Cellular/Molecular

Dual Roles of the C_2B Domain of Synaptotagmin I in Synchronizing Ca^{2+}-Dependent Neurotransmitter Release

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Although the vesicular protein synaptotagmin I contains two Ca^{2+}-binding domains (C_2A and C_2B), Ca^{2+} binding to the C_2B domain is more important for triggering synchronous neurotransmitter release. We have used point mutagenesis to determine the functional contributions of the five negatively charged aspartate (Asp) residues that constitute the Ca^{2+}-binding sites in the C_2B domain of synaptotagmin I. Transfecting wild-type synaptotagmin I DNA into cultured hippocampal neurons from synaptotagmin I knock-out mice rescued Ca^{2+}-dependent synchronous transmitter release and reduced a slower, asynchronous component of release, indicating that synaptotagmin I suppresses asynchronous release. Mutating either the second or third Asp residues of the C_2B domain potently inhibited the ability of synaptotagmin I to rescue synchronous release but did not change its ability to suppress asynchronous release. Synaptotagmin I with mutations in the first or fourth Asp residues of the C_2B domain partially rescued synchronous release and partially suppressed asynchronous release, whereas neutralizing the fifth Asp residue had no effect on the ability of synaptotagmin I to rescue transmitter release. Thus, we conclude that the C_2B domain of synaptotagmin I regulates neurotransmitter release in at least two ways. Synchronous release absolutely requires binding of Ca^{2+} to the second and third Asp residues in this domain. For the suppression of asynchronous release, Ca^{2+} binding to the C_2B domain of synaptotagmin I apparently is not necessary because mutation of the second Asp residue inhibits Ca^{2+} binding, yet still allows this protein to suppress asynchronous release.

Key words: **synaptic transmission; synaptic vesicle; calcium; exocytosis; SNARE; hippocampal neurons**

Introduction

Neurotransmitter release arises from the calcium (Ca^{2+})-dependent fusion of synaptic vesicles with the presynaptic plasma membrane. This fusion requires the v-soluble *N*-ethylmaleimide-sensitive factor attachment protein (SNAP) receptor (SNARE), synaptobrevin, found on vesicles, and the t-SNAREs, syntaxin 1 and SNAP-25, located on the plasma membrane (Rothman, 1994; Augustine et al., 1999; Chen and Scheller, 2001; Jahn et al., 2003). Although complexes between these SNARE proteins most likely drive membrane fusion, SNARE complex formation does not require Ca^{2+}. This means that some other molecule must serve as a Ca^{2+} sensor for neurotransmitter release.

The best candidate for this Ca^{2+} sensor is synaptotagmin I, an integral protein of synaptic vesicle membranes (Augustine, 2001; Chapman, 2002; Südhof, 2002; Koh and Bellen, 2003). A physiological function for synaptotagmin I is indicated by findings that Ca^{2+}-dependent evoked transmitter release is dramatically inhibited by presynaptic microinjection of peptides from synaptotagmin (Bommert et al., 1993; Fukuda et al., 2000) or anti-synaptotagmin antibodies (Mikoshiba et al., 1995), as well as by disruption of the synaptotagmin I gene (DiAntonio et al., 1993; Littleton et al., 1993; Nonet et al., 1993; Geppert et al., 1994). In cultured hippocampal neurons, Ca^{2+}-dependent evoked transmitter release consists of two kinetically distinct components: a fast synchronous component and a slower asynchronous component (Goda and Stevens, 1994). Loss of the synaptotagmin I gene causes the fast synchronous component of transmitter release to be severely inhibited (Geppert et al., 1994), whereas the amount of asynchronous release is increased to compensate (Yoshihara and Littleton, 2002; Shin et al., 2003; Nishiki and Augustine, 2004).

The cytoplasmic region of synaptotagmin I contains two copies of a Ca^{2+}-binding motif called the C_2 domain (Südhof, 2002). The two C_2 domains, termed C_2A and C_2B, have highly conserved structures consisting of a β sandwich with loops connecting the β strands (see Fig. 1*A*) (Sutton et al., 1995, 1999; Fernandez et al., 2001). Ca^{2+}-binding sites are formed by five negatively charged aspartate (Asp, D) residues in two loops at the top of the C_2 domains (see Fig. 1*A*,*B*) (Shao et al., 1996; Fernandez et al., 2001). Recent studies suggest that the two C_2 domains are not equivalent in their ability to support Ca^{2+}-dependent transmitter release. Although disruptions of the Ca^{2+}-binding sites of C_2A have small effects on transmitter release (Fernández-Chacón et al., 2002; Robinson et al., 2002; Stevens and Sullivan, 2003), similar mutations in the C_2B domain cause dramatic defects in neurotransmitter release (Mackler et al., 2002). Thus, Ca^{2+} binding to the C_2B domain apparently is most important for transmitter release.

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Here we have determined the physiological contributions of the Ca^{2+}-binding sites of the C_2B domain by systematically mutating each of the conserved Asp residues in the C_2B domain of synaptotagmin I. We found that these residues differ in their ability to support the synchronous component of neurotransmitter release from mouse hippocampal neurons. In addition, these mutations allow separation of the effects of synaptotagmin I on synchronous and asynchronous release, indicating that different properties of synaptotagmin I are responsible for triggering of synchronous release and suppression of asynchronous release.

Materials and Methods

Cell culture. Heterozygous synaptotagmin I knock-out mice (Geppert et al., 1994) were purchased from The Jackson Laboratory (Bar Harbor, ME). Animal breeding, maintenance, and use were done in accordance with protocols approved by the Institutional Animal Care and Use Committee of the Duke University Medical Center. Newborn pups (postnatal day 0) were genotyped and then decapitated to prepare hippocampal neurons as described by Nishiki and Augustine (2004). For electrophysiological experiments, micro-island cultures of hippocampal neurons were prepared and maintained as described by Bekkers and Stevens (1991) and Nishiki and Augustine (2004). For other experiments, cells were plated onto coverslips at a density of 4.0×10^4 cells/cm^2.

Electrophysiology. Whole-cell patch-clamp recordings were performed from single neurons on micro-islands, as described by Nishiki and Augustine (2004). Patch pipettes (2–3 MΩ) were filled with solution containing (in mM): 50 K-glutamate, 71 K-gluconate, 15 NaCl, 6 $MgCl_2$, 2 EGTA, 5 Na_2ATP, 5 Na_2GTP, 20 HEPES-KOH, pH 7.3 (285 mOsm). The extracellular solution contained (in mM): 150 NaCl, 3 KCl, 2 $CaCl_2$, 2 $MgCl_2$, 20 glucose, 10 HEPES-NaOH, pH 7.3 (300 mOsm). Neurons were voltage clamped at −70 mV with a PC-501 amplifier (Warner Instruments, Hamden, CT) and stimulated by depolarizing to +20 mV for 0.8 msec at 0.2 Hz. Postsynaptic currents were filtered at 3 kHz, digitized at 10 kHz, and analyzed as described by Feng et al. (2002). Only cells with series resistances <15 MΩ were analyzed, with 60–70% of this resistance compensated electronically. Fitting procedures were performed using Origin software (Microcal, Northampton, MA). Miniature postsynaptic currents were measured in the absence of stimulation and analyzed using the Mini Analysis Program (Synaptosoft, Decatur, GA).

Expression constructs and site-directed mutagenesis. A plasmid encoding synaptotagmin I was kindly provided by Masami Takahashi (Kitasato University School of Medicine, Sagamihara, Japan). The sequence of the protein encoded by this cDNA includes glycine at position 374 (in addition to aspartate at 188 and methionine at 393), which is identical to that of the synaptotagmin Ib subisoform (Osborne et al., 1999; Desai et al., 2000). The synaptotagmin I cDNA was amplified by PCR using the following primers: 5′-CGCCACCATGGTGAGTGCCAGT-3′ (sense) and 5′-GGGGATATCTTACTTCTTGACAGC-3′ (antisense). The amplified product was ligated into pCR-Script vector (Stratagene, La Jolla, CA) and then subcloned into pIRES2-EGFP vector (BD Biosciences–Clontech, Palo Alto, CA) with *Eco*R I and *Sac* II restriction enzyme sites. Single point mutations in the C_2B-domain of synaptotagmin I were generated by site direct mutagenesis with the QuikChange Site-Directed Mutagenesis kit (Stratagene), using the following mutagenic primers: D1N, 5′-CAAGAACCTGAAGAAGATGAATGTGGGTGGCTTATCTG-3′ (sense); D2N, 5′-GATGTGGGTGGCTTATCTAATCCCTACGTGAAGATTC-3′ (sense); D3N, 5′-GTGGTGGTAACTGTTTTGAACTATGACAAGATTGGC-3′ (sense); D4N, 5′-GTGGTAACTGTTTTGGACTATAACAAGATTGGCAAGAACG-3′ (sense); D5N, 5′-CAAGATTGGCAAGAACAACGCCATCGGCAAAGTC-3′ (sense). All antisense primers were designed as complementary to corresponding sense primers. The nucleotide sequences of all constructs were verified by DNA sequencing (Duke University DNA Sequencing Facility).

Transfection of neurons. Neurons were transfected 3 or 4 d after plating using Lipofectamine 2000 (Invitrogen, Carlsbad, CA) according to the manufacturer's instructions. In brief, before transfection, 1 ml of Neurobasal-A medium (Invitrogen) supplemented with GlutaMax-I (Invitrogen) and B-27 (Invitrogen), was added into each well of the 12-well plates containing neurons, and the same volume of the medium was removed and set aside for later use. DNA plasmid (0.2 μg) and Lipofectamine 2000 reagent (0.5 μl) were diluted separately with 25 μl of Opti-MEM I (Invitrogen), mixed, and then incubated with cells at 37°C for 3–4 hr in a CO_2 incubator.

Immunoblotting. SDS-PAGE and immunoblotting were performed as described by Nishiki et al. (1994). Cultured neurons were solubilized in 100 μl of SDS-PAGE sample buffer containing 20 mM dithiothreitol. After boiling, equal amounts of samples (20 μl) were loaded into each lane of gel. An anti-synaptotagmin I monoclonal antibody 1D12 (Takahashi et al., 1991; Leveque et al., 1992) was kindly provided by Dr. Masami Takahashi. Immunoreactive bands were visualized by enhanced chemiluminescence, using Kodak BioMax MR film and SuperSignal West Pico substrate (Pierce, Rockford, IL). Film images were digitized and band intensity was measured using the ImageJ software (National Institutes of Health, Bethesda, MD). All measurements were made within the linear range of this assay, as determined from a standard curve.

Results

Expressing synaptotagmin I with Ca^{2+}-binding site mutations

Nuclear magnetic resonance analysis (Fernandez et al., 2001) demonstrates that two Ca^{2+} ions bind to the C_2B domain of synaptotagmin I by associating with five negatively charged Asp residues located in two different loops (Fig. 1*B*): Asp^{303} (termed D1), Asp^{309} (D2), Asp^{363} (D3), Asp^{365} (D4), and Asp^{371} (D5). To elucidate the physiological contribution of these Asp residues, each was individually replaced with asparagine (Asn, N), and these mutations were designated D1N through D5N. Such mutations neutralize the negative charge of each Asp residue without major effects on the overall size of the side chain. These mutated synaptotagmin I constructs, as well as wild-type synaptotagmin I, were then transfected into cultured hippocampal neurons from synaptotagmin I knock-out mice (Geppert et al., 1994) and examined for their ability to rescue synaptic transmission (Stevens and Sullivan, 2003).

We used EGFP fluorescence as a reporter to identify transfected cells. Initially, synaptotagmin I was expressed as a chimeric protein fused to the N terminus of EGFP. Although this chimeric protein was expressed in neurons and appeared to target to synapses, it did not restore neurotransmitter release in knock-out neurons (data not shown). To avoid such impairment of synaptotagmin function by the fused EGFP, we expressed synaptotagmin I and EGFP as separate proteins by using a pIRES2-EGFP vector.

The expression of synaptotagmin I in transfected neurons was examined by immunoblotting experiments using an anti-synaptotagmin I monoclonal antibody (1D12). Endogenous synaptotagmin I was detected as a 65 kDa band in the wild-type and heterozygous neurons, but not in the knock-out neurons (Fig. 1*C*, arrow). Another band with molecular mass of ~46 kDa was faintly present in samples from the knock-out and heterozygous neurons, but not in wild-type cells (Fig. 1*C*, asterisk). This 46 kDa band is most likely the truncated product of the mutant synaptotagmin I gene, as reported previously (Geppert et al., 1994). The synaptotagmin I-deficient mice were generated by replacing an exon encoding the beginning part of the C_2B domain with a neomycin resistance cassette (Geppert et al., 1994) so that the epitope for the antibody, which resides between amino acid residues 105 and 275 in synaptotagmin I (M. Takahashi, personal communication), could still be expressed in the knock-out and heterozygous neurons.

When transfected knock-out neurons were subjected to immunoblotting, exogenous synaptotagmin I was detected as a very faint band, presumably because only a small fraction of the cells

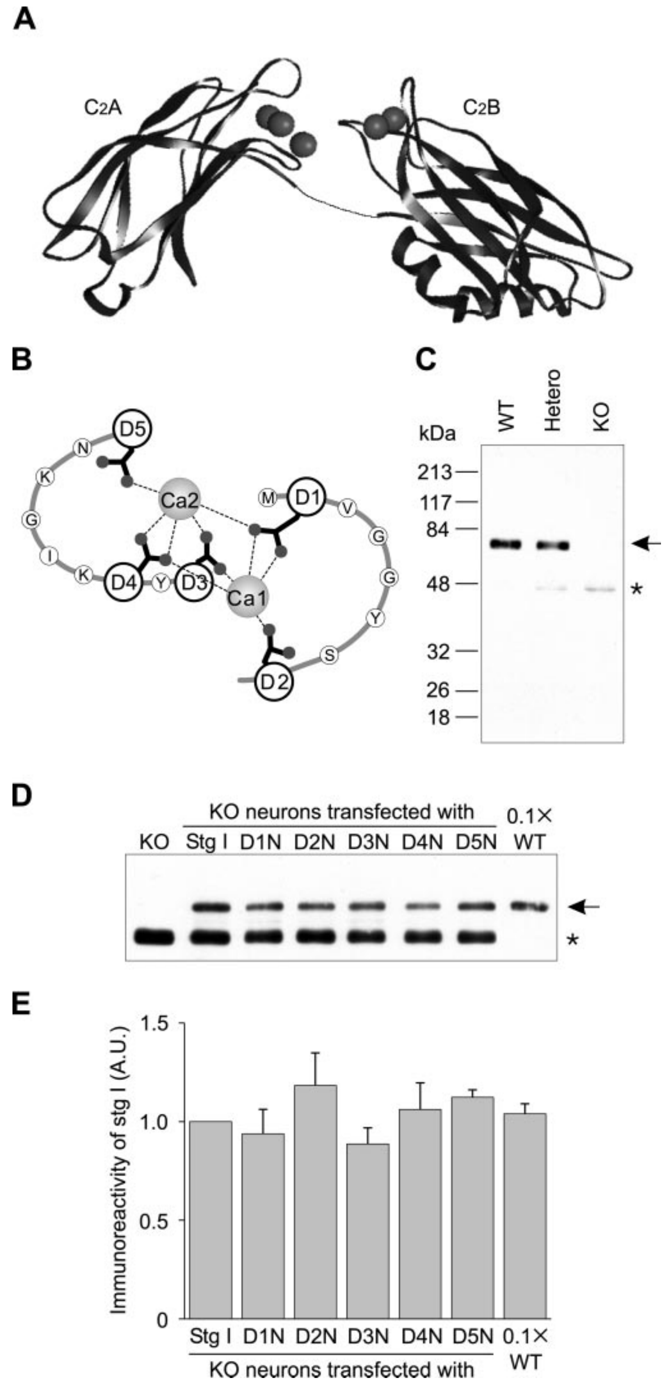

Figure 1. Expression of exogenous synaptotagmin I in the knock-out neurons. *A*, Vector NTI software (InfoMax, Frederick, MD) was used to generate structures of C_2A (Shao et al., 1998) and C_2B (Fernandez et al., 2001) domains in the presence of Ca^{2+} (spheres). The linker connecting the C_2 domains was added manually. *B*, Ca^{2+}-binding sites in the C_2B domain of synaptotagmin I, modified from Fernandez et al. (2001). Two Ca^{2+} (Ca1 and Ca2) are coordinated by five Asp residues (D1 through D5) on two loops. *C*, Expression of synaptotagmin I in neurons from wild-type (WT), heterozygous (Hetero), or knock-out (KO) mice. Cultured hippocampal neurons (2×10^3 cells) were subjected to immunoblotting. The migration position of synaptotagmin I is indicated by an arrow, and the truncated product weakly expressed in the synaptotagmin mutant is indicated by an asterisk. *D*, *E*, Expression of exogenous synaptotagmin I in the knock-out neurons. *D*, Knock-out neurons (2×10^4 cells) either untransfected (KO) or transfected with either wild-type (Stg I) or mutated synaptotagmin I (D1N–D5N) were analyzed via immunoblotting. Wild-type neurons (WT; 2×10^3 cells) were loaded as a control. *E*, Immunoreactivities were measured and normalized to wild-type synaptotagmin I expressed in the knock-out neurons. Data are the mean ± SEM from three experiments.

expressed synaptotagmin I. To enhance detection, samples from a high number of cells (2×10^4 cells) were loaded onto each lane. In addition to the truncated synaptotagmin I, which appeared as intense bands because of the large amount of sample loaded, bands migrating at the same position as endogenous synaptotagmin I were detected in the transfectants but not in untransfected knock-out neurons (Fig. 1*D*). These results indicate that exogenous wild-type and mutated synaptotagmin I proteins are expressed and stably exist in neurons. Quantitative analysis revealed that there were no significant differences in the amount of expression of wild-type and mutated forms of synaptotagmin I (Fig. 1*E*). This makes it possible to compare the efficacy of these proteins in rescue experiments. In addition, the amount of transfected synaptotagmin I that was expressed corresponded roughly to 1⁄10 of the amount of endogenous synaptotagmin I in cultures of wild-type neurons (Fig. 1*D*, right, *E*). Because the synaptotagmin constructs were transfected into ~2% of the neurons in these cultures under our experimental conditions (data not shown), we estimate that the level of exogenous synaptotagmin I expressed in individual knock-out neurons is approximately five times higher (0.1 divided by 0.02) than the level of endogenous synaptotagmin I in wild-type neurons.

Immunofluorescence imaging was also used to determine the localization of synaptotagmin I expressed in knock-out neurons (supplemental Fig. 1, available at www.jneurosci.org as supplemental material). Knock-out neurons transfected with either wild-type or mutated synaptotagmin I showed punctuate staining patterns along neurites similar to that observed for endogenous synaptotagmin I in wild-type neurons, which is characteristic of synaptic regions (Matthew et al., 1981; Gitler et al., 2004). As will be shown below, these transfected synaptotagmins also can alter synaptic transmission in the knock-out neurons. These results suggest that transfected synaptotagmin I proteins, both wild-type and mutated versions, are targeted to synaptic regions in knock-out neurons.

Rescue of synchronous neurotransmitter release in synaptotagmin I-deficient neurons

Loss of the synaptotagmin I gene causes a dramatic reduction in the fast synchronous component of transmitter release, whereas the slower asynchronous component of release increases to compensate (Fig. 2*A*, top and middle) (Geppert et al., 1994; Yoshihara and Littleton, 2002; Shin et al., 2003; Nishiki and Augustine, 2004). To determine whether this defect in the kinetics of neurotransmitter release could be restored by expressing exogenous synaptotagmin I, we transferred the gene into autaptic knock-out neurons.

When the wild-type synaptotagmin I gene was expressed in knock-out neurons, the fast component of transmitter release was recovered and the slow component of release was decreased (Fig. 2*A*, bottom). In the transfected neurons, the mean peak amplitude of EPSCs was 2.3 ± 0.7 nA ($n = 11$), which is seven times larger than the value of 0.34 ± 0.07 nA that was found for EPSCs in knock-out neurons ($n = 11$; $p < 0.006$). The peak amplitude of EPSCs in knock-out neurons expressing wild-type synaptotagmin I was quite similar to values reported for wild-type neurons under identical experimental conditions (2.1 ± 0.5 nA; $p > 0.1$) (Nishiki and Augustine, 2004), indicating that the exogenous wild-type synaptotagmin I rescues the deficits in EPSC amplitude produced by knocking out this protein.

To quantify the kinetics of transmitter release in the rescued neurons, we integrated EPSCs to determine the time course of postsynaptic charge transfer. Transmitter release from the res-

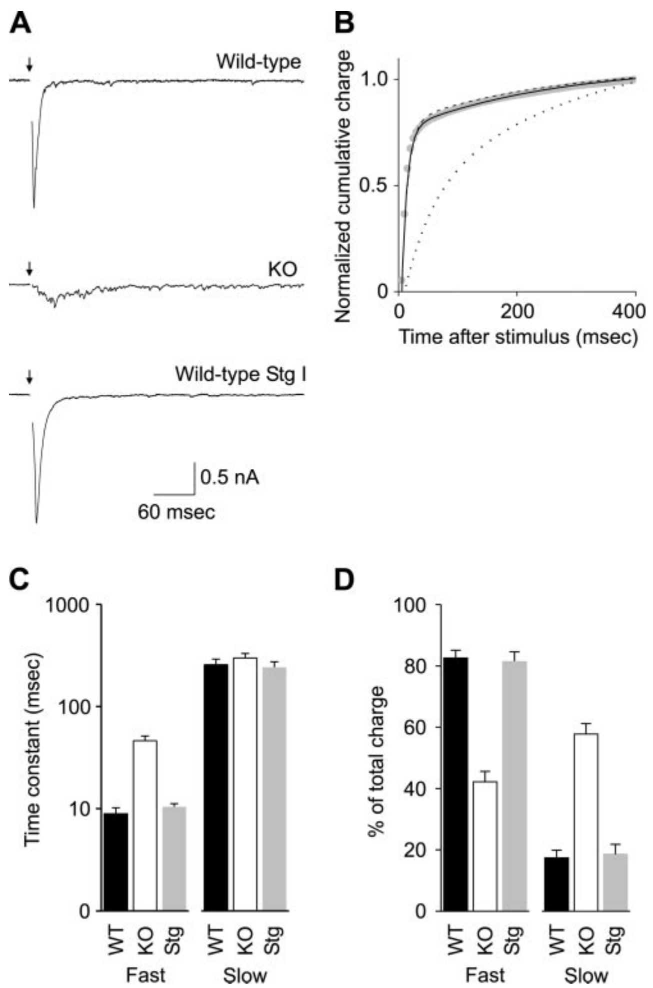

Figure 2. Restoration of synchronous release by expression of exogenous wild-type synaptotagmin I. *A*, Evoked EPSCs recorded from a wild-type neuron (top), an untransfected knock-out neuron (KO; middle), and a neuron transfected with wild-type synaptotagmin I (Stg I; bottom). Cells were held at −70 mV, and action potentials were induced by brief depolarizations (arrows); stimulus artifacts are blanked. *B*, Normalized cumulative charge released from the knock-out neurons transfected with wild-type synaptotagmin I (gray circles; $n = 11$). The solid line is a double exponential function fit to the data. For comparison, similar plots from wild-type (dashed) and knock-out (dotted) neurons are shown. *C*, Time constants of the fast, synchronous (left) and slower, asynchronous (right) components of transmitter release from knock-out neurons transfected with synaptotagmin I, as well as wild-type and knock-out neurons. *D*, Relative amplitudes of fast and slow components of transmitter release for the three types of neurons. Data for wild-type and knock-out neurons shown in *B–D* are from Nishiki and Augustine (2004).

cued knock-out neurons could be fitted by two exponential functions (Fig. 2*B*, solid line), with a fast time constant (τ_{fast}) of 8.9 ± 1.3 msec and a slow time constant (τ_{slow}) of 257 ± 33 msec (Fig. 2*C*). Although the τ_{slow} was not significantly changed by expressing synaptotagmin, the τ_{fast} was five times faster than that reported previously for knock-out neurons (Fig. 2*B*, dotted line, *C*) (Nishiki and Augustine, 2004) and almost identical to those observed in wild-type neurons in such conditions ($p > 0.34$) (Fig. 2*B*, dashed line, *C*) (Geppert et al., 1994; Goda and Stevens, 1994; Shin et al., 2003; Nishiki and Augustine, 2004). These measurements are unlikely to be affected by postsynaptic receptor desensitization, which would be a problem only if more than one quantum of glutamate is released onto the same cluster of postsynaptic receptors (Hartzell et al., 1975). In our experimental conditions, a single stimulus releases on the order of 300 quanta from ∼700 boutons (D. Gitler and G. J. Augustine, unpublished observations), so that the cumulative probability of release is so low that desensitization should be negligible. In the rescued neurons, the total charge released within 400 msec of the stimulus was 29.1 ± 7.8 pC, which is not significantly different from what is observed in wild-type or knock-out neurons under the same conditions ($p > 0.6$) (Nishiki and Augustine, 2004); however, expression of exogenous synaptotagmin I changed the relative amounts of charge in the fast and slow components in comparison with knock-out neurons, increasing the amplitude of the fast component and decreasing the relative amount of the slow component (Fig. 2*D*). The rescued neurons showed no significant differences ($p > 0.76$) in the relative amplitudes of the two components of release in comparison with wild-type neurons (Fig. 2*D*) (Nishiki and Augustine, 2004). In summary, defects in the kinetics of neurotransmitter release observed in synaptotagmin I-deficient neurons were completely rescued by expressing the exogenous wild-type synaptotagmin I gene.

Different effects of C_2B Ca^{2+}-binding motif mutants on neurotransmitter release

Our finding that transfection of synaptotagmin I can fully rescue the phenotype of synaptotagmin I knock-out neurons paves the way for a detailed structure–function study of the Ca^{2+}-binding sites of the C_2B domain. To examine the effects that mutations in these Ca^{2+}-binding sites have on transmitter release, EPSCs were measured from knock-out neurons transfected with synaptotagmin I possessing the Asn mutations described above. The fast synchronous component of transmitter release was rescued by synaptotagmin I harboring Asn mutations in the first, fourth, or fifth Asp residues of C_2B (Fig. 3). The peak amplitude of EPSCs recorded in neurons expressing the D1N mutation was 0.65 ± 0.15 nA ($n = 8$), which is two times larger than that of EPSCs recorded from knock-out neurons (Nishiki and Augustine, 2004) and is 30% of the peak amplitude of EPSCs recorded in neurons transfected with wild-type synaptotagmin I (Fig. 4*A*). This difference in the degree of rescue between wild-type and D1N mutant synaptotagmin I is significant ($p < 0.04$). Given that this mutant protein is expressed and targeted as well as the wild-type protein (Fig. 1 and supplemental Fig. 1, available at www.jneurosci.org as supplemental material), this difference in EPSC amplitude indicates that the D1N mutant is only partially capable of supporting the fast component of release. Knock-out neurons transfected with the D4N ($n = 6$) or D5N ($n = 7$) mutants had EPSCs with peak amplitudes slightly smaller than those recorded in neurons expressing wild-type synaptotagmin I (Fig. 4*A*), but these differences were not statistically significant ($p > 0.16$) because of the large cell-to-cell variance in EPSC amplitudes.

In contrast, mutating the second or third Asp residues caused defects in neurotransmitter release that were even more severe than the defects observed in neurons that completely lacked synaptotagmin I. The D2N and D3N mutations almost completely inhibited the ability of synaptotagmin I to rescue the fast synchronous component of synaptic transmission (Fig. 3). The peak amplitudes of EPSCs recorded from neurons expressing these mutants were only 3% of those for EPSCs recorded from neurons expressing wild-type synaptotagmin ($p < 0.03$) (Fig. 4*A*) and are even significantly smaller ($p < 0.02$) than the peak amplitude of EPSCs recorded from knock-out neurons (Nishiki and Augustine, 2004). Although the D2N and D3N mutants inhibited the fast synchronous release, these mutants markedly suppressed the slow asynchronous component of release seen in knock-out neurons (Fig. 3). As a result of these reductions in both synchronous

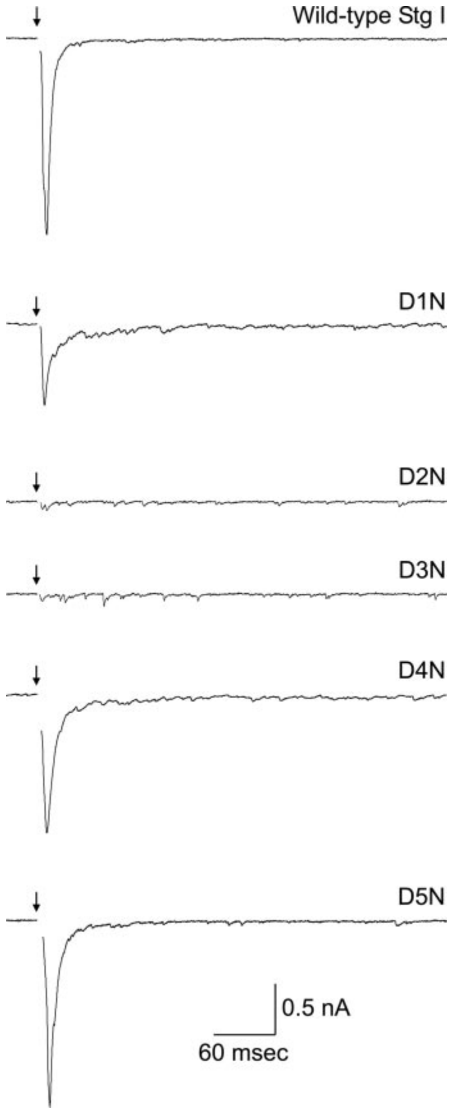

Figure 3. Effects of mutations in the C_2B Ca^{2+}-binding motifs on transmitter release. Evoked EPSCs were recorded from knock-out neurons expressing wild-type (Stg I) or mutant forms (D1N–D5N) of synaptotagmin I.

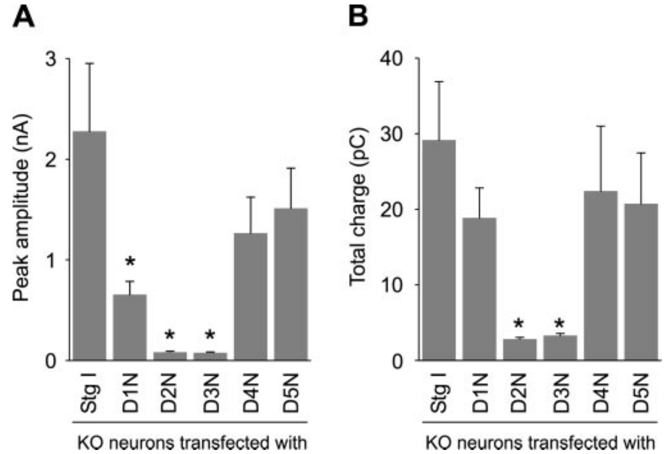

Figure 4. Magnitude of transmitter release in neurons expressing synaptotagmin I mutants. Peak amplitude of EPSCs (*A*) and cumulative amount of charge released (*B*) from knock-out neurons expressing either wild-type (Stg I) or mutated (D1N–D5N) synaptotagmin I. Total charge transfer was measured over 400 msec after the stimulus. Values are means ± SEM (Stg I, $n = 11$; D1N, $n = 8$; D2N, $n = 5$; D3N, $n = 5$; D4N, $n = 6$; D5N, $n = 7$). Asterisks indicate values that are significantly different ($p < 0.05$) from those obtained from cells transfected with wild-type synaptotagmin I.

and asynchronous release, expression of these mutants significantly reduced ($p < 0.03$) the total amount of EPSC charge by ~90% in comparison with values determined for neurons transfected with wild-type synaptotagmin I (Fig. 4*B*). This differs from EPSCs recorded from neurons expressing synaptotagmin I with mutations in one of the other three Asp residues, in which EPSC charge was not significantly different from that observed in neurons transfected with wild-type synaptotagmin I ($p > 0.3$) (Fig. 4*B*). Although the total charge measured in the D2N- or D3N-expressing neurons was small, ~3 pC, this still represented stimulus-evoked release because the amount of charge associated with spontaneous release from these neurons was <0.12 pC over 400 msec. These results indicate that the D2N and D3N mutations not only failed to restore the fast synchronous component of release but also inhibited the total amount of transmitter released in response to a stimulus. Although transfecting knock-out neurons with synaptotagmin I with D2N or D3N mutations reduced the total amount of transmitter release, synaptotagmin I knock-out neurons are fully capable of releasing neurotransmitter (Nishiki and Augustine, 2004). Thus, these two mutants must have dominant-negative effects on Ca^{2+}-dependent transmitter release from mouse hippocampal neurons.

Analysis of the kinetics of transmitter release from the transfected neurons revealed clear differences between the five synaptotagmin mutants. As described above (Fig. 2), the time course of EPSCs in neurons expressing wild-type synaptotagmin I could be described as the sum of two exponential functions (Fig. 5*A*, dashed line). The same was true for EPSCs from neurons expressing each of the five mutations (Fig. 5*A*). No significant differences in either τ_{fast} or τ_{slow} of transmitter release were found when comparing EPSCs from knock-out neurons expressing wild-type synaptotagmin I with those from neurons expressing the mutated synaptotagmins ($p > 0.09$) (Fig. 5*B*). In the D2N and D3N mutations, however, the relative amplitude of the fast component of release was markedly reduced ($p < 10^{-5}$), so that >90% of the total EPSC charge was contributed by the slow asynchronous component of release (Fig. 5*C*). The D1N and D4N mutations significantly reduced the relative amplitude of the fast synchronous component ($p < 10^{-5}$ and 0.002, respectively), whereas the D5N mutation had no effect on the kinetics of transmitter release compared with wild-type synaptotagmin I ($p > 0.75$). These data indicate that the second and third Asp residues in the C_2B domain of synaptotagmin I are essential for triggering the fast component of transmitter release. In addition, the data demonstrate that the first and fourth Asp residues are important for complete execution of the fast component of release and that the fifth Asp residue is not critical for the function of synaptotagmin I in neurotransmitter release.

In neurons expressing several of these synaptotagmin I mutants, as well as the knock-out neurons expressing no synaptotagmin I, there appeared to be a relationship between the synchronous and asynchronous components of release: all synapses with a large synchronous component of transmitter release had a relatively small amount of asynchronous release, whereas some of those with a small synchronous release component apparently had a compensatory increase in the asynchronous component. To define the relationship between the synchronous and asynchronous components of release, the amount of release associated with each of these components was quantified by determin-

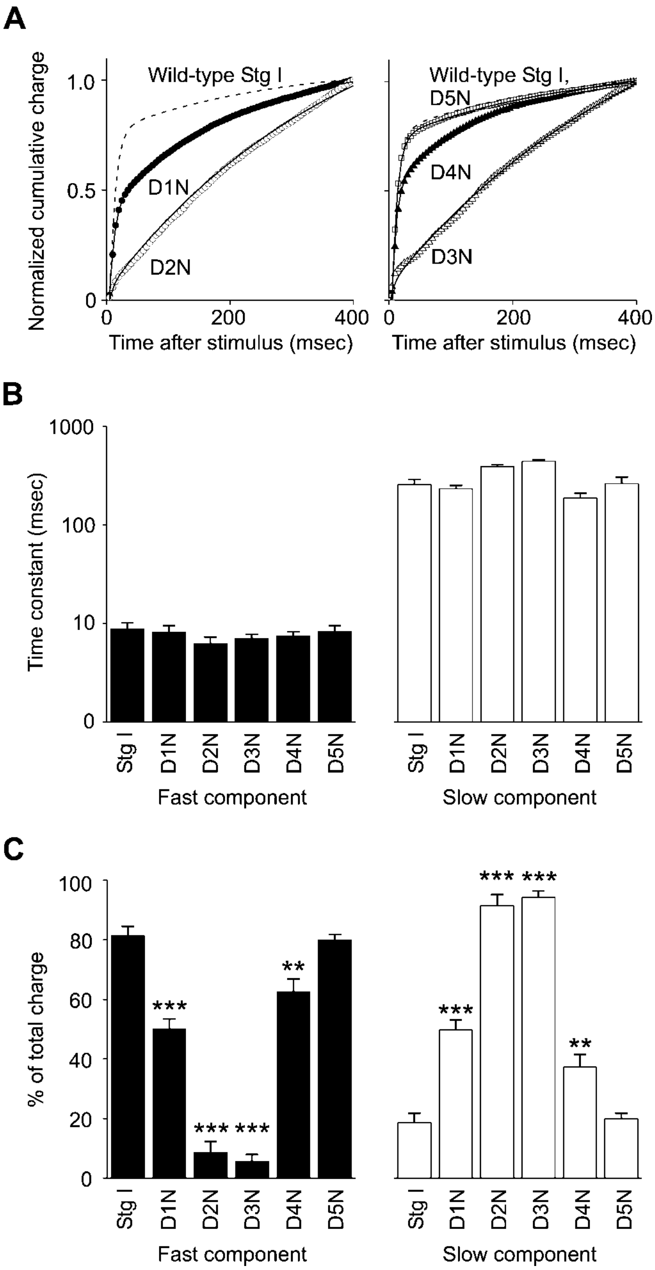

Figure 5. Kinetics of transmitter release in neurons expressing synaptotagmin I mutants. *A*, Normalized plots of cumulative charge released from the knock-out neurons transfected with the indicated synaptotagmin I mutants. Data are presented as in Figure 2*B*, and double exponential functions are fit to the data. Data for knock-out neurons expressing wild-type synaptotagmin I (dashed line) are replotted from Figure 2*B* for comparison. The number of replicates for each mutant are as follows: eight for D1N, five for D2N, five for D3N, six for D4N, and seven for D5N. *B*, *C*, Time constants (*B*) and relative amplitudes (*C*) of the fast synchronous (left) and slow asynchronous (right) components of transmitter release from knock-out neurons transfected with either wild-type (Stg I) or mutant (D1N–D5N) synaptotagmin I. The values are means ± SEM and were calculated by double exponential fitting of results obtained from individual cells. Asterisks indicate differences that are significantly different from those obtained from cells transfected with wild-type synaptotagmin I.

ing the magnitude of each component. Asynchronous release was increased when synchronous release was inhibited in knock-out neurons and in the D1N and D4N mutants. EPSC charge associated with asynchronous release was significantly larger for the D1N mutant than for wild-type synaptotagmin I ($p < 0.03$), as well as the D2N ($p < 0.01$), D3N ($p < 0.04$), or D5N ($p < 0.01$)

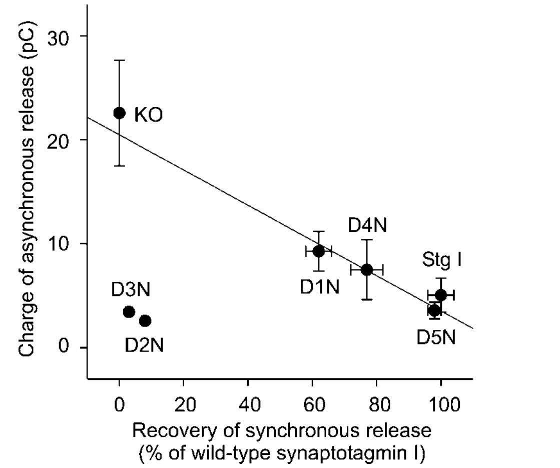

Figure 6. Inverse relationship between synchronous and asynchronous transmitter release. The relative amount of synchronous release measured for neurons expressing each type of synaptotagmin I mutant, as well as knock-out neurons, was normalized by the value measured for wild-type synaptotagmin I. The absolute amounts of asynchronous release were calculated from the product of the relative amplitude of this component (Fig. 5*C*) and total EPSC charge (Fig. 4*B*). Data are mean ± SEM. Straight line is a linear regression fit to all data points except for those for D2N and D3N.

mutants. The magnitude of asynchronous release was found to be negatively correlated with the amount of synchronous release (Fig. 6). Thus, the presence of synchronous release reduces the amount of asynchronous release. Remarkably, the D2N and D3N mutants deviated from this negative correlation, having small amounts of both synchronous and asynchronous release (Fig. 6). This indicates that each of these two mutations inhibits the synchronous component of release without increasing the amount of asynchronous release. A linear regression fit to the remaining data points yielded a high correlation coefficient ($r = 0.95$), indicating that there is a strong negative correlation between the amounts of synchronous and asynchronous release.

Discussion

We investigated the physiological function of the Ca^{2+}-binding sites of the C_2B domain of synaptotagmin I by generating a systematic series of point mutations in Ca^{2+}-binding Asp residues and examined the effects of these mutations on the synchronous and asynchronous triggering of transmitter release by Ca^{2+}. We found that D2 and D3 within the C_2B domain are more important than the others for the physiological sensing of Ca^{2+} during synchronous release. In addition, we found that mutating either of these two residues still allows synaptotagmin I to suppress asynchronous release. Thus, synchronizing release and suppressing asynchronous release require separate properties of synaptotagmin I.

Ca^{2+}-dependent triggering of synchronous release by the C_2B domain

Our results demonstrate that D2 and D3, but not D5, in the C_2B domain of synaptotagmin I are essential for triggering synchronous transmitter release. Although the structural consequences of these point mutations are not fully understood, the D2N mutation strongly impairs Ca^{2+} binding by both sites within the C_2B domain, whereas the D5N mutation decreases only the affinity of the second Ca^{2+}-binding site (Fig. 1*B*, Ca2) without significantly affecting the first Ca^{2+}-binding site (Fig. 1*B*, Ca1) (Fernandez et al., 2001). When these biochemical observations are combined with our physiological analysis, it appears that Ca^{2+} binding to Ca1 is more critical for the physiological function of synaptotagmin I.

Our results extend previous findings that simultaneous mutations in pairs of Asp residues in the C_2B domain (D1N–D2N or D3N–D4N) severely inhibit synchronous release in *Drosophila* (Mackler et al., 2002). Given that synaptotagmin I possessing single mutations of the D1 or D4 sites can trigger synchronous release whereas synaptotagmin I with mutations in D2 or D3 cannot, it is likely that the phenotype of the *Drosophila* double mutations is caused by defects in D2 or D3.

It is not yet clear how Ca^{2+} binding to synaptotagmin I synchronizes transmitter release. Ca^{2+} causes synaptotagmin I to interact with plasma membrane (Fernandez et al., 2001; Bai et al., 2004a). This could be important for synchronous release because the D2N mutation inhibits both synchronous release (Fig. 3) and Ca^{2+}-dependent binding to lipids (Fernandez et al., 2001). Ca^{2+} also causes oligomerization of synaptotagmin (Chapman et al., 1996; Damer and Creutz, 1996; Sugita et al., 1996; Wu et al., 2003). Although there are arguments against a functional role for oligomerization (Fukuda and Mikoshiba, 2000; Ubach et al., 2001; Wu et al., 2003), D2N or D3N mutations in the C_2B domain inhibit both oligomerization (Desai et al., 2000) and synchronous release (Fig. 3). Finally, Ca^{2+} could cause synchronous release by regulating binding of synaptotagmin I to syntaxin 1 and SNAP-25 (Chapman et al., 1995; Li et al., 1995; Gerona et al., 2000; Zhang et al., 2002; Bai et al., 2004b), although there are also arguments against this possibility (Shin et al., 2003). Additional analysis of the biochemical consequences of mutating the Asp residues of the C_2B domain could help distinguish among these possibilities.

Synaptotagmin I suppresses asynchronous release

Asynchronous transmitter release is increased in synaptotagmin I knock-out neurons (Yoshihara and Littleton, 2002; Shin et al., 2003; Nishiki and Augustine, 2004). This suggests that synaptotagmin I suppresses asynchronous release, although light-induced inactivation of synaptotagmin I reportedly changes only the amplitude, but not the half-width, of EPSPs in *Drosophila* (Marek and Davis, 2002). Our results provide additional support for the idea that synaptotagmin I suppresses asynchronous release by showing that asynchronous release is suppressed after expression of exogenous synaptotagmin I in knock-out neurons. This suppression is reduced after deletion of the C_2B domain (Yoshihara and Littleton, 2002), indicating a role for the C_2B domain in suppression of asynchronous release.

We have further clarified the mechanism of suppression by showing that D2N and D3N mutations of the C_2B domain still suppress asynchronous release, although these mutations do not support synchronous release (Figs. 3, 6). Because the D2N and probably D3N mutations bind Ca^{2+} very weakly, it is likely that the suppression is a Ca^{2+}-independent process. Although neutralization of the negative charges of these residues could mimic the Ca^{2+}-bound state of synaptotagmin I, it is unlikely that these mutants serve as a constitutive, Ca^{2+}-bound form of synaptotagmin I, because they are unable to bind phospholipids (Fernandez et al., 2001) or SNAP-25 (Bai et al., 2004b). We therefore conclude that synaptotagmin I suppresses asynchronous release at least partly through its C_2B domains and does so in a Ca^{2+}-independent manner. Thus, although synchronization of transmitter release after an action potential requires Ca^{2+} binding to the C_2B domain of synaptotagmin I, suppression of asynchronous release does not require Ca^{2+} binding to the C_2B domain.

Several possible molecular mechanisms could account for the Ca^{2+}-independent suppression of asynchronous release by synaptotagmin I. One potential mechanism is binding to phosphatidylinositol bisphosphate (Bai et al., 2004a). Many lines of evidence also suggest that Ca^{2+}-independent binding of synaptotagmin I to t-SNAREs suppresses asynchronous release. SNARE complexes are involved in asynchronous release because this component (as well as synchronous release) is abolished in syntaxin-null mutants (Schulze et al., 1995). Synaptotagmin I can bind syntaxin 1 and SNAP-25 in the absence of Ca^{2+} (Bennett et al., 1992; Yoshida et al., 1992; Söllner et al., 1993; Schiavo et al., 1997; Verona et al., 2000; Rickman and Davletov, 2003), and this requires the C_2B domain (Rickman et al., 2004). Mutations in the C-terminal cytoplasmic region of syntaxin 1, which is required for constitutive binding to synaptotagmin I (Kee and Scheller, 1996), strongly inhibit synchronous release and increase asynchronous release in *Drosophila* (Wu et al., 1999; Fergestad et al., 2001). Defects in the kinetics of transmitter release are also observed after microinjection of synprint peptides (Mochida et al., 1996), which bind to the C_2B domain in the absence of Ca^{2+} and inhibit the ability of synaptotagmin I to bind to syntaxin 1 (Sheng et al., 1997). Given these findings, we postulate that synaptotagmin I suppresses asynchronous release by binding to syntaxin 1 before Ca^{2+} entry.

Molecular mechanisms underlying Ca^{2+}-dependent neurotransmitter release

Figure 7*A* illustrates our model for the dual roles of synaptotagmin I in neurotransmitter release. This model proposes that Ca^{2+}-free synaptotagmin I binds to t-SNAREs and holds the fusogenic SNARE complex proteins in an intermediate state in resting terminals. Synchronous release would then arise from Ca^{2+} binding to the C_2B domain of synaptotagmin I (Fig. 7*A*, bottom left), thereby inserting synaptotagmin I into the plasma membrane and/or promoting the "zippering" of SNARE complexes (Chen and Scheller, 2001; Rettig and Neher, 2002; Jahn et al., 2003). The latter could arise from Ca^{2+}-triggered binding of synaptotagmin I to SNAP-25 (Gerona et al., 2000; Zhang et al., 2002; Bai et al., 2004b) or from synaptotagmin I dissociating from syntaxin 1 (Kee and Scheller, 1996; Leveque et al., 2000). Either could explain findings that Ca^{2+} is required to form full complexes between v-SNAREs in synaptic vesicles and t-SNAREs in liposomes (Hu et al., 2002) and that synaptotagmin I confers Ca^{2+} sensitivity to SNARE-dependent liposome fusion (Tucker et al., 2004). The inability of the D2N and/or D3N mutants of synaptotagmin I to trigger synchronous release would then be attributable to their inability to bind Ca^{2+} efficiently (Fernandez et al., 2001).

The inverse relationship between synchronous and asynchronous release (Fig. 6) suggests a competition between these two release mechanisms for exocytosis of synaptic vesicles from a common pool. It has been proposed that asynchronous release is regulated by another high-affinity Ca^{2+} sensor, perhaps another synaptotagmin isoform (Geppert et al., 1994; Goda and Stevens, 1994; Südhof, 2002; Yoshihara and Littleton, 2002; Nishiki and Augustine, 2004). Thus, synaptotagmin I may suppress asynchronous release by preventing this high-affinity Ca^{2+} sensor from binding to t-SNAREs in the resting presynaptic terminal. Ca^{2+} binding to the high-affinity Ca^{2+} sensor could then allow it to associate with t-SNAREs and cause asynchronous release by triggering the slow fusion of releasable vesicles that did not fuse rapidly in response to Ca^{2+} binding to synaptotagmin I (Fig. 7*A*, bottom right). Enhanced binding of this high-affinity Ca^{2+} sen-

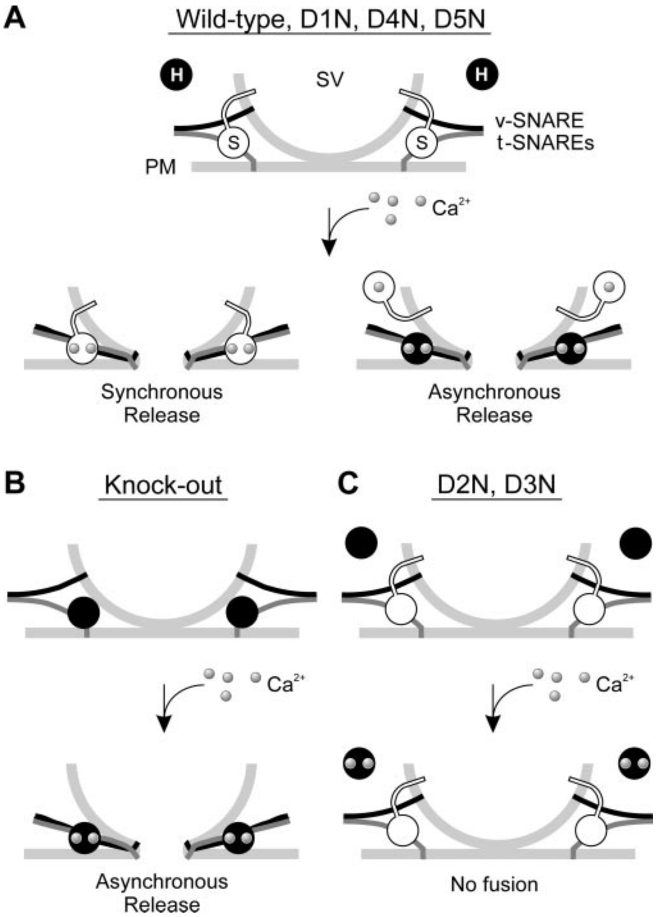

Figure 7. A model for the synchronous and asynchronous components of Ca^{2+}-dependent neurotransmitter release. Symbols represent synaptotagmin (S) and a high-affinity Ca^{2+} sensor (H); SV indicates a synaptic vesicle, and PM indicates the presynaptic plasma membrane; Ca^{2+} are shown as small, shaded spheres.

sor to t-SNAREs in the absence of synaptotagmin I could account for the increase in asynchronous release observed in knock-out neurons (Fig. 7*B*). Likewise, the increase in asynchronous release produced by the D1N or D4N mutants, compared with wild-type synaptotagmin, could arise from the reduced ability of these proteins to trigger synchronous release and compete with the high-affinity Ca^{2+} sensor (Fig. 7*A*). Although synaptotagmin I with D2N and/or D3N mutations greatly reduced Ca^{2+} binding, this mutant could remain bound to t-SNAREs and prevent the high-affinity Ca^{2+} sensor from taking its place, thereby reducing asynchronous release without causing synchronous release (Fig. 7*C*).

In summary, we found that various Asp residues in Ca^{2+}-binding sites of the C_2B domain of synaptotagmin I are critical for triggering efficient Ca^{2+}-dependent synchronous neurotransmitter release. We also demonstrated that asynchronous release is suppressed by synaptotagmin I and Ca^{2+} binding to the C_2B domain is not required for this suppression. Thus, in neurons, the synchrony of neurotransmitter release is maintained by two separate roles of synaptotagmin I that are differentially regulated by Ca^{2+}.

Note added in proof. In a paper that will soon appear, Cheng et al. (2004) use X-ray crystallography to examine the structure of Ca^{2+}-binding sites in the C_2B domain of synaptotagmin I. They report that the fifth Asp residue of C_2B does not contribute to Ca^{2+} binding, which is consistent with our observations that mutation of this residue does not affect the ability of synaptotagmin I to support synchronous transmitter release.

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