Role of Aberrant Spontaneous Neurotransmission in SNAP25-Associated Encephalopathies

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In Brief
Alten et al. show how SNAP25 variants give rise to clinically heterogeneous developmental and epileptic encephalopathies using structural and electrophysiological approaches. Their study identifies aberrant spontaneous neurotransmission as a culprit and suggests that therapies specifically targeting spontaneous release would be beneficial in treatment of these intractable disorders.

Highlights
- SNAP25 variants cause clinically variable developmental-epileptic encephalopathies
- Structurally clustered mutations give rise to similar synaptic phenotypes
- Haploinsufficiency and impaired evoked release cannot fully explain the pathology
- Aberrant spontaneous release phenotypes are associated with disease heterogeneity

Graphical Abstract

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SUMMARY

SNARE (soluble N-ethylmaleimide sensitive factor attachment protein receptor) complex, composed of synaptobrevin, syntaxin, and SNAP25, forms the essential fusion machinery for neurotransmitter release. Recent studies have reported several mutations in the gene encoding SNAP25 as a causative factor for developmental and epileptic encephalopathies of infancy and childhood with diverse clinical manifestations. However, it remains unclear how SNAP25 mutations give rise to these disorders. Here, we show that although structurally clustered mutations in SNAP25 give rise to related synaptic transmission phenotypes, specific alterations in spontaneous neurotransmitter release are a key factor to account for disease heterogeneity. Importantly, we identified a single mutation that augments spontaneous release without altering evoked release, suggesting that aberrant spontaneous release is sufficient to cause disease in humans.

INTRODUCTION

Developmental and epileptic encephalopathies (DEEs) of infancy and childhood is a group of heterogeneous and treatment-resistant disorders characterized by developmental slowing as a direct result of either the epileptic activity, the underlying cause independent from the epileptic activity, or a combination of both (Berg et al., 2010; Scheffer et al., 2017). Recent work has identified de novo mutations in genes mostly coding for proteins involved in synaptic transmission leading to these disorders, the cause of which was previously unknown (Claes et al., 2001; EuroEPINOMICS-RES Consortium et al., 2014; McTague et al., 2016; Thomas and Berkovic, 2014). Synaptic transmission relies on evoked release, in which depolarization of presynaptic terminal by an invading action potential causes neurotransmitter release. SNARE (soluble N-ethylmaleimide sensitive factor attachment protein receptor) complex composed of synaptobrevin-2 on synaptic vesicle, and SNAP25 and syntaxin-1 on plasma membrane, forms the essential fusion machinery for neurotransmitter release (Sollner et al., 1993a, 1993b; Weber et al., 1998). With the advent of next-generation sequencing, many de novo mutations in the genes coding for SNAP25 (Deciphering Developmental Disorders Study, 2015, 2017; Fukuda et al., 2018; Gabriel et al., 2016; Hamdan et al., 2017; Heyne et al., 2018; Liang et al., 2018; Rohena et al., 2013; Shen et al., 2014), synaptobrevin-2 (Salpietro et al., 2019; Simmons et al., 2020), syntaxin-1 (Vardar et al., 2020; Wolking et al., 2019), and synaptoTagmin-1 (Syt1) (Baker et al., 2018), the calcium sensor for fast synchronized release, have been identified in patients with DEEs. Among these, SNAP25 comprises the highest number of disease-causing mutations scattered throughout the SNARE complex, making SNAP25 an ideal model to explain how aberrant fusion machinery gives rise to DEEs with diverse clinical manifestations and varying severities. Here, we characterize SNAP25 variant-specific synaptic disturbances by structural, biochemical, and electrophysiological approaches. A total of 10 different heterozygous mutations in the evolutionarily conserved gene encoding SNAP25 have been reported to date (Table 1; Figure S1). All of these mutations are de novo in origin with the exception of R59P, which was inherited from an asymptomatic mosaic father and shared by two affected siblings (Fukuda et al., 2018). Alternative splicing of exon 5 (consisting of residues R59 and I67) of SNAP25 produces two different isoforms, SNAP25a and SNAP25b, which differ in only 9 of 206 amino acids (Bark and Wilson, 1994). The expression levels of these isoforms vary with the developmental stage. SNAP25a is the predominant isoform during embryonic and early postnatal development, whereas SNAP25b becomes the predominant isoform after early postnatal development through adulthood (Bark et al., 1995). The R59P and I67N mutations are only present in the
Table 1. Summary of Patients with SNAP25-Associated Developmental and Epileptic Encephalopathies

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<td>c.118A &gt; G</td>
<td>c.127G &gt; C</td>
<td>c.142G &gt; T</td>
<td>c.176G &gt; C</td>
<td>c.200T &gt; A</td>
<td>c.496G &gt; T</td>
<td>c.520C &gt; T</td>
<td>c.575T &gt; A</td>
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| Sex and age | Inheritance | Developmental delay | Intellectual disability | Seizure type | Seizure onset | Seizure type, treatment response to antiepileptic pharmacotherapy, severity of neurodevelopmental delay/intellectual disability, and associated cerebellar ataxia and facial dysmorphisms. Of note, the R59P variant is associated with the mildest clinical phenotype (Fukuda et al., 2018), whereas the Q174X and I192N variants were lethal in the first year of life (Deciphering Developmental Disorders Study, 2015, 2017, Gabriel et al., 2016). It is unknown whether these disorders are due to a simple loss-of-function or due to SNAP25 variants exerting specific synaptic impairments and interfering with the normal functioning of the wild-type (WT) SNAP25 in a dominant-negative manner.

**RESULTS**

**Effects of SNAP25 Haploinsufficiency on Synaptic Transmission**

Because loss of one normal allele of SNAP25 is common to all patients, we first investigated the effects of SNAP25 haploinsufficiency on synaptic transmission. The absence of both alleles of SNAP25 is lethal perinatally (Washbourne et al., 2002), and SNAP25+/− (Het) mice have increased susceptibility to kainate-induced seizures even though they do not seize spontaneously (Corradini et al., 2014). Our group previously showed that SNAP25 Het mice showed no significant behavioral impairments except for a marked hypoactivity (Monteggia et al., 2019). To complement our previous behavioral studies, we obtained field recordings from acute hippocampal slices prepared from either WT or Het mice (Figure 1A). With increasing stimulation intensities applied to Schaffer collaterals, we readily observed a comparable increase in the postsynaptic field potentials recorded from CA1 region of hippocampus in both WT and Het slices (Figure 1B), suggesting that haploinsufficiency does not change the overall efficiency of evoked synaptic transmission. We observed a small increase in the paired pulse ratio in Het slices, classically associated with a small decrease in release probability (Figure 1C). In addition, we did not detect alterations in the frequency nor the amplitude of the miniature spontaneous currents recorded from Het neurons (Figures 1D and 1E). Given the severity and diversity of the clinical manifestations, we concluded that haploinsufficiency of SNAP25 is unlikely the sole explanation of the disease pathophysiology but may aggravate the synaptic manifestations of individual SNAP25 variants. This conclusion is further supported by the clinical data from patients with heterozygous deletions of chromosome 20 including SNAP25. Among 7 patients whose phenotypes were reported in the DECIPHER database (Firth et al., 2009) at the time of the preparation of this manuscript, only one patient was reported to have seizures and only four had developmental delays and/or intellectual disability.

**Structural Investigation of SNAP25 Variants**

Synaptic SNAREs (Söllner et al., 1993a, 1993b; Weber et al., 1998) SNAP25, syntaxin-1, and synaptobrevin-2 zipper into a highly stable complex composed of four parallel intertwined α-helices (each called a SNARE motif) held together by 15 interacting layers of hydrophobic side chains and a central ionic...
layer (Figures 2A and 2B) (Sutton et al., 1998). Zippering of the individual SNAREs starts from the cytoplasmic N terminus and proceeds toward the C terminus in a stepwise manner, where it is coupled to fusion driven by the energy liberated from the formation of the complex (Gao et al., 2012; Lai et al., 2017; Ma et al., 2015; Sørensen et al., 2006). SNAP25 contributes two SNARE motifs to the complex and interacts with the Ca2+ sensor synaptotagmin for neurotransmitter release (Zhang et al., 2002; Zhou et al., 2015, 2017). In particular, Syt1 is the primary Ca2+ sensor for evoked synchronous release with fast kinetics (Bacaj et al., 2013; Brose et al., 1992; Fernández-Chacón et al., 2001; Geppert et al., 1994; Xu et al., 2007) and its C2B domain interacts with SNAP25 of SNARE complex extensively at the primary interface (Zhou et al., 2015; Zhou et al., 2017). Because the residues mutated in the K40E, V48F, and D166Y variants reside in the primary interface, we grouped these variants as SNARE-Syt1 primary interface variants. Interestingly, all of the remaining variants were mutated in either one of the 15 hydrophobic layers or the central ionic layer with the exception of R59P, emphasizing the importance of the hydrophobic interactions in SNARE-driven membrane fusion. Because the N- and C-terminal “sides” of the SNARE complex have differential functions (Gao et al., 2012; Sørensen et al., 2006; Zorman et al., 2014), we grouped these hydrophobic layer variants according to the side of the SNARE complex they reside in. Thus, G43R and L50S were grouped as N-terminal side hydrophobic layer variants, whereas I67N, I192N, and A199G variants were grouped as C-terminal side hydrophobic layer variants. As the gain of a stop codon causes loss of the complete C-terminal side hydrophobic layers of the second SNARE motif of SNAP25 in addition to the central ionic layer, we also included Q174X to the group of C-terminal side hydrophobic layer variants. R59P is the only variant that does not impair a known interaction interface or the hydrophobic interactions of the complex, however, it is expected to disrupt the regularity of the α-helical backbone conformation, as proline cannot donate a hydrogen bond to stabilize an α-helix. Thus, we categorized it as the C-terminal side non-hydrophobic layer variant.

Next, we assayed whether the SNAP25 variants are able to form SNARE complexes with the other cognate SNAREs syntaxin-1A and synaptobrevin-2/VAMP2, and if so, how stable the formed complexes are. We used a co-expression system to express all four SNARE motifs of aforementioned SNAREs in Escherichia coli, thus leading to the complex formation in the bacterial host (Zhou et al., 2015). After purification of the formed complexes, with an exception of the complex formed with the Q174X variant that failed to purify, we measured the melting temperature at which the SNARE complex loses α-helicity using circular dichroism (CD) spectroscopy, a measure of the stability of the complex (Figures 2C and S2). As expected, the SNARE-Syt1 primary interface variants do not change the melting temperature of the complex, consistent with the structure since the side chains of the mutated residues are facing outward and do not participate in the SNARE complex core. The N-terminal side hydrophobic layer variants, namely G43R and L50S, reduced the melting temperature, followed by the C-terminal side hydrophobic layer variants I67N, I192N, and A199G. The R59P variant impaired the SNARE complex stability the most, which is not surprising given that the proline substitution is considered to be an α-helix breaker. However, proline is occasionally found in the helices of both transmembrane (Perálvarez-Marín et al., 2006) and soluble (Karpusas et al., 1990) proteins, where it may cause helix bending and distort ideal helix geometry. If there was a substantial disruption of the structure of the ternary SNARE complex by interruption or distortion of one of the helices, one would expect a severe effect on function. Surprisingly, however, the R59P variant caused the mildest phenotype among all SNAP25 variants. To explain this observation, we determined the crystal structure of the R59P variant containing SNARE complex (Table S1). Consistent with the mild phenotype, the R59P variant retains the α-helical conformation of the SNARE motif of SNAP-25 with the mutant, and consequently, the structure of the mutant SNARE complex is similar to that of wild-type SNARE complex without distortion by the R59P mutation (Figure 2D; Videos S1 and S2). Considering that the overall geometry of the four-helix bundle is not affected in...
the R59P variant containing SNARE complex, the force available from C-terminal zippering may not be greatly affected by the mutation.

Effects of SNAP25 Variants on Evoked Release
To elucidate how these SNAP25 variants impact action potential triggered evoked neurotransmitter release, we expressed mutants using a lentiviral expression system on neuronal cultures prepared from SNAP25 knockout (KO) embryos. First, we immunostained variant-expressing KO neurons against both SNAP25 and synaptic marker synapsin-1 to show that all of the variants were expressed and trafficked normally (Figure S3). In addition, to investigate their potential dominant-negative effects, we overexpressed the variants using the same approach but on WT cultures (Figure 3A). As control, we overexpressed WT SNAP25 on WT cultures and showed that the overexpression of WT SNAP25 is electrophysiologically indistinguishable from WT cultures with endogenous SNAP25 only (Figure S4). Because SNAP25 functions similarly in both excitatory and inhibitory neurotransmission (Bronk et al., 2007), we recorded evoked inhibitory postsynaptic currents (eIPSCs) to prevent recurrent excitation from contaminating the excitatory recordings (Maximov et al., 2007).

SNAP25 KO virtually abolished the evoked release, whereas WT SNAP25 was able to rescue the evoked release with fast kinetics as expected (Figure 3) (Bronk et al., 2007). SNAP25-Syt1 primary interface variants were able to rescue the KO phenotype, however, the eIPSC amplitudes were smaller with slower desynchronized kinetics compared to WT, consistent with the premise that these mutants impair Syt1 interaction (Figures 3B and S5). When expressed on WT cultures, all three SNAP25-Syt1 primary interface variants exerted a dominant-negative effect on evoked release (Figure 3C). Previous work showed that the SNAP25-Syt1 interaction at the primary interface is essential for emergence of dominant-negative effects, because corresponding mutations in Syt1 suppressed a dominant-negative effect on evoked release of the so-called DN mutation of Syt1 C2B (Syt1-DN) in Drosophila (Guan et al., 2017). In response to 10 Hz repetitive stimulation, the K40E variant depressed less, consistent with a decrease in probability of release, whereas the V48F and D166Y variants depressed more, consistent with a small increase in probability of release (Figure 3C). Among the N-terminal side hydrophobic layer variants, expression of the G43R variant showed a similar but a much milder electrophysiological phenotype compared to the K40E variant in all evoked release parameters assayed (Figures 3D and 3E). Interestingly, expression of the L50S variant was able to rescue the KO phenotype of evoked release with fast kinetics, similar to the WT SNAP25, and did not exert any dominant-negative effects when expressed on WT cultures (Figures 3D and 3E). Expression of the C-terminal side hydrophobic layer variants showed the most severe impairments in evoked release, consistent with the role of C-terminal side acting as a power stroke to

See also Table S1 and Videos S1 and S2.
finalize the fusion. The Q174X and I192N variants failed to rescue evoked release at all, the I67N variant was nearly non-functional, and the A199G variant retained some rescue ability (Figure 3F). When expressed on WT cultures, Q174X exerted the greatest dominant-negative effect on evoked release, whereas the I67N, I192N, and A199G variants showed similar effects (Figure 3G). R59P, the non-hydrophobic layer C-terminal side variant, was able to rescue evoked release and did not exert a dominant-negative effect, suggesting that force generated from the zippering of the hydrophobic layers of the C-terminal half is essential for the final stages of membrane fusion, whereas the thermostability of the C-terminal half is less important.

Another important function of the SNARE complex assembly is to maintain a readily releasable pool (RRP) of vesicles that respond to Ca2+ influx induced by presynaptic depolarization. The aforementioned impairments in evoked release may arise from a decrease in RRP size, because the canonical SNAREs, including SNAP25 (Bronk et al., 2007), are necessary for the maintenance of the RRP size. In contrast, loss of both Syt1 and Syt7 is required to impair RRP size (Bacaj et al., 2015). Consistent with this premise, when overexpressed on WT cultures where Syt7 is abundant (Bacaj et al., 2013), the SNARE-Syt1 primary interface variants did not affect the size of the RRP measured by hyperosmotic stimulation, a standard Ca2+ influx-independent way to assess the RRP size (Figure 3H) (Rosenmund and Stevens, 1996). Furthermore, N-terminal side hydrophobic layer variants did not impair RRP size (Figure 3I), whereas C-terminal side variants, including R59P, significantly reduced it (Figure 3J), suggesting that overall thermostability of the C-terminal side regardless of the layer mutated is crucial to maintain the RRP.

Effects of SNAP25 Variants on Spontaneous Release

So far, we have shown that almost all of the SNAP25 variants impair either the evoked release or its associated readily releasable pool of vesicles, with the exception of L50S. This finding...
brings up two intriguing questions: (1) how do global impairments in evoked release give rise to this great heterogeneity in clinical manifestations? and (2) how does the L50S variant give rise to any kind of disease without affecting the action potential-triggered neurotransmitter release? To answer these questions, we focused on a less conventional mode of neurotransmitter release. Neurotransmitter containing synaptic vesicles can fuse with the presynaptic membrane spontaneously in the absence of action potentials in a quantal and regulated manner, hence called the spontaneous release (Kavalali, 2015). Spontaneous release shares certain key components of the fusion machinery with evoked release such as SNAP25 (Bronk et al., 2007). Even though the regulation and functions of spontaneous release are still a matter of ongoing research, it has been proposed to function in synaptic plasticity and homeostasis (Kavalali, 2015).

To examine the effects of SNAP25 variants on spontaneous neurotransmission, we utilized tetrodotoxin to block voltage-gated Na⁺ channels, preventing action potential firing (Figure 4A). Similar to its effect on the evoked release, SNAP25 KO almost completely abolished the frequency of spontaneous release events, which was rescued by reintroduction of WT SNAP25 (Figure 4). Even though the identity of the Ca²⁺ sensor for spontaneous release is still a matter of debate, Syt1 is thought to suppress spontaneous release by clamping a second Ca²⁺ sensor, which is more sensitive to Ca²⁺ but has a lower affinity for SNARE complex (Courtney et al., 2018; Xu et al., 2009). All SNARE-Syt1 primary interface variants rescued spontaneous release, and the V48F and D166Y variants caused substantial augmentation of spontaneous release, up to ~40 fold (Figure 4B), which is far greater than what has been previously reported in case of unclamping of spontaneous release by Syt1 loss-of-function (Bacaj et al., 2013). This trend was also observed when variants were expressed on WT cultures, exerting a dominant-positive effect in a similar fashion for both excitatory and inhibitory spontaneous miniature postsynaptic currents (Figures 4C and S6A).
preincubated neurons expressing V48F and D166Y variants with BAPTA-AM, an exogenous intracellular Ca\(^{2+}\) chelator, to examine whether this augmentation of spontaneous release is driven by an alternate Ca\(^{2+}\) sensor, similar to earlier studies in the case of Syt1 loss-of-function (Xu et al., 2009). However, BAPTA-AM preincubation was ineffective in reducing this robust increase in spontaneous release (Figure S6D), suggesting that the augmentation of spontaneous release by V48F and D166Y variants is due to an intrinsic increased propensity of spontaneous fusion, likely independent of an alternate Ca\(^{2+}\) sensor. The aforementioned DN-Syt1 caused enhanced spontaneous release as a dominant effect which failed to normalize upon disruption of the SNARE-Syt1 interaction at the primary interface through mutations of DN-Syt1 (Guan et al., 2017), further suggesting the V48F and D166Y variants do not simply elicit Syt1 loss-of-function effects to augment spontaneous release.

Expression of the N-terminal side hydrophobic layer variants, G43R and L50S, rescued spontaneous release with increased frequency and exerted a dominant-positive effect on WT cultures (Figures 4D, 4E, and S6B). The L50S variant was associated with greater increase compared to the G43R variant. Interestingly, the augmentation of spontaneous release by these variants was shown to require Ca\(^{2+}\), suggested by their BAPTA-AM responsivity (Figure S6D), possibly due to involvement of an alternate Ca\(^{2+}\) sensor. Given the close proximity of G43R and L50S to the primary interface and causing instability of the N-terminal side of the SNARE complex (Figure 2), which houses the primary interface and other important interfaces for Syt1 interaction (Zhou et al., 2015; Zhou et al., 2017), we speculate that these variants may alter the Syt1 interaction with the SNARE complex indirectly, leading to impaired evoked release with slow kinetics in case of G43R, and unclamping of spontaneous release and unchanged RRP size in case of both G43R and L50S. The additional effects on evoked release of G43R variant can be explained either by the mutated residues lying in different hydrophobic layers or the more dramatic difference in the side chains of glycine (small and hydrophobic) versus arginine (large and positively charged) compared to a more subtle difference in case of the L50S variant.

None of the C-terminal side hydrophobic layer variants were able to rescue spontaneous release when expressed on KO cultures (Figure 4F), consistent with C-terminal half providing a force for membrane fusion irrespective of the mode of release. I67N, I192N, and A199G variants showed a dominant-negative effect on spontaneous release, whereas Q174X did not (Figure 4G). The R59P variant retained the ability to rescue the spontaneous release similar to WT and showed no effect when expressed on WT cultures (Figures 4F and 4G).

In order to investigate the relationship between the variant-to-WT ratio of SNAP25 and observed dominant-negative (or dominant-positive) effects, we designed a simple titration experiment. The effect of D166Y on miniature excitatory postsynaptic current (mEPSC) frequency was the greatest among all parameters we studied so far, allowing enough room to conduct a sensitive titration. We utilized increasing infection volumes of non-tittered lentivirus from the same batch to gradually overexpress the D166Y variant on WT neuronal cultures and measured mEPSC frequency. We achieved the D166Y-to-WT ratio of 1, which mimics the expected expression pattern of heterozygous patients, when cultures were infected with 60 \(\mu\)L of supernatant (Figure 4H). Statistical analysis showed that mEPSC frequency is promptly saturated with increasing infection volumes, and the mEPSC frequency obtained with 60 \(\mu\)L of infection volume is no different than frequencies obtained with the other infection volumes (Figure 4I). This suggests that even a minor presence of mutant SNAP25 is sufficient for the dominant effects to emerge and we were able to mimic the synaptic phenotypes of patients when we overexpressed SNAP25 variants using WT neuronal cultures. For all the remaining variants, we achieved similar overexpression levels to the D166Y variant of the titration experiment (Figure S4A) with the same amount of lentivirus, suggesting that the stability of variants is comparable with each other. However, the biallelic expression pattern in heterozygous patients may be different than the stoichiometric 1:1 ratio. Our result suggests that, even sub-stoichiometric expression of SNAP25 variants may lead to dominant effects similar to that of the stoichiometric expression.

We also recorded evoked excitatory postsynaptic currents (eEPSCs) from WT neurons expressing the SNAP25 variants. To reduce the high background activity and recurrent excitation associated with eEPSC recordings, we lowered Ca\(^{2+}\) concentration as well as stimulus intensity. In this setting, we could show that all variants affect the evoked excitatory neurotransmission similar to their effects on evoked inhibitory neurotransmission, except for V48F and D166Y, where the decreasing trend in response amplitudes did not reach statistical significance (Figures S6E–S6G).

**Effects of SNAP25 Variants on Synapse Density**

Even though the brain of SNAP25 KO mouse develops normally (Washbourne et al., 2002), the cultured KO neurons undergo neuronal death within couple weeks. Our group has previously showed that the SNAP25 KO high density cultures have a comparable synapse density with WT neurons on days in vitro (DIV) 14, but subsequent degeneration causes a 20% reduction in synapse density on DIV21 (Bronk et al., 2007). This finding suggests that SNAP25 is not essential for initial brain development but necessary for neuronal survival in vitro and in vivo (also see Hoelder-Suabedissen et al., 2019). Therefore, we performed all the functional studies between DIV14 and DIV18, when the synapses are mature, the expression of SNAP25 variants plateaued, and synaptic loss due to SNAP25 loss-of-function is negligible. To address whether SNAP25 variants impact synapse numbers under our conditions, we quantified excitatory and inhibitory synapse densities. First, we showed that the expression of SNAP25 variants have already plateaued on DIV14 (Figure 5A). At the same time, we fixed another set of coverslips from the same set of cultures on DIV14 and co-immunostained with antibodies against the excitatory presynaptic protein vesicular glutamate transport (vGlut) and excitatory postsynaptic density scaffold protein PSD-95 to quantify excitatory synapse density or inhibitory presynaptic marker vesicular GABA transporter (vGAT) and inhibitory postsynaptic marker gephyrin to quantify inhibitory synapse density. When we counted the number of co-localized
puncta and normalized to MAP2 (dendritic marker) signal (Figure 5B), we found that expression of SNAP25 variants did not cause significant changes in either excitatory or inhibitory synapse density (Figures 5C and 5D), suggesting that the electrophysiological alterations are attributable to functional changes but not to changes in synapse density.

**Effects of Variant-Specific Alterations on Network Activity**

Plotting changes in amplitudes of evoked responses against changes seen in frequencies of spontaneous events reveals a distinct synaptic phenotype for each SNAP25 variant (Figures 6A and 6B). Notably, the L50S variant augmented only the spontaneous release without impairing the evoked release, whereas the V48F and D166Y variants augmented spontaneous release excessively in addition to relatively modest impairments in evoked release. In the hippocampal cultures, the frequency of mEPSCs is intrinsically higher than the frequency of mIPSCs. All variants augmenting spontaneous release retained this intrinsic ratio of excitatory-to-inhibitory spontaneous release, causing an absolute increase in the excitatory drive (Figure 6C).

To explore the changes in network activity secondary to the synaptic disturbances we identified, we recorded spontaneous action potential (AP) firings from SNAP25 variant overexpressing WT neurons in whole-cell current clamp mode and analyzed their firing patterns, resting membrane potentials, and kinetic characteristics of APs. The K40E variant, by simply impairing evoked release without altering spontaneous release, caused a shift toward a burst-like firing pattern (Figure 7A) without altering the resting membrane potential. G43R, by impairing evoked release and mildly augmenting spontaneous release, caused a halfway change in the firing pattern between burst-like pattern of the K40E variant and of the WT (Figure 7A).

The L50S, V48F, and D166Y variants caused a more depolarized resting membrane potential (Figure 7B), driven by the net increase in excitatory spontaneous neurotransmission (Figure 7C). Because voltage-gated Na⁺ channels require a hyperpolarized resting membrane potential to recover from inactivation and respond to the next round of depolarization, this more depolarized resting membrane potential decreased the availability of voltage-gated Na⁺ channels—and presumably voltage-gated K⁺ channels—leading to wider APs with smaller amplitudes (Figure 7D). Their effects on membrane potential, AP amplitude, and width were in parallel with their effects on spontaneous release, the D166Y variant causing the greatest disturbances followed by the V48F and L50S variants. In the case of the L50S variant, because evoked release is not impaired, subthreshold depolarizations can now trigger APs, as the membrane potential is closer to the threshold, which may explain the increased AP firing rate (Figures 7E and 7F). The V48F and D166Y variants were
expected to have similar firing patterns to the K40E variant only when their effects on evoked release are taken into consideration. However, membrane depolarization due to augmented spontaneous release normalized the firing pattern and rate compared to the K40E variant (Figures 7E and 7F). Interestingly, the D166Y variant showed a very similar firing pattern and firing rate relative to WT SNAP25, whereas the V48F variant was not able to normalize the firing pattern completely (Figure 7E). These observations parallel the severity of clinical manifestations between patients carrying the V48F and D166 variants. The patient with the V48F variant is globally delayed, nonverbal with severe intellectual disability, and seizures being refractory to more than 8 different antiepileptics in up to 3 drug combinations (Rohena et al., 2013). In contrast, the patient with the D166Y variant has milder developmental delays and has caught up with his peers in many of the domains delayed initially, has milder intellectual disability, and seizures in control with a single antiepileptic drug (Hamdan et al., 2017).

All of the randomly selected neurons expressing the SNARE-Syt1 primary interface and N-terminal side hydrophobic layer variants fired at least one AP during the recording period of 3 min. However, C-terminal side hydrophobic layer variants caused some of the neurons to be silent, suppressing the network activity (Figure 7G). The Q174X and I192N variants almost completely silenced the network, which correlates with the fact that the patients carrying these variants died in the first year of life. The I67N variant resulted in half of the recorded neurons to be silent, whereas the A199G variant caused only one-fourth of the neurons to be silent. Neither of the variants changed the membrane potential. As expected from the R59P variant, being able to carry out both evoked and spontaneous release similar to WT SNAP25, none of the recorded neurons were silent, and they all showed a similar firing rate and pattern with WT (Figures 7G and 7H). This finding also correlates with the R59P causing the mildest disease phenotype, probably contributed by haploinsufficiency and decreased RRP size.

Interestingly, wide variety of changes in the network activity driven by the changes in both evoked and spontaneous neurotransmission correlate with the heterogeneity and severity of the clinical manifestations of the patients, suggesting that besides evoked release, dysregulation of spontaneous release is a key factor in the phenotypic diversity among patients with SNAP25-associated encephalopathies.

**DISCUSSION**

The diagnosis of developmental and epileptic encephalopathies of infancy and childhood is clinically challenging; therefore, their overall incidence is probably underestimated. Whole-exome
sequencing and gene panels are offered to affected families to identify a genetic cause of these disorders in the hope of classifying patients into distinct subgroups of DEEs with specific electroclinical features, ultimately allowing application of tailored treatments. De novo mutations of the core fusion machinery components namely SNAP25, synaptobrevin-2 (Salpietro et al., 2019; Simmons et al., 2020), syntaxin-1 (Vardar et al., 2020; Wolking et al., 2019), and their key interaction partner synaptotagmin-1 (Baker et al., 2018; Bradberry et al., 2020), have been recently identified (Verhage and Sørensen, 2020). In 2018, only 3 out of 24 gene panels from academic and commercial providers for DEEs included SNAP25, and none included synaptobrevin-2 or synaptotagmin-1 (Heyne et al., 2018), showing a need to better recognize mutations of fusion machinery components as a cause of DEEs. We expect that further recognition of genes coding for components of fusion machinery and its interaction partners as a cause of DEEs would uncover more patients in the future.

In this study, we showed that heterozygous de novo mutations in SNAP25 lead to DEEs of infancy and childhood by impairing evoked release and/or modifying spontaneous release in both directions in a dominant manner, giving rise to distinct synaptic phenotypes contributing to the clinical heterogeneity. We found that structurally clustered mutations of SNAP25 give rise to related synaptic phenotypes by either impairing SNARE-synaptotagmin interactions or SNARE complex zippering that drives

![Figure 7. Effects of SNAP25 Variants on Network Activity](image-url)

(A) Representative traces of spontaneous AP firing patterns of WT SNAP25 and variants K40E and G43R in current-clamp mode along with cumulative histograms of interspike intervals and resting membrane potentials (compared against WT, two-tailed non-paired t-test). Cumulative histograms of variants were compared against WT using Kolmogorov-Smirnov test (WT versus K40E Kolmogorov-Smirnov D = 0.2762, p < 0.0001 and WT versus G43R Kolmogorov-Smirnov D = 0.2086, p < 0.0001).

(B) Representative traces of spontaneous AP firings of variants L50S, V48F, and D166Y in current-clamp mode along with resting membrane potentials (compared against WT, two-tailed non-paired t-test).

(C) Hyperpolarization of the depolarized resting membrane potential associated with L50S, V48F, and D166Y variants when perfused with CNQX (AMPA receptor blocker) and AP5 (NMDA receptor blocker) to block excitatory neurotransmission.

(D) Representative single action potentials along with the quantitative analysis of their amplitudes and half-width (compared against WT, two-tailed non-paired t-test).

(E) Cumulative histograms of variants were compared against WT using Kolmogorov-Smirnov test (WT versus L50S Kolmogorov-Smirnov D = 0.1872, p < 0.0001; WT versus V48F Kolmogorov-Smirnov D = 0.1337, p = 0.0001 and WT versus D166Y Kolmogorov-Smirnov D = 0.07846, p > 0.05) and spontaneous AP firing rates were analyzed (compared against WT, two-tailed non-paired t-test).

(F) Proposed mechanism to explain changes associated with variants L50S, V48F, and D166Y, all of which augments spontaneous neurotransmission significantly.

(G and H) Neuronal activity of C-terminal side variants were shown along with the resting membrane potentials (G, compared against WT, two-tailed non-paired t test) and cumulative histogram of interspike intervals (H) of the R59P variant (WT versus R59P Kolmogorov-Smirnov D = 0.07284, p > 0.05). Significance levels were stated as follows: *p < 0.05, **p < 0.01, ***p < 0.001, and ****p < 0.0001. ns denotes non-significance.
membrane fusion. Most importantly, we showed that specific dysregulation of the spontaneous release appears to be sufficient to cause disease, and to our knowledge, this is the first evidence implicating aberrant spontaneous neurotransmitter release as a cause of any disease. Identification of the L50S SNAP25 variant serves as a proof of concept that alterations in spontaneous release should not be overlooked in variants of SNARE complex components which alter spontaneous release in addition to impairing evoked release. However, we cannot fully exclude the possibility that L50S SNAP25 variant exerts additional effects on physiological processes other than neurotransmitter release, which may contribute to the disease phenotypes. Furthermore, some variants cause more profound alterations in spontaneous release compared to their effects on evoked release, such as V48F and D166Y. Given that these variants increase mEPSC amplitudes along with comparable eEPSC responses, potential postsynaptic effects of these variants may further augment the depolarizing effect of net increased excitatory postsynaptic neurotransmission on postsynaptic membrane.

We show that each variant has a distinct synaptic phenotype leading to distinct network patterns, both of which correlate with the clinical heterogeneity and severity. However, one possibility is that mutations of the alternatively spliced exon of SNAP25, namely R59P and I67N, universally give rise to milder phenotypes secondary to availability of WT SNAP25a along with mutant SNAP25b. Quantification of SNAP25 isoforms in post-mortem adult human brains showed that SNAP25b is expressed at least 2 times higher than SNAP25a, depending on the brain region (Prescott and Chamberlain, 2011), suggesting that mutant SNAP25b comprises ~70% of total SNAP25. Given that less than 1:1 stoichiometric expression of mutant SNAP25b is sufficient to cause emergence of dominant effects, the differential effects of R59P and I67N on synaptic transmission are the major determinants of the clinical severity, such as I67N causes a more severe phenotype by having a dominant-negative effect on both evoked and spontaneous release and silencing the network, whereas R59P has no dominant-negative effects on either forms of release and does not change the network firing characteristics, giving rise to a milder phenotype. These results clearly show that missense mutations of this alternatively spliced exon do not necessarily cause milder phenotypes, and availability of WT SNAP25a is not enough to rescue synaptic impairments associated with disease-associated SNAP25b variants.

As DEEs tend to be resistant to the conventional antiepileptic treatments (Berg et al., 2010), understanding the effects of mutations of fusion machinery on synaptic transmission is vital to develop novel targeted treatments. In particular, novel therapeutic strategies that directly target the presynaptic fusion machinery will likely provide significant treatment advance against these disorders (Li and Kavalali, 2017). This is highly crucial because early and aggressive seizure control correlates with greater cognitive outcomes later in life (Berg et al., 2012). We recently identified that aminopyridine class of K+ channel blockers are able to rescue impaired evoked release associated with VAMP2 variants in vitro by prolonging action potentials (Simmons et al., 2020). Furthermore, a patient with VAMP2 variant treated with off-label aminopyridine treatment showed significant clinical improvement assessed by both parental report and standardized cognitive measures (Simmons et al., 2020). In addition, this treatment approach is also shown in vitro as a viable option for patients harboring Syt1 variants (Bradberry et al., 2020). However, our results suggest that restoration of impaired evoked release alone in patients harboring variants associated with both aberrant spontaneous and impaired evoked release may not be an adequate approach. Here, a new generation of therapeutic approaches targeting spontaneous release will potentially be beneficial in treating these intractable disorders in addition to their potential benefits for other neurological and neuropsychiatric disorders (Kavalali and Monteggia, 2020). Additionally, because the mutations described here have dominant effects, our study suggests that eliminating the mutant allele instead of a gene addition strategy should be pursued in the future as a gene-based therapeutic approach.

**STAR METHODS**

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**SUPPLEMENTAL INFORMATION**

Supplemental Information can be found online at https://doi.org/10.1016/j.neuron.2020.10.012.

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**AUTHOR CONTRIBUTIONS**

B.A. designed, expressed, purified SNAP25 variant containing SNARE complexes, prepared primary neuronal cultures, performed whole cell voltage clamp and current clamp experiments, performed immunostaining, image acquisition, analysis, and western blotting, prepared the figures, and wrote the initial draft of the manuscript. Q.Z. supervised protein expression and purification, performed crystallization, collected diffraction data for R95P-SNAP25 containing SNARE complex, determined and refined its crystal structure, and edited the manuscript. O.S. designed lentiviral constructs to express SNAP25 variants. L.E. designed, expressed, purified SNAP25 variant containing SNARE complexes, and performed CD experiments. P.L. performed acute hippocampal slice electrophysiology. K.I.W. collected and reduced the diffraction data. R.S. assisted SNAP25 variant containing SNARE complex purification. W.K.C. provided clinical background and insight into the design of the project. L.M.M., A.T.B., and E.T.K. supervised the project and edited the manuscript. E.T.K. worked closely with B.A. in overall design and direction of the project.

**DECLARATION OF INTERESTS**

The authors declare no competing interests.

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**REFERENCES**


In this study, the authors investigated the clinical spectrum of STX1B-related epileptic disorders. They found that mutations in the STX1B gene, which encodes a synaptic vesicle membrane protein involved in neurotransmitter release, lead to a range of neurological conditions, including epilepsy. The study highlights the importance of understanding the molecular mechanisms underlying these disorders for developing targeted therapies.


## STAR METHODS

### KEY RESOURCES TABLE

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<th>SOURCE</th>
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RESOURCE AVAILABILITY

Lead Contact
Further information and requests for resources and reagents should be directed to and will be fulfilled by the Lead Contact, Ege T. Kavalali (ege.kavalali@vanderbilt.edu).

Materials Availability
This study did not generate new unique reagents.

Data and Code Availability
All data supporting the findings of this study are available from the lead author Ege T. Kavalali upon reasonable request. The accession number for the coordinates of the atomic model and corresponding structure factor of the R59P-SNAP25-containing SNARE complex reported in this paper is PDB: 6WVW.

EXPERIMENTAL MODEL AND SUBJECT DETAILS

Animals
Postnatal day 2–3 Sprague-Dawley rats of either sex were used for the SNAP25 overexpression experiments. Pregnant Sprague-Dawley rats were housed individually until they give birth to a litter. The rats were kept in 12 hours: 12 hours dark:light cycle. The pregnant rats were provided with treats as well as cardboard enrichments. Postnatal day 2-3 littermates were used to prepare primary dissociated neuronal cultures. SNAP25 (Washbourne et al., 2002) KO, Het and WT mice embryos of either sex were used for KO rescue and haploinsufficiency experiments. Briefly, each male heterozygous mouse is paired with two female heterozygous mice and housed together. They were provided with the same treats and the enrichments and kept in the same dark:light cycle as the previously described rats were subjected to. The embryonic day 18 embryos were surgically obtained from the pregnant mice and used to prepare primary dissociated neuronal cultures. All animal procedures were performed in accordance with the guide for the care and use of laboratory animals and were approved by the Institutional Animal Care and Use Committee at Vanderbilt University. Health status of the live animals were periodically checked and confirmed by the veterinary staff of animal facilities of the Vanderbilt University.

Primary dissociated neuronal cultures
Briefly, either hippocampi or cortical tissue were dissected in ice cold 20% fetal bovine serum (FBS) containing Hanks’ balanced salt solution. Tissues were then washed and treated with 10 mg/ml trypsin and 0.5 mg/ml DNase at 37°C for 10 mins. The tissues were washed again, dissociated using filtered P1000 tip and centrifuged at 1000 rpm for 10 mins at 4°C. Pellet containing neurons was
resuspended either in plating medium (for postnatal cultures) containing MEM (no phenol red), 5 g/l D-glucose, 0.2 g/l NaHCO3, 0.1 g/l transferrin, 10% FBS, 2 mM L-glutamine and 20 mg/l insulin or in Neurobasal Plus medium (for embryonic cultures) supplemented with GlutaMAX-I and B27 supplement. Neurons were plated onto 12 mm coverslips coated with either 1:50 MEM:Matrigel solution (for postnatal cultures) or poly-D-lysine (for embryonic cultures). Cultures were kept in humidified incubators at 37°C and gassed with 95% air and 5% CO2. On DIV (day in vitro) 1, the plating medium or the neurobasal plus medium was replaced with 4 μM cytosine arabinoside containing growth medium or neurobasal plus medium. Growth medium is similar to plating medium except for the following: 5% FBS, 0.5 mM L-glutamine, no insulin and supplemented with B27 supplement. On DIV4, the cytosine arabinoside concentration was dropped 2 μM by performing a half media change and lentivirus containing supernatant was added to express SNAP25 variants. Cultures were kept without any disruption until DIV14. All experiments were performed between DIV14-18, when synapses reached maturity and overexpression of the target protein was plateaued (Li et al., 2017). Sample size was not predetermined using statistical methods prior to experimentation. Sample sizes were based on previous studies in the field of molecular & cellular neuroscience.

**Cell lines**

Human embryonic kidney-293 (HEK293) cells (ATCC) were used to produce lentiviral particles to infect primary neuronal cultures. HEK293 cultures were kept in humidified incubators at 37°C and gassed with 95% air and 5% CO2. The cells were split and passaged when they reached 80% confluence. The culture medium consisted of 10% FBS containing Dulbecco’s Modified Eagle Medium supplemented with penicillin and streptomycin.

**In vitro studies**

SNAP25α cDNA sequence from rat is used to express SNARE motifs that were used in CD experiments as well as X-ray crystallography. NEB Turbo Competent E. coli (NEB) were used for standard molecular cloning methods, whereas BL21-Gold(DE3) Competent E. coli (Agilent) were used for protein expression.

**METHOD DETAILS**

**Cloning and lentivirus preparation**

SNAP25b was cloned into pFUGW vector containing human ubiquitin promoter and individual mutations were introduced using standard molecular biology methods and verified by sequencing. HEK293 cells were cotransfected by pFUGW and three packaging plasmids (pCMV-VSV-G) [Stewart et al., 2003], pMDLg/pRRE [Dull et al., 1998], pRSV-Rev [Dull et al., 1998]) using FuGENE 6 transfection reagent (Promega). 24 hours after the transfection, HEK cell media containing the transfection cocktail was replaced by either neuronal growth medium or Neurobasal Plus medium. 48 hours after the media change, the media containing lentiviral particles was collected and centrifuged at 2500 rpm for 15 mins at 4°C to get rid of debris. 200 μl of supernatant was directly added to cultures on DIV4 to infect the neurons.

**Electrophysiology and Data Analysis**

Whole-cell patch clamp recordings were performed on pyramidal cells using CV203BU headstage, Axopatch 200B amplifier, Digidata 1320 digitizer and Clampex 8.0 software (Molecular Devices). Recordings were filtered at 1 kHz and sampled at 100 μs. Experiments were conducted at room temperature. For external bath solution, a modified Tyrode’s solution containing the following was used: (in mM): 150 NaCl, 4 KCl, 1.25 MgCl2, 2 CaCl2, 10 glucose, 10 HEPES at pH 7.4. For eEPSCs, the CaCl2 concentration was decreased to 1.25 mM. To isolate mEPSCs, 1 μM TTX, 50 μM PTX, and 50 μM D-AP4 were added. To isolate mIPSCs, 1 μM TTX, 10 μM CNQX, and 50 μM D-AP5 were added. To isolate eIPSCs, 50 μM D-AP5, and 10 μM CNQX were added. To isolate eEPSCs, 50 μM PTX and 50 μM D-AP5 were added. For eIPSC recordings, the field stimulation was provided using a parallel bipolar electrode (FHC) immersed in the external bath solution, delivering 35 mA pulses via a stimulus isolation unit. For eEPSC recordings, the stimulus intensity was decreased to 33 mA. Throughout the evoked experiments where stimulations are delivered via a bipolar electrode, we used a programmable motorized Sutter micromanipulator to make sure that the location of the bipolar electrode is exactly the same in all experiments, making sure that a similar field of coverslip is stimulated each time. For BAPTA-AM experiments, coverslips were incubated either in 100 μM BAPTA-AM in 0.1% DMSO containing external bath solution with 0 mM Ca2+ or in vehicle for 20 mins at room temperature. For voltage clamp experiments, the membrane potential was held at −70 mV and the 3–5 MΩ borosilicate glass patch pipettes were filled with the internal solution contained the following (in mM): 115 Cs-MeSO3, 10 CsCl, 5 NaCl, 10 HEPES, 0.6 EGTA, 20 tetraethylammonium-Cl, 4 Mg-ATP, 0.3 Na2GTP, and 10 QX-314 [N-(2,6-dimethylphenylcarbamoylmethyl)-triethylammonium bromide] at pH 7.35 and 300 mOsm. For current clamp experiments, the 4–6 MΩ borosilicate glass patch pipettes were filled with the internal solution contained the following (in mM): 110 K-Gluconate, 20 KCl, 10 NaCl, 10 HEPES, 4 Mg-ATP, 0.3 Na2GTP, 0.6 EGTA at pH 7.3 and 284 mOsm. For all recordings included for the analysis, the membrane resistance was greater than 100 MΩ. When the access resistance was less than 20 MΩ and time constant (τ) was less than 1 ms. Miniature events and spontaneous AP firings were recorded for 3 mins. mPSC frequencies and amplitudes were analyzed and spike analysis was performed by using Mini Analysis software (Synaptosoft). Randomly picked equal number of events (or max number of event if the event number is less) from each
recording was included in the cumulative histograms of interevent intervals to make sure that the contribution of each recording is comparable. Evoked IPSCs and EPSCs were analyzed by using Clampfit (Molecular Devices).

Cloning, protein expression and purification
For SNAP25 variant containing SNARE complexes, the 10x-histidine-tagged and C-terminally truncated rat synaptobrevin-2 (amino-acids 28–89), the rat syntaxin-1A (amino-acids 191–256), the rat SNAP-25_N (amino-acids 7–83), and the rat SNAP-25_C (amino-acids 141-204) fragments were cloned into the Duet expression system (Novagen) as described previously (Zhou et al., 2015, 2017). As the structure of SNARE complex and its interaction interfaces with syntaptogamin are determined using SNAP25a (Zhou et al., 2015, 2017) and all the mutated residues are conserved in both of the SNAP25 isoforms, we used SNAP25a for protein experiments. The aforementioned four fragment constructs were co-expressed in Escherichia coli, leading to complex formation in the host. Specifically, E. coli BL21(DE3) were grown overnight at 37°C using auto-inducing LB medium (Studier, 2005). After harvesting the cells by centrifugation, the pellet was re-suspended in lysis buffer (50 mM Tris-HCl, pH 8.0, 300 mM NaCl, 20 mM imidazole, 0.5 mM TCEP) supplemented with lysozyme and DNase I, and subjected to sonication and centrifugation. The cleared lysate was bound to Ni-NTA agarose beads (Qiagen) equilibrated in the lysis buffer. Beads were harvested by centrifugation, poured into a column, and washed with the lysis buffer, urea buffer (50 mM Tris-HCl, pH 8.0, 300 mM NaCl, 60 mM imidazole, 0.5 mM TCEP, 7.5 M urea), and wash buffer (50 mM Tris-HCl, pH 8.0, 300 mM NaCl, 60 mM imidazole, 0.5 mM TCEP). The SNARE complex was then eluted with the lysis buffer supplemented with additional 330 mM imidazole. The fresh eluent of the Ni-NTA affinity purified SNARE complex was supplemented with tobacco etch virus protease and dialyzed against buffer A1 (50 mM Tris-HCl, pH 8.0, 50 mM NaCl, 0.5mM TCEP, 1 mM EDTA) overnight at 4°C. After removal of uncleaved protein, the His-tag-free complex was subjected to anion exchange chromatography (Buffer A1: 50 mM Tris-HCl, pH 8.0, 50 mM NaCl, 0.5 mM TCEP, 1 mM EDTA; Buffer B1: 50 mM Tris-HCl, pH 8.0, 500 mM NaCl, 0.5 mM TCEP, 1 mM EDTA) using a linear gradient of NaCl starting at 50 mM and ending at 500 mM. The protein eluted at ~280 mM NaCl. The peak fractions were pooled, concentrated and stored at ~80°C for crystallization and circular dichroism (CD) experiments.

CD spectroscopy
Circular dichroism measurements were conducted with circular dichroism spectrometer Model 202-01 (Aviv Biomedical) equipped with a temperature controller. Data were collected with 10 μL samples of WT and mutant SNARE complexes in 10 mM Tris-HCl (pH 8.0), 100 mM NaCl, 0.5 mM EDTA buffer over a wavelength range of 200–260 nm, with 1 nm increments, in a 1 nm path length cell at 25°C. Temperature melting scans were performed at a wavelength of 220 nm by increasing the temperature from 25 to 100°C in 3°C temperature increments, a 2 min temperature equilibration time, and a 3 s averaging time. The fraction of unfolded protein at each temperature was calculated by using the formula (I_{ob}-I)/I_u-I_f, where I_{ob} is the observed mean residue ellipticity, and I_u and I_f are the mean residue ellipticities of the unfolded and folded states, respectively. I_u and I_f were estimated by extrapolation of the linear regions of the extremes of the denaturation curves.

Crystallization
Purified protein sample of the R59P-SNAP25 containing SNARE complex was dialyzed against a buffer solution containing 20 mM HEPES (pH 7.4), 100 mM NaCl and 5 mM DTT. Crystals were grown by the hanging-drop vapor diffusion method at 20°C by mixing 2 μL protein solution (at a concentration of ~12 mg/ml) with equal volume of reservoir solution containing 100 mM MES (pH 6.0), 100 mM CaCl_2, and 20%–25% (v/v).

Data collection and structure determination
Crystals were flash-frozen in a cryo-protecting solution containing the same constituents as the crystallization condition supplemented with 25% (v/v) MPD. Diffraction data were collected at the Stanford Synchrotron Radiation Light Source (SSRL) beam line 9-2 (wavelength 0.9794 Å at 100 K). Diffraction data were indexed, integrated, scaled, and merged using HKL3000 (Minor et al., 2006). The phases for the diffraction data were determined by molecular replacement with Phaser (McCoy et al., 2007) using the rat WT SNARE complex (PDB accession number 1N7S) as a search model. The structure was iteratively rebuilt and refined using the programs Coot (Emsley and Cowtan, 2004) and Phenix (Adams et al., 2002) (Table S1). Ramachandran analysis with MolProbity (Chen et al., 2010) indicated that 100% of the residues are in the favored regions and none are in disallowed regions. MolProbity was used to evaluate the geometry and quality of the models (Table S1). All structure figures were prepared with PyMol (https://pymol.org/2/).

Immunofluorescence
Coverslips were fixed in 4% PFA/4% sucrose containing PBS solution for 20 mins at room temperature and permeabilized using 0.2% Triton-X containing PBS solution for 30 mins at room temperature. Coverslips were washed and incubated in blocking solution consisting of 1% bovine serum albumin and 2% goat serum in PBS for 2 hours at room temperature. Coverslips were incubated with primary antibodies diluted in the blocking solution: 1:500 Anti-SNAP25 (111-111, Synaptic Systems), 1:500 Anti-synapsin (106-103, Synaptic Systems), 1:1.000 Anti-VGlut (AB5905, EMD Millipore), 1:200 Anti-PSD-95 (7E3-1B8, ThermoFisher), 1:500 Anti-VGAT (131-008, Synaptic Systems), 1:200 Anti-gephyrin (147-021, Synaptic Systems) and anti-MAP2 (188-006, Synaptic Systems) in a humid
chamber overnight at 4 °C and with species-appropriate Alexa Fluor secondary antibodies diluted as 1:500 in the blocking solution in a humid chamber for 90 mins at room temperature. Coverslips were mounted on glass slides and imaged via a Zeiss LSM 710 with 63x objective at 1024 × 1024-pixel resolution. The synapse densities were analyzed using Intelllicount (Fantuzzo et al., 2017).

**Protein quantification**

To quantify protein levels, western blotting was carried out. Briefly, protein samples were prepared from coverslips using Laemmli Buffer containing protease and phosphatase inhibitor cocktails (Roche) and beta-mercaptoethanol. Samples were sonicated and boiled for 5 min at 95 °C to dissociate SNARE complexes and loaded on SDS-PAGE gels and transferred to nitrocellulose membranes. Membranes were incubated with primary antibodies at 4 °C overnight in following dilutions: 1:2000 Anti-SNAP25 mouse (111-111, Synaptic Systems), 1:10000 Anti-GAPDH rabbit (14C10, Cell Signaling), 1:2000 Anti-syntaxin-1 mouse (110-011, Synaptic Systems) and 1:2000 Anti-synaptobrevin-2 mouse (104-211, Synaptic Systems). After incubation with fluorescent secondary anti-rabbit and anti-mouse antibodies (IRDye Secondary Antibodies, Li-Cor), membranes were imaged using an Odyssey CLx imaging system (Li-Cor). Band intensities were analyzed using ImageJ and normalized to loading controls.

**Hippocampal slice preparation and field recording**

The mice were anesthetized with isoflurane before decapitation. The brains were removed and immersed in ice-cold dissection buffer containing the following (in mM): 2.6 KCl, 1.25 NaH₂PO₄, 26 NaHCO₃, 0.5 CaCl₂, 5 MgCl₂, 212 sucrose and 10 D-glucose for 2-3 min. The hippocampi were dissected out and cut with a vibratome into 400 μm thick transverse sections in ice-cold dissection buffer continuously aerated with 95% O₂ and 5% CO₂. Sections were recovered in oxygenated ACSF containing the following (in mM): 124 NaCl, 5 KCl, 1.25 NaH₂PO₄, 26 NaHCO₃, 2 CaCl₂, 2 MgCl₂ and 10 D-glucose at pH 7.4 (continuously equilibrated with 95% O₂ and 5% CO₂) for 2-3 hours at 30 °C. Hippocampal slices were transferred to the recording chamber and perfused with ACSF at a rate of 2-3 ml/min at 30 °C. Field EPSPs (fEPSPs) were evoked by inserting a concentric bipolar stimulating electrode (FHC) to Schaffer collateral/commissural afferents. 1-2 MΩ Extracellular recording electrodes filled with ACSF were inserted into the CA1 area proximally below the molecular layer. Baseline responses were collected every 30 s using an input stimulus intensity that induced 30%-40% of the maximum response. Paired-pulse facilitation (PPF) was elicited by giving paired-pulse stimulations at decreasing interstimulus intervals (ISIs) of 500, 400, 200, 100, 50, 30 and 20 ms and analyzed by dividing the fEPSP slope of pulse 2 (P2) by that of pulse 1 (P1).

**QUANTIFICATION AND STATISTICAL ANALYSIS**

The data was presented as mean ± standard error of mean, unless stated otherwise in the figure legends. The sample size for each experiment was stated either in the figure or in the legend and was not predetermined using statistical methods prior to experimentation. For patch-clamp electrophysiology experiments, the sample size corresponds to number of cells patched. Sample sizes were based on previous studies in the field of molecular & cellular neuroscience. To ensure reproducibility of experimental findings, each set of experiments was performed at least twice (and at least in two different sets of neuronal cultures if appropriate) to confirm the results. Prism 8 (Graphpad) was used to run statistical tests. A test of normality was not conducted and throughout the study, two-tailed non-paired t test was used to compare effects of SNAP25 variants against either WT SNAP25 or SNAP25 KO. Field recordings were analyzed using multiple t tests corrected with Holm-Sidak correction for multiple comparisons were used. Kolmogorov-Smirnov test was used to compare cumulative histograms of interevent intervals. Outliers were identified with Robust regression and Outlier removal (ROUT) method. p value less than 0.05 was considered as statistically significant. Significance levels were stated as follows: *p < 0.05, **p < 0.01, ***p < 0.001 and ****p < 0.0001. ns denotes non-significance.
Supplemental Information

Role of Aberrant Spontaneous Neurotransmission in SNAP25-Associated Encephalopathies

**Supplementary Figure 1**

<table>
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<tr>
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**Supplementary figure 1.** Sequence alignments of SNAP25 from multiple species, related to Table 1. Mutated residues from patients were shown in yellow columns. The alignment was performed using ClustalW2 and the figure was prepared with Boxshade3.21. List of Uniprot accession numbers as follows: P60880 (Homo sapiens, human), P60879 (Mus musculus, mouse), P60881 (Rattus norvegicus, rat), Q17QQ3 (Bos taurus, bovine), Q5R1X1 (Pan troglodytes, chimpanzee), P60877 (Macaca mulatta, rhesus macaque), P60878 (Gallus gallus, chicken), A0A151MGV1 (Alligator mississippiensis, alligator), Q640W4 (Xenopus laevis, frog) and S4RGG0 (Petromyzon marinus, sea lamprey).
Supplementary Figure 2

Supplementary figure 2. CD spectra of SNARE complexes formed with SNAP25 variants, related to Figure 2. Mean residue ellipticity values at different wavelengths from 200 nm to 260 nm are shown for individual SNARE complexes composed of different SNAP25 variants.
Supplementary Figure 3

A

Representative western blotting immunolabeled against SNAP25 and GAPDH with quantification. Note that Q174X gives rise to a truncated SNAP25 variant of which molecular weight is less than 25 kDa.

B

Coimmunostaining of SNAP25 and synapsin (presynaptic marker) to show that variants were expressed and trafficked normally by lentiviral infected neurons. The scale bar in the KO images are valid for all images and represents 5 μm.

Supplementary Figure 3. Expression of SNAP25 variants on SNAP25 KO cultures, related to Figure 3 and Figure 4. A. Representative western blotting immunolabeled against SNAP25 and GAPDH with quantification. Note that Q174X gives rise to a truncated SNAP25 variant of which molecular weight is less than 25 kD. B. Coimmunostaining of SNAP25 and synapsin (presynaptic marker) to show that variants were expressed and trafficked normally by lentiviral infected neurons. The scale bar in the KO images are valid for all images and represents 5 μm.
Supplementary Figure 4

Supplementary figure 4. Overexpression of WT SNAP25 on WT cultures is electrophysiologically indistinguishable from WT cultures expressing WT SNAP25 only, related to Figure 3, Figure 4 and Figure 7.

A. Representative western blot with quantification of SNAP25 levels obtained from WT overexpression experiments shown in Figures 3, 4 and 7. pFUGW denotes empty lentiviral construct, whereas the others denote the SNAP25 variant that the pFUGW construct carried.  

B. Representative western blot showing WT SNAP25 overexpression without any changes in other canonical SNAREs, syntaxin-1a and synaptobrevin-2.  

C-H. WT denotes cultures without WT SNAP25 overexpression, OE denotes cultures overexpressing WT SNAP25 in addition to endogenous SNAP25. All experiments performed with SNAP25 variant overexpression (Figures 3, 4 and 7) were also performed with WT SNAP25 overexpression and compared to WT cultures without SNAP25 overexpression. WT overexpression does not affect any of the parameter we studied with the variants and we used WT overexpression as the control group. For comparison of the cumulative histogram of interevent intervals, Kolmogorov-Smirnov test was used (Kolmogorov-Smirnov D=0.04194, p>0.05).
Supplementary Figure 5

A. eIPSC recordings obtained from variant expressing KO neurons (from Fig. 3)

B. SNARE-Syt1 Primary Interface Variants

C. N-terminal Side Hydrophobic Layer Variants

D. C-terminal Side Variants

Supplementary figure 5. Representative and averaged traces of eIPSCs to show event kinetics, related to Figure 3. A. Explanation of the trace conversion. B-D. Scaled eIPSCs along with quantification of rise slopes and 20-80% rise times, and their averaged logarithmic traces to emphasize the differences in the rising phase of the eIPSCs.
Supplementary Figure 6

Supplementary figure 6. Effects of SNAP25 variants on inhibitory spontaneous neurotransmission and excitatory evoked neurotransmission, and BAPTA-AM responsiveness of the augmented spontaneous release, related to Figure 4 and Figure 6. A-C. Representative mIPSC recordings along with quantitative analysis of event frequencies and amplitudes. D. mEPSC frequencies of variants augmenting spontaneous release after incubated with either BAPTA-AM or vehicle. E-G. Effects of SNAP25 variants on evoked excitatory postsynaptic currents (eEPSCs).
## Supplementary Table 1

<table>
<thead>
<tr>
<th>Data collection</th>
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</tr>
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<td>Space group</td>
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</tr>
<tr>
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<td></td>
</tr>
<tr>
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<tr>
<td>(\alpha, \beta, \gamma) (°)</td>
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<tr>
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<td>(R_{merge})</td>
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<td>(R_{FOM})</td>
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<tr>
<td>(I / \sigma I)</td>
<td>11.2/0.8 (0.6/1.3)</td>
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<td>Completeness (%)</td>
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<tr>
<td>Redundancy</td>
<td>11.4 (4.8)</td>
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</tbody>
</table>

## Refinement

| Resolution (Å)                  | 42.29 – 2.11 (2.186 – 2.11) |
| No. reflections                 | 19820 (498)                 |
| \(R_{work} / R_{free}\)        | 0.2003 / 0.2440 (0.2619 / 0.3445) |
| No. atoms                       | 4654                       |
| Protein                         | 4351                       |
| Ligand/ion                      | 20                         |
| Water                           | 283                        |
| B-factors                       | 34.61                      |
| Protein                         | 34.48                      |
| Ligand/ion                      | 59.48                      |
| Water                           | 34.90                      |
| Ramachandran                    |                            |
| Favor (%                        | 100                        |
| Allowed (%)                     | 0                          |
| Outliers (%)                    | 0                          |
| Rotamer outliers (%)            | 2.10                       |
| Clashscore                      | 6.58                       |
| R.m.s. deviations               |                            |
| Bond lengths (Å)                | 0.003                      |
| Bond angles (°)                 | 0.57                       |

*Values in parentheses are for highest-resolution shell.

**Supplementary table 1.** Data collection and refinement statistics for R59P-SNAP25 containing SNARE complex, related to Figure 2.