Genetic analysis of synaptotagmin-7 function in synaptic vesicle exocytosis

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Synaptotagmin-7 is a candidate Ca^{2+} sensor for exocytosis that is at least partly localized to synapses. Similar to synaptotagmin-1, which functions as a Ca^{2+} sensor for fast synaptic vesicle (SV) exocytosis, synaptotagmin-7 contains C_2A and C_2B domains that exhibit Ca^{2+}-dependent phospholipid binding. However, synaptotagmin-7 cannot replace synaptotagmin-1 as a Ca^{2+} sensor for fast SV exocytosis, raising questions about the physiological significance of its Ca^{2+}-binding properties. Here, we examine how synaptotagmin-7 binds Ca^{2+} and test whether this Ca^{2+} binding regulates Ca^{2+}-triggered SV exocytosis. We show that the synaptotagmin-7 C_2A domain exhibits a Ca^{2+}-binding mode similar to that of the synaptotagmin-1 C_2A domain, suggesting that the synaptotagmin-1 and -7 C_2 domains generally employ comparable Ca^{2+}-binding mechanisms. We then generated mutant mice that lack synaptotagmin-7 or contain point mutations inactivating Ca^{2+} binding either to both C_2 domains of synaptotagmin-7 or only to its C_2B domain. Synaptotagmin-7-mutant mice were viable and fertile. Inactivation of Ca^{2+} binding to both C_2 domains caused an ≈70% reduction in synaptotagmin-7 levels, whereas inactivation of Ca^{2+} binding to only the C_2B domain did not alter synaptotagmin-7 levels. The synaptotagmin-7 deletion did not change fast synchronous release, slow asynchronous release, or short-term synaptic plasticity of release of neurotransmitters. Thus, our results show that Ca^{2+} binding to the synaptotagmin-7 C_2 domains is physiologically important for stabilizing synaptotagmin-7, but that Ca^{2+} binding by synaptotagmin-7 likely does not regulate SV exocytosis, consistent with a role for synaptotagmin-7 in other forms of Ca^{2+}-dependent synaptic exocytosis.

asynchronous release | calcium-binding protein | neurotransmitter release | synaptic plasticity

At the synapse, neurotransmitter release is mediated by Ca^{2+}-dependent exocytosis of synaptic vesicles (SVs) (1, 2). In mammals, Ca^{2+} triggers exocytosis by binding to two distinct classes of Ca^{2+} sensors: synaptotagmin-1, -2, or -9, which mediate fast synchronous release; and an unknown Ca^{2+} sensor, which mediates asynchronous release (3–9). A similar situation appears to operate in *Drosophila*, except that only a single synaptotagmin isoform seems to be involved (10, 11).

Synaptotagmins constitute a family of membrane-trafficking proteins composed of a single transmembrane domain, a variable linker sequence, and two C_2 domains (the C_2A and C_2B domains) (reviewed in refs. 12–15). Of 15 synaptotagmins identified in the mammalian genome, 8 exhibit Ca^{2+}-dependent phospholipid binding (synaptotagmin-1, -2, -3, -5, -6, -7, -9, and -10), and 7 do not (see references cited in ref. 8). Four synaptotagmins are localized to SVs: synaptotagmin-1, -2, and -9, which function as Ca^{2+} sensors for fast exocytosis, and synaptotagmin-12, which does not bind Ca^{2+}, but regulates release (16). The remaining 11 synaptotagmins are probably present on other types of secretory vesicles and/or the plasma membrane. In synaptotagmin-1, the C_2A and C_2B domains bind two and three Ca^{2+} ions, respectively, via similar Ca^{2+}-binding sites (17–19). Based on their comparable phospholipid-binding properties and putative Ca^{2+}-binding sequences, other synaptotagmins are thought to bind Ca^{2+} by similar mechanisms. However, this hypothesis has not been directly tested. Sequence analyses can be misleading, as exemplified by synaptotagmin-4, which was believed to bind Ca^{2+} based only on sequence analyses (20) until biophysical studies revealed that it is unable to do so (21). Thus, direct measurements of Ca^{2+} binding are essential to define whether and how a particular synaptotagmin binds Ca^{2+}.

Among synaptotagmins, synaptotagmin-7 is particularly interesting because it is initially expressed ubiquitously during development but is later restricted to dividing cells (including immortalized cancer cells), postmitotic neurons, and neuroendocrine cells (22). Moreover, synaptotagmin-7 is extensively alternatively spliced in a developmentally regulated pattern in its linker region connecting the C_2 domains with the membrane (23, 24). Ubiquitous synaptotagmin-7 contains only a relatively short alternatively spliced sequence, whereas neuronal synaptotagmin-7 exhibits a baroque variety of alternative splice variants. Ubiquitous synaptotagmin-7 is present on intracellular organelles and/or the plasma membrane (22, 25), whereas neuronal synaptotagmin-7 is enriched in synapses, but is excluded from SVs (23, 26). Finally, synaptotagmin-7 in neuroendocrine cells is localized to large secretory vesicles (27–29).

Compared with synaptotagmin-1, the C_2 domains of synaptotagmin-7 display a higher apparent Ca^{2+} affinity during phospholipid binding (30–33), suggesting that synaptotagmin-7 might function as a Ca^{2+} sensor for exocytosis (30). Four types of studies were performed to test this hypothesis: (*i*) addition of dominant-negative recombinant C_2 domain fragments to permeabilized secretory cells (23, 34), (*ii*) overexpression of synaptotagmin-7 in secretory cells or cultured neurons (26, 29), (*iii*) suppression of synaptotagmin-7 levels by RNAi in nonneuronal cells (27–29), and (*iv*) analysis of nonneuronal cells from synaptotagmin-7 KO mice (35, 36). These studies supported a role for synaptotagmin-7 as a Ca^{2+} sensor for exocytosis but produced conflicting results. For example, in neuroendocrine PC12 cells, recombinant synaptotagmin-7 C_2-domain fragments are potent inhibitors of Ca^{2+}-dependent exocytosis, which also is impaired by RNAi knockdown of synaptotagmin-7 (23, 27–29). However, these experiments did not address the puzzling competing observation that synaptotagmin-9 is the primary Ca^{2+} sensor for exocytosis in PC12 cells (37, 38). In another example, synaptotagmin-7 was proposed to act as a Ca^{2+} sensor for lysosome exocytosis (25, 35). However, an independent study suggested that synaptotagmin-7 acts as a regulator of lysosomal fusion-

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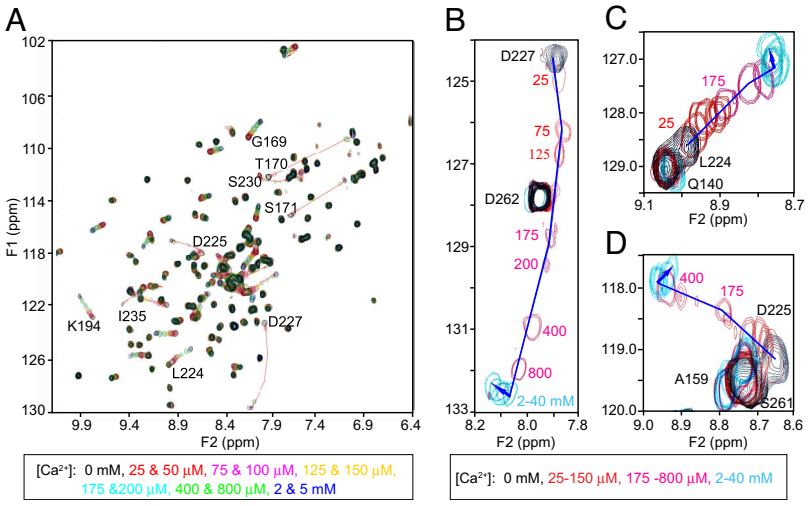

Fig. 1. Analysis of intrinsic Ca^{2+} binding to the synaptotagmin-7 C_2A domain by NMR spectroscopy. (*A*) Superposition of ^1H-^{15}N HSQC spectra of the ^{15}N-labeled synaptotagmin-7 C_2A domain acquired at Ca^{2+} concentrations of 0–5 mM. The spectrum obtained in the absence of Ca^{2+} is shown in black; rainbow coloring was used for the remaining spectra (red, lowest Ca^{2+} concentrations; blue, highest Ca^{2+} concentrations). Selected cross-peak assignments are indicated (red arrows, Ca^{2+}-dependent movement of some cross-peaks). (*B–D*) Superpositions of expansions of analogous ^1H-^{15}N HSQC spectra acquired in the presence of 0–40 mM Ca^{2+}, illustrating the Ca^{2+}-dependent movement of the cross-peaks from D227, L224, and D225 (other cross-peaks in the expansions that exhibit little or no Ca^{2+}-dependent changes are also labeled). The Ca^{2+} concentrations used are indicated next to some of the cross-peaks and in the box below the spectra.

pore expansion and not as an exocytic Ca^{2+} sensor (36). Indeed, it is puzzling that Ca^{2+}-induced lysosome exocytosis persists in cells lacking synaptotagmin-7. Moreover, synaptic transmission has not been tested in synaptotagmin-7 KO neurons despite the fact that neurons contain the highest synaptotagmin-7 levels in mature mice, although exocytosis appears to be normal in neuromuscular junctions of flies that lack a possible synaptotagmin-7 ortholog (39).

To address the question of whether Ca^{2+} binding to synaptotagmin-7 plays a role in SV exocytosis, we have now studied the intrinsic Ca^{2+}-binding properties of the synaptotagmin-7 C_2A domain and examined the physiological importance of Ca^{2+} binding to the C_2A and C_2B domain of synaptotagmin-7 in synaptic exocytosis. We show that the synaptotagmin-7 C_2A domain exhibits Ca^{2+}-binding properties that are similar to those of the synaptotagmin-1 C_2 domains and that inactivation of Ca^{2+} binding to both C_2 domains of synaptotagmin-7 destabilizes the protein *in vivo*, whereas inactivation of Ca^{2+} binding to only the C_2B domain does not. Finally, we demonstrate that deletion of synaptotagmin-7 does not affect spontaneous or evoked inhibitory neurotransmission and short-term synaptic plasticity in mice despite its dramatic effect on Ca^{2+}-dependent catecholamine release in chromaffin cells (40). These observations suggest that Ca^{2+} binding to synaptotagmin-7 is physiologically important but does not regulate synaptic exocytosis.

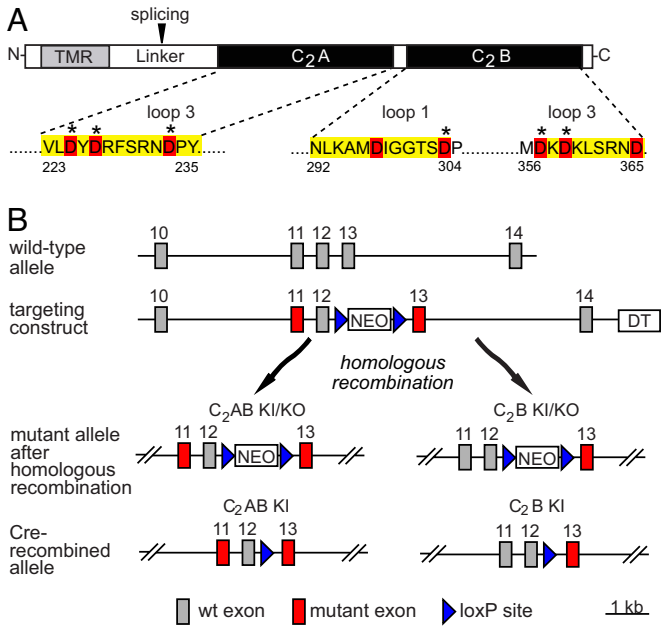

Fig. 2. Strategy for generation of synaptotagmin-7-mutant mouse lines. (*A*) Diagram of the synaptotagmin-7 domain structure (*Upper*: TMR, transmembrane region; arrowhead, position of the alternatively spliced linker region) and sequences of the Ca^{2+}-binding sites in the two C_2 domains (C_2A and C_2B) that were mutated in the knockin (KI) mice (*Lower*: red boxes, aspartate residues essential for Ca^{2+} binding; asterisks, aspartate residues replaced with alanine residues in synaptotagmin-7 knockin mice). (*B*) Homologous recombination strategy for synaptotagmin-7 C_2B- and C_2AB-domain mutant mice. A NEO-resistance gene cassette flanked by LoxP sites (blue triangles) was introduced into an EcoRI site in intron 12, and a diphtheria toxin gene (DT) was inserted downstream of the short vector arm. The coding sequences of exons 11 and 13 (red boxes) were mutated to replace six aspartate residues that bind Ca^{2+} in the C_2A and C_2B domains with alanines (D225, D227, and D233 in exon 11; D303, D357, and D359 in exon 13) (see asterisks in *A*). Diagrams on the bottom depict the mutant synaptotagmin-7 alleles containing alanine substitutions in both exon 11 and 13 (C_2AB KI/KO) or only in exon 13 (C_2B KI/KO) and the subsequent Cre-recombined alleles in which the NEO cassettes were excised.

Results

Ca^{2+}-Binding Properties of the Synaptotagmin-7 C_2A Domain. Both C_2 domains of synaptotagmin-7 bind to phospholipids as a function of Ca^{2+}, suggesting that they directly bind Ca^{2+} (23, 30, 31). However, only the Ca^{2+}-binding mode of a single synaptotagmin isoform, synaptotagmin-1, was previously determined directly. To test whether synaptotagmin-7 binds Ca^{2+} in a mode similar to that of synaptotagmin-1, we analyzed its C_2A domain by NMR spectroscopy.

Using the purified, uniformly ^{15}N,^{13}C-labeled C_2A domain of synaptotagmin-7, we used standard triple resonance spectra (HNCACB and CBCACONH) to assign its backbone resonances (Fig. 1*A*). We then performed Ca^{2+} titrations monitored by ^1H-^{15}N HSQC spectra to determine its Ca^{2+}-binding mode (Fig. 1 *B–D*) as described for the synaptotagmin-1 C_2A domain (17, 18). The C_2A-domain spectra exhibited excellent cross-peak dispersion, showing that the domain is folded. Marked Ca^{2+}-dependent movements were observed for many ^1H-^{15}N HSQC cross-peaks from residues in the predicted Ca^{2+}-binding loops. This behavior was similar to that observed for the synaptotagmin-1 C_2A domain. Indeed, the locations of many cross-peaks in the spectrum and the directions of the Ca^{2+}-dependent movements were analogous (examples are the cross-peaks of T170, S171, K194, D227, and S230 of the synaptotagmin-7 C_2A domain, which correspond to T176,

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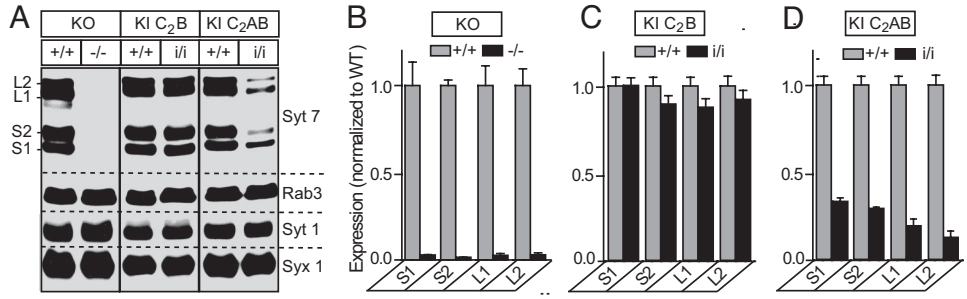

Fig. 3. Expression of synaptotagmin-7 in mutant mice. (*A*) Representative immunoblots of synaptotagmin-7 (Syt 7), Rab 3, synaptotagmin-1 (Syt 1), and syntaxin-1A (Syx 1) in brain proteins (≈40 μg per lane) from synaptotagmin-7 KO and Cre-recombined C_2B and C_2AB knockin mice and their WT littermate controls. Symbols on the left indicate the four major brain synaptotagmin-7 splice variants (S, short form; L, long form). (*B–D*) Expression levels of individual synaptotagmin-7 splice variants in the brains of adult (>P30) KO (*B*) and Cre-recombined C_2B- and C_2AB-domain KI mice (*C* and *D*, respectively) determined by quantitative immunoblotting with ^{125}I-labeled secondary antibodies and normalized to expression in their WT littermates (means ± SDs; $n \geq 3$).

S177, K200, D232, and S235 of the synaptotagmin-1 C_2A domain) (Fig. 1 *B–D* and data not shown). Clear curved movements were observed for Ca^{2+} concentrations ranging from 0–800 μM for multiple cross-peaks (e.g., D227 and S230) (Fig. 1). Based on the similar behavior of the two domains, these curved movements can be unambiguously attributed to Ca^{2+} binding to the first two of three Ca^{2+}-binding sites (Ca1 and Ca2). The curves are less pronounced in the synaptotagmin-7 C_2A domain than in the synaptotagmin-1 C_2A domain because the Ca^{2+} affinities of the Ca1 and Ca2 sites are more similar to each other. This fact hinders the measurement of accurate K_ds, allowing only an approximate estimate of the Ca1 affinities (Ca1 $K_d \approx 150$ μM Ca^{2+} based particularly on the K194 NH cross-peaks that are primarily affected by Ca^{2+} binding to this site; Ca2 $K_d = 200$–300 μM).

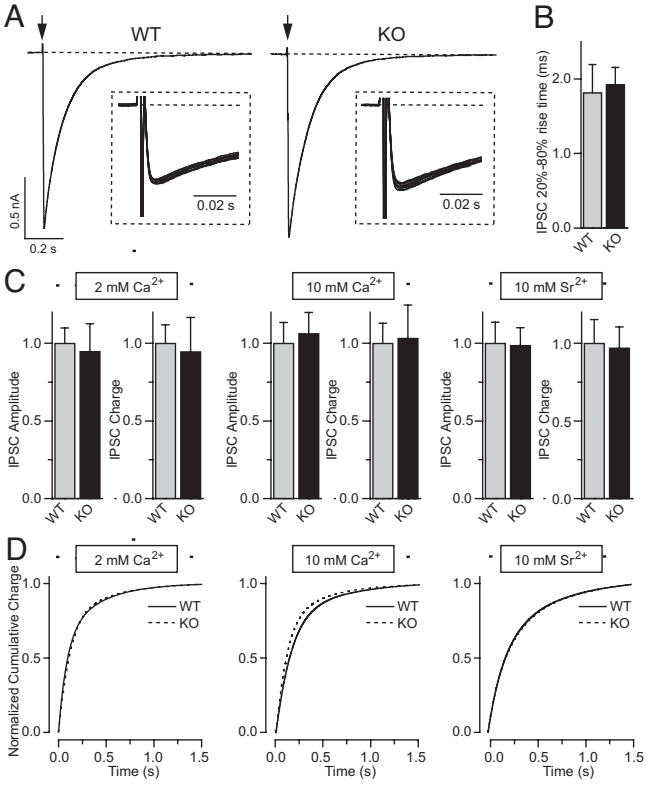

Fig. 4. Fast synaptic transmission in neurons lacking synaptotagmin-7. (*A*) Representative IPSCs monitored in cortical neurons from littermate WT and synaptotagmin-7 KO mice cultured 14 days *in vitro*. IPSCs were recorded in 2 mM extracellular Ca^{2+} in the presence of AP5 and CNQX. Synaptic responses were evoked by single action potentials applied at 0.1 Hz (arrows). Scale bars apply to both traces. (*Insets*) Initial phases of multiple responses collected from the same neuron to illustrate the kinetics of the IPSC onset. (*B*) Average rise times of IPSCs recorded from WT and synaptotagmin-7-deficient neurons in 10 mM extracellular Ca^{2+} (means ± SDs; WT, $n = 9$; KO, $n = 10$). (*C*) Amplitudes and synaptic charge transfer (integrated over 1.5 s) of individual IPSCs in WT and synaptotagmin-7-deficient neurons monitored in 2 mM or 10 mM Ca^{2+} or 10 mM Sr^{2+} as indicated. Data are plotted as a fraction of the WT response (means ± SDs; 2 mM Ca^{2+}, WT, $n = 9$; KO, $n = 8$; 10 mM Ca^{2+}, WT, $n = 14$; KO, $n = 14$; 10 mM Sr^{2+}, WT, $n = 12$; KO, $n = 10$; from two to four cultures from littermate WT and KO mice). (*D*) Time course of individual IPSCs monitored from WT (solid lines) and synaptagmin-7-deficient neurons (dashed lines). Plots exhibit the cumulative charge transfer over 1.5 s after an action potential (means ± SDs; 2 mM Ca^{2+}, WT, $n = 15$; KO, $n = 9$; 10 mM Ca^{2+}, WT, $n = 11$; KO, $n = 12$; 10 mM Sr^{2+}, WT, $n = 11$; KO, $n = 10$).

At the highest Ca^{2+} concentrations used (2 and 5 mM), we observed an additional change in the movement direction for a few cross-peaks (e.g., S171 and D227) (Fig. 1). Analogous to the synaptotagmin-1 C_2A domain (18), this additional component can be attributed to Ca^{2+} binding to a third site (Ca3) with a very low affinity. Overall, these data demonstrate that the Ca^{2+}-binding mechanism of the synaptotagmin-7 C_2A domain is similar to that of the synaptotagmin-1 C_2A domain, although differences exist in the intrinsic Ca^{2+}-binding affinities as predicted from their distinct Ca^{2+}-dependent phospholipid-binding properties (31). It seems likely that this conclusion also applies to the C_2B domain, and we thus conclude that the similar sequences and Ca^{2+}-dependent phospholipid-binding properties of the synaptotagmin-1 and -7 C_2 domains correlate with similar intrinsic Ca^{2+}-binding properties of these domains.

Generation of Synaptotagmin-7-Mutant Mice. To determine the role of synaptotagmin-7 in Ca^{2+}-dependent exocytosis, we generated three lines of mutant mice by using a single homologous recombination experiment (Fig. 2): (*i*) mice in which expression of synaptotagmin-7 was abolished, (*ii*) mice in which Ca^{2+} binding to both C_2 domains was inactivated, and (*iii*) mice in which Ca^{2+} binding only to the C_2B domain was inactivated.

The initial homologous recombination experiment used a targeting vector that contained a NEO-resistance cassette in intron 12 for positive selection, a diphtheria toxin cassette flanking the short arm for negative selection, and alanine substitutions in essential aspartates residues in the C_2A- and C_2B-domain Ca^{2+}-binding sites to inactivate Ca^{2+} binding (Fig. 2). After homologous recombination, we identified mutant ES cells that contained the NEO cassette and included either the mutations in both C_2 domains or only in the C_2B domain, with the latter presumably arising from spontaneous repair of the C_2A-domain mutations during homologous recombination. We generated mice from these mutant ES cells and bred them to homozygosity. Quantitative immunoblotting demonstrated that in mice that were homozygous for the C_2B- or the C_2AB-domain knockin and contained the NEO-resistance cassette, the expression of synaptotogmin-7 was undetectable (Fig. 3 *A* and *B*) (data not shown), prompting us

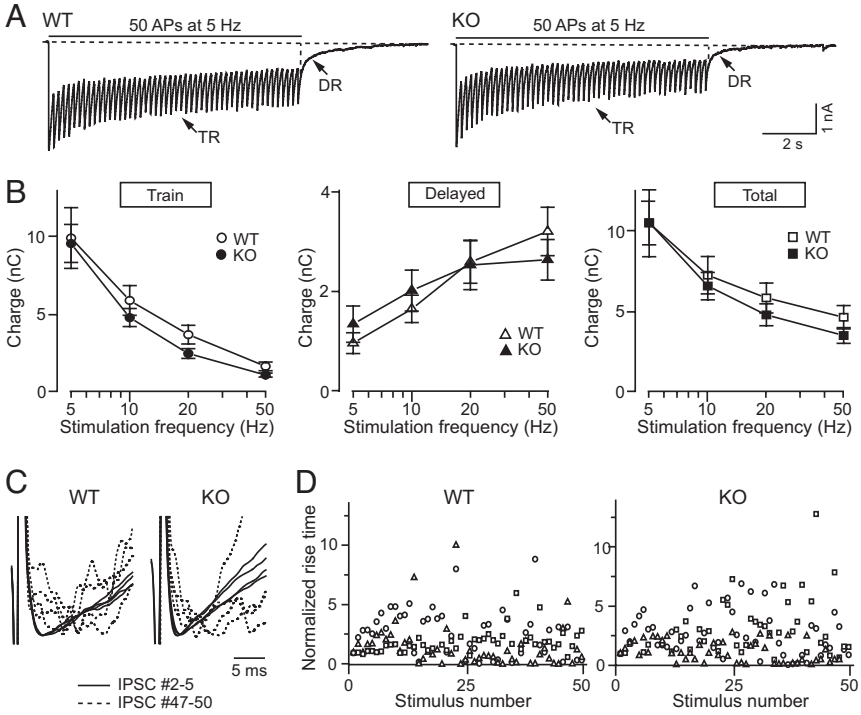

Fig. 5. Properties of inhibitory synaptic responses triggered by trains of action potentials. (*A*) Representative IPSCs recorded from WT and synaptotagmin-7-deficient neurons during a 5-Hz, 10-s action potential train in 2 mM extracellular Ca^{2+}. Arrows indicate the areas defined as train release (TR, release during the stimulus train) and delayed release (DR, tail currents observed after the end of the stimulus train). Scale bars apply to both traces. (*B*) Average synaptic charge transfer induced by stimulus trains of 50 action potentials applied at 5–50 Hz in WT and synaptotagmin-7-deficient neurons during the train (train release), after the train (delayed release), and over the entire experiment (total charge transfer) plotted as a function of the stimulation frequency. IPSCs were triggered in 10 mM extracellular Ca^{2+} (means ± SDs; WT, $n = 14$; KO, $n = 12$). (*C*) Examples of individual IPSCs elicited by 50 action potentials applied at 20 Hz in WT and synaptotagmin-7 KO neurons. In the examples, aligned and normalized IPSCs from the beginning (solid lines, IPSCs 2–5) and end of the stimulus train (dashed lines, IPSCs 47–50) are depicted. (*D*) Rise times of IPSCs during a train of 50 action potentials applied at 20 Hz, plotted as a function of stimulus number. Rise times were normalized to the first response (data represent individual points from three different neurons).

to refer to these mice as KO mice. Consistent with a previous report (35), homozygous synaptotagmin-7 KO mice were viable and fertile and did not display significant abnormalities in body weight (data not shown).

To remove the NEO cassette, we crossed the mutant mice to transgenic mice with germ-line expression of Cre recombinase. Quantitative immunoblotting showed that excision of the NEO-resistance cassette fully restored the expression of synaptotagmin-7 when only the C_2B-domain Ca^{2+}-binding sites were mutated (Fig. 3 *A* and *C*). However, excision of the NEO cassette only increased the expression of synaptotagmin-7 to only 20–30% of WT levels when both C_2 domains were mutated (Fig. 3 *A* and *D*). Because the only difference between the two C_2B- and the C_2AB-domain knockin lines after excision of the NEO cassette is the presence or absence of point mutations in the C_2A domain (all other intronic and exonic sequences are identical), the mutation in the C_2A-domain Ca^{2+}-binding sites must have destabilized the synaptotagmin-7 protein.

Synaptic Transmission in Synaptotagmin-7-Deficient Neurons. To determine whether synaptotagmin-7 is a Ca^{2+} sensor for SV exocytosis, we performed systematic analyses of GABAergic synaptic transmission in neurons isolated from synaptotagmin-7 KO mice and their WT littermates. In this study, we carried out measurements of GABAergic transmission in mixed cultured cortical neurons by using focal stimulation (41). We first analyzed the properties of evoked synaptic responses triggered by single-action potentials in the presence of 2 mM Ca^{2+}, 10 mM Ca^{2+}, or 10 mM Sr^{2+} in the extracellular bath. We found that under all three experimental conditions inhibitory postsynaptic currents (IPSCs) monitored from WT and synaptotagmin-7-deficient neurons displayed similar sizes and kinetics of onset and decay (Fig. 4), indicating that deletion of synaptotagmin-7 did not have a significant effect on fast-evoked SV exocytosis and the Ca^{2+} dependence of release. In addition, we found that deletion of synaptotgmin-7 did not alter the frequency and amplitudes of miniature IPSC events (minis), suggesting that synaptotagmin-7 is not involved in triggering or regulating minis (data not shown).

To probe for a potential role of synaptotagmin-7 in the regulation of short-term synaptic plasticity, fast SV recycling, and/or asynchronous release, we analyzed the properties of synaptic responses triggered by trains of 50 action potentials applied at stimulation frequencies of 5–50 Hz. Again, we found that deletion of synaptotagmin-7 did not have a significant effect on charges transferred by high-frequency inhibitory responses during the stimulus train (train release) or after the stimulus train (delayed release), suggesting that both synchronous and asynchronous components of neurotransmitter release were unaffected (Fig. 5 *A* and *B*). Consistent with these observations, WT and synaptotagmin-7-deficient neurons displayed similar kinetics of synchronous IPSC depression during action potential trains [supporting information (SI) Fig. 7] and became increasingly desynchronized during the stimulus train (Fig. 5 *C* and *D*).

Together, our data indicate that synaptotagmin-7 is unlikely to be involved in the regulation of SV exocytosis and probably has no role in short-term synaptic plasticity. However, it is possible that synaptotagmin-1 is redundant with synaptotagmin-7 in asynchronous but not synchronous release. To examine this possibility, we crossed synaptotagmin-7 and synaptotagmin-1 KO mice with each other and analyzed cortical cultures from mice that lack synaptotagmin-7 and either express or lack synaptotagmin-1. These experiments demonstrated that the robust asynchronous release present in synaptotagmin-1 KO neurons (42) is not impaired by the additional deletion of synaptotagmin-7, providing further support for the conclusion that synaptotagmin-7 is not involved in this process (Fig. 6).

Discussion

In this study, we employ biophysical, genetic, and electrophysiological analyses to test the general role of synaptotagmin-7 in the regulation of neurotransmitter release and the specific hypothesis that it functions as a Ca^{2+} sensor for asynchronous release. We demonstrate that Ca^{2+} binding to the synaptotagmin-7 C_2A domain operates by a similar mechanism as Ca^{2+} binding to the synaptotagmin-1 C_2A domain, confirming that these molecules are structurally similar. We show that, consistent with a previous report (35), homozygous mice lacking synaptotagmin-7 are viable, fertile, and

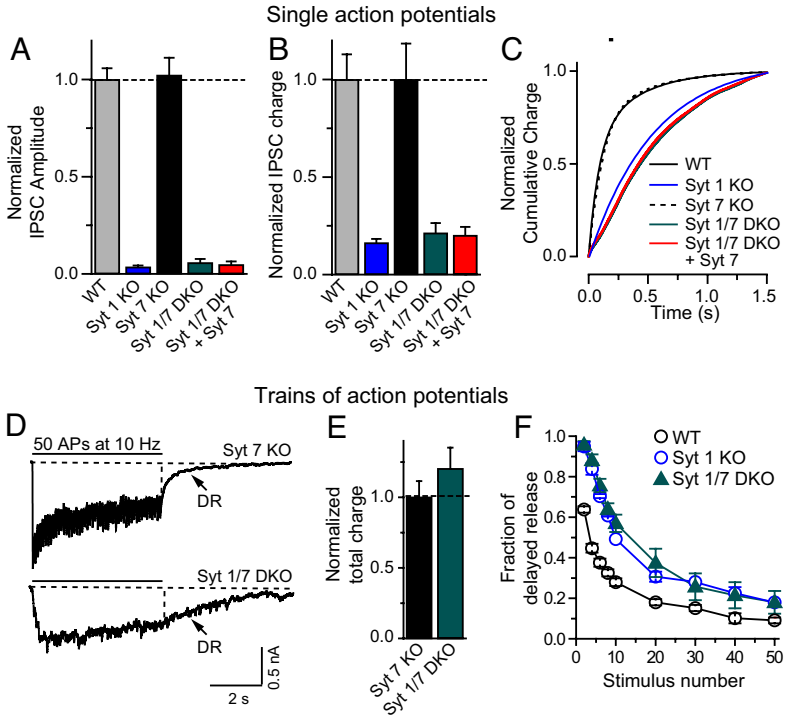

Fig. 6. Synaptotagmin-7 deletion does not suppress asynchronous release in synaptotagmin-1-deficient neurons. (*A* and *B*) Normalized amplitudes (*A*) and charges (*B*) of IPSCs triggered by isolated action potentials in WT neurons ($n = 24$) or neurons lacking synaptotagmin-1 (Syt 1KO, $n = 20$), synaptotagmin-7 (Syt 7 KO, $n = 14$), or both synaptotagmin-1 and -7 (Syt 1/7 DKO, $n = 10$). In addition, neurons lacking synaptotagmin-1 and -7 were infected with lentivirus encoding the short splice variant of synaptotagmin-7 to test rescue (Syt 1/7 DKO + Syt 7, $n = 3$). Note that the *ns* apply to all panels of this figure; data are from two independent cultures, with the rescue experiment performed for one of the two cultures. (*C*) Time course of single IPSCs triggered by isolated action potentials in WT neurons or neurons lacking synaptotagmin-1 (Syt 1 KO), synaptotagmin-7 (Syt 7 KO), synaptotagmin-1 and -7 (Syt 1/7 DKO), or Syt 1/7 DKO neurons infected with the lentivirus encoding the short synaptotagmin-7 splice variant (Syt 1/7 DKO + Syt 7). The time course is depicted as a cumulative plot of the synaptic charge transfer. (*D* and *E*) Representative traces (*D*) and mean synaptic charge transfer (normalized to that observed in synaptotagmin-7-deficient neurons) of inhibitory responses triggered by high-frequency action potential trains in synaptotagmin-7 KO and in synaptotagmin-1 and -7 double KO neurons. (*F*) Contribution of delayed asynchronous release to total synaptic charge transfer in WT neurons and neurons lacking synaptotagmin-1 alone or both synaptotagmin-1 and -7. IPSCs were triggered by trains of 2–50 action potentials applied at 10 Hz. The charges of delayed responses are represented as a fraction of total response and plotted as a function of the stimulus number. Data shown in all panels are means ± SDs.

do not display significant abnormalities in body weight. Moreover, we find that mutations in synaptotagmin-7 that prevent Ca^{2+} binding to either one or both of its C_2 domains also do not affect its viability or fertility. We observe, however, that inactivation of Ca^{2+} binding to both synaptotagmin-7 C_2 domains destabilizes the protein, whereas inactivation of Ca^{2+} binding to only the C_2B domain does not. Please note that the destabilization of synaptotagmin-7 by the Ca^{2+}-binding site mutations is not a simple reflection of a functional inactivation because, at least in chromaffin cells, inactivation of Ca^{2+} binding to the C_2B domain alone impairs the function of synatotagmin-7 as a Ca^{2+} sensor for exocytosis (40). Please also note that the inactivating mutations we introduced into the C_2A and C_2B domains are single amino acid substitutions that are unlikely to have an effect on the folding of the C_2 domains, but that we cannot completely rule out a misfolding effect at elevated physiological temperatures.

The fact that inactivation of Ca^{2+} binding to synaptotagmin-7 destabilizes the protein suggests that synaptotagmin-7 physiologically functions as a Ca^{2+}-binding protein and validates its candidacy as a Ca^{2+}-sensor protein. To test whether this putative function operates in SV exocytosis, we examined the effect of the deletion of synaptotagmin-7 on neurotransmitter release. Surprisingly, we found in inhibitory neurons that synaptotagmin-7 is not essential for triggering synchronous or asynchronous release or for regulating presynaptic short-term plasticity. This conclusion is based on the observations that synaptotagmin-7-deficient neurons display normal synaptic responses triggered by single-action potentials, a normal Ca^{2+} dependence of release, and an unchanged amount of release induced by high-frequency stimulus trains. In particular, the magnitude and kinetics of synaptic responses triggered by the stimulus train and of delayed release that correspond to a form of asynchronous release were not altered by the synaptotagmin-7 deletion, indicating that synaptotagmin-7 is not involved in triggering asynchronous release, the regulation of the size of readily releasable pool of SVs, or the speed of SV recycling. The absence of an effect of the synaptotagmin-7 deletion on SV exocytosis is especially notable in view of the essential role of synaptotagmin-7 in chromaffin cell exocytosis (40).

Neurons display two distinct modes of neurotransmitter release that are driven by distinct Ca^{2+} sensors: fast synchronous and slow asynchronous release (4, 5, 9, 43–46). Three synaptotagmins that are differentially expressed in the brain, synaptotagmin-1, -2, and -9, function as Ca^{2+} sensors for fast synchronous SV exocytosis (3–9). In contrast, the molecular nature of a slow asynchronous Ca^{2+} sensor remains elusive despite extensive electrophysiological analyses in various synapse preparations (42–46). Because of its unique biochemical properties and partially synaptic localization, synaptotagmin-7 was proposed as a candidate for such a Ca^{2+} sensor (30), but our observations argue against this possibility. Indeed, we show that WT and synaptotagmin-7-deficient neurons display similar magnitudes of delayed asynchronous release triggered by high-frequency trains and similar kinetics of IPSC desynchronization during train stimulation, suggesting that asynchronous release operates normally in the absence of synaptotagmin-7. Our conclusions are in agreement with the functional characterization of a slow Ca^{2+} sensor in Calyx of Held synapses, which suggests that the Ca^{2+} cooperativity of an asynchronous sensor is inconsistent with the intrinsic Ca^{2+}-binding properties of synaptotagmin-7 (9). As revealed by Schonn *et al.* (40), deletion of synaptotagmin-7 or inactivation of Ca^{2+} binding to the synaptotagmin-7 C_2B domain indeed impairs Ca^{2+}-induced chromaffin granule exocytosis in chromaffin cells, supporting the role of synaptotagmin-7 as a Ca^{2+} sensor for dense-core vesicle exocytosis. Similarly, the studies by Gustavsson *et al.* (47) demonstrated that synaptotagmin-7 is essential for normal insulin exocytosis in pancreatic β-cells. On the background of a lack of an effect of the same mutations on SV exocytosis, these observations suggest that, in neurons, synaptotagmin-7 may be involved in the control of synaptic neuropeptide release or some other form of Ca^{2+}-induced exocytosis, a hypothesis that needs to be further elucidated before it can be accepted.

Methods

NMR spectroscopy was performed essentially as described previously (17, 18) by using uniformly ^{15}N,^{13}C-labeled recombinant proteins. For a detailed description of the methods, see *SI Methods*.

Generation of Synaptotagmin-7-Mutant Mice. We produced mutant synaptotagmin-7 mice containing the C_2A- and C_2B-domain mutations or only the C_2B-domain mutations and the NEO cassette by homologous recombination (see Fig. 2) essentially as described previously (6). For *in vivo* NEO-cassette excision, the heterozygous offspring of chimera/C57B/6 parents were crossed to a transgenic line expressing Cre recombinase.

Neuronal Cultures and Electrophysiology. Analyses of inhibitory synaptic transmission in cultured cortical neurons were performed as described previously (41, 42). All data are shown are means ± SDs or SEMs as indicated. Statistical significance was assessed by Student's *t* test.

Miscellaneous Procedures. SDS/PAGE electrophoresis and quantitative immunoblotting were performed by using standard procedures.

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