

Calcium Control of Neurotransmitter Release

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Upon entering a presynaptic terminal, an action potential opens Ca^{2+} channels, and transiently increases the local Ca^{2+} concentration at the presynaptic active zone. Ca^{2+} then triggers neurotransmitter release within a few hundred microseconds by activating synaptotagmins Ca^{2+} . Synaptotagmins bind Ca^{2+} via two C2-domains, and transduce the Ca^{2+} signal into a nanomechanical activation of the membrane fusion machinery; this activation is mediated by the Ca^{2+} -dependent interaction of the synaptotagmin C2-domains with phospholipids and SNARE proteins. In triggering exocytosis, synaptotagmins do not act alone, but require an obligatory cofactor called complexin, a small protein that binds to SNARE complexes and simultaneously activates and clamps the SNARE complexes, thereby positioning the SNARE complexes for subsequent synaptotagmin action. The conserved function of synaptotagmins and complexins operates generally in most, if not all, Ca^{2+} -regulated forms of exocytosis throughout the body in addition to synaptic vesicle exocytosis, including in the degranulation of mast cells, acrosome exocytosis in sperm cells, hormone secretion from endocrine cells, and neuropeptide release.

Synaptic transmission is initiated when an action potential invades a nerve terminal, opening Ca^{2+} channels, which gate a highly localized, transient increase in intracellular Ca^{2+} at the active zone (Fig. 1A). Ca^{2+} triggers synaptic vesicle exocytosis, thereby releasing the neurotransmitters contained in the vesicles and initiating synaptic transmission. This fundamental mechanism was discovered in pioneering work on the neuromuscular junction by Katz and Miledi (1967). Its precise time course was studied in many “model” synapses, including giant squid axon synapses (Augustine et al. 1985) and rat cerebellar parallel fiber synapses (Sabatini and Regehr 1996), but characterized in greatest detail in the calyx of Held synapses

in the brainstem (Fig. 1B; reviewed in Meinrenken et al. 2003). Overall, these high-resolution electrophysiological studies on neurotransmitter release revealed that a presynaptic action potential is tightly coupled to Ca^{2+} influx and synaptic vesicle fusion, such that under physiological conditions, Ca^{2+} triggers fusion in a few hundred microseconds, or possibly even in less than 100 microseconds (Sabatini and Regehr 1996).

The amazing speed and precision of Ca^{2+} -triggered neurotransmitter release raised the question of how such speed might be possible—how can Ca^{2+} induce exocytosis in a few hundred microseconds, on the same timescale as the gating of an ion channel? As we will

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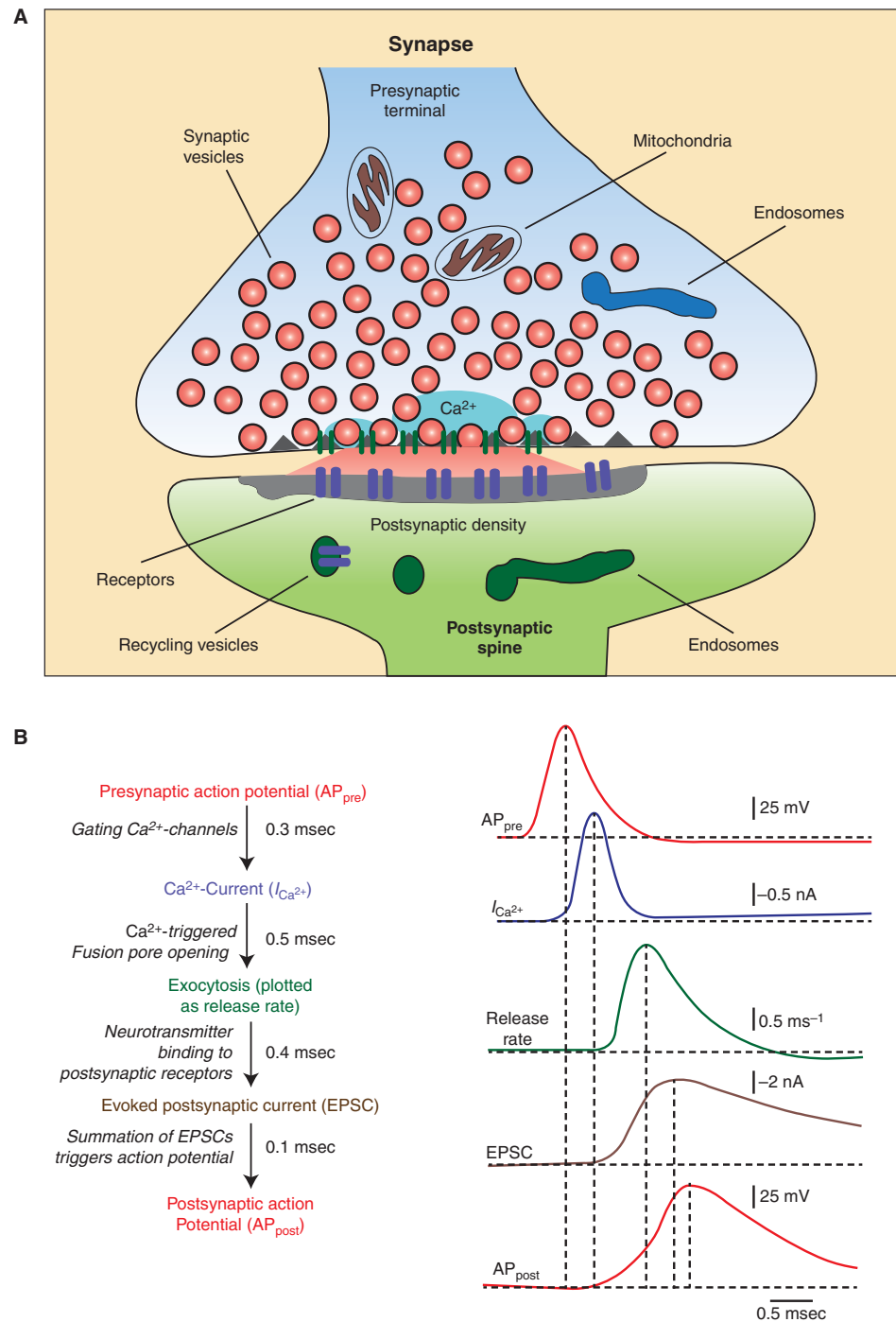


Figure 1. Principle and time course of Ca^{2+} -triggered synaptic transmission. (A) Schematic diagram of a synapse illustrating the localized influx of Ca^{2+} at the active zone (red = secreted neurotransmitters). (B) Schematic illustration of the sequence and time course of synaptic transmission as measured by simultaneous pre- and postsynaptic patch-clamp recordings at the calyx of Held synapse. Note that Ca^{2+} currents and EPSC are shown inverted. (Images are modified from Südhof 2004 and Meinrenken et al. 2003.)

describe in this review, the speed and precision of release are mediated, at least in part, by the properties of the Ca^{2+} sensor synaptotagmin (Syt) and its cofactor complexin. Moreover, increasing evidence indicates that the principal mechanism of Ca^{2+} -triggered exocytosis, although not the organization of this mechanism, is conserved in other, slower forms of Ca^{2+} -induced exocytosis. Thus, the Syt-based fusion apparatus emerges as a general paradigm that accounts for most Ca^{2+} -regulated exocytosis in eukaryotic cells.

In addition to normal action potential-evoked neurotransmitter release, synapses exhibit two additional forms of Ca^{2+} -dependent synaptic exocytosis: spontaneous “mini” release and asynchronous release (Pang and Südhof 2010). Spontaneous mini release was discovered by Katz (Fatt and Katz 1952), and is thought to represent the background activity of a nerve terminal that is triggered by spontaneous Ca^{2+} fluctuations. Although the biological significance of spontaneous release remains debated, its presence can be observed in all neurons. When calculated on a per-synapse basis, spontaneous release occurs only once every 2–3 hours at an individual excitatory synapse of a pyramidal neuron in the CA1 region of the hippocampus, and approximately once every 3 min at individual inhibitory synapses. Asynchronous release is undetectable in most synapses under physiological conditions, but becomes apparent when the principal Syt Ca^{2+} sensor for regular release is ablated (Gepert et al. 1994). Some inhibitory synapses exhibit a slow form of release on repetitive stimulation (Hefft and Jonas 2005), which may represent either genuine physiological asynchronous release or simply synaptotagmin-dependent delayed release.

STRUCTURE AND BIOCHEMICAL PROPERTIES OF SYNAPTOTAGMINS

Syts are defined as proteins containing a short amino-terminal noncytoplasmic sequence followed by a transmembrane region, a central linker sequence of variable length, and two carboxy-terminal C2-domains that account for the

majority of the protein (Fig. 2A; Perin et al. 1990). Sixteen genes encoding canonical Syts are expressed in mammals; in addition, mammals contain a Syt-related gene encoding a similar protein (the so-called B/K protein) that lacks a transmembrane region, and is occasionally called Syt17. Synaptotagmins are evolutionarily conserved, and invertebrates also express multiple Syt isoforms. However, synaptotagmins are absent from plants and unicellular eukaryotes, suggesting that they evolved at the same time as animals. Moreover, a large family of Syt-like proteins is expressed in animals; these proteins also contain two C2-domains, but will not be discussed here further (see Pang and Südhof 2010). Syt1, Syt2, Syt9, and Syt12 are present on synaptic vesicles and on secretory granules in neuroendocrine cells; the latter also contain Syt7. In addition, Syt10 was recently found on secretory vesicles containing IGF-1 in olfactory mitral neurons (Cao et al. 2011); Syt4 was localized to the trans-Golgi complex, synaptic vesicles, and postsynaptic organelles, and may be widely distributed in neuronal organelles (reviewed in Südhof 2004); and Syt7 was additionally localized to “secretory lysosomes” in nonneuronal and non-endocrine cells (Andrews and Chakrabarti 2005). Other Syts appear to be primarily localized to transport vesicles, although some Syt isoforms are enriched on the plasma membrane (Butz et al. 1999), and the identity of many of the Syt-containing vesicles remains unknown.

C2-domains were named as sequence motifs in protein kinase C isoforms (Coussens et al. 1986), but their functional activity as autonomously folded Ca^{2+} -binding domains was first identified in Syt1 (Davletov and Südhof 1993). The atomic structures of the C2A-domain of Syt1, the first C2-domain structure determined (Sutton et al. 1995), revealed that C2-domains are composed of a stable β -sandwich with flexible loops emerging from the top and bottom (Fig. 2B); all subsequently determined C2-domain structures described a similar fold. Ca^{2+} binds exclusively to the top loops of the C2-domains, which form binding sites for two to three Ca^{2+} ions in close vicinity (Shao et al. 1996). Although not all C2-domains

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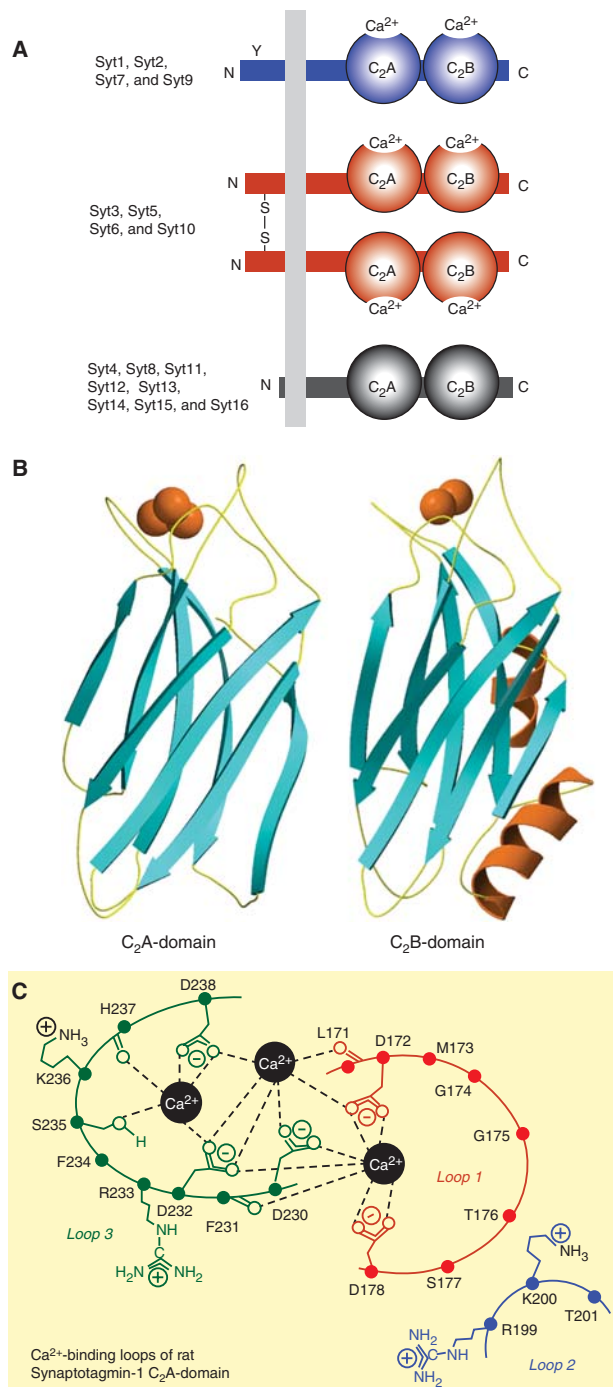


Figure 2. Structures and Ca²⁺-binding properties of synaptotagmins. (A) Canonical domain structures and classification of synaptotagmins. Mammals express 16 synaptotagmins composed of an amino-terminal transmembrane region preceded by a short noncytoplasmic sequence and followed by a variable linker sequence and two C2-domains; in addition, a 17th related protein called B/K-protein contains the same domain structure but an amino-terminal lipid anchor instead of the transmembrane region (see Pang and Südhof 2010, for a discussion of additional synaptotagmin-related proteins). (See facing page for legend.)

bind Ca^{2+} (see below), all of those that do and have been studied in detail contain the same Ca^{2+} -binding site architecture, with the Ca^{2+} ions ligated primarily by aspartate side chains (Fig. 2C). C2-domains are now recognized as general Ca^{2+} -binding modules which are found in a large number of signal transduction and membrane trafficking proteins.

Approximately half of all C2-domains lack the canonical aspartate residues required for Ca^{2+} binding, including the C2A-domains of Syt4 and Syt11 (von Poser et al. 1997), and all C2-domains of Syt8 and Syt12–Syt16, which are thus thought to be Ca^{2+} independent. Even the C2B-domains of Syt4 and Syt11 which include all of the requisite Ca^{2+} -binding sequences were found to be unable to bind Ca^{2+} (Dai et al. 2004). This initially surprising result was explained by the atomic structure of the Syt4 C2B-domain, which showed that the canonical Ca^{2+} -binding residues in the top loops of the domain are too far apart to be able to ligate Ca^{2+} (Dai et al. 2004). Thus, the 16 mammalian Syts can be broadly classified into Ca^{2+} -dependent and Ca^{2+} -independent groups (Fig. 2A). The first group can be further subclassified based on whether or not their amino-terminal noncytoplasmic sequences contain disulfide-bonded cysteine residues (Fukuda et al. 1999). Similarly, the second group can be subclassified based on evolutionary conservation and sequence homologies, which reveal two sets of evolutionarily conserved synaptotagmins formed by Syt4 and Syt11 and by Syt14–16, and into a

heterogeneous set of nonconserved synaptotagmins (Syt8, Syt12, and Syt13).

Initial studies on Syt1 revealed that both of its C2-domains bind to phospholipids in a Ca^{2+} -dependent manner (Davletov and Südhof 1992; Fernandez et al. 2001), and that they bind to SNARE proteins (Bennett et al. 1992; Chapman et al. 1995; Li et al. 1995). Syt1 binding to phospholipids requires Ca^{2+} and negatively charged phospholipids, with phosphatidyl-inositolphosphates being most effective (Pang et al. 2006). Interestingly, the intrinsic Ca^{2+} affinity of the Syt1 C2-domains is relatively low (millimolar), but the presence of negatively charged phospholipids increases their apparent Ca^{2+} affinity by two to three orders of magnitude. This probably occurs because the Ca^{2+} -coordination spheres of the Syt1 C2-domains are incomplete, but are completed by the negatively charged phospholipid headgroups during Ca^{2+} -dependent phospholipid binding (Fernández-Chacón et al. 2001; Fernandez et al. 2002). In contrast to the Ca^{2+} -dependent interaction of Syt1 with phospholipids, its binding to SNARE proteins and SNARE complexes is less well understood. There seems to be a direct interaction of the Syt1 C2-domains with syntaxin-1 that is greatly enhanced by Ca^{2+} (Li et al. 1995); moreover, Syt1 also binds to assembled SNARE complexes in a Ca^{2+} -dependent manner (Pang et al. 2006). However, the interaction of Syt1 with SNARE complexes is highly sensitive to ionic strength in that it becomes Ca^{2+} independent at lower ionic strength, and at least some

Figure 2. (Continued) Eight synaptotagmins bind Ca^{2+} (Syt1, 2, 3, 5, 6, 7, 9, and 10; blue and red); the remaining synaptotagmins do not (black). The eight Ca^{2+} -binding synaptotagmins fall into two broad classes that differ in the absence (Syt1, 2, 7, and 9; blue) or presence (Syt3, 5, 6, and 10; red) of disulfide-bonded cysteine residues in their amino-terminal sequences. Note that Syt1 and 2 include an N-glycosylated sequence at the amino terminus (indicated by a “Y”), and that Syt7 is extensively alternatively spliced in the linker sequence (Han et al. 2004). (B) Atomic structures of the C2A- and C2B-domains of Syt1 containing bound Ca^{2+} ions (red spheres) bound to flexible loops formed by an eight-stranded β -sandwich (from Sutton et al. 1995; Ubach et al. 1998; Fernandez et al. 2001). (C) Structure of the Ca^{2+} -binding sites of the Syt1 C2A-domain (modified from Fernández-Chacón et al. 2001). The Syt1 C2A-domain contains three Ca^{2+} -binding sites, of which the central two are canonical for all Ca^{2+} -binding C2-domains, whereas the third Ca^{2+} site on the left is variably observed in C2-domains, and is absent from the Syt1 C2B-domain. A further Ca^{2+} -binding site that localizes to loop1 on the right is observed in some C2-domains, but is absent from Syt1 and is not shown. Loops refer to the flexible sequences on the top of the Syt1 C2-domain sandwich; the structures of some of the side chains are indicated.

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Ca^{2+} -independent SNARE-complex binding by Syt1 may occur physiologically (Pang et al. 2006).

All Syt isoforms that bind Ca^{2+} appear to bind to phospholipids and to SNARE proteins in a Ca^{2+} -dependent manner (Li et al. 1995; Sutton et al. 1999; Sugita et al. 2002; Hui et al. 2005). Interestingly, at least during Ca^{2+} -dependent phospholipid binding, different Syts exhibit quite different apparent Ca^{2+} affinities, suggesting that they normally differentially respond to Ca^{2+} signals (Sugita et al. 2002). The biochemical properties of most Ca^{2+} -independent Syts remain unknown, apart from Syt12, which has been shown to bind to Syt1 on the synaptic vesicle membrane (Maximov et al. 2007).

FUNCTION OF SYNAPTOTAGMINS IN NEUROTRANSMITTER RELEASE

From the very beginning, Syt1 was hypothesized to function as a Ca^{2+} sensor for neurotransmitter release (Perin et al. 1990; Brose et al. 1992; Davletov and Südhof 1993). Knockout (KO) of Syt1 confirmed this hypothesis, showing that the Syt1 deletion abolished fast synchronous neurotransmitter release (Fig. 3A; Geppert et al. 1994). Strikingly, not all Ca^{2+} -stimulated release was abolished in Syt1 KO neurons, which retained a delayed asynchronous form of release that is increased on repetitive stimulation (Maximov and Südhof 2005). Moreover, release induced by hypertonic sucrose, which is thought to cause the Ca^{2+} -independent exocytosis of all vesicles in the readily releasable pool of a synapse was unchanged, indicating that the Syt1 KO did not interfere with vesicle fusion as such, but only with the Ca^{2+} triggering of fusion (Geppert et al. 1994).

Given that eight Ca^{2+} -binding Syts are expressed in neurons, which of these Syts function in neurotransmitter release? A systematic screen of Ca^{2+} -binding Syts for rescue of the Syt1 KO phenotype revealed that only Syt1, Syt2, and Syt9 were capable of rescue (Fig. 3B; Xu et al. 2007). This result is surprising given the close similarity of Ca^{2+} -binding Syts with each other, but is consistent with the differential expression patterns of Syt1, Syt2, and Syt9, and

also explains why Syt1 KO neurons lose all release despite expressing high levels of Syt7, Syt3, and Syt5. Strikingly, Syt1, Syt2, and Syt9 were found to trigger release with quite distinct kinetics, with Syt2 exhibiting the fastest onset and the fastest decline in release, and Syt9 the slowest onset and the slowest decline (Fig. 3C,D; Xu et al. 2007). The distinct kinetic properties of Syt1, Syt2, and Syt9 fit well with the expression of Syt9 primarily in the limbic system, whereas Syt2 is primarily expressed in synapses requiring very fast synaptic transmission, such as the auditory system or the neuromuscular junction.

The demonstration that Syt1 is essential for fast Ca^{2+} -triggered neurotransmitter release was consistent with the notion that Syt1 functions as a Ca^{2+} sensor for release, but did not represent proof that Ca^{2+} binding to Syt1 triggers release, nor did it tell us which of the principal two biochemical activities of Syt1— Ca^{2+} -dependent phospholipid- and SNARE-binding—was involved in its function. Definitive evidence that Syt1, and by analogy other Syts, is indeed a Ca^{2+} sensor for release came from knockin experiments in which point mutations that selectively alter the Ca^{2+} affinity and specificity of Ca^{2+} -dependent interactions were introduced into the endogenous mouse Syt1 (Fernández-Chacón et al. 2001; Pang et al. 2006). Biochemical studies revealed that the D232N mutation in the C2A-domain of Syt1 increases the strength of the Ca^{2+} -dependent binding of the double C2-domain fragment to SNARE complexes without altering phospholipid binding, whereas both the R233Q mutation and, to a lesser degree, the D238N mutation decrease the apparent Ca^{2+} affinity of the double C2-domain fragment of Syt1 during phospholipid binding without major effects on SNARE-complex binding (Fernández-Chacón et al. 2001; Pang et al. 2006). Strikingly, the D232N mutation increased the apparent Ca^{2+} affinity of evoked neurotransmitter release (i.e., decreased the K_d for Ca^{2+}), whereas the R233Q and the D232N decreased the apparent Ca^{2+} affinity of evoked release without changing the apparent Ca^{2+} cooperativity of release (Fig. 4A). Thus, changes in the apparent Ca^{2+} affinity of Syt1 lead to corresponding

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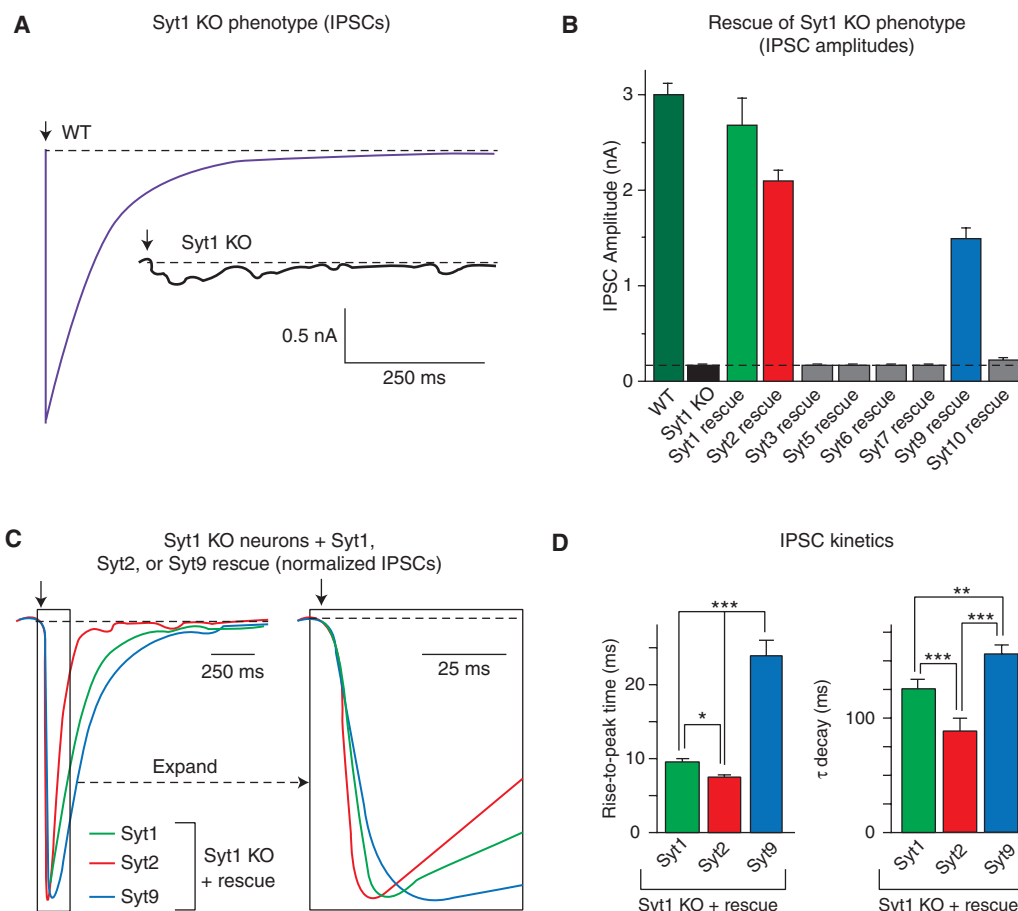


Figure 3. Syt1, Syt2, and Syt9 function as synaptic Ca^{2+} sensors for neurotransmitter release. (A) Deletion of Syt1 in cortical neurons blocks fast synchronous neurotransmitter release. Panels depict representative inhibitory postsynaptic currents (IPSCs) monitored in cortical neurons cultured from littermate wild-type (WT) and Syt1 knockout (Syt1 KO) neurons (arrow = action potential). These data and those shown in panels B–D were modified from Xu et al. (2007). (B) Screen of all Ca^{2+} -binding synaptotagmin isoforms for their ability to rescue the loss of fast neurotransmitter release in Syt1 KO neurons. Data shown are means IPSC amplitudes (\pm S.E.M.); note that only Syt1, 2, and 9 are capable of rescue. (C) Syt1, Syt2, and Syt9 mediate Ca^{2+} triggering of release with distinct kinetics. Data shown are representative IPSCs monitored in Syt1 KO neurons expressing Syt1, 2, or 9. IPSCs were normalized for the maximal amplitude. The traces on the right display an expanded part of the overall IPSCs depicted on the left. (D) Quantitation of the kinetics of the increase in the IPSC (left, as mean rise time) and the decay of the IPSC (right, as mean time constant of the IPSC decay) triggered by Ca^{2+} binding to Syt1, 2, or 9. Note that IPSCs mediated by Syt2 exhibit the fastest rise and decay kinetics, whereas IPSCs mediated by Syt9 are twofold slower. All error bars indicate S.E.M.; asterisks indicate statistically significant differences as assessed by Student's *t*-test (* $p < 0.05$; ** $p < 0.01$; *** $p < 0.001$).

changes in the apparent Ca^{2+} affinity of release, proving that Syt1 is a true Ca^{2+} sensor. Moreover, these experiments show that both SNARE- and phospholipid-binding by Syt1 are involved in release, suggesting that Ca^{2+} binding to Syt1 triggers release by inducing a fast simultaneous

interaction of Syt1 with both SNARE complexes and with phospholipids.

Ca^{2+} binding to both C2-domains of Syt1 contributes to triggering release, although the two C2-domains are not equivalent. Mutations in the C2B-domain Ca^{2+} -binding sites block

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release similarly to the Syt1 KO, whereas mutations in the C2A-domain Ca^{2+} -binding sites have a much less severe effect (Mackler and Reist 2001; Stevens and Sullivan 2003; Shin et al. 2009). Initially it was hypothesized that the C2A-domain Ca^{2+} -binding sites are of little importance, but careful Ca^{2+} titrations revealed that blocking Ca^{2+} binding to the C2A-domain not only decreases the magnitude of release, but also decreases the apparent Ca^{2+} cooperativity of release (Shin et al. 2009). Among others, these experiments confirmed that the two C2-domains of Syt1 function cooperatively, and that Ca^{2+} binding to both C2-domains triggers release to achieve the high apparent cooperativity of Ca^{2+} triggering of release (Meinrenken et al. 2003).

SYNAPTOTAGMINS AS Ca^{2+} SENSORS FOR SPONTANEOUS RELEASE

In a typical central neuron, spontaneous release events add up over time to a sizable signal, and may have an important physiological role (McKinney et al. 1999; Carter and Regehr 2002; Sutton et al. 2006). Most spontaneous release is Ca^{2+} dependent, suggesting it is regulated (Xu et al. 2009). Although spontaneous release was traditionally considered to be determined primarily if not exclusively by the number of synapses and the release probability of these synapses, increasing evidence indicates that spontaneous release may be derived from a vesicle pool that is different from that used for evoked release (Sara et al. 2005), and that it does not always, and possibly not even in most cases, correlate with the number of synapses and their release probability.

The Ca^{2+} dependence of spontaneous release suggested that a Ca^{2+} sensor should trigger it. The logical choice for such a Ca^{2+} sensor would be Syt1 and its homologs. Indeed, analysis of the Ca^{2+} dependence of spontaneous release in knockin mutant mice in which the affinity and binding-specificity of the Syt1 Ca^{2+} -binding sites were altered revealed that increases in the Ca^{2+} -dependent interaction of Syt1 with SNARE complexes induced by the D232N mutation caused an increase in the

apparent Ca^{2+} affinity of spontaneous release (Fig. 4B; Xu et al. 2009). Conversely, decreases in the apparent Ca^{2+} affinity of Syt1 during phospholipid binding induced by the R233Q or the D238N mutation caused a decrease in the apparent Ca^{2+} affinity of spontaneous release (Fig. 4B). These results indicated that Syt1 functions as a Ca^{2+} sensor for spontaneous release, consistent with the previous finding that other elements of the canonical release machinery (e.g., SNARE proteins, Munc18-1, Munc13, and RIMs) are similarly involved.

However, deletion of Syt1 produces a large increase in spontaneous release frequency instead of blocking spontaneous release (Littleton et al. 1994; Maximov and Südhof 2005), suggesting that Syt1 is a clamp for spontaneous release instead of a Ca^{2+} sensor. This apparently contradictory observation was explained when it was found that the increased spontaneous release in Syt1 KO neurons was also completely Ca^{2+} dependent, but exhibited a higher apparent Ca^{2+} affinity and a lower apparent Ca^{2+} cooperativity than spontaneous release triggered by Ca^{2+} binding to Syt1 (Xu et al. 2009). Thus, deletion of Syt1 unmasked a secondary Ca^{2+} sensor with different properties that was normally clamped by Syt1. The nature of this Ca^{2+} sensor remains unclear, but it may be the same as the Ca^{2+} sensor that triggers asynchronous release and that has similar properties (Sun et al. 2007).

In addition to Syts, Doc2 proteins were implicated in regulating spontaneous release. KO or shRNA-mediated knockdown of Doc2 proteins, which are Ca^{2+} -binding proteins related to Syts that also bind to SNAREs and to phospholipids, decreases spontaneous release significantly (Groffen et al. 2010; Pang et al. 2011). Interestingly, the Doc2 KO and knockdown had no effect on evoked release, confirming the differential nature of spontaneous and evoked release. Although the Ca^{2+} -binding properties of Doc2 proteins suggested that they may function as Ca^{2+} sensors for spontaneous exocytosis (Groffen et al. 2010), the decrease in mini frequency induced by Doc2 knockdown was fully rescued by expression of Doc2 proteins lacking Ca^{2+} -binding sites,

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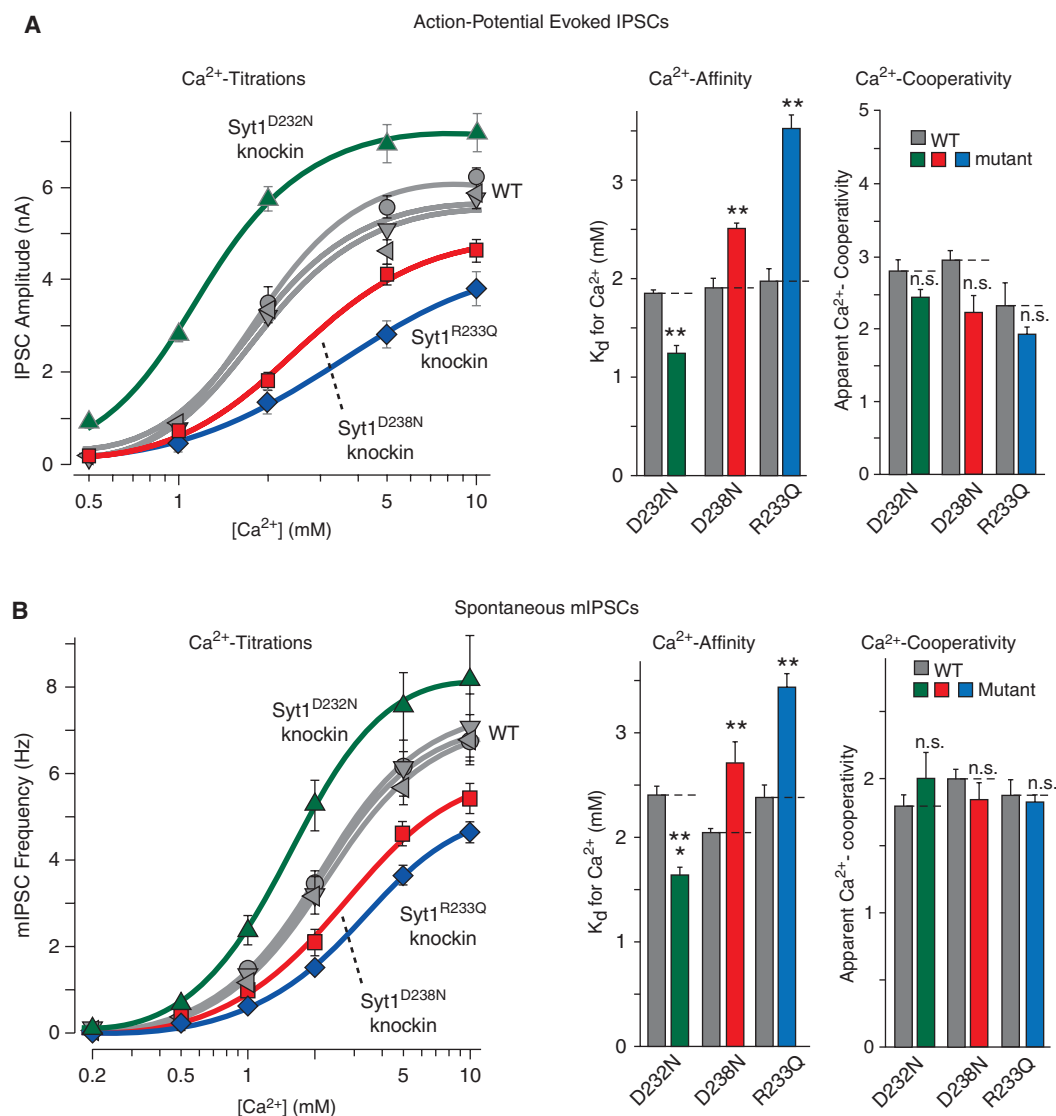


Figure 4. Syt1 Ca²⁺-binding site point mutations illustrate Syt1 function as a Ca²⁺ sensor for evoked and spontaneous neurotransmitter release. (A) Evoked IPSCs measured in neurons cultured from knockin mice carrying single amino-acid substitutions in the Ca²⁺-binding site of the Syt1 C2A-domain. Three knockin mice, each with an individual littermate control (gray) were analyzed: mice with the D232N substitution that increases the amount of Ca²⁺-stimulated SNARE-complex binding by Syt1 (green; Pang et al. 2006); the R233Q substitution that greatly decreases the apparent Ca²⁺ affinity of Syt1 during phospholipid binding (blue; Fernández-Chacón et al. 2001); and the D238N substitution that modestly decreases the apparent Ca²⁺ affinity of Syt1 (red; Pang et al. 2006). Action-potential evoked IPSCs were measured at the indicated concentrations of extracellular Ca²⁺; the plot on the left depicts the absolute IPSC amplitude as a function of the extracellular Ca²⁺ concentration, whereas the summary graphs on the right display the parameters for the apparent Ca²⁺ affinity and Ca²⁺ cooperativity of release obtained by fitting individual Ca²⁺ titration experiments to a Hill function. (B) Same as (A), except that the frequency of spontaneous release events was measured. For both (A) and (B), data are means \pm S.E.M. (Figure is adapted from Xu et al. 2007.)

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indicating that Doc2 proteins do not function as Ca^{2+} sensors for spontaneous exocytosis (Pang et al. 2011). Moreover, the Doc2 knock-down had no effect on the increased spontaneous release observed in Syt1 KO neurons, and also did not affect asynchronous release, suggesting that Doc2 proteins do not function in these types of release.

COMPLEXINS AS SYNAPTOTAGMIN SIDEKICKS

Complexins were identified as SNARE-complex binding proteins, giving rise to their name (McMahon et al. 1995). Complexins are small soluble proteins of approximately 120 residues that are evolutionarily conserved in all animals similar to synaptotagmins. The crystal structure of complexin bound to the assembled SNARE complex revealed that complexin contains a central short ~ 20 -residue α -helix that nestles into the groove formed by the synaptobrevin and syntaxin SNARE motifs in the SNARE complex (Chen et al. 2002). In addition, complexin contains a similarly short accessory amino-terminal α -helix that does not contact the SNARE complex in the crystal structure, with amino- and carboxy-terminal unstructured sequences flanking the two helices. Interestingly, complexin binds to the SNARE complex in an antiparallel orientation at a membrane-proximal site of the complex, suggesting that complexin can only bind to the SNARE complex after it has assembled to more than 50%.

Mammals express four complexin isoforms, two that are widely distributed in the body and abundant in brain (complexin-1 and -2; McMahon et al. 1995), and two additional isoforms that are enriched in retina (complexin-3 and -4; Reim et al. 2005). Complexin-3 and -4 include a carboxy-terminal isoprenylation sequence that is absent from complexin-1 and -2 and likely attaches complexin-3 and -4 to the plasma membrane. Deletion of complexin-1 and -2 in mice led to a striking phenotype that represented a milder phenocopy of the Syt1 knockout phenotype, suggesting that complexins act in the same pathway as synaptotagmins (Reim et al. 2001). Specifically, these

mice exhibited a selective but only partial loss of synchronous neurotransmitter release, without a major decrease in asynchronous release. Subsequent knockdown experiments using shRNAs confirmed this phenotype, but extended it to reveal an increase in spontaneous release analogous to that observed in Syt1 knockout neurons, with the same shift to a higher apparent Ca^{2+} affinity and lower apparent Ca^{2+} cooperativity (Maximov et al. 2009; Yang et al. 2010). Thus, complexins act both as activators and as clamps of neurotransmitter release. Moreover, unlike the Syt1 KO, which had no effect on the size of the readily-releasable pool, the complexin knockdown produced a $\sim 40\%$ decrease in the readily-releasable pool (Yang et al. 2010). Rescue experiments revealed that SNARE-complex binding by complexin is essential for its function (Maximov et al. 2009). Moreover, such experiments showed that the complexin amino terminus is crucial for its activating role (Xue et al. 2007), whereas its accessory α -helix mediates its clamping function (Maximov et al. 2009; Yang et al. 2010). Considerable controversy developed at one point about whether or not complexins have a clamping function, in that such function was not obvious in autapses (Reim et al. 2001), but was the only function observed in in vitro fusion assays (Giraud et al. 2009). Although the activation function of complexin is likely more important overall than its clamping function, the fact that the two functions can be localized to distinct autonomously acting sequences in complexin (Maximov et al. 2009; Yang et al. 2010) and that both phenocopy a function observed for Syts strongly supports their physiological importance.

How do complexin and Syts act in promoting Ca^{2+} triggering of synaptic vesicle fusion? Biochemical data indicate that the central α -helix of complexin and Syt1 bind to SNARE complexes at overlapping sites, and that Ca^{2+} binding to Syt1 triggers the displacement of the central α -helix of complexin from its binding site on SNARE complexes (Tang et al. 2006). Based on these electrophysiological and biochemical studies, a plausible current model is that complexin binding to primed synaptic

vesicles containing partially assembled SNARE complexes “superprimes” these vesicles into an activated state, and simultaneously clamps them (Fig. 5). Ca^{2+} binding to synaptotagmin then triggers synaptotagmin binding to the SNARE complex and the phospholipid bilayer, dislodging the complexin clamp and pulling on the SNARE complex, thereby opening the fusion pore. This model accounts for most of the existing data, although it offers no explanation for the clamping function of synaptotagmin, and thus is unlikely to be definitive.

SYNAPTOTAGMINS AS Ca^{2+} SENSORS FOR OTHER FORMS OF EXOCYTOSIS

Many forms of exocytosis are induced by Ca^{2+} , including hormone exocytosis in endocrine cells such as chromaffin cells, neuropeptide

secretion in neurons, mast cell degranulation, and the acrosome reaction in sperm. Strikingly, for most of these forms of exocytosis the same Syts that mediate synaptic vesicle fusion (Syt1, Syt2, and Syt9) appear to be involved; in addition, Syt7 was also shown to play a central role. Specifically, for chromaffin granule exocytosis in chromaffin cells, nearly all Ca^{2+} triggering is mediated in equal parts by Syt1 and Syt7, such that only the double Syt1/7 KO has a major phenotype in exocytosis (Schonn et al. 2008). In contrast, in pancreatic insulin- and glucagon-secreting cells, Syt7 appears to be the dominant Ca^{2+} sensor (Gustavsson et al. 2008, 2009), whereas in mast cell degranulation, Syt2 is the most important Ca^{2+} sensor (Mellor et al. 2009). In addition, Syt7 has been linked to exocytosis in non-neuronal cells, although it is not quite clear whether this is a physiological

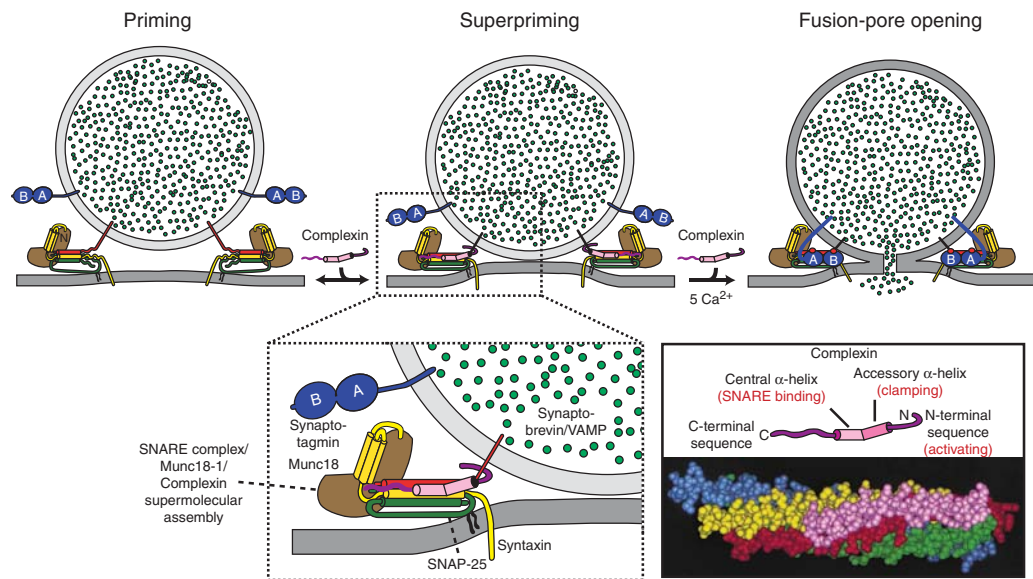


Figure 5. Schematic diagram of the mechanism of action of synaptotagmin and its complexin cofactor in Ca^{2+} -triggered exocytosis. The *top* diagram displays the sequential priming of synaptic vesicles by partial SNARE/SM protein complex assembly, superpriming by binding of complexin to partially assembled SNARE complexes, and Ca^{2+} triggering of fusion-pore opening by Ca^{2+} binding to synaptotagmin. The objects in the diagram approximate real-size relationships. The diagram on the *bottom left* depicts a cartoon of a partially assembled SNARE/SM protein complex with complexin bound. The functional domain structure of complexin is shown on the *right*, with a space-filling model of complexin bound to a SNARE complex at the bottom (pink, the two α -helices of complexin; yellow, syntaxin-1; red, synaptobrevin; green and blue, SNAP-25). (The model and the functional assignment of complexin sequences are modified from Maximov et al. 2009; the complexin/SNARE complex crystal structure is modified from Chen et al. 2002.)

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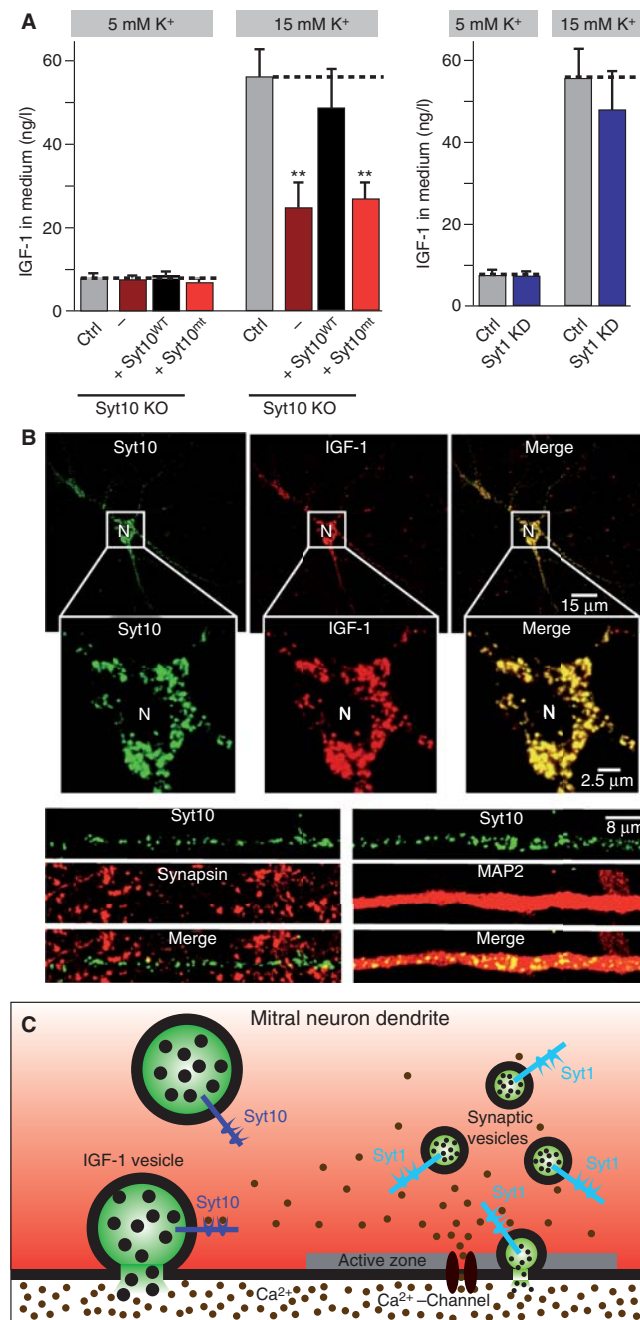


Figure 6. Illustration of distinct but similar functions of different synaptotagmins: Syt10 acts as a Ca²⁺ sensor for IGF-1 containing vesicles in mitral neurons from olfactory bulb. (A) IGF-1 secretion from cultured olfactory bulb neurons is stimulated by depolarization with 15 mM KCl, but impaired by deletion of Syt10 (this and the following panels were modified from Cao et al. 2011). (B) Syt10 colocalizes with IGF-1 containing vesicles in the somato-dendritic regions of cultured mitral neurons. Images show double immunofluorescence labeling of tagged Syt10 and either IGF-1, synapsin, or MAP2 as indicated. (C) Illustration of the distinct functions of Syt1 and Syt10 as Ca²⁺ sensors exclusively for synaptic and IGF-1-containing vesicles, respectively, in mitral neurons.



event (Martinez et al. 2000; Jaiswal et al. 2004; Wang and Hilgemann 2008). At least in chromaffin cells, complexins are obligatory cofactors for Syt-triggered exocytosis (Cai et al. 2008). Thus, despite the different kinetics of various types of exocytosis and the different localizations of various Syts, the same Syt paradigm appears to widely operate for all of these forms of exocytosis.

Until recently, no definitive function was identified for the second class of Ca^{2+} -binding Syts that contain amino-terminal disulfide bonds, which lead to constitutive dimerization (Fig. 2A). These Syts exhibit a higher apparent Ca^{2+} affinity than Syt1, and also interact with phospholipids and SNAREs in a Ca^{2+} -dependent manner (Li et al. 1995; Sugita et al. 2002), suggesting that they may also function as Ca^{2+} sensors in exocytosis. This hypothesis was recently confirmed for Syt10, which was identified as the Ca^{2+} sensor for exocytosis of IGF-1 containing secretory vesicles in mitral neurons from the olfactory bulb (Cao et al. 2011). Interestingly, in the same neurons Syt1 functions as the Ca^{2+} sensor for synaptic vesicle exocytosis, and the two Syts act completely independently of each other (Fig. 6). Thus, different synaptotagmin isoforms can operate in the same cell to mediate the Ca^{2+} -regulated fusion of distinct types of secretory vesicles.

CONCLUSIONS

Over the last two decades, a universal mechanism by which Ca^{2+} binding to Syts triggers fusion has emerged. This process involves a pas de deux of Syts and complexin, which act simultaneously on SNARE complexes and phospholipid membranes. The mechanism ensures the millisecond control of neurotransmitter release from presynaptic terminals on arrival of an action potential, but goes far beyond the synapse. The mechanism similarly applies to many other Ca^{2+} -induced secretory events, as exemplified by the role of Syts and complexins in neuroendocrine secretion including IGF-1 exocytosis, and might further extend to all Ca^{2+} -regulated membrane trafficking events in general.

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