator in the clamping of spontaneous vesicle fusion (Vardar et al., 2022). We discuss 3 possible mechanisms through which the C-terminus of the SNARE domain of STX1 might contribute to the stability of the primed state of synaptic vesicles and the clamping of spontaneous release.

395
396 **The interaction of the C-terminus of de SNARE domain of STX1A with Munc18-1 in the stabilization of**
397 **the primed pool of vesicles.**
398
399 Previous studies have attributed the vesicle priming function to the N-terminal half of the SNARE domain
400 (Sørensen et al., 2006; Weber et al., 2010) and to proteins involved in the N-terminal nucleation of the SNARE
401 complex, such as Munc13 and Munc18-1 (Ma et al., 2011; Wang et al., 2020). One line of thought proposes that
402 these proteins prime vesicles to the plasma membrane by forming a SYB2-STX1 acceptor-complex for SNAP-25
403 (Jiao et al., 2018; Lai et al., 2017; Parisotto et al., 2012; Stepien et al., 2022; Wang et al., 2019). Thus, changes in
404 Munc18-1 levels in STX1 and STX2 chimeras is one of the first hypotheses that comes to mind as an underlying
405 mechanism of the altered primed state of the vesicles. Notably, we did not observe changes in the levels of
406 Munc18-1 when exchanging N-terminal halves of the SNARE domain between syntaxins. However, all the
407 chimeras that contained the C-terminal half of the SNARE domain of the non-cognate isoform showed an increase
408 in levels of Munc18-1 compared to their native form (Figure 4C, Figure 5F) while manifesting opposing effects
409 in the stabilization of primed vesicles: STX2 chimeras showed a better stabilized primed state reflected in the
410 increase of the RRP and a decrease in the fusogenicity parameters (Figure 5H-J and Figure 7), whereas STX1A-
411 chimeras showed signs of a destabilized primed state (Figure 4F-H and Figure 7). These opposing phenotypes
412 both accompanied by an increase in Munc18-1 levels makes the scenario of Munc18-1 levels as the direct cause
413 in the stabilization of the primed state unlikely. On the other hand it is important to note that Munc18-1 was shown
414 to maintain an interaction with the C-terminus of the SNARE domain of STX1 during the N-terminal nucleation
415 of the SNARE complex (Stepien et al., 2022). Therefore, it is tempting to speculate that Munc18-1 might have a
416 qualitative effect on the vesicle stabilization rather than a quantitative one. In this case, it can be argued that an
417 intact C-terminal half of the SNARE domain is a conditional requirement in order for Munc18-1, even if it is
418 present in abundance, to fulfill its nucleation function on the N-terminus in a physiological setting, thus leading
419 to a reduced RRP (Figure 4F).
420
421 **The participation of STX1A in the electrostatic regulation of the fusion energy barrier model.**
422
423 Besides the destabilization of the RRP, we found that the clamping of the spontaneous release is particularly
424 susceptible to changes in single or double consecutive residues in the C-terminus of the SNARE domain (Figure
425 6). The degree of the vesicle fusion energy barrier is largely regulated by the electrostatic landscape of the fusion
426 apparatus (Shao et al., 1997; Trimbuch et al., 2014; Williams et al., 2009) which will try to neutralize the
427 negatively charged approaching membranes so they can fuse. This is also influenced by the total charge of the
428 SNARE domain complex (Ruiter et al., 2019). Thus, changes in the net charge of the SNARE complex can cause
429 altered vesicle-fusogenicity and as a result, altered release rates at the synapse (Ruiter et al., 2019). The
430 observation of SNAP-25 charge-reversal mutations proposed a model by which a positive net charge increase
431 lowerers the fusion energy barrier and increases fusion rates because it neutralizes the negative charges of the
432 membranes, while a negative net charge increase increases the negative electrostatic fusion energy barrier causing
433 a decrease in fusion rates (Kádková et al., 2023; Ruiter et al., 2019). In this light, it is plausible to argue that the
434 differences in the release behavior led by the single or double-point mutations in the C-terminal half of the SNARE

435 domain of STX1A (Figure 6) might be due to the altered electrostatic net charge. The net charge difference in the
436 C-terminal half of the SNARE domain between STX1A and STX2 is only one positive charge that would only
437 lead to a minor change in the release rate. However, the release rate we observed are 15-fold (in the native form)
438 (Figure 1K) or 17-fold and 8-fold (in STX1A chimeras) (Figure 2H) increased. Additionally, the mutations
439 $STX1A^{D231N,R232N}$ and $Stx1A^{V248K,S249E}$ which result in a null net charge difference, increased the spontaneous
440 release rate 4-fold compared to STX1A WT, or mutation $STX1A^{S249E}$ that should reduce the release rate according
441 to this hypothesis as it possesses one negative net charge difference increases the spontaneous release rate 3-fold
442 (Figure 6G). Therefore, our results suggest that the changes in release rate in STX1A point-mutations are not
443 caused merely by electrostatic changes but by an independent mechanism from the electrostatic model of
444 regulation of releas.
445
446 **<u>The interaction of STX1A with the C2B domain of SYT1 though the primary interface.</u>**
447
448 What can be the mechanism through which the C-terminal half of the SNARE domain regulates vesicle fusion if
449 it is not solely through electrostatic changes? Interestingly, neurons expressing STX2 showed a slowed down
450 Ca^{2+}-evoked release, a dramatic increase in the spontaneous release rate, and decrease in the RRP (Figure1).
451 Additionally, STX1A-chimeras which contained the C-terminus of the SNARE domain of STX2 showed a
452 decrease in the RRP and a dramatic unclamping of the spontaneous release rate (Figure 2). This is reminiscent of
453 the well-established phenotype of SYT1-null mouse neurons (Bouazza-Arostegui et al, 2022; Stepien et al., 2022;
454 Xu et al, 2007; Xue et al., 2009) and suggests a possible alteration in the function of SYT1 in these neurons.
455 Moreover, mutating individual residues in the C-terminal half of the SNARE domain which differ between
456 STX1A and STX2 showed a particular effect on the clamping of spontaneous release and the RRP in the case of
457 D231 and R232 (Figure 6E), two functions associated to SYT1-SNARE interaction (Schupp et al., 2016). Some
458 of these residues (D231 and E238) have been identified by crystal structure studies to be involved in the putative
459 interaction between the C2B domain of SYT1 and the C-terminal half of the SNARE domain of STX1 and SNAP-
460 25, referred to as the primary interface (Zhou et al., 2015). Mutations of the residues at the primary interface in
461 SYT1 and SNAP-25 identified this putative interaction as a key regulation site for the clamping of spontaneous
462 release and Ca^{2+}-triggered release (Chang et al., 2018; Schupp et al., 2016; Zhou et al., 2015). The partial rescue
463 of the speed of Ca^{2+}-evoked release, the RRP and the clamping of the spontaneous release in STX2 chimeras
464 which contained the C-terminus of the SNARE domain of STX1A (Figure 3), suggested a gain of function though
465 the interaction with SYT1. However, none of the changes in the C-terminus of the SNARE domain of STX1A
466 resulted in a change in the speed of release (Figure 2 and Figure 6) and the release rates obtained in STX1A-
467 2(SNARE) and STX1A-2(Cter) were dramatically higher (Figure 2) than in SYT1-KO neurons (Bouazza-
468 Arostegui et al., 2022). In this light, our data support the primary interface as a key site for the regulation of
469 spontaneous release, in addition to further unidentified mechanisms involving other residues in the C-terminal
470 half of the SNARE domain of STX1A. Notably, it argues against STX1 as part of the Ca^{2+}-evoked release
471 mechanisms through its interaction with the C2B domain of SYT1, supporting our previous findings that identified
472 regions outside of the SNARE domain of STX1, such as the juxtamembrane and transmembrane domain, as
473 regulators of the synchronization of neurotransmitter release (Vardar et al., 2022).
474

475 ## Materials and methods

476

477 **Animal maintenance and generation of mouse lines**

478 All animal-related procedures and experiments were performed in accordance with the guidelines and approved
479 by the animal welfare committee of Charité-Universitätsmedizin and the Berlin state government Agency for
480 Health and Social Services, under license number T0220/09. To generate the STX1-null mouse model, we bred
481 the conventional *Stx1A*- knockout (KO) line, in which exon 2 and 3 were deleted (Gerber et al., 2008), with the
482 *Stx1B* conditional-KO line in which exons 2–4 were flanked by loxP sites (Wu et al., 2015).

483

484 **Neuronal cultures**

485 Primary hippocampal neuronal cultures were prepared from STX1A/1B cDKO mice at postnatal day 0-1 and were
486 seeded onto a continental astrocyte feeder layer (for immunocytochemistry 40K neurons/well in 12-well plates)
487 or astrocyte micro-dot islands (for the electrophysiology 4K neurons/well in 6-well plates) as previously described
488 (Vardar et al., 2016; Xue et al., 2007). Cortical neuronal cultures were prepared from the same mice and plated
489 onto continental astrocyte feeder layer for Western Blot experiments (500K neurons/ well in 6 well plates).
490 Cultures were incubated in NeuroBasal-A (NBA) medium (Invitrogen) supplemented with B-27 (invitrogen), 50
491 IU/ml penicillin and 50µg/ml streptomycin) at 37°C before and during the experiments. Neuronal cultures were
492 incubated for 13-20 days *in vitro* (DIV).

493

494 **Lentiviral construct production and viral infection**

495 Point mutations were introduced into *Stx1A* or *Stx2* using a TA cloning vector with the QuickChange Site-Directed
496 Mutagenesis Kit (Stratagene). Chimeric constructs of both syntaxin isoforms were obtained using Gibson
497 Assembly (NEB). For the viral infection the cDNA of mouse *Stx1A* (NM_016801.4), *Stx2* (NM_007941.2),
498 chimeric and point-mutation constructs were cloned into a lentiviral shuttle vector containing a nuclear
499 localization signal (NLS) GFP- P2A expression cassette under the human synapsin-1 promoter. For obtaining the
500 *Stx1B*-KO of in the *Stx1A*(-/-);*Stx1B*(*flox/flox*) neurons (Stx1-null), we used a lentiviral construct which induces
501 the expression of improved Cre-recombinase (iCre) fused to NLS-RFP-P2A under the control of human synapsin-
502 1 promoter. All viral particles were made by the Viral Core Facility of Charité-Universitätsmedizin as previously
503 described (Lois et al., 2002). Empty NLS-GFP-P2A constructs were used as control. For all experiments cultures
504 were infected with lentiviral particles at DIV1-2.

505

506 **Neuron viability**

507 For neuron survival assays we quantified the number of the STX1-null hippocampal neurons transduced with
508 *Stx1A, Stx2 and GFP* (as control) at DIV 15, 22, and 29, and compared this to the number of neurons at DIV8.
509 Two wells per group were plated and fifteen random ROIs of 1.23mm2 per well were imaged at the different time
510 points (thirty total ROIs per group at each time point per culture). Phase-contrast bright field images and
511 fluorescent images with excitatory lengths of 488 and 555 nm were acquired on a DMI 400 Leica microscope,
512 DFC 345 FX camera (Leica), HCX PL FlUPTAR 10x objectives (Leica) and LASAD soft-ware (Leica). The
513 neurons were counted offline witht the 3D Object Counter function in Fiji software (Vardar et al., 2016). For the

514 example images the cultures were fixed at the corresponding time points in 4% paraformaldehyde (PFA) in 0.1M
515 phosphate-buffered saline, pH 7.4 (PBS) and immunocytochemistry was done as follows.
516
517 **Immunocytochemistry and image acquisition**
518 High-density hippocampal neuron cultures (40 x 103) were plated onto the astrocyte-feeder layer and infected at
519 DIV1-2. At DIV14-15 the cultures were fixed in 4% PFA in PBS for 10 min, permeabilized in 0.1% Tween-
520 20 PBS (PBS-T) for 45 min at room temperature (RT) and blocked in 5% normal donkey serum in PBS-T for 1h
521 at RT. To detect our proteins of interest the neurons are incubated with the primary antibodies in PBS-T at 4°C
522 over-night (O/N). For protein expression analysis neurons were incubated with guinea-pig polyclonal anti-
523 VGlut1(1:4000; Synaptic Systems), mouse monoclonal anti-STX1A (1:1000; Synaptic Systems) and rabbit
524 polyclonal anti-STX2 (1:1000; Synaptic Systems) or rabbit polyclonal Munc18-1 (1:1000; Sigma-Aldrich).
525 Secondary antibodies conjugated with rhodamine red, or Alexa Fluor 488, or 647 (1:500; Jackson
526 ImmunoResearch) in PBS-T were applied for 1h at RT in the dark. For the example images for survival assay
527 neurons were incubated with chicken polyclonal anti-MAP2 (1:2000; Merck Milipore) and secondary antibody
528 Alexa Fluor 488. The coverslips were mounted on glass slides with Mowiol mounting agent (Sigma-Aldrich). For
529 quantitative analysis, every comparable group in each culture is treated with the same antibody solution. Images
530 are acquired with an Olympus IX81 epifluorescence-microscope with MicroMax 1300YHS camera (Princeton
531 Instruments) and with MetaMorph software (Molecular Devices). Images for the example images of the survival
532 assay and for the quantitative analysis of protein expression were taken with an optical magnification of 10X and
533 60X, respectively. The exposure times of each wavelength are kept constant for all experimental groups in one
534 culture. Overexposure and photobleaching is avoided by monitoring the fluorescent saturation level at the synapse.
535 To image glutamatergic neurons 7-9 images that had a fluorescent signal for VGlut1 staining are taken from each
536 group in each experimental replicate. Data was analyzed offline on ImageJ. Three to five regions per image were
537 selected which showed neuronal projections marked by VGlut1-positive labelling. Vglut1-positive puncta are
538 selected using MaxEntropy threshold function of the selected regions, creating a VGlut1-positive ROI. The
539 intensities of VGlut1, STX1A, STX2 and Munc18-1 are measured using the corresponding ROIs. For the
540 quantification of relative protein expression at glutamatergic synapses, the ratio between STX1A, STX2 or
541 Munc18-1 to VGlut1 is calculated in each selected region. To compare between groups the data is normalized to
542 STX1A group or STX2 group.
543
544 **Electrophysiology**
545 To assess synaptic function, whole-cell patch-clamp recordings in autaptic neurons were performed at DIV13-
546 DIV19 at room temperature. Synaptic currents were recorded using a MultiClamp 700B amplifier (Molecular
547 Devices) and data was digitally sampled at 10kHz and low-pass filtered at 3kHz with an Axon Digidata 1440A
548 digitizer (Molecular Devices). Data acquisition was controlled by Clampex10 software (Molecular Devices).
549 Series resistance was compensated at 70% and only neurons with a series resistance lower than 10MΩ were further
550 recorded. Neurons are continuously perfused using a fast perfusion system (1-2ml/min) with an extracellular
551 solution (unless stated otherwise) that contains 140mM NaCl, 2.4mM KCl, 10mM HEPES, 10mM glucose, 2mM
552 CaCl2 and 4mM MgCl (295-305mOsm, pH7.4). Borosilicate glass pipettes are pulled yielding a resistance
553 between 2-5MΩ. Pipettes are filled with a KCl-based intracellular solution containing 136mM KCl, 17.8mM

554 HEPES, 1mM EGTA, 4.6mM MgCl2, 4mM Na2ATP, 0.3mM Na2GTP, 12mM creatine phosphate, and 50 Uml1
555 phosphocreatine kinase (300mOsm; pH7.4). Only neurons that express RFP and GFP are selected for recording
556 and cells with a leak current higher than 300pA were excluded. Neurons are clamped at -70mV during all protocols
557 of electrophysiological recording. For triggering action potential (AP) evoked release neurons are stimulated by
558 a 2ms depolarization to 0mV and excitatory postsynaptic currents (EPSCs) are registered. To measure the
559 synchronicity and the kinetics of the AP-evoked responses we inverted the EPSC change and integrated our signal
560 For measuring spontaneous release (mEPSC) we recorded at -70mV for 48s and for 24s in the presence of
561 the AMPA-receptor antagonist NBQX (3µM) (Tocris Bioscience) diluted in extracellular solution. To calculate
562 the frequency of mEPSC we filtered at 1kHz and analyzed using a template-based miniature event detection
563 algorithm implemented in the AxoGraph X software. We then subtracted the measured mEPSC in NBQX to the
564 mEPSCs measured in extracellular solution. The release of the readily releasable pool (RRP) of synaptic vesicles
565 is triggered by the application of a 500mM hypertonic sucrose solution diluted in extracellular solution for 5s
566 (Rosenmund and Stevens, 1996), and the RRP size is estimated by integrating the area of the sucrose-evoked
567 current setting as baseline the steady-state current. The vesicular release probability (PVR) of each neuron is
568 determined by the ratio between the charge of the EPSC and the size of the RRP. The spontaneous release rate is
569 calculated as the ratio between the mEPSC frequency and the number of primed vesicles. This determines the
570 fraction of the RRP which is spontaneously released per second. To measure the cumulative charge transfer of the
571 synaptic responses we inverted the EPSC charge and integrated the signal. Short term plasticity was examined
572 either by evoking 2 AP with 25 ms interval (40 Hz) or a train of 50 AP at an interval of 100 ms (10 Hz). Data
573 were analyzed offline using Axograph10 (Axograph Scientific)
574
575 **Western Blot**
576 Protein lysates from cortical mass cultures were prepared by lysing neurons in 200 µl lysis buffer containing 50
577 mm Tris/HCl, pH 7.9, 150 mm NaCl, 5 mm EDTA, 1% Triton X- 100, 0.5% sodium deoxycholate, 1% Nonidet
578 P- 40, and 1 tablet of Complete Protease Inhibitor (Roche) for 30 min on ice. Samples are boiled for 5min at 95ºC.
579 Equal amounts of protein were loaded onto a 12% SDS-PAGE and run in electrophoresis buffer at 80V for 30
580 min and then 120mV for 1h. Proteins were transferred onto a nitrocellulose membrane at 50mA O/N. The
581 membranes were blocked in 5% milk for 1h at room temperature and incubated with the corresponding primary
582 antibody diluted in PBS for 1h at room temperature. Membranes were incubated with mouse monoclonal anti-
583 STX1A (1:10,000; Synaptic Systems), rabbit polyclonal anti-STX2 (1:10,000; Synaptic Systems), rabbit
584 polyclonal anti-STX3A (1:10,000; Synaptic Systems), rabbit polyclonal anti-Munc18-1 (1:10,000; Sigma-
585 Aldrich) and mouse monoclonal anti-betaTubIII (1:10,000; Sigma) (as loading control). Secondary antibodies
586 conjugated with HRP-conjugated goat secondary antibodies (1:10,000; Jackson ImmunoResearch) diluted in PBT
587 were applied for 1h at RT. Membranes are then incubated with ECL Plus Western Blotting Detection Reagents
588 (GE Healthcare Biosci- ences) and luminal signal was visualized and imaged in Fusion FX7 image and analytics
589 system (Vilber Lourmat).
590
591 **Statistical analysis**
592 Data presented in box-whisker plots are compiled as single observations, median, quartiles and outliers. Data in
593 bar graphs and X-Y plots present means ± SEM. All data were tested for normality with D'Agostino-Pearson test.

Data from two groups with non- parametric distribution were subjected to Mann- Whitney test. Data from two groups with normal distribution were subjected to unpaired two-tailed t-test. Data from more than two groups were subjected to Kruskal-Wallis followed by Dunn's *post hoc* test when at least one group showed a non-parametric distribution. Data from more than two groups were subjected to Ordinary one-way ANOVA when all groups showed a parametric distribution. STP was subjected to two-way ANOVA. Statistical analyses were performed using Prism 7 (GraphPad).

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825
826 **Figure Legends**
827
828 **Figure 1. STX2 supports neuronal viability but does not rescue synchronous evoked release, the RRP or**
829 **the clamping of spontaneous release**.
830
831 A. STX1A and STX2 domain structure scheme and SNARE domain sequence alignment (68% homology).
832 Layers are highlighted in gray.
833 B. Example images of high-densitiy cultured STX1-null hippocampal neurons rescued with GFP (STX1-
834 null), STX1A or STX2 (from top to bottom) at DIV8, DIV15, DIV22 and DIV29 (from left to right).
835 Immunofluorescent labelling of MAP2. Scale bar: 500μm.
836 C. Quantification of total number of neurons at DIV8 of each group.
837 D. Quantification of the percentage of the surviving neurons at DIV8, DIV15, DIV22 and DIV29
838 normalized to the number of neurons at DIV8 in the same group.
839 E. Example traces (left) and quantification of EPSC amplitude (right) from autaptic Stx1A-null
840 hippocampal mouse neurons rescued with STX1A or STX2.
841 F. Quantification of the cumulative charge transfer of the EPSC from the onset of the response until 0.55s
842 after.
843 G. Example traces of normalized EPSC to their peak amplitude (left) and quantification of the half width of
844 the EPSC (right).
845 H. Example traces (left) and quantification of the response induced by a 5s 0.5M application of sucrose,
846 which represents the readily releasable pool of vesicles (RRP).
847 I. Example traces (left) and quantification of the frequency of the miniature excitatory postsynaptic currents
848 (mEPSC) (rights).
849 J. Quantification of the vesicle release probability (PVR) as the ratio of the EPSC charge over the RRP
850 charge (PVR).
851 K. Quantification of the spontaneous vesicular release rate as the ratio between the of mEPSC frequency
852 and number of vesicles in the RRP.
853 L. Example traces (left) and the quantification of a 40Hz paired-pulse ratio (PPR).
854
855 Data information: In (D) data points represent mean ± SEM. In (D, E-L) data is show in a whisker-box plot.
856 Each data point represents single observations, middle line represents the median, boxes represent the
857 distribution of the data and external data points represent outliers. In (C, D) significances and P values of data
858 were determined by non-parametric Kruskal-Wallis test followed by Dunn's post hoc test; *$p \leq 0.05$,
859 **$p \leq 0.001$, ***$p \leq 0.001$, ****$p \leq 0.0001$. In (E-L) Significances and P values of data were determined by non-
860 parametric Mann-Whitney test and unpaired two-tailed t-test; *$p \leq 0.05$, **$p \leq 0.001$, ***$p \leq 0.001$,
861 ****$p \leq 0.0001$.
862
863
864 **Figure 2. The C-terminal half of the SNARE domain of STX1A has a regulatory effect on the RRP,**
865 **spontaneous release and both, N- and C-terminus have a role in the regulation of efficiency of Ca^{2+}-evoked**
866 **release**.
867 A. STX1A WT and chimera domain structure scheme, sequence alignment of STX1A and STX2 SNARE
868 domain and percentage homology between both SNARE domains.
869 B. Example traces (left) and quantification of the EPSC amplitude (right) from autaptic STX1A-null
870 hippocampal mouse neurons rescued with STX1A, STX1A-2(SNARE), STX1A-2(Nter) or STX1A-
871 2(Cter).
872 C. Quantification of the cumulative charge transfer of the EPSC from the onset of the response until 0.55s
873 after.
874 D. Example traces of normalized EPSC to their peak amplitude (left) and quantification of the half width of
875 the EPSC (right).
876 E. Example traces (left) and quantification of the frequency of the miniature excitatory postsynaptic currents
877 (mEPSC) (right).
878 F. Example traces (left) and quantification of the response induced by a 5s 0.5M application of sucrose,
879 which represents the readily releasable pool of vesicles (RRP).
880 G. Quantification of the vesicle release probability (PVR) as the ratio of the EPSC charge over the RRP
881 charge (PVR).

H. Quantification of the spontaneous vesicular release rate as the ratio between the of mEPSC frequency and number of vesicles in the RRP.
I. Example traces (left) and the quantification of a 40Hz paired-pulse ratio (PPR).
J. Quantification of STP measured by 50 stimulations at 10Hz.

Data information: In (B, D-I) data is show in a whisker-box plot. Each data point represents single observations, middle line represents the median, boxes represent the distribution of the data, where the majority of the data points lie and external data points represent outliers. In (J) data represents the mean ± SEM. Significances and P values of data were determined by non-parametric Kruskal-Wallis test followed by Dunn's post hoc test; *p≤0.05, **p≤0.001, ***p≤0.001, ****p≤0.0001.

Figure 3. The C-terminal half of the SNARE domain of STX1A has a regulatory effect on the RRP, spontaneous release and speed of release and Ca^{2+}-evoked release depends on the integrity of the SNARE domain.

A. STX2 WT and chimera domain structure scheme, sequence alignment of STX2 and STX1A SNARE domain and percentage homology between both SNARE domains.
B. Example traces (left) and quantification of the EPSC amplitude (right) from autaptic STX1A-null hippocampal mouse neurons rescued with STX2, STX2-1A(SNARE), STX2-1A(Nter) or STX2-1A(Cter).
C. Quantification of the cumulative charge transfer of the EPSC from the onset of the response until 0.55s after.
D. Example traces of normalized EPSC to their peak amplitude (left) and quantification of the half width of the EPSC (right).
E. Example traces (left) and quantification of the frequency of the miniature excitatory postsynaptic currents (mEPSC) (right).
F. Example traces (left) and quantification of the response induced by a 5s 0.5mM application of sucrose, which represents the readily releasable pool of vesicles (RRP).
G. Quantification of the vesicle release probability (PVR) as the ratio of the EPSC charge over the RRP charge (PVR).
H. Quantification of the spontaneous vesicular release rate as the ratio between the of mEPSC frequency and number of vesicles in the RRP.
I. Example traces (left) and the quantification of a 40Hz paired-pulse ratio (PPR).
J. Quantification of STP measured by 50 stimulations at 10Hz.

Data Information: In (E-L) data is show in a whisker-box plot. Each data point represents single observations, middle line represents the median, boxes represent the distribution of the data, where the majority of the data points lie and external data points represent outliers. Significances and P values of data were determined by non-parametric Kruskal-Wallis test followed by Dunn's post hoc test; *p≤0.05, **p≤0.001, ***p≤0.001, ****p≤0.0001.

Figure 4. **Quantification of Stx1A and Munc18-1 levels at the synapse**.

A. Example images of Stx1-null neurons plated in high-density cultures and rescued with STX1A, STX1A-2(SNARE), STX1A-2(Nter) or STX1A-2(Cter) or GFP (STX1-null) as negative control. Neurons were fixed between DIV14-16. Cultures were stained with fluorophore-labeled antibodies that recognize VGlut1 (red in merge), STX1A (green in merge) and Munc18-1(blue in merge), from left to right. Scale bar: 10μm.
B. Quantification of the immunofluorescent intensity of STX1A normalized to the intensity of the same VGlut1-labeled ROIs. Values where normalized to values in STX1A rescue group.
C. Quantification of the immunofluorescent intensity of Munc18-1 normalized to the intensity of the same VGlut1-labeled ROIs. Values where normalized to values in STX1 rescue group.
D. SDS-PAGE of the electrophoretic analysis of neuronal lysates obtained from each experimental group. Proteins were detected using antibodies that recognize β-Tubuline III as loading control, STX1A and Munc18-1.
E. Correlation between Munc18-1 values and EPSC amplitude of Stx1-null neurons expressing STX1A, STX2, STX2-1A(SNARE), STX2-1A(Nter) or STX2-1A(Cter).
F. Correlation between Munc18-1 values and RRP.
G. Correlation between Munc18-1 values and PVR.

H. Correlation between Munc18-1 values and spontaneous vesicular release rate

Data information: In (B, C) data is show in a whisker-box plot. Each data point represents single ROIs, middle line represents the median, boxes represent the distribution of the data, where the majority of the data points lie and external data points represent outliers. In (E-H) each data point is the correlation of the mean ±SEM. Significances and P values of data were determined by non-parametric Kruskal-Wallis test followed by Dunn's post hoc test; *p≤0.05, **p≤0.001, ***p≤0.001, ****p≤0.0001.

Figure 5. **Quantification of STX2 and Munc18-1 levels at the synapse**.

A. Example images of Stx1-null neurons plated in high-density cultures and rescued with STX1A, STX2, STX2-1A(SNARE), STX2-1A(Nter), STX2-1A(Cter) or GFP (STX1-null) as negative control. Between DIV14-16 neurons were fixed**. (A)** Example images of neurons stained with fluorophore-labeled antibodies that recognize VGlut1 (red in merge) and STX2 (green in merge), from left to right. Scale bar: 10μm.
B. Quantification of the immunofluorescent intensity of STX2 normalized to the intensity of the same VGlut1-labeled ROIs. Values where normalized to values in STX2 rescue group.
C. SDS-PAGE of the electrophoretic analysis of neuronal lysates obtained from each experimental group. Proteins were detected using antibodies that recognize β-Tubuline III as loading control and STX2.
D. SDS-PAGE of the electrophoretic analysis of neuronal lysates obtained from each experimental group Proteins were detected using antibodies that recognize β-Tubuline III and Munc18-1.
E. Example images of neurons stained with fluorophore-labeled antibodies that recognize VGlut1 (red in merge) and Munc18-1(blue in merge), from left to right. Scale bar: 10μm.
F. Quantification of the immunofluorescent intensity of Munc18-1 normalized to the intensity of the same VGlut1-labeled ROIs. Values where normalized to values in STX2 rescue group.
G. Correlation between Munc18-1 values and EPSC amplitude of STX1-null neurons expressing STX1A, STX2, STX2-1A(SNARE), STX2-1A(Nter) or STX2-1A(Cter).
H. Correlation between Munc18-1 values and RRP.
I. Correlation between Munc18-1 values and PVR.
J. Correlation between Munc18-1 values and spontaneous vesicular release rate.

Data information: In (B, F) data is show in a whisker-box plot. Each data point represents single ROIs, middle line represents the median, boxes represent the distribution of the data, where the majority of the data points lie and external data points represent outliers. In (G-J) each data point is the correlation of the mean ±SEM. Significances and P values of data were determined by non-parametric Kruskal-Wallis test followed by Dunn's post hoc test; *p≤0.05, **p≤0.001, ***p≤0.001, ****p≤0.0001.

Figure 6. The charge of the outer-surface residues in the C-terminal half of the SNARE domain is important for clamping spontaneous release and D231,R232 are important in the stabilization of the pool and the efficiency of Ca^{2+}-evoked release.

A. Sequence of the C-terminal half of STX1A and STX2 and single- and double-point mutations in the sequence of STX1A WT.
B. Example traces (left) and quantification of the EPSC amplitude (right) from autaptic STX1A-null hippocampal mouse neurons rescued with STX1A, $STX1A^{D231N}$, $STX1A^{R232N}$, $STX1A^{Y235R}$, $STX1A^{E238V}$, $STX1A^{V248K}$, $STX1A^{S249E}$ $STX1A^{D231N,R232N}$ and $STX1A^{V248K,S249E}$.
C. Example traces (left) and quantification of the response induced by a 5s 0.5M application of sucrose, which represents the readily releasable pool of vesicles (RRP).
D. Quantification of the vesicle release probability (PVR) as the ratio of the EPSC charge over the RRP charge (PVR).
E. Example traces (left) and quantification of the frequency of the miniature excitatory postsynaptic currents (mEPSC) (right).
F. Quantification of the spontaneous vesicular release rate as the ratio between the of mEPSC frequency and number of vesicles in the RRP.
G. Quantification of the spontaneous vesicular release rate as the ratio between the of mEPSC frequency and number of vesicles in the RRP but STX1A-2(SNARE) and STX1A-2(Cter) chimeras were removed.
H. Correlation between the RRP and the spontaneous vesicular release rate. Both values are normalized to STX1A WT.

I. Correlation between the PVR and the spontaneous vesicular release rate. Values are normalized to STX1A WT.

Data information: (B-H) All electrophysiological recording were done on autaptic neurons. Between 30-35 neurons per group from 3 independent cultures where recorded. Values from STX1A-2(SNARE) and STX1A-2(Cter) groups were taken from the experiments done in Figure 2 and normalized to their own STX1A WT control and used here for visual comparison. Data points represent the mean ±SEM. Significances and P values of data were determined by non-parametric Kruskal-Wallis test followed by Dunn's post hoc test; *p≤0.05, **p≤0.001, ***p≤0.001, ****p≤0.0001.

Figure 7. Speculative model based on most important changes in electrophysiological properties found in STX1A-chimeras or STX2-chimeras compared to their WT isoforms.

A. Energy landscape for priming and fusion of synaptic vesicles that have SNARE complexes formed with STX1A WT (black line), STX1A-2(SNARE or Cter) (red dotted line), STX2 WT (blue line); STX2-1A(Cter) (light green dotted line).
B. Summarized conclusions of our results based on the speculative model in (A).
C. Changes in the equilibrium between the unprimed/primed state (reflecting the stability of the primed state) and the rate of fusion (energy landscape for fusion adapted from "Sørensen, 2009").

Figure 1 – figure supplement 1. STX2 is expressed in Stx1-null hippocampal neurons.

A. SDS-PAGE electrophoresis of lysates from STX1-null neurons infected with STX1A, STX2 or GFP (STX1-null) and STX3A as negative controls. Proteins were detected using antibodies that recognize β-Tubuline III as loading control STX1A, STX2 and STX3A.
B. Example images of mass-culture hippocampal neurons stained with fluorophore-labeled antibodies that recognize VGlut1 (red in merge) and STX2 (green in merge) from left to right. Scale bar: 10μm.
C. Quantification of the immunofluorescent intensity of STX2 normalized to the intensity of the same VGlut1-labeled ROIs.

Data information: In (C) data is show in a whisker-box plot. Each data point represents single ROIs, middle line represents the median, boxes represent the distribution of the data, where the majority of the data points lie and external data points represent outliers. Significances and P values of data were determined by non-parametric Kruskal-Wallis test followed by Dunn's post hoc test; *p≤0.05, **p≤0.001, ***p≤0.001, ****p≤0.0001.

Figure 2 – figure supplement 1. Quantification of kinetic parameters of the EPSC and the mEPSC of autaptic STX1A-null hippocamapal mouse neurons rescued with STX1A, STX1A-2(SNARE), STX1A-2(Nter) or STX1A-2(Cter).

A. Quantification of the rise time (20-80%) of the EPSC neurons
B. Quantification of the decay time (80-20%) of the EPSC.
C. Quantification of the mEPSC charge.
D. Quantification of the mEPSC amplitude.
E. Quantification of the rise time (20-80%) of the mEPSC.
F. Quantification of the half width of the mEPSC.
G. Quantification of the decay time of the mEPSC.
H. All electrophysiological recording were done on autaptic neurons.

Data information: Data in (A-G) is show in a whisker-box plot. Each data point represents single observations, middle line represents the median, boxes represent the distribution of the data, where the majority of the data points lie and external data points represent outliers. Significances and P values of data were determined by non-parametric Kruskal-Wallis test followed by Dunn's post hoc test or by Ordinary one-way ANOVA; *p≤0.05, **p≤0.001, ***p≤0.001, ****p≤0.0001.

Figure 3 – figure supplement 1. Quantification of kinetic parameters of the EPSC and the mEPSC of autaptic STX1A-null hippocamapal mouse neurons rescued with STX2, STX2-1A(SNARE), STX2-1A(Nter) or STX2-1A(Cter).

A. Quantification of the rise time (20-80%) of the EPSC.
B. Quantification of the decay time (80-20%) of the EPSC.
C. Quantification of the mEPSC charge.
D. Quantification of the mEPSC amplitude.
E. Quantification of the rise time (20-80%) of the mEPSC.
F. Quantification of the half width of the mEPSC.
G. Quantification of the decay time of the mEPSC.

Data information: Data in (A-G) is show in a whisker-box plot. Each data point represents single observations, middle line represents the median, boxes represent the distribution of the data, where the majority of the data points lie and external data points represent outliers. Significances and P values of data were determined by non-parametric Kruskal-Wallis test followed by Dunn's post hoc test or by Ordinary one-way ANOVA; **$p \leq 0.05$, **$p \leq 0.001$, ***$p \leq 0.001$, ****$p \leq 0.0001$.

Figure 6 – figure supplement 1. **Quantification of STX2 levels at the synapse**.

A. Example images of Stx1-null neurons plated in high-density cultures and rescued with STX1A, STX2, STX2-1A(SNARE), STX2-1A(Nter), STX2-1A(Cter) or GFP (STX1-null) as negative control. Between DIV14-16 neurons were fixed**.** Neurons were stained with fluorophore-labeled antibodies that recognize VGlut1 (red in merge), STX1A (green in merge) and Munc18-1 (blue in merge) from left to right. Scale bar: 10μm.
B. Quantification of the immunofluorescent intensity of STX1A normalized to the intensity of the same VGlut1-labeled ROIs. Values where normalized to STX1A WT values.
C. Quantification of the immunofluorescent intensity of Munc18-1 normalized to the intensity of the same VGlut1-labeled ROIs. Values where normalized to STX1A WT values.
D. SDS-PAGE of the electrophoretic analysis of neuronal lysates obtained from each experimental group. Proteins were detected using antibodies that recognize β-Tubuline III as loading control and STX1A and Munc18-1.

Data information: In (B, C) data points represent the mean ±SEM. 7-9 images per group per culture were obtained and 3-5 ROI per image where analyzed. 3 replicates per group. Significances and P values of data were determined by non-parametric Kruskal-Wallis test followed by Dunn's post hoc test; *$p \leq 0.05$, **$p \leq 0.001$, ***$p \leq 0.001$, ****$p \leq 0.0001$.

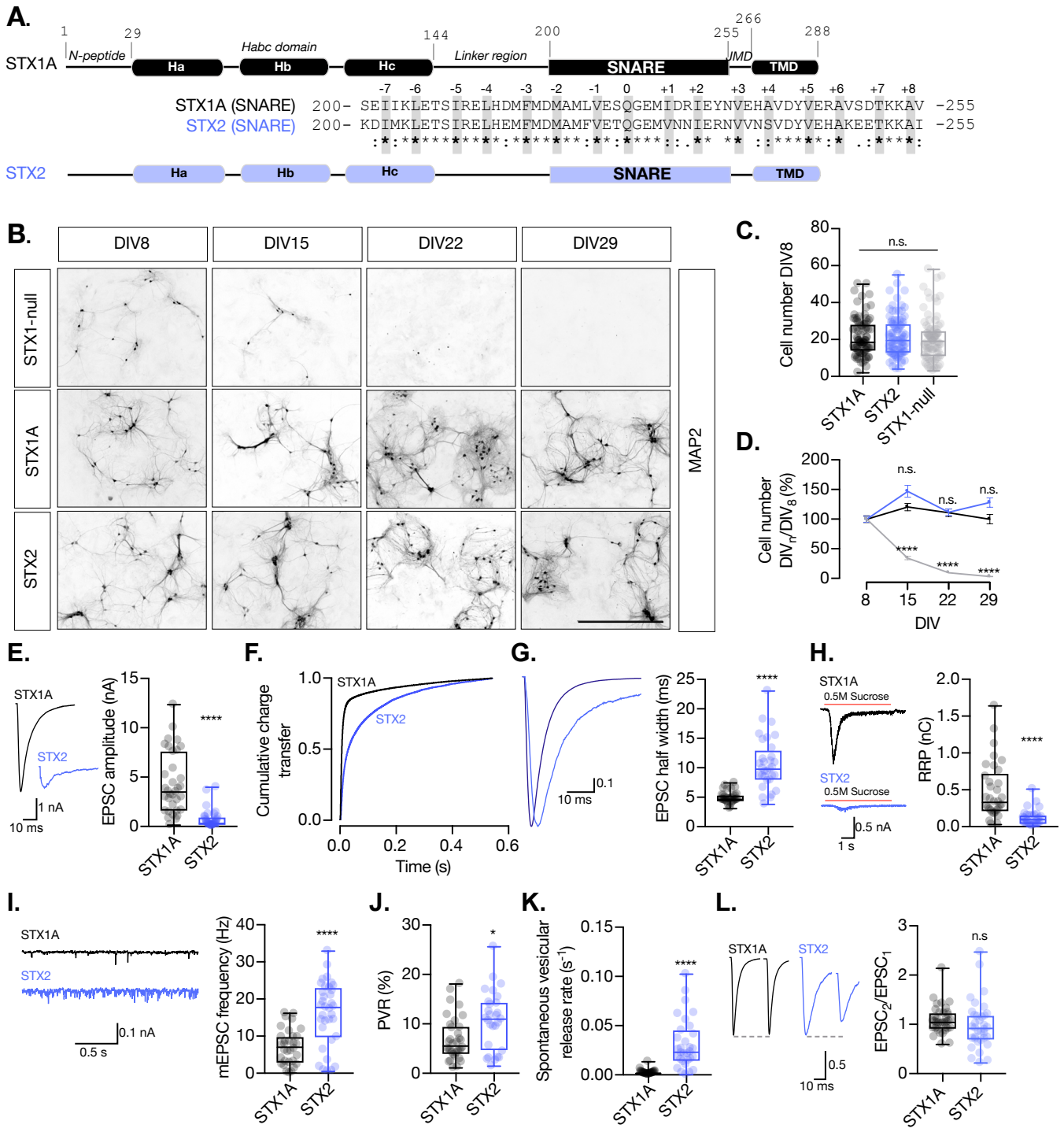

A.
STX1A
N-peptide
Ha
Habc domain
Hb
Hc
Linker region
SNARE
JMD
TMD
STX1A (SNARE) 200- SEIIKLETSIRELHDMFMDMAMLVESQGEMIDRIEYNVEHAVDYVERAVSDTKKAV -255
STX2 (SNARE) 200- KDIMKLETSIRELHEMFMDMAMFVETQGEMVNNIERNVVNSVDYVEHAKEETKKAI -255
STX2
B.
DIV8
DIV15
DIV22
DIV29
STX1-null
STX1A
STX2
MAP2
C.
Cell number DIV8
n.s.
STX1A
STX2
STX1-null
D.
Cell number DIV8 (%)
n.s.
****
DIV
E.
STX1A
STX2
1 nA
10 ms
EPSC amplitude (nA)
****
F.
Cumulative charge transfer
Time (s)
G.
0.1
10 ms
EPSC half width (ms)
****
H.
0.5M Sucrose
0.5 nA
1 s
RRP (nC)
****
I.
0.1 nA
0.5 s
mEPSC frequency (Hz)
****
J.
PVR (%)
*
K.
Spontaneous vesicular release rate (s-1)
****
L.
0.5
10 ms
EPSC2/EPSC1
n.s

Figure 1

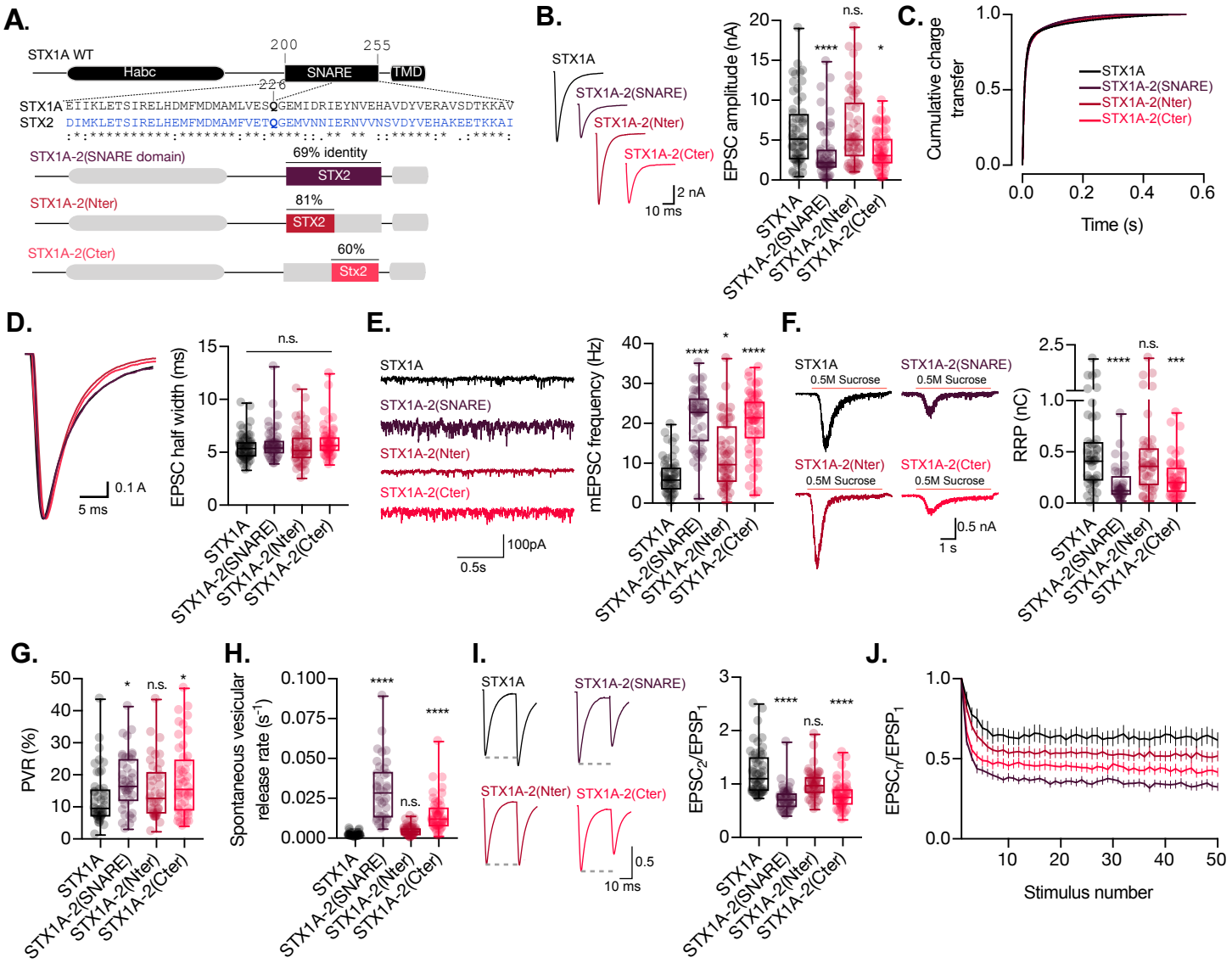

A.
STX1A WT
Habc
SNARE
TMD
200
255
226
STX1A EIIKLETSIRELHDMFMDMAMLVESQGEMIDRIEYNVEHAVDYVERAVSDTKKAV
STX2 DIMKLETSIRELHEMFMDMAMFVETQGEMVNNIERNVVNSVDYVEHAKEETKKAI
STX1A-2(SNARE domain)
69% identity
STX2
STX1A-2(Nter)
81%
STX2
STX1A-2(Cter)
60%
Stx2
B.
STX1A
STX1A-2(SNARE)
STX1A-2(Nter)
STX1A-2(Cter)
2 nA
10 ms
EPSC amplitude (nA)
n.s.
****
*
C.
Cumulative charge transfer
Time (s)
STX1A
STX1A-2(SNARE)
STX1A-2(Nter)
STX1A-2(Cter)
D.
0.1 A
5 ms
EPSC half width (ms)
n.s.
E.
STX1A
STX1A-2(SNARE)
STX1A-2(Nter)
STX1A-2(Cter)
100pA
0.5s
mEPSC frequency (Hz)
F.
STX1A
0.5M Sucrose
STX1A-2(SNARE)
0.5M Sucrose
STX1A-2(Nter)
0.5M Sucrose
STX1A-2(Cter)
0.5M Sucrose
0.5 nA
1 s
RRP (nC)
n.s.
***
G.
PVR (%)
n.s.
H.
Spontaneous vesicular release rate (s-1)
n.s.
I.
STX1A
STX1A-2(SNARE)
STX1A-2(Nter)
STX1A-2(Cter)
0.5
10 ms
EPSC2/EPSP1
n.s.
J.
EPSCn/EPSP1
Stimulus number
STX1A
STX1A-2(SNARE)
STX1A-2(Nter)
STX1A-2(Cter)

Figure 2

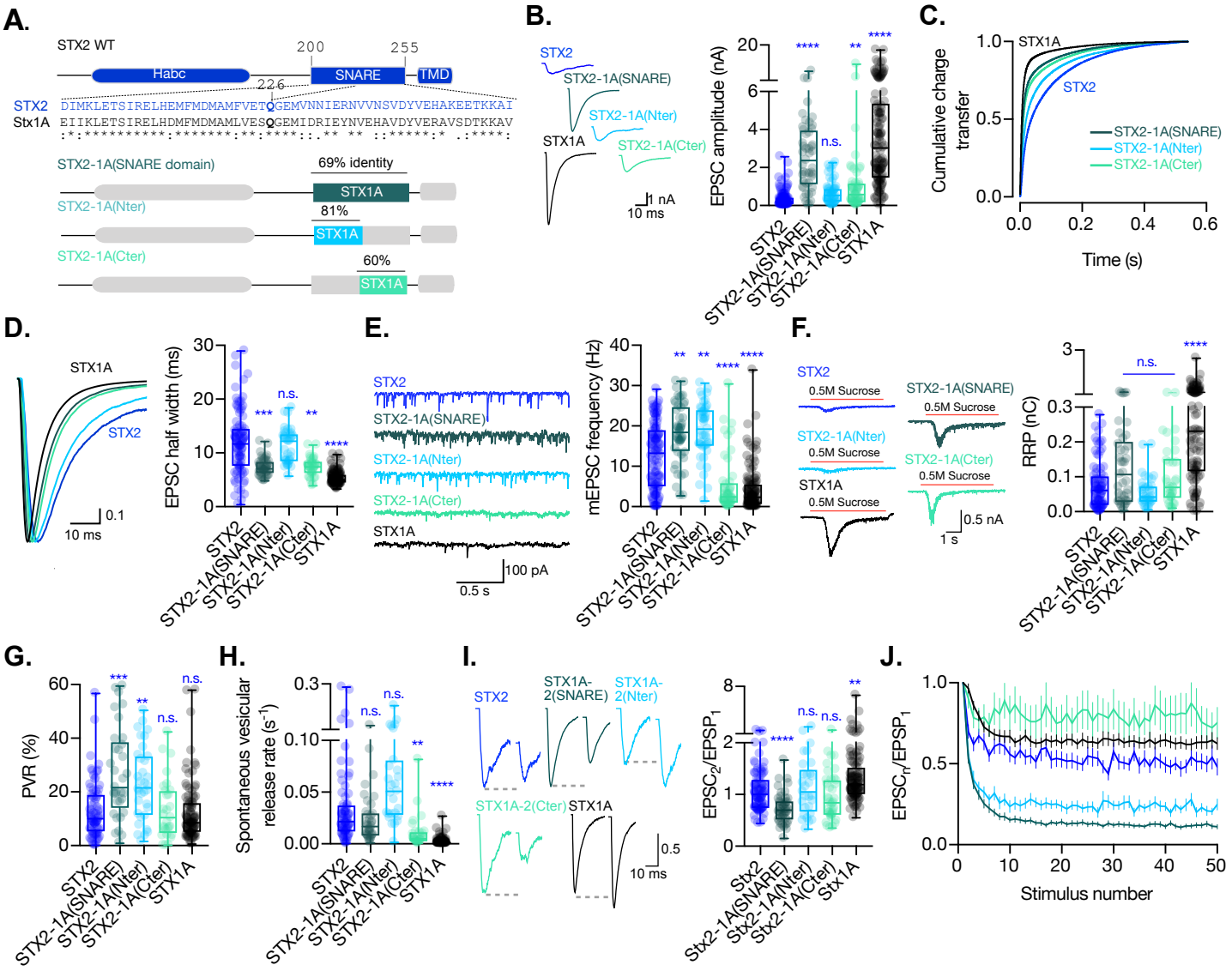

A.
STX2 WT
200
255
Habc
SNARE
TMD
226
STX2 DIMKLETSIRELHEMFMDMAMFVETQGEMVNNIERNVVNSVDYVEHAKEETKKAI
Stx1A EIIKLETSIRELHDMFMDMAMLVESQGEMIDRIEYNVEHAVDYVERAVSDTKKAV
STX2-1A(SNARE domain)
69% identity
STX1A
STX2-1A(Nter)
81%
STX1A
STX2-1A(Cter)
60%
STX1A
B.
STX2
STX2-1A(SNARE)
STX2-1A(Nter)
STX1A
STX2-1A(Cter)
1 nA
10 ms
EPSC amplitude (nA)
n.s.
STX2
STX2-1A(SNARE)
STX2-1A(Nter)
STX2-1A(Cter)
STX1A
C.
STX1A
STX2
Cumulative charge transfer
STX2-1A(SNARE)
STX2-1A(Nter)
STX2-1A(Cter)
Time (s)
D.
STX1A
STX2
0.1
10 ms
EPSC half width (ms)
n.s.
E.
STX2
STX2-1A(SNARE)
STX2-1A(Nter)
STX2-1A(Cter)
STX1A
100 pA
0.5 s
mEPSC frequency (Hz)
F.
STX2
0.5M Sucrose
STX2-1A(SNARE)
0.5M Sucrose
STX2-1A(Nter)
0.5M Sucrose
STX2-1A(Cter)
0.5M Sucrose
STX1A
0.5M Sucrose
0.5 nA
1 s
RRP (nC)
n.s.
G.
PVR (%)
n.s.
H.
Spontaneous vesicular release rate (s-1)
n.s.
I.
STX2
STX1A-2(SNARE)
STX1A-2(Nter)
STX1A-2(Cter)
STX1A
0.5
10 ms
EPSC2/EPSP1
n.s.
J.
EPSCn/EPSP1
Stimulus number

Figure 3

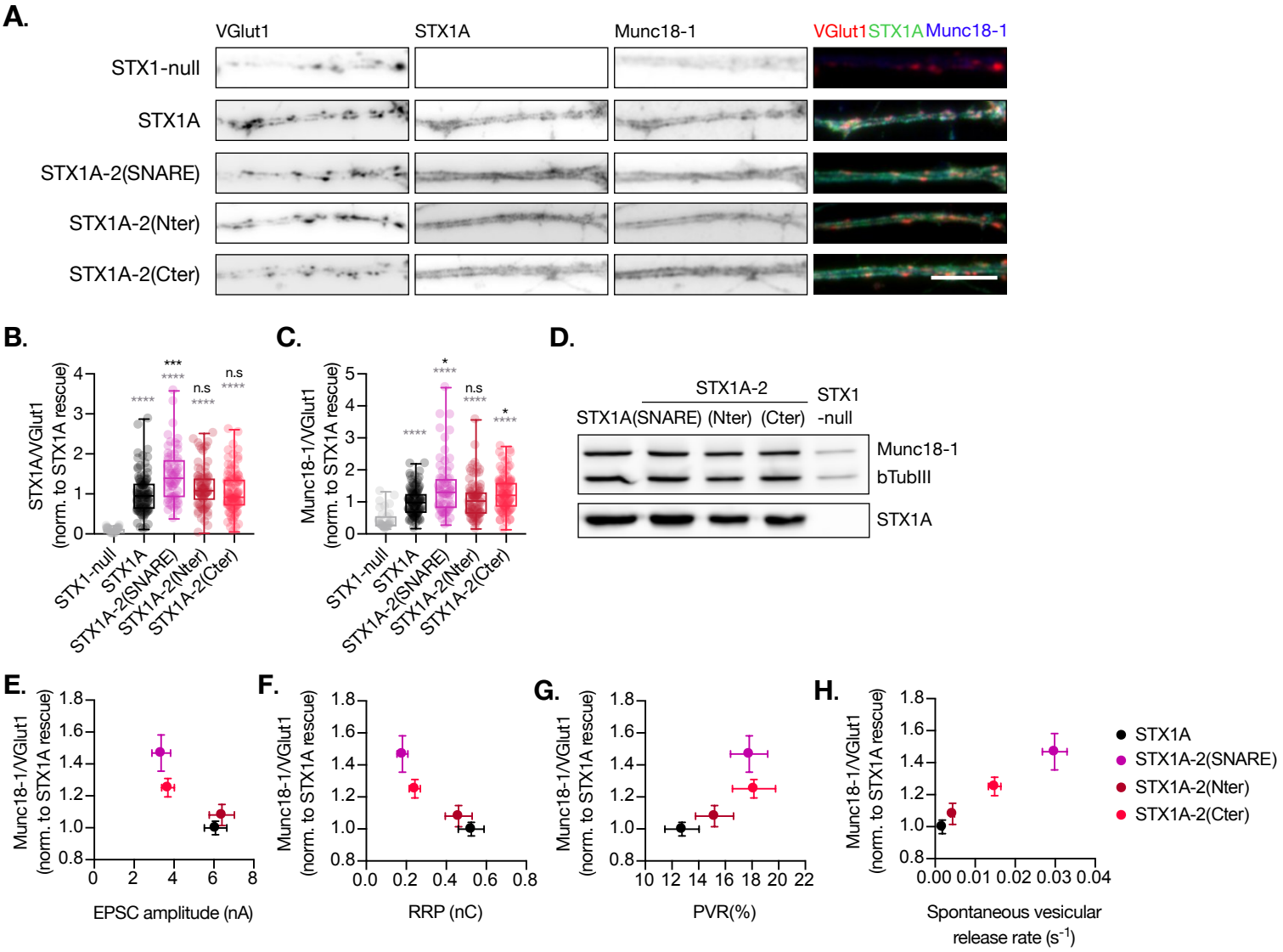

A.
VGlut1
STX1A
Munc18-1
VGlut1STX1AMunc18-1
STX1-null
STX1A
STX1A-2(SNARE)
STX1A-2(Nter)
STX1A-2(Cter)
B.
STX1A/VGlut1 (norm. to STX1A rescue)
C.
Munc18-1/VGlut1 (norm. to STX1A rescue)
D.
STX1A-2
STX1A(SNARE) (Nter) (Cter)
STX1 -null
Munc18-1
bTubIII
STX1A
E.
EPSC amplitude (nA)
F.
RRP (nC)
G.
PVR(%)
H.
Spontaneous vesicular release rate (s-1)
STX1A
STX1A-2(SNARE)
STX1A-2(Nter)
STX1A-2(Cter)

Figure 4

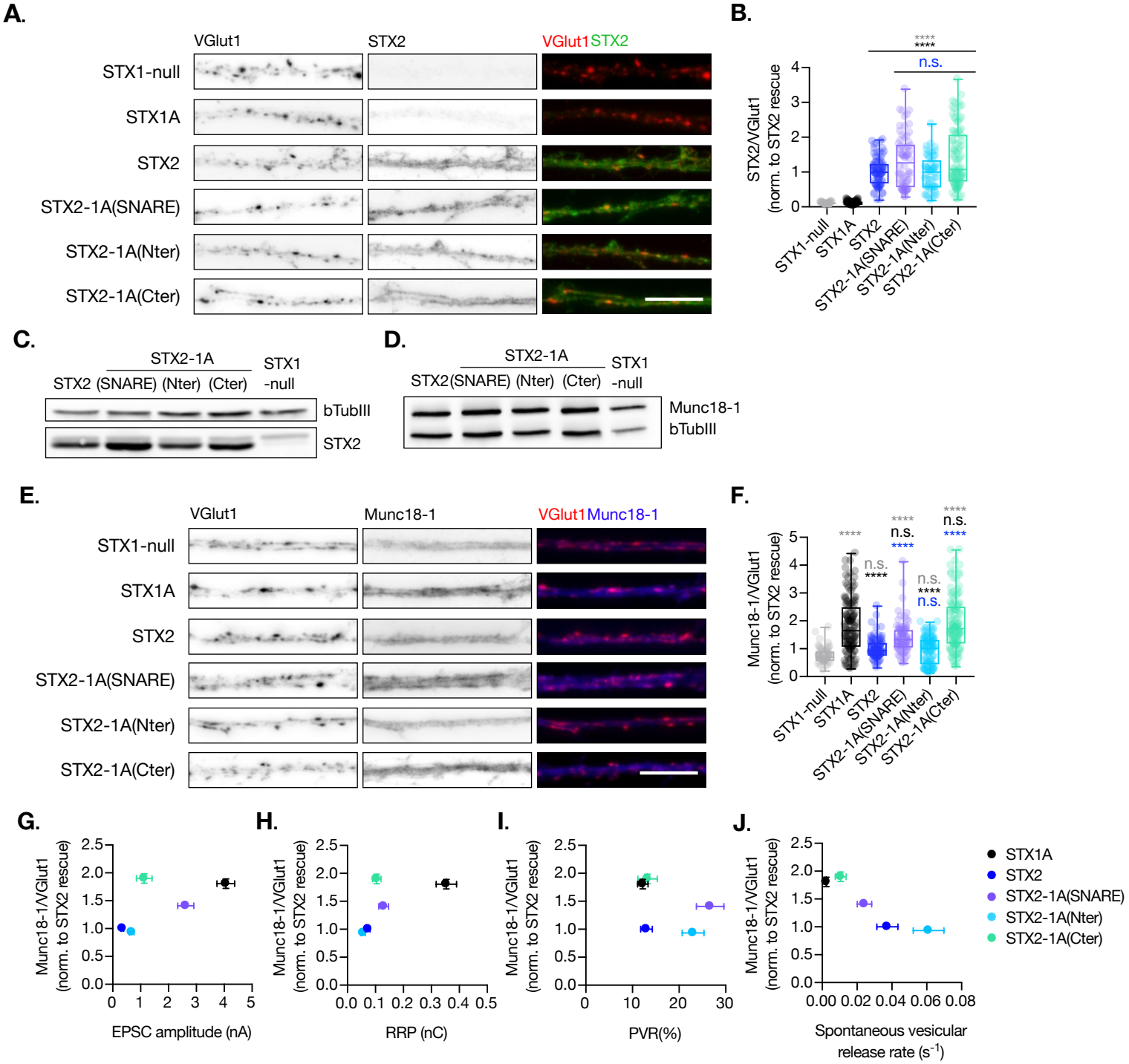

A.
VGlut1
STX2
VGlut1 STX2
STX1-null
STX1A
STX2
STX2-1A(SNARE)
STX2-1A(Nter)
STX2-1A(Cter)
B.
STX2/VGlut1 (norm. to STX2 rescue)
****
****
n.s.
STX1-null
STX1A
STX2
STX2-1A(SNARE)
STX2-1A(Nter)
STX2-1A(Cter)
C.
STX2-1A
STX2 (SNARE) (Nter) (Cter)
STX1 -null
bTubIII
STX2
D.
STX2-1A
STX2(SNARE) (Nter) (Cter)
STX1 -null
Munc18-1
bTubIII
E.
VGlut1
Munc18-1
VGlut1 Munc18-1
STX1-null
STX1A
STX2
STX2-1A(SNARE)
STX2-1A(Nter)
STX2-1A(Cter)
F.
Munc18-1/VGlut1 (norm. to STX2 rescue)
****
n.s.
****
n.s.
****
n.s.
****
n.s.
****
n.s.
****
STX1-null
STX1A
STX2
STX2-1A(SNARE)
STX2-1A(Nter)
STX2-1A(Cter)
G.
Munc18-1/VGlut1 (norm. to STX2 rescue)
EPSC amplitude (nA)
H.
Munc18-1/VGlut1 (norm. to STX2 rescue)
RRP (nC)
I.
Munc18-1/VGlut1 (norm. to STX2 rescue)
PVR(%)
J.
Munc18-1/VGlut1 (norm. to STX2 rescue)
Spontaneous vesicular release rate (s-1)
STX1A
STX2
STX2-1A(SNARE)
STX2-1A(Nter)
STX2-1A(Cter)

Figure 5

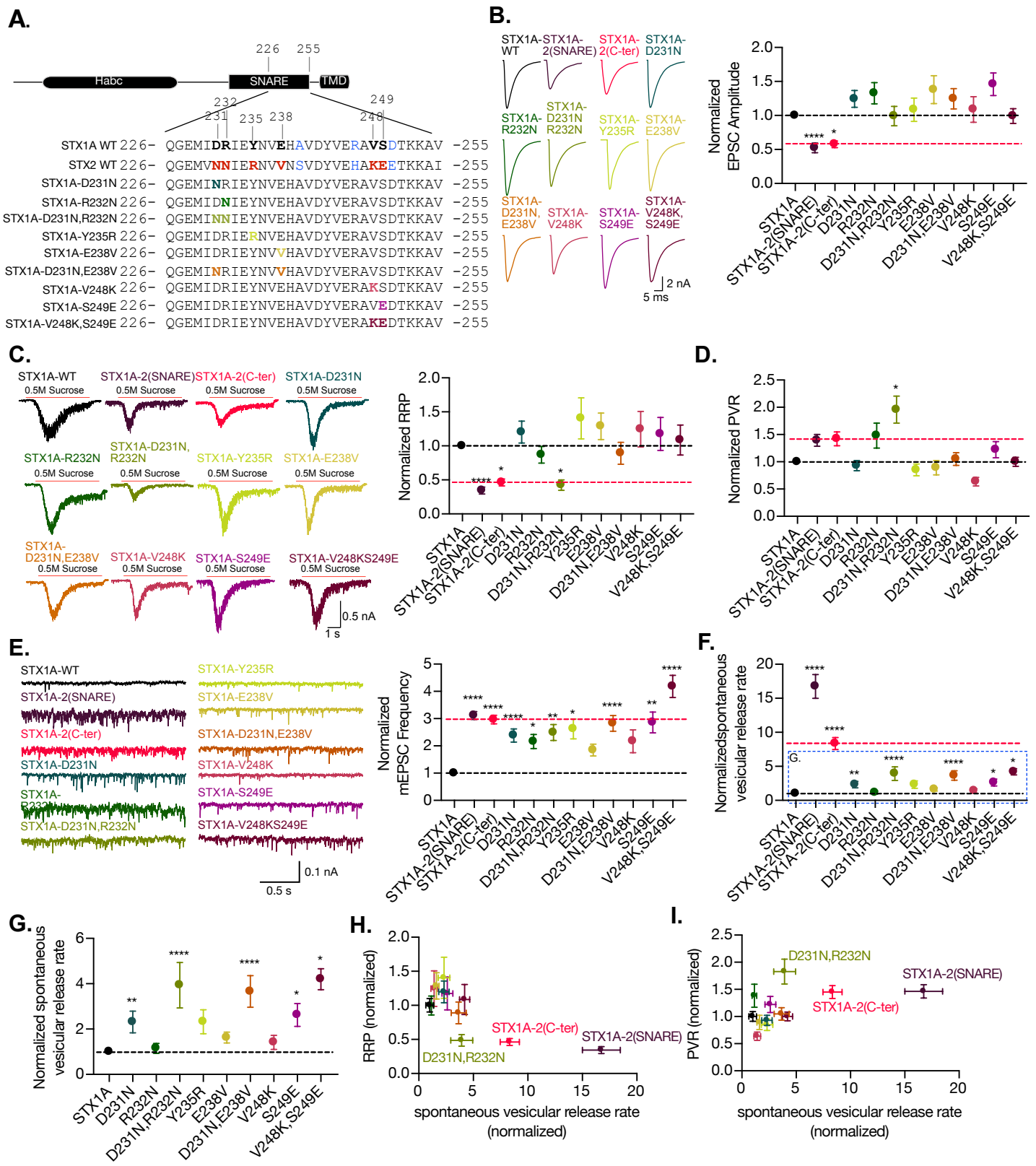
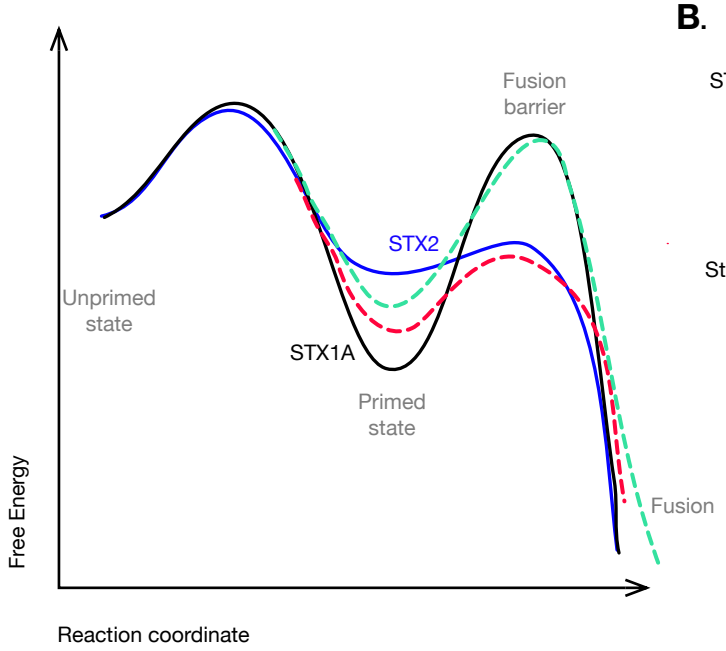

A.
Habc
SNARE
TMD
226
255
232
231
235
238
248
249
STX1A WT 226- QGEMIDRIEYNVEHAVDYVERAVSDTKKAV -255
STX2 WT 226- QGEMVNNIERNVVNSVDYVEHAKEETKKAI -255
STX1A-D231N 226- QGEMINRIEYNVEHAVDYVERAVSDTKKAV -255
STX1A-R232N 226- QGEMIDNIEYNVEHAVDYVERAVSDTKKAV -255
STX1A-D231N,R232N 226- QGEMINNIEYNVEHAVDYVERAVSDTKKAV -255
STX1A-Y235R 226- QGEMIDRIERNVEHAVDYVERAVSDTKKAV -255
STX1A-E238V 226- QGEMIDRIEYNVVHAVDYVERAVSDTKKAV -255
STX1A-D231N,E238V 226- QGEMINRIEYNVVHAVDYVERAVSDTKKAV -255
STX1A-V248K 226- QGEMIDRIEYNVEHAVDYVERAKSDTKKAV -255
STX1A-S249E 226- QGEMIDRIEYNVEHAVDYVERAVEDTKKAV -255
STX1A-V248K,S249E 226- QGEMIDRIEYNVEHAVDYVERAKEDTKKAV -255
B.
STX1A-WT
STX1A-2(SNARE)
STX1A-2(C-ter)
STX1A-D231N
STX1A-R232N
STX1A-D231N R232N
STX1A-Y235R
STX1A-E238V
STX1A-D231N, E238V
STX1A-V248K
STX1A-S249E
STX1A-V248K, S249E
2 nA
5 ms
Normalized EPSC Amplitude
C.
0.5M Sucrose
STX1A-WT
STX1A-2(SNARE)
STX1A-2(C-ter)
STX1A-D231N
STX1A-R232N
STX1A-D231N, R232N
STX1A-Y235R
STX1A-E238V
STX1A-D231N,E238V
STX1A-V248K
STX1A-S249E
STX1A-V248KS249E
0.5 nA
1 s
Normalized RRP
D.
Normalized PVR
E.
STX1A-WT
STX1A-2(SNARE)
STX1A-2(C-ter)
STX1A-D231N
STX1A-R232N
STX1A-D231N,R232N
STX1A-Y235R
STX1A-E238V
STX1A-D231N,E238V
STX1A-V248K
STX1A-S249E
STX1A-V248KS249E
0.1 nA
0.5 s
Normalized mEPSC Frequency
F.
Normalizedspontaneous vesicular release rate
G.
Normalized spontaneous vesicular release rate
H.
RRP (normalized)
D231N,R232N
STX1A-2(C-ter)
STX1A-2(SNARE)
spontaneous vesicular release rate (normalized)
I.
PVR (normalized)
D231N,R232N
STX1A-2(SNARE)
STX1A-2(C-ter)
spontaneous vesicular release rate (normalized)

Figure 6

A.



B.

STX1A-2(Cter) vs. Stx1A WT

- Decrease in the stability of the RRP
- Decrease in the height of the energy barrier for fusion (increase in PVR and release rates)
- Increase in the fusogenicity of the vesicles

Stx2-1A(Cter) vs. STX2 WT

- Increase in the stability of the RRP
- Increase in the height of the energy barrier for fusion (decrease in release rates)
- Decrease in the fusogenicity of the vesicles

C.

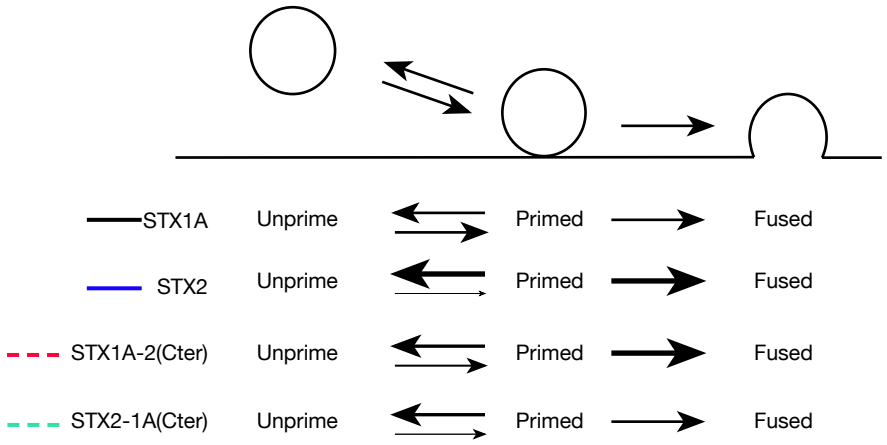


Figure 7

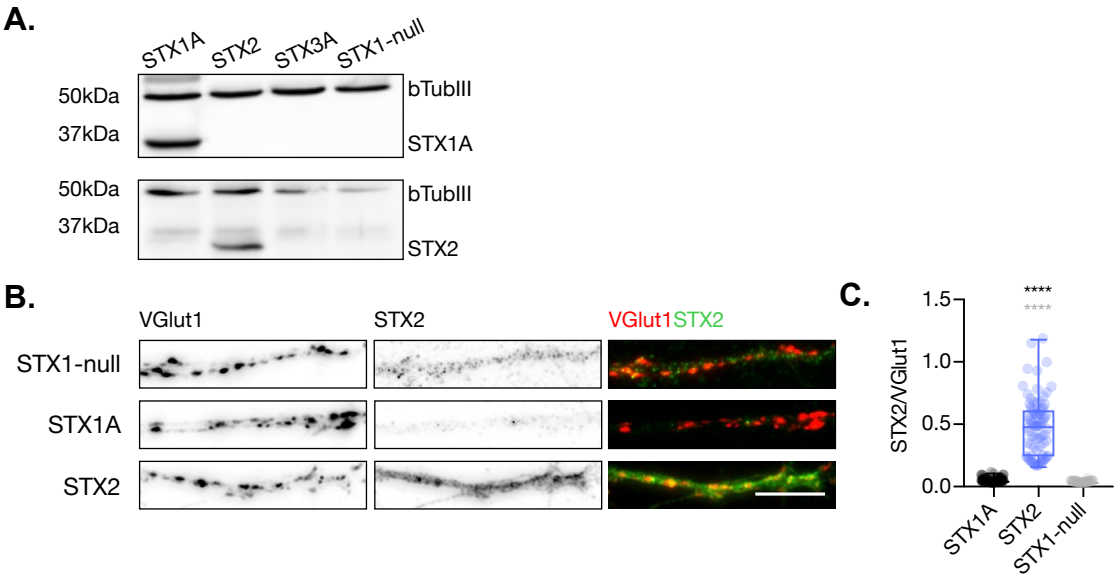


Figure 1- figure supplement 1

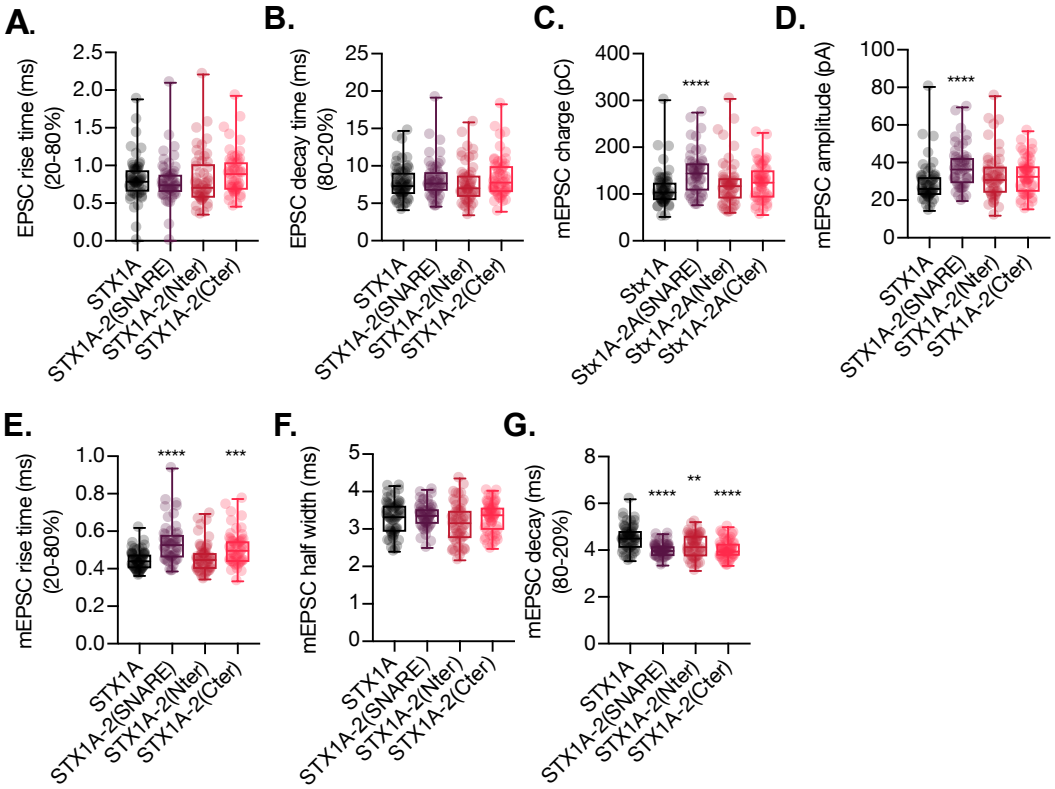


Figure 2- figure supplement 1

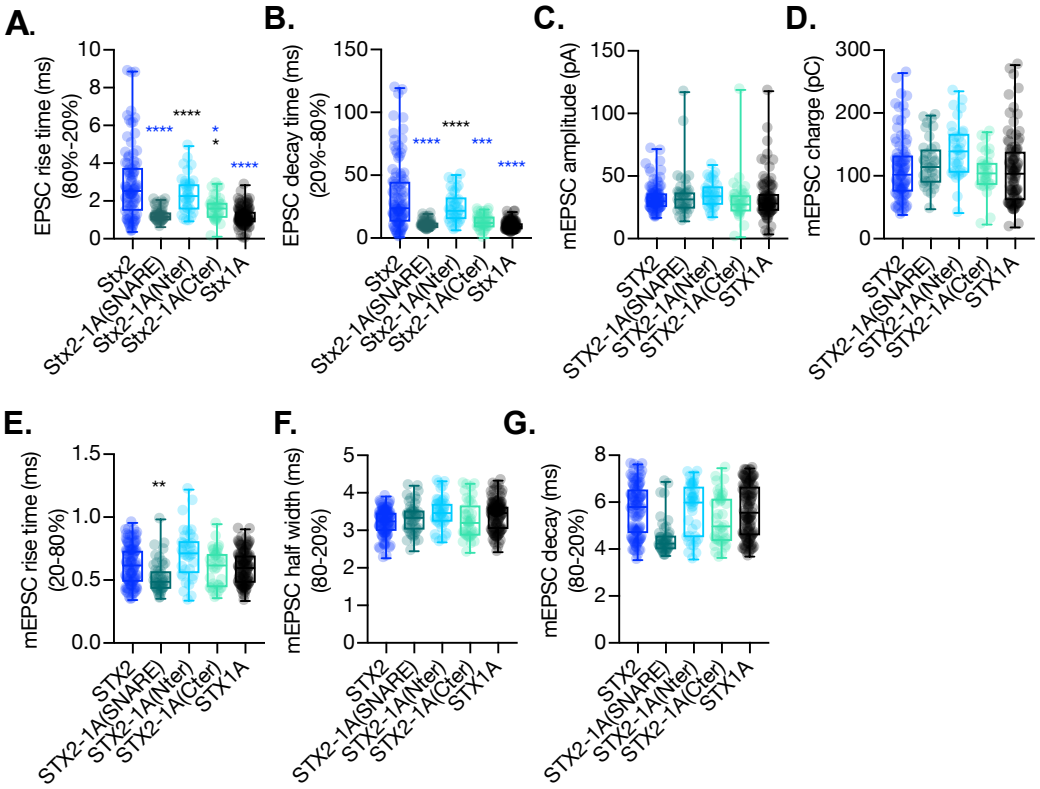


Figure 3- figure supplement 1

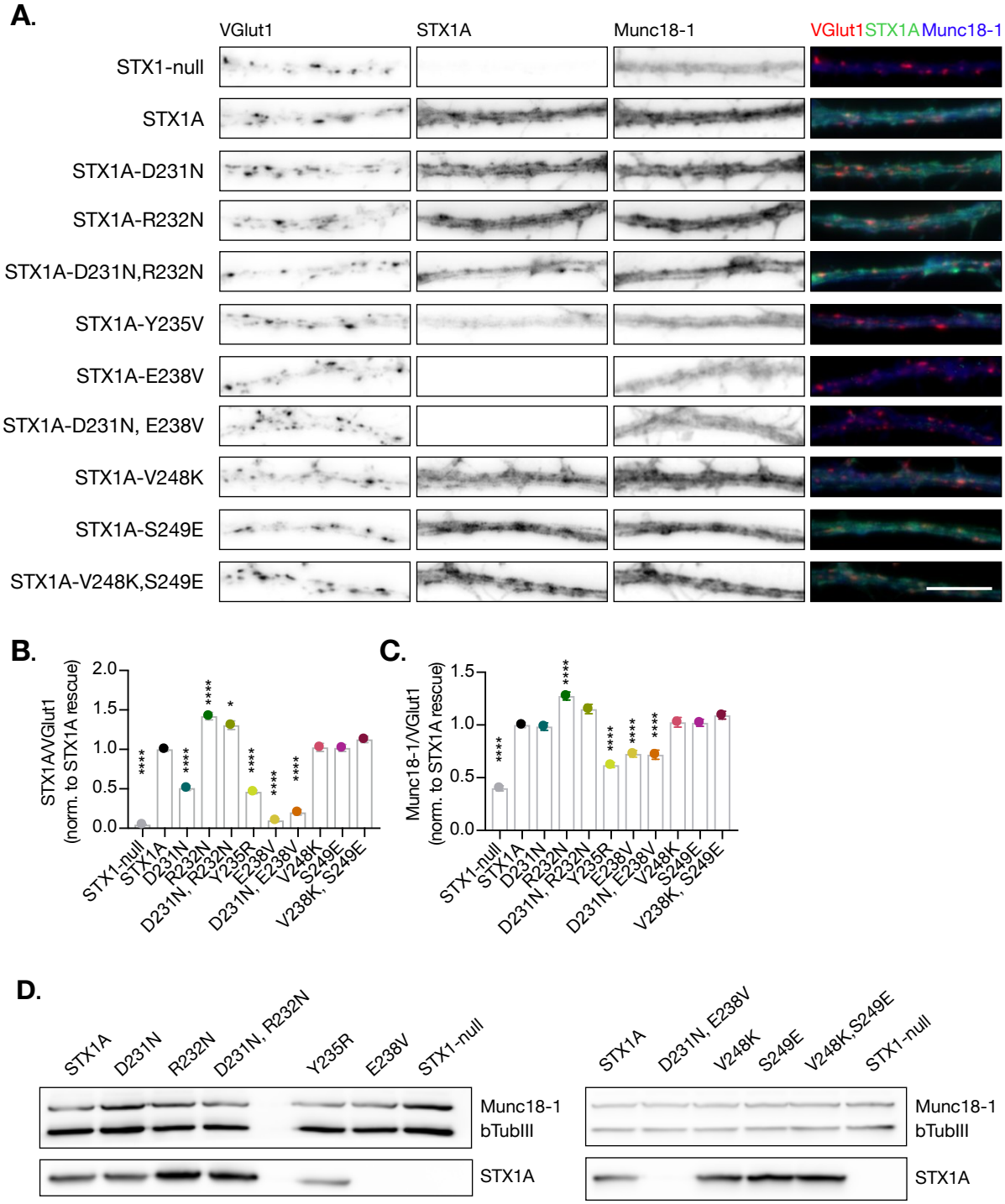


Figure 6- figure supplement 1