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 | synaptic vesicle protein sorting | calcium sensor | endocytosis | knockout mice

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–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 SV2 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 data (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. S1*A*), 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 live. 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 littermates 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 hippocampus of DKO mice, and this reduction was further aggravated in TKO (Fig. 1 *E* and *F*). No significant alterations in the levels of synaptobrevin 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. S1*D* 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. S2*D*), similar although slightly less pronounced to what we observed in the brain from SV2A/B DKO mice (compare Fig. 1*A* and Fig. 1*C*). Partial loss of Syt1 in SV2A-depleted neurons was rescued by plasmid-based reexpression of siRNA-resistant SV2A (Fig. S2*D*). Recent data show that repartitioning 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 ($\tau_{KO} = 35.1 \pm 1.2$ s) or depleted of SV2A by siRNA ($\tau_{KD} = 34.5 \pm$

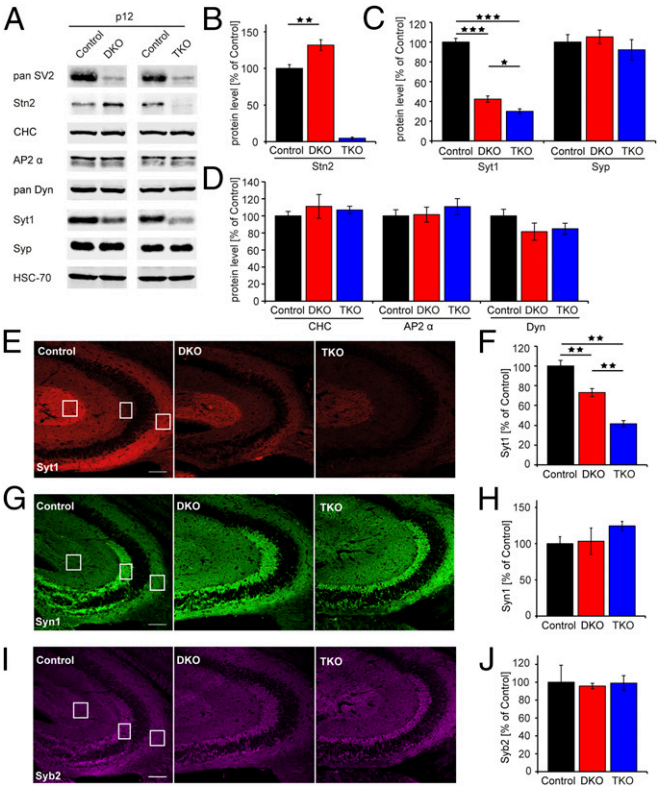

Fig. 1. Exacerbated loss of Syt1 upon combined deletion of Stn2 and SV2A/B. (*A*) Levels of synaptic and endocytic proteins in total brain lysates from p12 control, SV2A/B DKO, and SV2A/B/Stn2 TKO mice probed by immunoblotting with specific antibodies. AP-2 α, adaptor protein 2α; CHC, clathrin heavy chain; Hsc70, heat shock cognate protein of 70 kDa; pan-Dyn, dynamin 1–3; Syp, synaptophysin; Syt1, synaptotagmin 1. The band remaining in DKO and TKO lysates decorated with pan-SV2 antibodies corresponds to the SV2C isoform. (*B*) Stn2 is up-regulated in the absence of SV2A/B (control, 100.0 ± 6.9%; DKO, 131.8 ± 7.2%; TKO, 4.7 ± 1.5%; *n* control/DKO = 6; *n* control/TKO = 5; $^{**}P < 0.01$). (*C*) Syt1 levels are significantly reduced in DKO (42.3 ± 3.2%) and in TKO (29.9 ± 2.6%) compared with control (100.0 ± 6.0%; *n* control/DKO = 6; *n* control/TKO = 5; $^{*}P < 0.05$, $^{**}P < 0.01$, $^{***}P < 0.001$), and Syp levels are unaffected (control, 100.0 ± 7.6%; DKO, 105.3 ± 6.8%; TKO, 92.3 ± 10.21%; *n* control/DKO = 6; *n* control/TKO = 5). (*D*) Levels of clathrin (CHC), AP-2α, and dynamin 1–3 (Dyn) in total brain lysates derived from p12 control, DKO, and TKO mice (CHC—control, 100.0 ± 5.2%; DKO, 111.2 ± 14.0%; TKO, 106.9 ± 4.4%; AP2—control, 100.0 ± 7.1%; DKO, 101.5 ± 8.7%; TKO, 110.9 ± 9.3%; Dyn—control, 100.0 ± 7.7%; DKO, 81.5 ± 10.1%; TKO, 84.9 ± 6.5%; *n* control/DKO = 6; *n* control/TKO = 5). (*E*–*J*) Total levels of Syt1 (*E* and *F*), Syn1 (*F* and *H*), and Syb2 (*I* and *J*) in the hippocampus of control, DKO, and TKO mice revealed by immunostaining. These are representative confocal images of perfused sagittal brain sections. (Scale bar, 100 μm.) (*F*) Syt1 intensity levels are reduced in DKO and even further decreased in TKO mice compared with controls (control, 100.0 ± 5.6%; DKO, 71.4 ± 3.5%; TKO, 41.7 ± 3.0%; *n* control/DKO = 4; *n* control/TKO = 3, $^{*}P < 0.05$, $^{**}P < 0.01$). (*H* and *J*) Syn1 and Syb2 expression levels are not significantly different in DKO and TKO mice compared with controls (control Syn1, 100.0 ± 9.6%; DKO Syn1, 103.4 ± 18.1%; TKO Syn1, 124.5 ± 6.3%; control Syb2, 100,0 ± 19.0%; DKO Syb2, 95.7 ± 3.1%; TKO Syb2, 99.0 ± 8.4%; *n* WT/DKO = 4; *n* WT/TKO = 3). Regions of interest (ROI) in *E*, *G*, and *I* indicate areas taken for quantification. All data represent mean ± SEM.

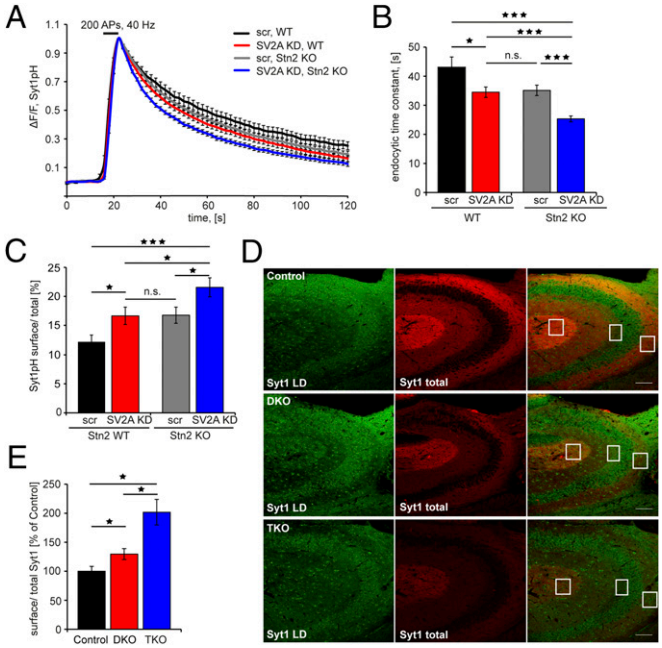

Fig. 2. Combined deficiency of SV2 and Stn2 causes additive defects in Syt1 sorting. (*A* and *B*) Time course (*A*) and endocytic time constants (τ) (*B*) of Syt1-pHluorin endocytosis/reacidification in WT and Stn2 KO neurons coexpressing Syt1-pHluorin and scr or SV2A siRNA (KD) in response to 200 APs (40 Hz). Endocytosis is facilitated in the absence of either SV2A $\tau_{WT,scr} = 43.1 \pm 3.6$ s ($\tau_{WT,SV2A\ KD} = 34.5 \pm 1.8$ s) or Stn2 ($\tau_{KO,scr} = 35.1 \pm 1.2$ s), an effect further aggravated by depletion of both proteins ($\tau_{KO,SV2A\ KD} = 25.3 \pm 1.0$ s; $^*P < 0.05$, $^{***}P < 0.0001$; $n = 3$; >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; $^*P < 0.05$, $^{***}P < 0.0001$). (*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%) compared with controls (control, 100 ± 8.4%; *n* control/DKO = 4; *n* control/TKO = 3; $^*P < 0.05$). Data represent mean ± SEM.

1.8 s), compared with controls ($\tau_{scr} = 43.1 \pm 3.6$ s). This phenotype was further aggravated by combined deficiency of both SV2A and Stn2 ($\tau_{KO,SV2KD} = 25.3 \pm 1.0$ s) (Fig. 2*B*), possibly by shunting the pathway of SV membrane retrieval toward a fast clathrin-independent mechanism (9). Similar results were obtained in SV2A KD neurons expressing Syp-pHluorin (Fig. S2*F*), 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. 2*C*). 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. 2 *D* and *E*), consistent with our data in cultured neurons (Fig. 2*C*). These results demonstrate that SV2A and Stn2 execute overlapping functions with respect to selective sorting of Syt1 to SVs.

SV2 Regulates Syt1 Sorting to SVs During Neuronal Activity. Syt1 repartitioning 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. 3*A*) 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 KD, 1.62 ± 0.13; $P < 0.001$], a phenotype rescued by reexpression of siRNA-resistant SV2A (Fig. S2*E*). Syt1 surface stranding was completely eliminated by silencing neuronal activity in the presence of TTX (Fig. 3*B*). Surface levels of Syp, a SV protein that neither associates with SV2 nor is sorted by Stn2, taken as a control were unaltered (Fig. 3*B*). Thus, loss of SV2A selectively impairs endocytic retrieval of Syt1 to SVs during neuronal activity. As a consequence of impaired Syt1 sorting in 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. S2*J*). Neurons were stimulated first in the absence, then in the presence of folimycin, and the relative number of exocytosed Syt1-pHluorin molecules was quantified (ΔF) (Fig. 3 *C–F*). Depletion of SV2A significantly reduced the apparent number of exocytosed Syt1-pHluorin molecules (Fig. 3 *C*, *D*, and *G*), whereas the number of exocytosed Syp-pHluorin molecules was unaffected (Fig. 3 *E*, *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 missorting 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 DKO or SV2A/B/Stn2 TKO mice. For comparison we measured evoked neurotransmission and paired-pulse responses from SV2A-WT/SV2B-KO/Stn2-WT (WT/KO/WT) or SV2A-WT/SV2B-KO/Stn2-HET (WT/KO/HET) littermates as controls (Fig. S3 *F* and *G*). 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. 4 *A* and *B*). In slices from SV2A/B DKO mice, the size of maximal

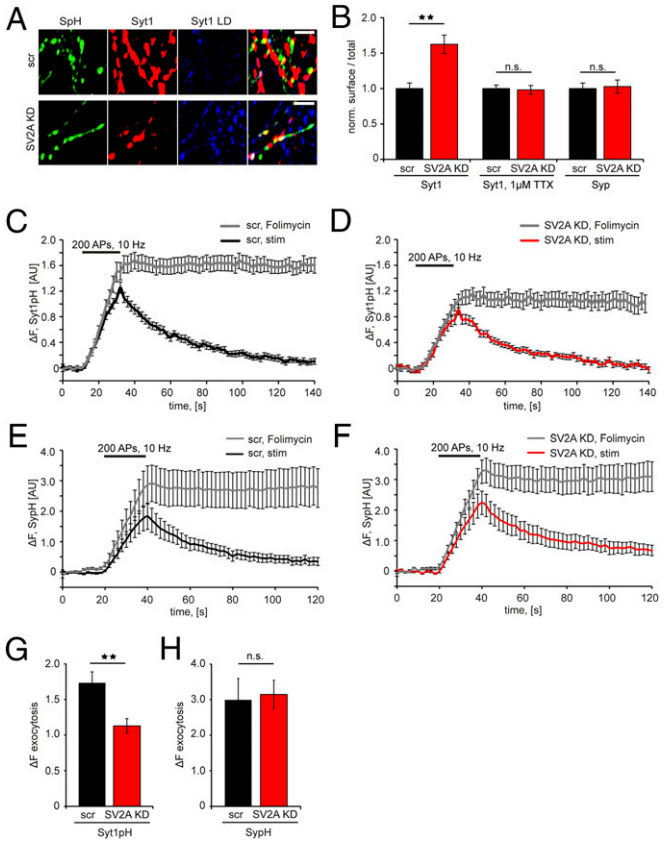

Fig. 3. SV2 regulates Syt1 sorting to SVs during neuronal activity. (*A* and *B*) Surface accumulation of Syt1 in SV2A-deficient hippocampal neurons. (*A*) Representative confocal images of mouse hippocampal neurons coexpressing Syp-pHluorin (SpH, green) with scr or SV2A siRNA (SV2A KD) and immunostained for total Syt1 (red) and surface Syt1 (Syt1 LD, blue). (Scale bar, 5 µm.) (*B*) Elevated normalized ratio of surface/total Syt1 in neurons treated with SV2A siRNA compared with scr siRNA-treated controls (set to 1; scr, 1.0 ± 0.08; SV2A KD, 1.62 ± 0.13; $n = 3$; >1,800 boutons per condition; $^{**}P < 0.001$). Syp surface/total ratio is unaffected (scr, 1.0 ± 0.08; SV2A KD, 1.03 ± 0.09; $n = 3$; >1,700 boutons per condition). Surface accumulation of Syt1 is rescued in TTX-silenced neurons (scr, 1.0 ± 0.5; SV2AKD, 0.98 ± 0.06; $n = 2$; >4,500 boutons per condition). (*C* and *D*) Loss of SV2A 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 folimycin. Neurons were cotransfected with Syt1-pH and scr (*C*) or SV2A siRNA (KD, *D*). (*E* and *F*) Depletion of SV2A does not affect Syp-pHluorin (SypH) sorting fidelity during stimulation. Average traces of hippocampal neurons cotransfected with SypH and scr (*E*) or SV2A siRNA (*F*) in response to 200 APs (10 Hz) in the absence or presence of folimycin. (*G*) Reduced 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 SypH molecules (*H*) is unaffected (scr, 3.0 ± 0.6; SV2AKD, 3.1 ± 0.4; $n = 8$; >300 boutons per genotype). Data represent mean ± SEM.

fEPSPs was decreased about twofold compared with controls (Fig. 4*A*). This effect was exacerbated in slices from TKOs where maximal fEPSP sizes were reduced additionally by 20% compared with the DKO amplitude (Fig. 4*B*). 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. S3 *A* and *B*). Moreover, CA1 synapses from DKO or TKO mice also displayed a normal overall morphology with unaltered numbers of SVs or length of the active zone (Fig. S1*D* and Table S1). In contrast to evoked neurotransmission, neither the frequency (Fig. S3 *C* and *D*) nor the amplitude of mEPSCs (Fig. S3 *C* and *E*) was altered in slices from TKO mice, akin to what has been observed at hippocampal synapses from SV2A single KOs or SV2A/B DKOs (29) but distinct from Syt1 null mice (30).

Next, we analyzed short-term plasticity at the excitatory Schaffer collateral to CA1 pyramidal cell synapse in acute brain slices from control, DKO, and TKO mice. Measurement of synaptic responses

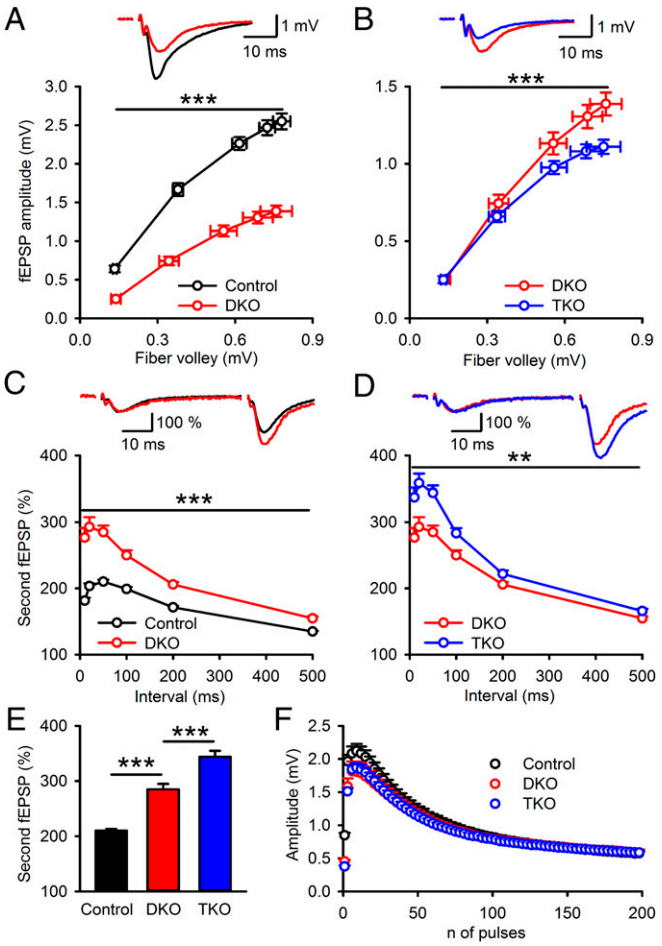

Fig. 4. Combined deficiency of SV2 and Stn2 aggravates impairments in synaptic strength and short-term plasticity caused by deletion of SV2. (*A* and *B*) Reduced basal transmission at excitatory Schaffer collateral to CA1 pyramidal cell synapses in SV2A/B DKO (red trace) and SV2A/B/Stn2 TKO (blue trace) mice in response to increasing stimulus intensities. (*A*) fEPSP amplitudes are significantly reduced in DKO compared with control ($^{***}P < 0.001$). *Inset* illustrates sample traces of maximal fEPSPs in DKO (red) and control (black). (*B*) fEPSP amplitudes in TKO are significantly lower compared with DKO ($^{***}P < 0.001$). *Inset* illustrates sample traces of maximal fEPSPs in TKO (blue) and in DKO (red). (*C–E*) Altered paired-pulse facilitation (PPF) at excitatory Schaffer collateral to CA1 pyramidal cell synapses in DKO and TKO mice. (*C*) PPF measured at 10, 20, 50, 100, 200, and 500 ms interpulse intervals (ISIs) reveals increased facilitation in DKO compared with control ($^{***}P < 0.001$). *Inset* depicts sample traces of fEPSPs at 50 ms ISI normalized to the first response of DKO (red) and control (black) synapses. (*D*) Significantly elevated PPF (fEPSP2/fEPSP1) at 10, 20, 50, 100, 200, and 500 ms ISI at synapses of TKO, compared with DKO ($^{**}P = 0.004$). *Inset* illustrates sample traces of fEPSPs at 50 ms ISI normalized to the first response at DKO (red) and TKO (blue) synapses. (*E*) PPF at 50 ms ISI is significantly increased in DKO compared with control ($^{***}P < 0.001$) and is further increased in TKO ($^{***}P < 0.001$). Note that maximal facilitation of the second response is detected at 50 ms in control slices (control $n = 20$, $n = 8$; DKO $n = 9$, $n = 4$; TKO $n = 14$, $n = 5$). (*F*) Normal steady-state level of neurotransmission in DKO and TKO mice probed by high-frequency stimulation (20 Hz). Each value is an average of three consecutive responses except for $n = 1$. Only the first 200 out of 500 pulses are presented.

to paired stimuli given at various interstimulus intervals (ISIs) revealed increased facilitation in slices from DKO 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 P_r in SV2A/B DKO and more pronounced reduction in SV2A/B/Stn2 TKO synapses (consistent with ref. 24) 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*I*) 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 repeated stimulation (Fig. S3*H*). The facilitation in DKO and TKO synapses we observed is, thus, unlikely to be caused by alterations in 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 repartitioning to the neuronal plasma membrane (Figs. 2 and 3), Syt1 levels (Fig. 1), and neurotransmission (Fig. 4) further argues that Stn2 and SV2 operate via distinct genetically separable mechanisms to facilitate Syt1 sorting to SVs, in agreement with their distinctive molecular properties. Such a model is consistent with prior observations that SVs isolated from SV2A/B KO mice contain reduced levels of Syt1 (25) and with impaired sorting of Syt1 but not other SV proteins to internal vesicular pools in the absence of Stn2 in vivo (14). The overlapping function of Stn2 and SV2A/B in sorting of Syt1 to SVs explains why loss of stonin expression in invertebrate neurons results in a dramatic loss of Syt1 expression and corresponding defects in neurotransmission (12, 13, 32), whereas KO of Stn2 expression in mammalian central neurons exhibits comparably mild phenotypes (14). We therefore propose that mammalian neurons, in addition to sorting SV proteins based on cargo-specific endocytic adaptors such as Stn2, have evolved piggy-back mechanisms to safeguard SV protein sorting to maintain evoked neurotransmission. For example, recent data indicate that the SV SNARE Syb2 not only is recognized by its specific endocytic adaptors AP180 and CALM (10, 33) but also uses a complex formation with Syp for efficient sorting in vivo (34, 35). The fact that invertebrates do not express close homologs of SV2 or Syp further suggests that sorting of SV proteins by complex formation between vesicle proteins (36) may be a unique specialization of the mammalian central nervous system.

Consistent with earlier studies (22–24) we find that loss of SV2A/B impairs the efficiency of stimulus-evoked SV exocytosis, a phenotype that is accompanied by the loss of Syt1 from SVs. Strikingly, the efficacy of neurotransmission is further reduced at synapses from SV2A/B/Stn2 TKO mice (Table S2), and this correlates with exacerbated missorting and loss of Syt1 (Fig. S4). Several studies have suggested that SV2 proteins govern synaptic plasticity by regulating residual calcium (22, 23, 37), release probability, and/or the size of the readily-releasable pool of SVs (24). Our results agree with these findings (Table S2) and suggest that SV2 proteins modulate the efficacy of neurotransmitter release at least in part via chaperoning Syt1 sorting during activity-dependent SV recycling. Interestingly, it appears that maintenance of high vesicular Syt1 levels by SV2 and Stn2 is particularly crucial for evoked neurotransmission, whereas the comparably few copies of Syt1 on SVs of TKO animals suffice to repress spontaneous fusion events.

What is the possible advantage of this apparent complexity of SV protein sorting in mammalian neurons? First, the use of multiple parallel mechanisms for sorting of SV proteins such as Syt1 likely increases both the efficiency of sorting as well as the robustness of the system to disturbances. Second, the formation of SV protein assemblies (18, 36) may alleviate the need to specifically sort each SV component during every round of exo-/endocytosis and SV reformation. Rather, it would allow rapid bulk retrieval of SV membranes—that is, by ultrafast or bulk endocytosis (6, 38)—followed by clathrin- and adaptor-mediated SV reformation from internal endosome-like structures (5, 9) during phases of high-frequency synaptic activity. In agreement with the view that endocytic membrane retrieval and SV protein sorting occur by largely distinct mechanisms, we failed to observe major kinetic defects in Syt1 retrieval even in SV2A-deficient Stn2 KO neurons (Fig. 2 and Fig. S2), where Syt1 is extensively missorted to the neuronal surface. In this model, endocytic sorting adaptors such as Stn2, 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 several genotypically identical littermates. All animal experiments have been approved by the Landesamt für Gesundheit und Soziales (LAGeSo, Berlin) and have been conducted in compliance with the German Genetic Engineering Act and EU-directive 2010/63/EU on the protection of animals used for scientific purposes as monitored by the institute's Animal Care Officer.

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

SI Materials and Methods includes the following: breeding of DKO and generation of TKO KO mice, plasmids and siRNA, antibodies (Table S3), analysis of tissue extracts, immunohistochemical analysis, pHluorin imaging, immunocytochemistry analysis of cultured neurons, cell lysates of HEK293 cells, electrophysiology, electron microscopy, and statistical analysis.

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