Overlapping functions of stonin 2 and SV2 in sorting of the calcium sensor synaptotagmin 1 to synaptic vesicles

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Neurotransmission involves the calcium-regulated exocytic fusion of synaptic vesicles (SVs) and the subsequent retrieval of SV membranes followed by reformation of properly sized and shaped SVs. An unresolved question is whether each SV protein is sorted by its own dedicated adaptor or whether sorting is facilitated by association between different SV proteins. We demonstrate that endocytic sorting of the calcium sensor synaptotagmin 1 (Syt1) is mediated by the overlapping activities of the Syt1-associated SV glycoprotein SV2A/B and the endocytic Syt1-adaptor stonin 2 (Stn2). Deletion or knockdown of either SV2A/B or Stn2 results in partial Syt1 loss and missorting of Syt1 to the neuronal surface, whereas deletion of both SV2A/B and Stn2 dramatically exacerbates this phenotype. Selective missorting and degradation of Syt1 in the absence of SV2A/B and Stn2 impairs the efficacy of neurotransmission at hippocampal synapses. These results indicate that endocytic sorting of Syt1 to SVs is mediated by the overlapping activities of SV2A/B and Stn2 and favor a model according to which SV protein sorting is guarded by both cargo-specific mechanisms as well as association between SV proteins.

Neurotransmission is based on the calcium-triggered fusion of neurotransmitter-filled synaptic vesicles (SVs) with the presynaptic plasma membrane. To sustain neurotransmitter release, neurons have evolved mechanisms to retrieve SV membranes and to reform SVs locally within presynaptic nerve terminals. How SVs are reformed and maintain their compositional identity (1, 2) is controversial (3–5). One possibility is that upon fusion SV proteins remain clustered at the active zone—that is, by association between SV proteins—and are retrieved via “kiss-and-run” or ultrafast endocytosis (6), thereby alleviating the need for specific sorting of individual SV proteins. Alternatively, if SVs lose their identity during multiple rounds of exo-/endocytosis (7, 8), specific mechanisms exist to orchestrate high-fidelity SV protein sorting, either directly at the plasma membrane via slow clathrin-mediated endocytosis (CME) or at endosome-like vacuoles generated by fast clathrin-independent membrane retrieval (5, 9). Endocytic adaptors for SV protein sorting include the heterotetrameric adaptor protein complex 2 (AP-2) (9), the synaptobrevin 2/VAMP2 adaptor AP180 (10), and the AP-2μ-μ-related protein stonin 2 (Stn2), a specific sorting adaptor for the SV calcium sensor synaptotagmin 1 (Syt1) (8, 11). Although genetic inactivation of the Stn2 orthologs Stoned B and Unc41 in flies and worms is lethal due to defective neurotransmission caused by degradation and missorting of Syt1 (12, 13), Stn2 knockout (KO) mice are viable and able to internalize Syt1, albeit with reduced fidelity of sorting (14). Thus, mammalian synapses, in contrast to invertebrates, have evolved mechanisms to sort Syt1 in the absence of its specific sorting adaptor Stn2. One possibility is that Syt1 sorting in addition to its direct recognition by Stn2 is facilitated by complex formation with other SV proteins. Likely candidates for such a piggyback mechanism are the SV2 family of transmembrane SV glycoproteins (15, 16), which might regulate Syt1 function either via direct interaction (17, 18) or by facilitating its binding to AP-2 (19). Apart from the distantly related SVOP protein (20), no close SV homologs exist in invertebrates, suggesting that SV2 fulfills a unique function at mammalian synapses. KO of SV2A or combined loss of its major A and B isoforms in mice causes early postnatal lethality due to epileptic seizures (21, 22), impaired neurotransmission (23, 24), and defects in Syt1 trafficking (25), whereas SV2B KO mice are phenotypically normal (17). Given that SV2A in addition to its association with Syt1 binds to endocytic proteins including AP-2 and Eps15 (25), SV2 would be a likely candidate for mediating Syt1 sorting to SVs.

Here we demonstrate that endocytic sorting of Syt1 is mediated by the overlapping activities of SV2A/B and Stn2. Deletion or knockdown of either SV2A/B or Stn2 results in partial Syt1 loss and missorting of Syt1 to the neuronal surface, whereas deletion of both SV2A/B and Stn2 dramatically exacerbates this phenotype, resulting in severely impaired basal neurotransmission. Our results favor a model according to which SV protein sorting is guarded by both cargo-specific mechanisms as well as association between SV proteins.

Significance

Brain function depends on neurotransmission, and alterations in this process are linked to neurological disorders. Neurotransmitter release requires the rapid recycling of synaptic vesicles (SVs) by endocytosis. How synapses maintain the molecular composition of SVs during recycling is poorly understood. We demonstrate that overlapping functions of two completely distinct proteins, the vesicle protein SV2A/B and the adaptor stonin 2, mediate endocytic sorting of the vesicular calcium sensor synaptotagmin 1. As SV2A is the target of the commonly used antiepileptic drug levetiracetam and is linked to late onset Alzheimer’s disease, our findings bear implications for the treatment of neurological and neurodegenerative disorders.

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Results

Exacerbated Loss of Syt1 Upon Combined Deletion of Stn2 and SV2A/B. Previous work has suggested putative functions of both Stn2 and SV2 in sorting of Syt1 to SVs with Syt1 levels being reduced in brain sections from KO mice lacking Stn2 (14) and in SVs isolated from brains of SV2A/B KO mice (17, 25). Given this phenotypic similarity, we hypothesized that Stn2 and SV2A/B fulfill overlapping roles with respect to Syt1 sorting and maintenance in the mammalian brain. To test this hypothesis, we analyzed the expression levels of endocytic proteins including Stn2 in double knockout (DKO) mice lacking SV2A and B. Indeed, Stn2 levels were significantly increased in brain extracts from SV2A/B DKO mice (Fig. 1 A and B), whereas Syt1 expression was reduced (Fig. 1 C), in agreement with earlier findings (17, 25). The levels of other endocytic proteins such as clathrin, AP-2, and dynamin1-3, or of synaptophysin (Syp), another major SV protein, were not significantly different (Fig. 1 A, C, and D). Thus, loss of SV2A/B results in a compensatory increase in the steady-state levels of Stn2 in vivo. To further corroborate these data and to address whether the comparably mild phenotype of Stn2 loss in mice is due to functional compensation by SV2 family members, we crossed SV2A heterozygous (HET) and SV2B KO mice (22) with KO mice lacking Stn2 (14) (Fig. S1A), resulting in offspring that lack both SV2A/B and Stn2 (hereafter referred to as triple KO; TKO). TKOs appeared normal at birth, but ~70% of the offspring died within the first 3 d of their postnatal life. This phenotype is more severe than that of DKO, with about 50% lethality in our hands. The remaining TKO mice survived for up to 2 wk but lagged behind their control littersmates in postnatal development and weight gain (Fig. S1 B and C) and experienced major neurological defects, as revealed by poor motor coordination and spontaneous epileptic seizures. We then analyzed the effect of deleting SV2A/B alone or in combination with Stn2 on the steady-state levels of Syt1. TKO mice displayed a significant further reduction of Syt1 expression compared with control or DKO, whereas Syp levels were normal (Fig. 1 A and C), consistent with the selective association of Stn2 with Syt1 but not with other SV proteins (8, 11, 14). As expected, Syt1 levels were reduced in the hippocampi of DKO mice, and this reduction was further aggravated in TKO (Fig. 1 E and F). No significant alterations in the levels of synaptophysin 2/VAMP2 or of the SV-associated phosphoprotein synapsin 1 were observed in hippocampal sections from either DKO or TKO (Fig. 1 G–J). Consistent with this, Schaffer collateral synapses from DKO and TKO mice displayed normal numbers, morphology, and organization of SVs compared with controls (Fig. S1D and Table S1). These data suggest that SV2A/B and Stn2 fulfill overlapping functions in maintaining vesicular Syt1 at central synapses.

Combined Deficiency of SV2 and Stn2 Causes Additive Defects in Syt1 Sorting. The findings described above suggest that SV2 and Stn2, despite their distinct molecular features, execute shared overlapping functions with respect to Syt1 maintenance at mammalian synapses in vivo. To corroborate this hypothesis and to bypass the problem that the majority of DKO mice experience severe seizures and die soon after birth, we used siRNA-mediated gene knockdown to deplete SV2A in cultured hippocampal neurons (Fig. S2 A–D). SV2A knockdown reduced the levels of endogenous SV2A levels to about 35% of that observed in controls (Fig. S2 A–C), and this was accompanied by a roughly 20% decrease in the levels of Syt1 (Fig. S2D), similar although slightly less pronounced to what we observed in the brain from SV2A/B DKO mice (compare Fig. 1 J and Fig. 1 C). Partial loss of Syt1 in SV2A-depleted neurons was rescued by plasmid-based reexpression of siRNA-resistant SV2A (Fig. S2D). Recent data show that repositioning of Syt1 to the neuronal surface in Stn2 KO neurons is accompanied by an increased rate of SV membrane retrieval during high-frequency stimulation (14). We therefore studied whether combined depletion of both Stn2 and SV2 further accelerates SV membrane retrieval by analyzing the kinetics of Syt1 exo-/endocytosis using a chimera carrying the pH-sensitive green fluorescent protein pHluorin (Syt1-pHluorin) in response to high-frequency stimulation with 200 APs at 40 Hz (Fig. 2 A and B). As expected, retrieval of Syt1-pHluorin was significantly facilitated in neurons derived from Stn2 KO mice (τK0 = 35.1 ± 1.2 s) or depleted of SV2A by siRNA (τKd = 34.5 ±
SV2 Regulates Syt1 Sorting to SVs During Neuronal Activity. Syt1 partitioning between vesicular and surface pools in the absence of SV2 and/or Stn2 likely is a consequence of its defective endocytic sorting postexocytosis and thus should depend on synaptic activity within the neuronal network. To test this hypothesis we analyzed the levels of surface-stranded Syt1 in cultured control or SV2A-deficient neurons (Fig. 3A) in the absence or presence of the sodium channel blocker tetrodotoxin (TTX). As expected, loss of SV2A led to elevated Syt1 surface-to-total pool ratios compared with controls [scrambled (scr), 1.0 ± 0.08; SV2A KO, 1.62 ± 0.13; P < 0.001], a phenotype rescued by reexpression of siRNA-resistant SV2A (Fig. S2E). Syt1 surface stranding was completely eliminated by silencing neuronal activity in the presence of TTX (Fig. 3B). Surface levels of Syp, a SV protein that neither associates with SV2 nor is sorted by Stn2, taken as a control were unaltered (Fig. 3B). Thus, loss of SV2A selectively impairs endocytic retrieval of Syt1 to SVs during neuronal activity. As a consequence of impaired Syt1 sorting, the absence of SV2A and/or Stn2, recycled SVs are expected to contain reduced Syt1 copy numbers. To monitor the relative copy number of Syt1 on SVs actively undergoing recycling (9), we subjected neurons expressing Syt1-pHluorin to sustained stimulation with a train of 200 APs applied at 10 Hz, a stimulation paradigm known to trigger the rapid reuse of SVs (26) and to increase release probability due to the accumulation of residual calcium (27). When combined with the application of the vesicular ATPase blocker folimycin, which prevents reacidification of internalized membranes (28) (i.e., blinding pHluorin signals for endocytosis), this allowed us to selectively monitor the relative number of pHluorin molecules undergoing exocytosis (28) under conditions that bypass the exocytosis defects reported at synapses of DKO mice (22–24). We imaged boutons from control or SV2A-deficient neurons expressing plasmid-encoded Syt1-pHluorin at near identical levels (Fig. S2D). Neurons were stimulated first in the absence, then in the presence of folimycin, and the relative number of exocytosed Syt1-pHluorin molecules was quantified (AF) (Fig. 3C–F). Depletion of SV2A significantly reduced the apparent number of exocytosed Syt1-pHluorin molecules (Fig. 3C, D, and G), whereas the number of exocytosed Syp-pHluorin molecules was unaffected (Fig. 3E, F, and H), suggesting that SVs undergoing exocytosis contain a reduced Syt1 copy number. These data show that loss of SV2A causes a partial depletion of Syt1 from recycling SVs, consistent with biochemical data from SV2A/B-deficient mice (Fig. 1) (25).

Combined Deficiency of SV2 and Stn2 Aggravates Impairments in Synaptic Strength and Short-Term Plasticity Caused by Deletion of SV2. The drastic loss of Syt1 from SVs and its misorting to the plasma membrane in synapses from SV2A/B DKO, and more pronouncedly in TKO synapses, might impact the efficacy of neurotransmitter release. To address this, we measured synaptic responses of hippocampal CA1 pyramidal neurons in acute brain slices derived from p12 SV2A/B KO or SV2A/B/Stn2 TKO mice. For comparison we measured evoked neurotransmission and paired-pulse responses from SV2A/B WT (WT/KO/WT) or SV2A-WT/SV2B-KO/Stn2-HET (WT/KO/HET) littermates as controls (Fig. S3 B and F). First, we analyzed evoked neurotransmission [field excitatory postsynaptic potential (fEPSP) amplitudes] in response to increasing stimulus intensities and plotted the data as input–output curves (Fig. 4A and B). In slices from SV2A/B DKO mice, the size of maximal

1.8 s), compared with controls (τ_{ex} = 43.1 ± 3.6 s). This phenotype was further aggravated by combined deficiency of both SV2A and Stn2 (τ_{ex} = 25.3 ± 1.0 s) (Fig. 2B), possibly by shunting the pathway of SV membrane retrieval toward a fast clathrin-independent mechanism (9). Similar results were obtained in SV2A KO neurons expressing Syp-pHluorin (Fig. S2F), indicating that the increased rate of membrane retrieval is independent of the pHluorin sensor used.

Next, we studied the role of SV2A and Stn2 in Syt1 sorting. To directly probe Syt1 sorting between internal vesicular and surface-stranded pools in the absence of SV2A and Stn2, we performed acid quenching–dequenching experiments (8) of cultured neurons expressing Syt1-pHluorin. Combined deficiency of SV2A and Stn2 further increased the ratio between surface-stranded and total Syt1-pHluorin compared with neurons depleted either of SV2A or Stn2 alone (Fig. 2C). To probe for sorting of endogenous Syt1, we applied antibodies that specifically recognize the luminal domain (LD) of Syt1 without prior membrane permeabilization to detect surface-stranded Syt1 and compared their relative levels to the total pool of Syt1. As expected, SV2A loss increased the surface accumulation of endogenous Syt1 in neurons from Stn2 KO mice (Fig. S2 G–I). Furthermore, the ratio of endogenous surface-stranded/total Syt1 was elevated in hippocampal slices from SV2A/B DKO mice, and this defect was further aggravated in TKO mice lacking SV2A/B and Stn2 (Fig. 2D and E), consistent with our data in cultured neurons (Fig. 2C). These results demonstrate that SV2A and Stn2 execute overlapping functions with respect to selective sorting of Syt1 to SVs.

Fig. 2. Combined deficiency of SV2 and Stn2 causes additive defects in Syt1 sorting. (A and B) Time course (A) and endocytic time constants (F) of Syt1-pHluorin endocytosis/reacidification in WT and Stn2 KO neurons coexpressing Syt1-pHluorin and scr or SV2A siRNA (KD) in response to 200 APs (40 Hz). Endocytosis is facilitated in the absence of either SV2A (τ_{ex} = 43.1 ± 3.6 s) or Stn2 (τ_{ex} = 34.3 ± 1.8 s) or both proteins (τ_{ex} = 25.3 ± 1.0 s; **P < 0.05, ***P < 0.001; n = 3; >950 boutons per condition). Data represent mean ± SEM. (C) Surface/total ratios of Syt1-pHluorin expressed in hippocampal wild-type (WT) or Stn2 KO neurons cotransfected with scr or SV2A siRNA (KD). The surface/total Syt1-pHluorin ratio was significantly increased in the absence of either SV2A or Stn2 (Stn2 WT, scr, 12.2 ± 0.1%; Stn2 WT, SV2A KD, 16.6 ± 0.1%; Stn2 KO, scr, 16.8 ± 0.1%) and further aggravated by depletion of both proteins (Stn2 KO, SV2A KD, 21.6 ± 0.1%; n = 5; >510 boutons per condition; **P < 0.05, ***P < 0.001). (D) Total (red) and surface levels (green) of Syt1 in the hippocampus of p12 control, SV2A/B DKO, and SV2A/B/Stn2 TKO mice (green). (Scale bar, 100 μm.) ROIs in D indicate areas taken for quantification. (E) Elevated Syt1 surface levels in SV2A/B DKO and more pronouncedly in SV2A/B/Stn2 TKO mice (DKO, 129.6 ± 9.4%; TKO, 201.8 ± 22.2%).
IEPSPs was decreased about twofold compared with controls (Fig. 4A). This effect was exacerbated in slices from TKOs where maximal IEPSP sizes were reduced additionally by 20% compared with the DKO amplitude (Fig. 4B). These reduced postsynaptic responses in DKO and TKO mice were not a result of decreased presynaptic input, as evident in measurements of fiber volley amplitudes plotted as a function of increasing stimulus intensity (Fig. S3A and B). Moreover, CA1 synapses from DKO or TKO mice also displayed a normal overall morphology with unaltered presynaptic input, as evident in measurements of fiber volley amplitudes (Fig. S3A and B). (C and D) Loss of SV2 selectively impairs Syt1-pHluorin (Syt1pH) sorting to SVs during repetitive rounds of exo-/endocytosis. Average traces in response to 200 APs (10 Hz) in the absence or presence of tetrodotoxin (TTX-silenced neurons) (scr, 1.0 ± 0.5; SV2AKD, 0.98 ± 0.06; n = 2; >4,500 boutons per condition). (E and F) Depletion of SV2A does not affect Syt1pHluorin (Syt1pH) sorting to SVs during repetitive rounds of exo-/endocytosis. Average traces of hippocampal neurons cotransfected with Syt1pH and scr (C) or SV2A siRNA (KD, D). (E and F) Depletion of SV2A does not affect Syt1pHluorin (Syt1pH) sorting fidelity during stimulation. Average traces of hippocampal neurons cotransfected with Syt1pH and scr (E) or SV2A siRNA (F) in response to 200 APs (10 Hz) in the absence or presence of tetrodotoxin (TTX-silenced neurons) (scr, 1.0 ± 0.5; SV2AKD, 0.98 ± 0.06; n = 2; >4,500 boutons per condition). The apparent number of exocytosed Syt1-pHluorin molecules in SV2A-deficient neurons (SV2AKD, 1.13 ± 0.10) compared with control (scr, 1.73 ± 0.15; **P < 0.001; n = 13; >630 boutons per genotype). The apparent number of exocytosed Syt1pH molecules (H) is unaffected (scr, 3.0 ± 0.6; SV2AKD, 3.1 ± 0.4; n = 8; >300 boutons per genotype). Data represent mean ± SEM.
to paired stimuli given at various interstimulus intervals (ISIs) revealed increased facilitation in slices from TKO mice, a phenotype further aggravated in slices from TKO mice compared with controls (Fig. 4 C–E). As the analysis of short-term plasticity is often taken as a surrogate measure for release probability (31), these data are most likely explained by a reduction of release probability P0 in SV2A/B DKO and more pronounced reduction in SV2A/B/Stn2 TKO synapses (consistent with ref. 25) that correlates with the depletion of Syt1 from SVs. To exclude that the altered short-term plasticity in DKO and TKO synapses is caused by alterations in the refilling of the releasable SV pool, we analyzed responses of DKO and TKO synapses during a train of APs applied at high stimulation frequency (20 Hz, 500 APs). This stimulation paradigm elevates release probability and depletes the readily releasable pool of vesicles, thereby unmasking possible defects in its refilling once exocytosis reaches a steady state. During the initial phase of the train, responses of DKO and, more pronouncedly, those of TKO were reduced followed by strong hyperfacilitation (Fig. S3 J) to eventually reach amplitudes that were similar to those elicited from controls (−2 mV) (Fig. 4 F). Following this initial facilitation phase, amplitudes declined to eventually reach baseline steady-state-level neurotransmission that was similar between genotypes (Fig. 4 F). Analyses of cumulative amplitudes revealed an unaltered SV pool challenged by increased stimulation (Fig. S3 H). The facilitation in DKO and TKO synapses we observed thus uniquely be caused by alterations in the refilling of the releasable SV pool and may rather be a consequence of the reduced ability of SVs from DKO or TKO to fuse in response to calcium (23, 24).

Collectively these data show that loss of SV2A/B (24) and, more pronouncedly, of SV2A/B/Stn2 impairs basal neurotransmission and reduces release probability, most likely as a consequence of impaired Syt1 sorting to SVs, although additional functions of SV2A/B (i.e., in SV priming to acquire competence for calcium-induced exocytosis) (21–24) and/or Stn2 may contribute to the phenotype.

**Discussion**

Our data reported here unravel a crucial overlapping function of the endocytic adaptor Stn2 and the SV protein SV2A/B in Syt1 sorting and maintenance at mammalian synapses. The observation that loss of Stn2 and SV2 has additive effects with respect to Syt1 partitioning to the neuronal plasma membrane (Figs. 2 and 3) is consistent with the view that endocytic membranes—rather than being essential components of the machinery for membrane retrieval, may serve a “proofreading” function to monitor the compositional identity of newly formed SVs, whereas surface-stranded SV proteins may be targeted for degradation by autophagy and/or the endolysosomal pathway. Whether the reduced levels of Syt1 observed in SV2A/B-Stn2 TKO neurons are indeed the result of facilitated Syt1 degradation will need to be addressed in future studies. Given the putative genetic association of Stn2 with neuropsychiatric disorders including schizophrenia and autism spectrum disorders (24, 39, 40) and the role of SV2A in late onset Alzheimer’s disease (41) and as a major target of the antiepileptic drug levetiracetam (42, 43), our results may also be of relevance for the diagnosis and treatment of neurological disorders in humans.

**Materials and Methods**

**Neuronal Cell Culture, Transfections, and Animal Experiments.** Hippocampal neurons (p1–p4 mice) were prepared in mass culture according to a previously described protocol and transfected at 7–9 days in vitro (DIV) by calcium phosphate transfection (14). For siRNA knockdowns, plasmid DNA was cotransfected with equal amounts of siRNA. Hippocampi were pooled from generation of TKO KO mice, plasmids and siRNA, antibodies (Table S2), Neuronal Cell Culture, Transfections, and Animal Experiments.

**Phlorizin Imaging of Living Neurons.** Imaging was performed at 12–15 DIV as described before (9). See SI Materials and Methods for further details.

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