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Molecular Underpinnings of Synaptic Vesicle Pool Heterogeneity

Devon C. Crawford¹ and Ege T. Kavalali^{1,2,*}

¹Department of Neuroscience, UT Southwestern Medical Center

²Department of Physiology, UT Southwestern Medical Center

Abstract

Neuronal communication relies on chemical synaptic transmission for information transfer and processing. Chemical neurotransmission is initiated by synaptic vesicle fusion with the presynaptic active zone resulting in release of neurotransmitters. Classical models have assumed that all synaptic vesicles within a synapse have the same potential to fuse under different functional contexts. In this model, functional differences among synaptic vesicle populations are ascribed to their spatial distribution in the synapse with respect to the active zone. Emerging evidence suggests, however, that synaptic vesicles are not a homogenous population of organelles, and they possess intrinsic molecular differences and differential interaction partners. Recent studies have reported a diverse array of synaptic molecules that selectively regulate synaptic vesicles' ability to fuse synchronously and asynchronously in response to action potentials or spontaneously irrespective of action potentials. Here we discuss these molecular mediators of vesicle pool heterogeneity that are found on the synaptic vesicle membrane, on the presynaptic plasma membrane, or within the cytosol and consider some of the functional consequences of this diversity. This emerging molecular framework presents novel avenues to probe synaptic function and uncover how synaptic vesicle pools impact neuronal signaling.

Keywords

vesicle pools; spontaneous neurotransmission; asynchronous neurotransmission; synchronous neurotransmission; vesicle exocytosis; synaptic vesicle; synaptic transmission; neurotransmitter release; SNARE proteins

Introduction

Synaptic connections between neurons are vitally important for information transfer throughout the nervous system. At chemical synapses action potentials cause calcium influx into the presynaptic terminal, which typically triggers the fusion of synaptic vesicles with the presynaptic active zone membrane and the release of neurotransmitter; however, neurotransmitter can also be released in the absence of action potentials. The neurotransmitter then travels across the synaptic cleft to bind postsynaptic receptors, which subsequently send an excitatory or inhibitory signal to the postsynaptic neuron. It is

*Corresponding Author: Ege Kavalali, Department of Neuroscience, UT Southwestern Medical Center, 5323 Harry Hines Blvd., Dallas, TX 75390-9111, Phone: 214-648-1628, Fax: 214-648-1801, Ege.Kavalali@UTSouthwestern.edu.

becoming clear that synaptic vesicle trafficking and exocytosis require many interwoven molecular components that have yet to be fully untangled (1). Many molecular mediators of trafficking and exocytosis have been identified, but the contributions of each molecule to various types of exocytosis and the resulting effects on information flow remain incompletely elucidated.

Historically synaptic vesicles have been divided into a number of “pools” based on the contexts under which they travel to the presynaptic membrane and exocytose. For example, vesicles have been categorized as part of the readily releasable pool, the recycling pool, the reserve pool, or the resting pool depending on their relative rates of trafficking in response to stimulation (2-5). Readily releasable vesicles are generally assumed to be both docked and primed at the presynaptic active zone membrane and probabilistically fuse in response to a single action potential while the recycling pool consists of all vesicles that are recruited for release during strong or prolonged stimulation. Vesicles in the reserve pool, in contrast, are extremely reluctant to traffic to the presynaptic membrane for fusion, and their function in neuronal signaling under physiological conditions remains unclear (2). This pool of vesicles, therefore, has also been referred to as the “resting pool” (3, 5). Few molecular mediators that differentiate vesicles into these various pools have been identified; instead, these pools remain defined by their functional properties and, in some cases, ultrastructurally defined distances between vesicles and the active zone (2, 3, 5-7, but see 8).

Recent emphasis has been placed on defining vesicle pools by their molecular components and the temporal relationship of vesicle release to the arrival of presynaptic action potentials (Figure 1). This classification divides vesicles into those that are released synchronously with the arrival of an action potential, those that are released asynchronously in the wake of an action potential, and those that are released spontaneously in the absence of action potentials (9-11). In addition to functional studies demonstrating these forms of release, molecular evidence suggests that a concurrent molecular framework underlies this vesicle pool classification (11-14). Here we will describe key molecular players that sort vesicles into functionally distinct pools and discuss potential implications for nervous system function.

Functional Segregation of Vesicle Pools

Researchers have been measuring postsynaptic responses to presynaptic activity since the mid-twentieth century (15), although synaptic vesicles themselves were not yet discovered (16, 17). In response to electrical stimulation, vesicles release neurotransmitters that, in turn, induce large, synchronous currents in the postsynaptic cell that can be measured using electrophysiological techniques (11). These “evoked” synchronous events often precede asynchronous currents that, although induced by stimulation, are produced by delayed vesicle fusion events (11, 18). In contrast small, so-called “miniature” postsynaptic events, first detected in 1952 (19), still occur in the absence of action potential stimulation and represent small numbers of vesicles fusing with the presynaptic membrane (10, 11). These miniature events exhibit a seemingly random temporal pattern and have been, therefore, referred to as spontaneous events despite their sensitivity to presynaptic calcium signaling (20).

These three forms of vesicle release – synchronous evoked, asynchronous evoked, and spontaneous – can be studied separately even when an experimental preparation is capable of all forms of release, but distinguishing the behavior of individual synapses and vesicles has not been possible until recently. For example early work using the neuromuscular junction preparation disagreed on whether spontaneously released vesicles and vesicles released in response to stimulation resided at the same release sites (21-24), but now it is clear that, although some synapses favor one form of release over the other, many boutons produce both types of release (25-29). The ability to tease apart how these types of release are modulated and produced has become paramount to understanding synaptic transmission in the nervous system.

Many studies have described differential modulation of each form of fusion, suggesting that these vesicle pools are molecularly distinct (14, 30). It has been difficult to distinguish asynchronous and synchronous evoked release with a functional assay, besides observing their respective timing in response to action potentials, because these two pools may share vesicles and activate the same postsynaptic receptor populations (9, 26, 31, 32), but vesicles released spontaneously often can be modulated independently from those that are released in response to stimulation. For example, *Drosophila shibire* mutants undergo temperature-sensitive inhibition of neurotransmission and recover evoked neurotransmission more easily than spontaneous neurotransmission, with functional recovery correlating with different stages of presynaptic ultrastructural recovery (33). The $G_{i/o}$ protein-coupled GABA_B receptor agonist baclofen inhibits evoked excitatory, spontaneous excitatory, and evoked inhibitory events but not spontaneous inhibitory events in hippocampal slice cultures and cerebellar slices (34, 35). Additionally cadmium, a calcium channel blocker, inhibits evoked but not spontaneous neurotransmission in some preparations, suggesting that spontaneous and evoked events are differentially dependent upon calcium influx (34). Application of nitrosonium donors to cultured hippocampal neurons simultaneously inhibits evoked excitatory neurotransmission while enhancing spontaneous excitatory neurotransmission (36). Similarly a recent study in rat hindbrain slices demonstrated that $G_{i/o}$ protein-coupled cannabinoid receptor 1 activation selectively inhibits evoked but not spontaneous neurotransmission while TRPV1 receptor activation inhibits evoked neurotransmission and facilitates spontaneous neurotransmission (37, 38). These studies make it clear that spontaneous and evoked neurotransmission are functionally separable under particular experimental contexts.

The differential modulation of these forms of vesicle release suggests that they originate from distinct vesicle pools. Investigators have tagged spontaneously recycled and stimulation-evoked vesicles independently with fluorescent probes or dyes and shown that the release properties of these tagged vesicles are substantially different (39-42), although other studies have detected complete overlap between the pools (43-46). Blocking excitatory postsynaptic receptors activated by evoked neurotransmission does not inhibit receptors activated by spontaneous events and *vice versa* (26), suggesting that vesicles from different pools fuse to the plasma membrane at distinct locations, allowing their neurotransmitter molecules to bind separate postsynaptic receptors. To determine whether these vesicle pools are truly distinct, it has become necessary to molecularly dissect vesicle trafficking

pathways. Proteins involved in endocytosis likely contribute to this vesicle sorting (47-49), and vesicle biogenesis mechanisms have been reviewed in this *Traffic* review series and elsewhere (50-52). This review, however, will focus on how vesicles are differentially trafficked to the plasma membrane for exocytosis.

During exocytosis, vesicular soluble NSF attachment protein receptor (v-SNARE) proteins bind to target membrane SNARE (t-SNARE) proteins to form a complex that allows the vesicular and plasma membranes to fuse. Canonically the v-SNARE synaptobrevin 2 (syb2; also known as vesicle-associated membrane protein 2 or VAMP2) binds to t-SNAREs syntaxin 1 and synaptosomal-associated protein of 25 kDa (SNAP-25) to bring the membranes together for fusion and release of neurotransmitter, a process that is catalyzed by calcium binding to the canonical calcium sensor synaptotagmin 1 (syt1) (53). Increasing the number of SNARE proteins facilitates vesicle fusion synchrony *in vitro*, but only a small number of SNARE proteins is required for fusion to occur (54, 55), so many cooperative factors modulate the synchrony of exocytosis. A variety of synaptic proteins have been identified that regulate fusion, including proteins located in the vesicle membrane, the plasma membrane, and the cytosolic space between the membranes (Figure 2) (53). Molecular, genetic, and functional studies are now identifying which of these proteins sort vesicles into synchronous, asynchronous, and spontaneous vesicle pools.

Vesicle-associated Proteins that Segregate Vesicle Pools

The vesicle membrane in particular contains a variety of proteins important for vesicle exocytosis (56-58). Evidence suggests that some v-SNAREs, calcium sensors, and other vesicular proteins participate in trafficking vesicles to segregated vesicle pools for release (Figure 2), so vesicles may have distinct molecular identities germane to their functional properties.

Vesicular SNAREs

There are multiple v-SNAREs that participate in synaptic vesicle trafficking and fusion with the plasma membrane (Figure 1; Table 1), but the canonical v-SNARE syb2 is the primary v-SNARE responsible for synchronous evoked vesicle release. Syb2 is one of the most abundant vesicle proteins (56, 58), so it is unsurprising that it drives the majority of neurotransmission. In cultured mouse hippocampal neurons deficient in syb2, evoked neurotransmission is all but abolished while spontaneous neurotransmission persists, although at a decreased event frequency (59, 60). This phenotype is also observed in *Drosophila* neurons deficient in the synaptobrevin-like protein n-syb (61-65). Similarly, vesicles deficient in syb2 recycle styryl dye with kinetics resembling control vesicles during spontaneous neurotransmission (39), suggesting that only spontaneous neurotransmission remains. Tetanus toxin, which cleaves syb2 (66), severely impairs evoked neurotransmission while decreasing but not eliminating spontaneous neurotransmission (61, 67-69). Additionally, vesicles tagged with a membrane probe during stimulation selectively co-label with antibody against syb2 compared to vesicles tagged during spontaneous neurotransmission (70), suggesting that syb2 is found in a higher proportion of evoked vesicles than spontaneously recycling vesicles. Recently, studies using mutated syb2 have found that juxtamembrane and transmembrane regions of the protein control the strength of

evoked neurotransmission and its balance with spontaneous neurotransmission (71-77). Altogether, these studies suggest that syb2 primarily traffics vesicles to the plasma membrane for release during evoked synchronous neurotransmission. It is possible that the small number of syb2-containing vesicles that is released spontaneously interacts stochastically with the spontaneous release machinery, but these vesicles could also contain a combination of additional vesicular proteins that is distinct from vesicles that swiftly respond to stimulation.

Synaptobrevin 1 (syb1, also known as VAMP1) is a v-SNARE related to syb2 that also promotes synchronous vesicle fusion. Syb1 is expressed on synaptic vesicles and is found widely throughout the nervous system, but syb1 expresses at relatively different levels than syb2 in most brain regions and is more highly expressed than syb2 peripherally (56, 58, 78-81). Null mutations in mouse syb1 cause both spontaneous and synchronous evoked release to decrease with a marked increase in asynchronous release at the neuromuscular junction (82). In cultured, autaptic hippocampal neurons from syb2 knockout mice, heterologous syb1 expression rescues synchronous evoked release to a greater degree than spontaneous release (83). Although syb1 promotes both evoked and spontaneous fusion, these results suggest that it functions primarily to synchronize evoked fusion events.

In contrast to syb2 and syb1, which facilitate synchronous evoked release, v-SNARE VAMP4 facilitates asynchronous evoked release. VAMP4 is better known for its trafficking role in endosomes and the trans-Golgi network (84-88), but it also localizes to synaptic vesicles (84, 89, 90), although less abundantly than syb2 (58). Knockdown of VAMP4 in cultured hippocampal neurons reduces the asynchronous phase of inhibitory postsynaptic currents after strong stimulation, and slow calcium chelation inhibits VAMP4-, but not syb2-, mediated neurotransmission when these proteins are overexpressed in syb2-deficient neurons (90). Additionally, vesicles expressing pH-sensitive fluorescent tags fused to VAMP4 are more reluctant to release in response to stimulation than vesicles expressing tagged syb2 (90). Although VAMP4 also drives a small amount of synchronous evoked and spontaneous neurotransmission (70, 90), the majority of release maintained by VAMP4-containing vesicles occurs in a desynchronized manner.

Recently it was discovered that the v-SNARE vps10p tail interactor 1a (vti1a) participates in spontaneous vesicle recycling. Vti1a localizes mostly to the Golgi apparatus (85, 88, 89), but at least one splice variant has been identified in synaptic vesicles (58, 91, 92). Vti1a deficiency does not produce global protein trafficking or developmental deficits (93, 94), but loss of vti1a in adrenal chromaffin cells reduces the number of dense core vesicles available for hormone secretion (95). Vti1a knockdown in cultured hippocampal neurons reduces the frequency of spontaneous neurotransmission events measured via electrophysiology, and fluorescently tagged vti1a-expressing vesicles do not traffic in response to stimulation as effectively as tagged syb2-expressing vesicles (70, 96, 97), suggesting that this decrease in neurotransmission is not caused solely by vesicle depletion. Spontaneously fused vti1a-containing vesicles are also trafficked independently of syb2-containing vesicles within the same synaptic bouton, further suggesting that these two v-SNAREs drive different pools of vesicles (97). Vti1a-containing synaptic vesicles, therefore, appear to traffic spontaneously to the plasma membrane for fusion.

VAMP7 (also known as tetanus toxin-insensitive VAMP, or TI-VAMP) is another v-SNARE that participates in protein trafficking but also drives spontaneous synaptic transmission (98). VAMP7 is widely expressed and localizes mainly to endosomes, lysosomes, and somatodendritic compartments (91, 99, 100), a pattern that is driven by its longin domain (101, 102). VAMP7 also promotes neurite outgrowth during development (103-105) and is enriched in synaptic vesicles of mature hippocampal mossy fiber terminals (106, 107). VAMP7 knockout mice, however, develop normally and express only subtle behavioral phenotypes (108, 109). When VAMP7 is tagged with a pH-sensitive pHluorin and overexpressed in cultured hippocampal neurons, electrical stimulation induces less vesicle fusion than when syb2-pHluorin is used (97, 110). Also, a much smaller fraction of VAMP7-containing vesicles than syb2-containing vesicles participates in the stimulation-driven recycling pool (97, 110). Recently reelin, a glycoprotein previously shown to signal postsynaptically, was found to drive presynaptic spontaneous vesicle fusion by recruiting VAMP7-containing vesicles but not syb2-, VAMP4-, or vti1a-containing vesicles for exocytosis (111). VAMP7-containing synaptic vesicles, therefore, can be recruited for action potential-independent fusion events.

Vesicle-Associated Calcium Sensors

Calcium sensors are proteins that bind calcium and subsequently catalyze vesicle fusion (112, 113). There are multiple calcium sensors that contribute to specific forms of neurotransmission (Table 2), but one of the most-studied is syt1, which promotes synchronous evoked vesicle fusion. Syt1 is the most abundant synaptotagmin at mammalian synapses (56-58) and is widely expressed in the rodent brain (114-117). In cultured neurons from syt1 knockout mice, synchronous evoked release measured via electrophysiology is abolished while asynchronous release and the frequency of spontaneous events are increased (118-121), an effect that is also seen in *Drosophila* neurons deficient for syt1 (122-125). Vesicles tagged during evoked neurotransmission are also enriched with syt1 compared to vesicles tagged during spontaneous neurotransmission (70), confirming syt1's propensity for synchronously fused vesicles. Interestingly, some inhibitory neurons in hippocampal slice culture and excitatory autaptic neurons in dissociated culture are less affected by syt1 deficiency (126, 127), suggesting that syt1 is not the primary calcium sensor for all synchronous release even within the same brain region. Many studies have been performed to determine how the C2A and C2B domains of syt1 bind calcium and differentially modulate synchronous, asynchronous, and spontaneous release (128-138), but there is an emerging consensus around the notion that these domains cooperate to promote synchronous fusion and clamp delayed fusion (139-144). Overall, these studies suggest that vesicles containing syt1 are likely to be trafficked to the plasma membrane for synchronous evoked release.

Syt2 is another calcium sensor for synchronous evoked vesicle fusion. Syt2 is expressed in rodent brain less abundantly than syt1 and in relatively different regions (58, 114, 115, 117, 145). It also exhibits calcium-dependent binding to phospholipids and both calcium-dependent and calcium-independent binding to t-SNAREs via its C2 domains (146-148). Syt2 knockout or knockdown reduces evoked but increases spontaneous neurotransmission at the neuromuscular junction (145, 149), and Syt2 expression in cortical syt1 knockout

neurons rescues synchronous evoked neurotransmission (121). In calyx of Held synapses, which normally express syt2 rather than syt1, synchronous evoked neurotransmission is reduced when syt2 is knocked out or mutated (150-152). Syt2, therefore, promotes evoked synchronous release and clamps spontaneous release in a similar manner as syt1, but these two sensors are differentially expressed throughout the nervous system.

Other synaptotagmins also coordinate vesicle trafficking and fusion. Of the multiple known synaptotagmins expressed in brain tissue (117, 153), however, only a small minority have been shown both to localize to synaptic vesicle membranes and to differentially modulate these three forms of vesicle fusion. In addition to syt1 and syt2, syt9 and syt12 appear to meet these criteria. Syt9 expression in cortical syt1 knockout neurons rescues synchronous evoked neurotransmission, and syt9 deficiency in striatal neurons reduces synchronous evoked responses while leaving asynchronous fusion events unaffected (121). Overexpression of syt12, one of the few synaptotagmins that does not exhibit calcium-dependent binding to its targets (146, 154, 155), increases the frequency of inhibitory spontaneous events in cultured cortical neurons in a protein kinase A-dependent manner but does not alter synchronous or asynchronous evoked responses (156). The synaptotagmin protein family, therefore, exhibits diversity in its effects on neurotransmission.

Other Vesicle-associated Proteins

In addition to v-SNAREs and calcium sensors, other vesicular proteins also have reported roles in guiding vesicles to segregated release pools. For example, mouse hippocampal neurons deficient in rab3a, a GTP-binding protein, exhibit subtly increased evoked but normal spontaneous release while rab3a-deficient neuromuscular junctions produce reduced spontaneous yet normal evoked release (157-159). Synapsin 1, in contrast, differentially alters inhibitory and excitatory evoked neurotransmission without changing spontaneous neurotransmission (160, 161). Additionally inhibitory hippocampal neurons from synapsin 2 knockout mice display increased synchronous evoked and decreased asynchronous evoked and spontaneous neurotransmission (162). These proteins, therefore, may alter the likelihood that vesicles will release synchronously.

Plasma Membrane-associated Proteins that Segregate Vesicle Pools

Vesicular proteins interact with target membrane proteins to complete vesicle trafficking to the plasma membrane for subsequent fusion (53). The target membrane contains a variety of molecules (57, 58), including t-SNAREs and calcium channels, that affect vesicle release properties and, therefore, vesicle pool membership (Figure 2; Table 2; Table 3).

Target Membrane SNAREs

Syntaxin 1 is one of two canonical t-SNAREs recruited during neurotransmission. Syntaxin 1 is highly expressed throughout the nervous system, especially at presynaptic terminals (58, 163). This membrane-anchored protein dimerizes with the other canonical t-SNARE SNAP-25 before binding to v-SNAREs, and this dimer is capable of binding to VAMP4 and VAMP7, although less efficiently than syb2 (90, 164, 165). Syntaxin 1 knockout in *Drosophila* neuromuscular junctions abolishes all forms of neurotransmission except for

rare asynchronous fusion events (61, 64, 166), but syntaxin 1 hypomorphs exhibit small evoked responses while retaining spontaneous neurotransmission (166). Interference with binding sites on syntaxin 1A's H3 domain, which destabilizes SNARE complex formation, in *Drosophila* neuromuscular junction or mammalian hippocampal neurons severely impairs evoked neurotransmission with smaller effects on spontaneous neurotransmission (167, 168). Additionally, expression of syntaxin 1 mutants with residues added between the transmembrane domain and the SNARE binding domain reduces evoked but not spontaneous fusion (77). Knockdown of syntaxin 1 in cultured cortical neurons impairs all neurotransmission, but interestingly overexpression of syntaxin 1 with a mutated H_{abc} domain, which alters syntaxin 1's ability to bind Munc18-1, in syntaxin 1 knockdown neurons rescues inhibitory synchronous evoked neurotransmission but not spontaneous event frequency (169). Similarly hippocampal neurons deficient in syntaxin 1B exhibit decreased excitatory and inhibitory spontaneous event frequencies but normal excitatory and inhibitory synchronous evoked responses (170). Syntaxin 1A and 1B double knockout neurons from cortex, however, display impaired synchronous evoked and spontaneous neurotransmission but increased asynchronous evoked release (170). These studies suggest that syntaxin 1 is vital for neurotransmission, but different regions of the molecule or its different isoforms may be responsible for modulating each form of neurotransmission and, therefore, for guiding vesicles from different pools to the membrane for fusion.

Another syntaxin with the potential to differentially modulate these forms of neurotransmission is syntaxin 13. Syntaxin 13 is expressed in brain and is usually localized to endosomes (171). When syntaxin 13 is tagged with pHluorin and overexpressed in hippocampal neurons, it traffics to presynaptic terminals and participates in synaptic vesicle recycling (96). Co-expressing syntaxin 13-pHluorin with a soluble fragment of syntaxin 13, which acts dominant-negatively, decreases exocytosis after a short electrical stimulus train but increases exocytosis after longer stimulus trains and during a period of spontaneous neurotransmission (96). Although it is unclear whether syntaxin 13 participates directly in neurotransmission under physiological conditions, it is possible that this molecule promotes synchronous fusion events and clamps delayed and spontaneous events.

SNAP-25, the canonical t-SNARE that dimerizes with syntaxin 1, is also important for neurotransmission. SNAP-25 is almost exclusively expressed in the brain (172). It does not contain a transmembrane domain, but it is anchored to the membrane through palmitoylated cysteine residues and its interaction with syntaxin 1 (173-175). Inhibiting the C-terminus of SNAP-25 by introducing mutations or applying exogenous peptides reduces vesicular secretion from chromaffin cells (176, 177). Botulinum neurotoxin A, which cleaves SNAP-25 (178, 179), reduces both action potential-dependent and spontaneous neurotransmission in neurons from hippocampal slice culture (180). Temperature-sensitive SNAP-25 *Drosophila* mutants, however, exhibit smaller synchronous evoked response amplitudes but similar spontaneous event frequency changes at destabilizing temperatures when compared to wild type flies (181). Homozygous knockout of SNAP-25 in mouse, which is embryonically lethal, abolishes evoked muscle contractions at embryonic neuromuscular junctions while maintaining spontaneous neurotransmission (182). In cortical slices from embryonic SNAP-25 knockout mice, inhibitory evoked responses are absent

while some spontaneous neurotransmission remains (183). Similarly, in cultured neurons from SNAP-25 knockout mice, both excitatory and inhibitory evoked responses are abolished while a small amount of excitatory and inhibitory spontaneous neurotransmission remains (184), and these deficits are rescued by genetic reintroduction of SNAP-25 (185). A recent study has also reported that excitatory evoked neurotransmission is increased in SNAP-25 heterozygous neurons while inhibitory evoked responses are decreased and spontaneous neurotransmission is normal (186), suggesting that heterozygosity unmasks functions of SNAP-25 that are undetected in knockout neurons. C-terminal deletions in SNAP-25 severely impair synchronous and spontaneous forms of release, but interestingly N-terminal deletions and central mutations unclamp spontaneous release without altering synchronous evoked release, suggesting that different regions of the protein differentially modulate these forms of neurotransmission (187). Altogether, these studies suggest that SNAP-25 is essential for evoked neurotransmission but less vital for spontaneous neurotransmission.

SNAP-23 may also play a role in differentially promoting particular forms of neurotransmission. SNAP-23, which is structurally related to SNAP-25, is expressed in a wide variety of tissues, including the brain (172). SNAP-23-dependent vesicle docking occurs at lower calcium concentrations than SNAP-25-dependent docking *in vitro*, and SNAP-23 has a lower affinity for syt1 than SNAP-25 (188). Chromaffin cells from SNAP-25 knockout mice exhibit increased sustained release when SNAP-23 is overexpressed but stimulated bursts when SNAP-25 is reintroduced (189). Similarly, SNAP-25 knockout neurons overexpressing SNAP-23 exhibit excitatory and inhibitory asynchronous vesicle fusion, which contrasts with the rescue of synchronous evoked neurotransmission by SNAP-25 (185, 190). Although it is unclear whether SNAP-23 modulates physiological neurotransmission, it may function to promote asynchronous neurotransmission under defined contexts.

Plasma Membrane Calcium Sensors

Syt7, in contrast to syt1, syt2, and syt9, does not promote synchronous vesicle fusion and is not clearly present in synaptic vesicle membranes (191). It is widely expressed in the rodent brain (117, 192) and is found in lysosomes and at synapses (193-197), where it is much less abundant than syt1 (58). Although syt7 is reportedly found in large dense core vesicles of PC12 cells (197-199), electron microscopy studies of hippocampal neurons suggest that it localizes to the plasma membrane at synapses rather than to synaptic vesicles (192, 200). Syt7 exhibits a higher calcium affinity than syt1 (146, 201, 202), and its slow disassembly kinetics suggest that it is better suited for coordinating delayed rather than synchronous fusion events (148). This is consistent with syt7's ability to bind SNAP-23, the t-SNARE involved in asynchronous fusion events (188). Interestingly, syt7 has multiple splice variants that differentially modulate vesicle endocytosis (192, 200). Syt7 knockdown in neuromuscular junctions of zebrafish and *Drosophila* has no effect on synchronous evoked release as measured via electrophysiology, but the asynchronous component of release is reduced in zebrafish (149, 155). Inhibitory cortical neurons from syt7 knockout mice, however, do not display consistent deficits in evoked or spontaneous neurotransmission (203). In contrast inhibitory hippocampal syt1 knockout neurons, which lack synchronous

release, display markedly reduced asynchronous release when syt7 is knocked down (204). Additionally inhibitory spontaneous neurotransmission in wild type and syt1 knockout hippocampal neurons is not affected by syt7 knockdown, but overexpression of syt7 in syt1 knockout neurons reduces spontaneous neurotransmission (204). These data suggest that syt7 promotes asynchronous evoked release and possibly clamps spontaneous fusion, but these phenotypes are not apparent in mammalian neurons expressing an abundance of syt1.

Calcium Channels

Calcium channels also seem to be important for determining the temporal dynamics of vesicle fusion. Presynaptic calcium channels are known to interact directly with presynaptic release machinery, and this close interaction facilitates the coupling of stimulation-evoked calcium entry and vesicle fusion (205, 206). Calcium influx through voltage-gated calcium channels like N-type, P/Q-type, and R-type channels also mediates a portion of spontaneous fusion events (207). For example, cholecystokinin-expressing interneurons that synapse onto hippocampal dentate granule neurons exhibit higher levels of spontaneous neurotransmission than parvalbumin-expressing interneurons, and this effect has been linked to different relative amounts of N-type and P/Q-type calcium channels (208). It is becoming clear, however, that spontaneous and asynchronous vesicle pools do not always depend on these voltage-gated calcium channels for release (209). Blocking calcium channels with cadmium in cerebellar slices does not reduce inhibitory spontaneous event frequencies (210), and application of peptides that prevent calcium channel binding to SNARE proteins reduces evoked but increases asynchronous release in superior cervical ganglion neurons (211). Similarly, mutations in the *Drosophila* gene *fuseless*, which prevent calcium channel recruitment to presynaptic terminals of the neuromuscular junction, cause reduced evoked excitatory junctional currents but increased frequency and amplitude of spontaneous events (212). Phorbol ester-induced potentiation of evoked release in cultured hippocampal neurons does not appear to depend on L-type calcium channels while the phorbol ester-induced increase in spontaneous event frequency does (213, 214). Interestingly, excitatory spontaneous neurotransmission in cultured neocortical neurons appears to depend on a G protein-coupled calcium-sensing receptor while inhibitory spontaneous neurotransmission in the same preparation depends on nanodomain calcium entry from voltage-gated calcium channels (215, 216). These studies provide evidence that vesicle release from different pools can be guided by differential dependence on calcium influx from calcium channels located in the plasma membrane.

Cytosolic Proteins that Segregate Vesicle Pools

Synapses contain many soluble molecules that interact with vesicular and plasma membrane proteins and are important for modulating vesicle fusion (53, 58). Some of these proteins, as discussed below, also appear to be important for maintaining segregated vesicle pools (Figure 2; Table 2; Table 4).

Cytosolic Calcium Sensors

Potential cytosolic calcium sensors include the double C2 domain (doc2) protein family, which contains three proteins structurally similar to syt1: doc2 α , doc2 β , and doc2 γ . Doc2

proteins have two calcium-binding C2 domains and translocate to the plasma membrane after calcium influx to interact with the Munc13-1 priming protein, t-SNAREs, or the membrane itself (217-225). Excitatory hippocampal neurons and inhibitory cerebellar neurons deficient in doc2 α or both doc2 α and doc2 β exhibit normal synchronous evoked neurotransmission measured via electrophysiology (226, 227), but the frequency of spontaneous neurotransmission is reduced in doc2 α /doc2 β double knockout neurons (227). Additionally, asynchronous neurotransmission measured in the presence or absence of syt1 is decreased in excitatory hippocampal neurons with doc2 α , but not doc2 β , knocked down (228). Knocking down all three doc2 proteins along with the related protein rabphilin reduces spontaneous neurotransmission in excitatory and inhibitory cortical neurons but does not alter asynchronous release in inhibitory neurons (229). The precise role of doc2 proteins as calcium sensors in synaptic transmission is debated, but these proteins likely promote spontaneous and possibly asynchronous neurotransmission.

Active Zone Proteins

Biochemical priming of vesicles after docking at the presynaptic active zone is vitally important for vesicle fusion (53). One such priming protein, Munc13-1, is presynaptically localized and is perinatally lethal when knocked out in mice (58, 230, 231). Munc13-1 has also been proposed to function as a calcium sensor at the presynaptic terminal (232). Cultured Munc13-1 knockout neurons from hippocampus exhibit impaired evoked excitatory neurotransmission but relatively spared spontaneous event frequencies (233, 234). In mice doubly deficient for Munc13-1 and the related protein Munc13-2, neuromuscular junction synapses exhibit decreased evoked response amplitudes and increased spontaneous event frequencies (235). Knocking in a Munc13-1 mutation that cannot bind phorbol esters onto a Munc13-2 knockout background produces murine hippocampal neurons with higher basal spontaneous event frequencies without altering evoked response amplitudes, suggesting that vesicle pools can be differentially modulated by the phorbol ester binding region of Munc13-1 (236). In *Caenorhabditis elegans*, neurons express two forms of unc13: a long form that is analogous to mammalian Munc13-1 and ubMunc13-2 and a short form that is analogous to mammalian bMunc13-2 and Munc13-3 (237). The long form of *C. elegans* unc13 localizes closer to active zones than the short form, and expression of the long form in a double deficiency background rescues evoked synchronous release while expression of the short form rescues asynchronous evoked release, with either form rescuing spontaneous event frequency (238).

Similarly, removing the N-terminal C2A domain of the long form of unc13 impairs synchronous evoked and spontaneous release, and acute ablation of a diffusely localized unc13 isoform selectively impairs asynchronous release (239). This is consistent with mammalian calyx of Held, in which Munc13-2 and Munc13-3 double knockout produces reduced asynchronous release, but introducing a dominant-negative Munc13-1 into the double knockout background reduces overall evoked response amplitudes (240). Overall, Munc13-1 promotes synchronous evoked vesicle fusion while other Munc13 isoforms facilitate asynchronous fusion events.

Munc13-1 is known to interact with additional proteins in the presynaptic terminal like Rim1. Rim1 α is found near active zones (241, 242), and knocking out Rim1 α in mice is perinatally lethal when combined with knockout of the related protein Rim2 α (243). Double knockout of Rim1 α and Rim2 α reduces both evoked and spontaneous release in neuromuscular junctions, hippocampal neurons, and calyx of Held synapses, putatively due to reduced vesicle docking near calcium channels (243-245). Munc13-1 levels are decreased in Rim1 α single knockout mouse neurons (246), and hippocampal autaptic as well as slice neurons from Rim1 α knockout mice exhibit reduced excitatory evoked neurotransmission, with a relatively more reduced asynchronous component, and normal spontaneous event frequency (246, 247). Neuromuscular junction synapses from unc10/Rim1 *C. elegans* mutants also express decreased evoked responses but instead exhibit decreased spontaneous event frequencies (248). Rim1 α , therefore, appears to promote synchronous evoked release in general but also asynchronous evoked and spontaneous release in particular contexts.

Tomosyn, another soluble active zone protein, is a SNARE protein that differentially affects vesicle fusion events. *In vitro* studies suggest that tomosyn binds to t-SNAREs to create a SNARE complex that clamps membrane fusion until fusion-promoting v-SNAREs displace it (249-253). Tomosyn inhibits both synchronous and asynchronous evoked responses in *C. elegans* neuromuscular junction and murine excitatory synapses without changing the spontaneous event frequency, and some of these effects seem to occur through clamping unc13/Munc13 activity (254-256). Tomosyn, therefore, appears to selectively modulate evoked fusion events.

Complexins

Complexins are another family of proteins that differentially modulate synchronous, asynchronous, and spontaneous forms of neurotransmission. Complexin I and complexin II are soluble proteins expressed in the nervous system both presynaptically and postsynaptically (58, 257-259). Complexin III is also widely expressed in the nervous system, albeit at lower levels than complexin I and complexin II, but complexin IV is restricted to ribbon synapses of the retina (260). Complexin III and complexin IV, unlike complexin I and complexin II, are anchored to membranes by their C-terminal domains (260-262). In cultured cortical neurons from mice doubly deficient in complexin I and complexin II, excitatory synchronous evoked response amplitudes measured via electrophysiology are reduced while excitatory spontaneous event frequencies are increased (262-265). Complexin deficiency in *Drosophila* neuromuscular junction, *C. elegans* neuromuscular junction, mouse auditory pathway, mouse olfactory bulb, and zebrafish retinal bipolar cells also produces these effects on neurotransmission (266-272). Interestingly autaptic hippocampal neurons deficient in both complexin I and complexin II exhibit reduced EPSC amplitudes but no increase in spontaneous event frequency (273-275). Complexin deficiency may also increase the relative amount of asynchronous release present in the evoked response (263, 267, 269, 273), even though complexin does not bind VAMP4-containing SNARE complexes that select for asynchronous release (90). Various *in vitro* vesicle fusion assays and complexin mutants have been employed to determine the biochemical basis of complexin's differential effects. The central α -helix of the protein seems to interact with SNARE machinery to dock and clamp vesicles from synchronous and

spontaneous pools to await calcium influx (263, 264, 270, 274, 276-278) while the negative electrical charges on the accessory α -helix function to clamp neurotransmission (263, 264, 270, 272, 274, 279). The N-terminus promotes fusion events normally suppressed by the accessory α -helix (264, 270, 274, 280), and the C-terminus, which differs between the soluble complexins I and II and the membrane-anchored complexins III and IV, promotes docking of vesicles while clamping spontaneous release (261, 262, 265, 272, 281, 282). Overall complexins seem to promote synchronous evoked vesicle fusion, while in some circumstances they also clamp spontaneous release.

Membrane Lipid Components that Modulate Vesicle Pools

There are additional molecules found in membranes that modulate the form of fusion a vesicle undergoes (Figure 2). For example, cholesterol content at the presynaptic terminal appears to promote synchronous evoked release. At crayfish and frog neuromuscular junction and in cultured hippocampal neurons, pretreating neurons with methyl- β -cyclodextrin to remove cholesterol inhibits evoked vesicle fusion as measured by electrophysiological and vesicle labeling techniques (283-285). Despite this reduction in evoked release, removing cholesterol from membranes increases the frequency of spontaneous fusion events (283-286). The effects of cholesterol on evoked, but not spontaneous, events are absent in hippocampal *syb2* knockout neurons (284). Because *syb2* is preferentially expressed in vesicles that fuse synchronously in response to stimulation (70, 97), this further suggests that cholesterol differentially affects vesicles belonging to different vesicle pools.

Functional Consequences of Segregated Vesicle Pools

The presence of molecularly and functionally distinct vesicle pools suggests that each form of neurotransmission has differential downstream effects on neuronal function. Information transfer throughout the nervous system has been classically assumed to depend on synchronous evoked release, due to its temporal precision, while asynchronous evoked and spontaneous release have been thought to modulate the information carried by the synchronous evoked signal (11). A recent study, however, showed that reducing synchronous evoked release in hippocampus, entorhinal cortex, or prefrontal cortex by knocking down *sytl* had only subtle effects on fear conditioning memory tests in mice (287), suggesting that asynchronous release is sufficient to deliver the majority of the informational signal. Spontaneous neurotransmission, in turn, modulates action potential generation in postsynaptic neurons (288-290) and homeostatically scales postsynaptic receptor levels (291, 292), which is thought to occur via activation of postsynaptic signaling cascades that are distinct from those activated by evoked release (10, 14). Inhibiting spontaneous neurotransmission also appears to be the mechanism by which glutamate receptor blockers induce fast-acting antidepressant behaviors in rodents (293-295), suggesting that spontaneous neurotransmission indeed exerts potent effects on neurotransmission. Each form of neurotransmission, therefore, works to refine the signal being propagated through the circuit. Because of these distinct contributions to neurotransmission, determining the molecular basis of different vesicle pools is important for understanding nervous system function.

Conclusions

Evidence suggests that a complex array of presynaptic factors guides synaptic vesicles toward different modes of release. Even though classical vesicle pools have been defined by their release characteristics or ultrastructural localization (2-6), a molecular framework is now emerging where distinct vesicle populations can be functionally and molecularly distinguished (11, 12). Vesicular membrane proteins like v-SNAREs and calcium sensors give each vesicle a molecular identity while soluble factors like Munc13-1 or doc2 and plasma membrane proteins like t-SNAREs guide vesicles through segregated, though likely partially overlapping, fusion pathways. These molecularly defined vesicle pools are unlikely to be a static property of the synapse, however, due to activity-dependent rearrangements that arise from bulk vesicle endocytosis, lipid mixing, and exogenous factors that recruit particular vesicles under defined contexts. It is becoming increasingly clear that synaptic vesicle populations and their interaction partners exhibit greater heterogeneity than previously appreciated, and future work is needed to clarify the molecular and functional distinctions between synaptic vesicle pools.

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Synopsis

In this review, Crawford and Kavalali discuss the various molecular components required to traffic synaptic vesicles to the presynaptic plasma membrane for synchronous evoked, asynchronous evoked, or spontaneous fusion. It is becoming clear that synaptic vesicles exhibit distinct molecular identities and a variety of molecules interact with vesicles to modulate their exocytosis in relation to neuronal action potentials. These distinct forms of neurotransmission, in turn, are critical for proper information transfer and synaptic signaling homeostasis throughout the nervous system.

Figure 1. Presynaptic vesicle pools

A. Classically vesicles have been considered a homogenous population within the presynaptic terminal. Under this model synchronous evoked, asynchronous evoked, and spontaneous fusion events arise from a single population of presynaptic vesicles, and other factors besides vesicle heterogeneity (e.g. distance from the active zone) determine whether a vesicle is in the readily releasable, recycling, reserve, or resting pools. **B.** Recently molecules like vesicular SNARE proteins have been hypothesized to confer heterogeneity to presynaptic vesicle populations. For example, synaptobrevin 2 (syb2) vesicles mainly drive synchronous evoked release, vesicle-associated membrane protein 4 (VAMP4) vesicles primarily drive asynchronous evoked release, and vps10p tail interactor 1a (vti1a) and vesicle-associated membrane protein 7 (VAMP7) vesicles mostly drive spontaneous release. Some studies have also shown that neurotransmitter released from evoked and spontaneous vesicles activates separate populations of postsynaptic receptors, so fusion sites for these vesicle pools may be spatially segregated along the active zone.

Figure 2. Molecules contributing to presynaptic vesicle pool heterogeneity

Vesicles are shuttled to the presynaptic active zone for fusion and release of neurotransmitter synchronously in response to the arrival of an action potential, asynchronously after an action potential, or spontaneously in the absence of action potentials. Vesicular proteins (a) confer heterogeneity to synaptic vesicle populations while cytosolic and plasma membrane molecules (b) coordinate with vesicular proteins to determine the vesicle's ultimate fusion parameters.

Table 1

Vesicular SNAREs that segregate vesicle pools

Molecule	Manipulation	Experimental System	Synchronous Fusion	Asynchronous Fusion	Spontaneous Fusion	References
Synaptobrevin 2/n-synaptobrevin	KO/null mutation	Cultured mouse hippocampal neurons; <i>Drosophila</i> NMJ	↓↓	↓	↓	(59-64, 71)
	Juxtamembrane region mutations	Cultured mouse hippocampal neurons	↓	↓	↓	(71, 74)
	TMR mutations	Cultured mouse cortical neurons	↓	↓	=	(77)
	Tetanus toxin	<i>Drosophila</i> NMJ; Crayfish NMJ; <i>Torpedo</i> electric organ	↓↓	↓	↓	(61, 67-69)
Synaptobrevin 1	KO/null mutation	Mouse NMJ	↓	↑	↓	(82)
VAMP4	KD	Cultured inhibitory mouse hippocampal neurons	↓	↓↓	↓	(90)
Vti1a	KD	Cultured rat hippocampal neurons	=	ND	↓	(97)
	N-terminal deletion mutation	Cultured rat hippocampal neurons	=	ND	↑	(97)
VAMP7	reelin	Cultured rat hippocampal neurons	=	ND	↑	(111)
	KD + reelin	Cultured rat hippocampal neurons	ND	ND	=	(111)

Abbreviations: knockdown (KD), knockout (KO), neuromuscular junction (NMJ), not determined (ND), transmembrane region (TMR), vesicle-associated membrane protein (VAMP), vps10p tail interactor 1a (vti1a), abolished or nearly abolished (↓↓), significantly reduced (↓), similar to control (=), significantly increased (↑).

Table 2

Calcium sensors that segregate vesicle pools

Molecule	Manipulation	Experimental System	Synchronous Fusion	Asynchronous Fusion	Spontaneous Fusion	References
Synaptotagmin 1	KO/null mutation	Autaptic/low density mouse hippocampal neurons	↓↓ or ↓	↑	=	(118, 119, 127, 132, 133)
		Cultured mouse hippocampal or cortical neurons	↓↓ or ↓	↑ or =	↑	(120, 121, 127, 140, 150)
		Cultured mouse striatal neurons	↓	ND	ND	(121)
		Organotypic mouse hippocampal slice cultures	↓↓ or ↓	=	↑	(126)
	Acute ablation	<i>Drosophila</i> NMJ	↓↓ or ↓	↑	= or ↑	(122-124, 131, 134, 141)
		<i>Drosophila</i> NMJ	↓	ND	=	(125)
		Autaptic mouse hippocampal neurons	↓ or =	ND	=	(129, 132)
	C2A domain mutations	Cultured mouse cortical neurons; acute mouse cortical slices	↓ or ↑	ND	↓ or ↑	(139, 140)
		<i>Drosophila</i> NMJ	= or ↓	= or ↑	= or ↑	(136, 141-143)
		Autaptic mouse hippocampal neurons	= or ↓ or ↓↓	= or ↑	ND	(133, 137)
Synaptotagmin 2	C2B domain mutations	Cultured mouse cortical neurons	↓↓	ND	↑	(139, 140)
		<i>Drosophila</i> NMJ	↓↓ or ↓	= or ↑	= or ↑	(130, 131, 134, 135, 141-143)
		<i>Drosophila</i> NMJ	↑	ND	=	(143)
		<i>Drosophila</i> NMJ	↓↓	ND	↑	(143)
	C2A and C2B domain mutation + WT protein	Cultured mouse cortical neurons	↓	ND	=	(140)
		Cultured mouse hippocampal neurons	↓	ND	=	(144)
		Mouse NMJ; mouse calyx of Held	↓	↑	↑	(145, 151, 152)
	C2A and C2B domain mutation + KO/null mutation	Cultured mouse dorsal neostriatum neurons	=	ND	ND	(145)
		Zebrafish NMJ	↓	=	↑	(149)
		Mouse calyx of Held	↓	↑	↑	(150)
Synaptotagmin 2	Point mutation	Mouse NMJ	ND	ND	↑	(150)

Molecule	Manipulation	Experimental System	Synchronous Fusion	Asynchronous Fusion	Spontaneous Fusion	References
Synaptotagmin 7	C2A domain mutations	Autaptic mouse hippocampal neurons	=	ND	ND	(132)
	C2B domain mutations	Mouse calyx of Held	= or ↓	=	= or ↑	(152)
	Lentiviral expression + synaptotagmin 1 KO	Cultured mouse cortical neurons	=	ND	ND	(121)
	KO/null mutation	Cultured mouse hippocampal or inhibitory cortical neurons	=	= or ↓	=	(190, 203, 204)
	KO/null mutation + synaptotagmin 1 KO	Cultured mouse inhibitory cortical neurons	↑↑	↑	ND	(203)
	KO/null mutation + SNAP-25 KO + SNAP-23 lentiviral expression	Autaptic excitatory mouse hippocampal neurons	↑	↑	↑	(190)
	KD	Zebrafish NMJ	=	↓	=	(149)
		<i>Drosophila</i> NMJ	=	ND	ND	(155)
		Cultured mouse hippocampal neurons	=	=	=	(204)
		Acute mouse hippocampal slices	=	ND	ND	(204)
		Cultured hippocampal neurons from synaptotagmin 1 KO mouse	↑	↓	=	(204)
		Acute mouse hippocampal slices with synaptotagmin 1 KD	=	↓	ND	(204)
Synaptotagmin 9	C2A domain mutations	Cultured hippocampal neurons from synaptotagmin 1 KO mouse	↑	↓	=	(204)
	C2B domain mutations	Cultured hippocampal neurons from synaptotagmin 1 KO mouse	=	=	↓	(204)
	C2A and C2B domain mutations	Cultured mouse hippocampal neurons with synaptotagmin 1 KD	ND	↓	ND	(204)
	Lentiviral expression	Cultured hippocampal neurons from synaptotagmin 1 KO mouse	↑	↓	=	(204)
		Cultured hippocampal neurons from WT mouse	ND	ND	=	(204)
		Cultured hippocampal neurons from synaptotagmin 1 KO mouse	=	↑	↓	(204)
		Cultured mouse cortical neurons	↑	=	ND	(121, 203)
	Conditional KO	Cultured mouse striatal neurons	↓	=	ND	(121)
	Lentiviral expression + synaptotagmin 1 KO	Cultured mouse cortical neurons	=	↑	ND	(121)
	Lentiviral expression	Cultured mouse cortical neurons	=	=	↑	(156)

Molecule	Manipulation	Experimental System	Synchronous Fusion	Asynchronous Fusion	Spontaneous Fusion	References
Doc2 proteins	Linker mutation between TMR and C2 domains	Cultured mouse cortical neurons	ND	ND	=	(156)
	KO or KD of doc2 α	Cultured mouse hippocampal neurons; acute mouse hippocampal slices	=	↓	ND	(226, 228)
	C2A and C2B domain mutations of doc2 α	Cultured hippocampal neurons from synaptotagmin 1 KO mouse	ND	↑	ND	(228)
	KO of doc2 β	Acute mouse cerebellar slices	ND	ND	↓	(227)
		Cultured hippocampal neurons from synaptotagmin 1 KO mouse	=	=	ND	(228)
	Double KO of doc2 α and doc2 β	Autaptic mouse hippocampal neurons; mouse calyx of Held	=	=	↓	(227)
	Lentiviral expression of doc2 α or doc2 β	Cultured mouse hippocampal neurons	=	↑	ND	(228)
	Quadruple KD of doc2 α , doc2 β , doc2 γ , and rabphilin	Cultured mouse cortical neurons	=	=	↓	(229)
	Quadruple KD of doc2 α , doc2 β , doc2 γ , and rabphilin	Cultured cortical neurons from synaptotagmin 1 KO mouse	ND	=	=	(229)
	C2 domain mutation of doc2 β + quadruple KD of doc2 α , doc2 β , doc2 γ , and rabphilin	Cultured mouse cortical neurons	ND	ND	=	(229)

Abbreviations: double C2 domain (doc2), knockdown (KD), knockout (KO), neuromuscular junction (NMJ), not determined (ND), transmembrane region (TMR), wild type (WT), abolished or nearly abolished (↓), significantly reduced (↓), similar to control (=), significantly increased (↑).

Table 3

Target membrane SNAREs that segregate vesicle pools

Molecule	Manipulation	Experimental System	Synchronous Fusion	Asynchronous Fusion	Spontaneous Fusion	References
<i>t</i> -SNAREs: Syntaxin 1	KO/null mutation of syntaxin 1A	<i>Drosophila</i> NMJ	↑↑	↑	↑↑	(61, 64, 166)
	KO of syntaxin 1B	Cultured mouse hippocampal neurons	=	ND	↑	(170)
	Double KO of syntaxin 1A and syntaxin 1B	Autaptic mouse cortical neurons	↑	↑	= or ↓	(170)
	KD of syntaxin 1B	Cultured cortical neurons from syntaxin 1A KO mouse	↑	↑	↑	(169)
	SNARE motif mutation	<i>Drosophila</i> NMJ	↑↑	↑	↑	(167)
	Inhibitory peptide against SNARE motif	Cultured rat hippocampal neurons	↑	ND	ND	(168)
	N-terminal deletion mutation of syntaxin 1A + syntaxin 1B KD	Cultured cortical neurons from syntaxin 1A KO mouse	↑	↑	↑	(169)
	H _{abc} domain deletion mutation of syntaxin 1A + syntaxin 1B KD	Cultured cortical neurons from syntaxin 1A KO mouse	=	=	↑	(169)
	Linker mutation between SNARE motif and TMR of syntaxin 1A + syntaxin 1B KD	Cultured mouse cortical neurons	↑	↑	=	(77)
	TMR mutation + syntaxin 1B KD	Cultured mouse cortical neurons	=	=	=	(77)
Syntaxin 13	TMR mutation + linker mutation between SNARE motif & TMR + syntaxin 1B KD	Cultured mouse cortical neurons	↑	↑	↑	(77)
	Inhibitory peptide	Cultured rat hippocampal neurons	↑	ND	↑	(96)
	KO/null mutation	Cultured mouse hippocampal, inhibitory cortical, or striatal neurons	↑↑	ND	↑	(182-185)
	KO/null mutation heterozygote	Mouse NMJ	↑↑	ND	↑	(182)
		Cultured mouse excitatory hippocampal neurons	↑	ND	=	(186)
		Cultured mouse inhibitory hippocampal neurons	↑	ND	=	(186)
	Temperature-sensitive mutation	<i>Drosophila</i> NMJ at 22°C	↑	ND	↑	(181)
	Central α-helix mutation	<i>Drosophila</i> NMJ at 37°C	↑	ND	=	(181)
	C-terminal deletion mutation	Autaptic mouse hippocampal neurons	=	=	↑	(187)
	N-terminal deletion mutation	Autaptic mouse hippocampal neurons	↑↑	=	↑↑	(187)
SNAP-25		Autaptic mouse hippocampal neurons	=	↑	↑	(187)
		Autaptic mouse hippocampal neurons	=	↑	↑	(187)

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Molecule	Manipulation	Experimental System	Synchronous Fusion	Asynchronous Fusion	Spontaneous Fusion	References
SNAP-23	Botulinum toxin A	Organotypic rat hippocampal slice cultures	↑	ND	↑	(180)
	Lentiviral expression + SNAP-25 KO	Autaptic excitatory mouse hippocampal neurons	↓	=	↑	(190)
		Cultured mouse hippocampal neurons	↓	↑	=	(185)
	Lentiviral expression + double KO of SNAP-25 and synaptotagmin 7	Autaptic excitatory mouse hippocampal neurons	↑↑	↑	↑	(190)

Abbreviations: knockdown (KD), knockout (KO), neuromuscular junction (NMJ), not determined (ND), transmembrane region (TMR), abolished or nearly abolished (↓↓), significantly reduced (↓), similar to control (=), significantly increased (↑).

Table 4

Cytosolic active zone proteins that segregate vesicle pools

Molecule	Manipulation	Experimental System	Synchronous Fusion	Asynchronous Fusion	Spontaneous Fusion	References
Munc13	KO/null mutation of Munc13-1	Autaptic or cultured mouse excitatory hippocampal neurons	↓↓	ND	↑	(233, 234)
		Autaptic mouse inhibitory hippocampal neurons	=	ND	ND	(233)
	C1 domain mutation of Munc13-1	Autaptic mouse excitatory hippocampal neurons	=	=	↑	(236)
	KO or acute ablation of unc13-long	<i>Caenorhabditis elegans</i> NMJ	↑	=	↑	(238, 239)
	C2A domain deletion mutation of unc13-long	<i>Caenorhabditis elegans</i> NMJ	↑	↑	↑	(239)
	N-terminal deletion mutation of unc13-long	<i>Caenorhabditis elegans</i> NMJ	=	↑	↑	(239)
	Acute ablation of unc13-long with N-terminal deletion mutation	<i>Caenorhabditis elegans</i> NMJ	=	↑	=	(239)
	KO/null mutation of Munc13-2	Autaptic mouse excitatory or inhibitory hippocampal neurons; mouse calyx of Held	=	ND	=	(233, 240)
	Double KO of Munc13-1 and 2	Autaptic mouse excitatory or inhibitory hippocampal neurons	↓↓	↓↓	↓↓	(233)
		Mouse NMJ	↑	ND	↑	(235)
	KO/null mutation of unc13-short	<i>Caenorhabditis elegans</i> NMJ	↑	↑	=	(238)
	Double KO of Unc13-long and unc13-short	<i>Caenorhabditis elegans</i> NMJ	↓↓	ND	↓↓	(238, 239)
	KO/null mutation of Munc13-3	Mouse calyx of Held	=	ND	ND	(240)
	Double KO of Munc13-2 and 3	Mouse calyx of Held	=	↑	ND	(240)
	Dominant negative Munc13-1 + double KO of Munc13-2 and 3	Mouse calyx of Held	↑	ND	ND	(240)
Rim1a	KO/null mutation	Autaptic mouse excitatory hippocampal neurons; acute mouse hippocampal slices	↑	↑	=	(246, 247)
		<i>Caenorhabditis elegans</i> NMJ	↑	ND	↑	(248)
Tomosyn	KO/null mutation	Acute mouse hippocampal slices	↑	ND	ND	(256)
		<i>Caenorhabditis elegans</i> NMJ	= or ↑	↑	=	(238, 254, 255)
Complexins	KO/null mutation of complexin 1 or complexin	Autaptic mouse excitatory hippocampal neurons	=	ND	=	(273)

Molecule	Manipulation	Experimental System	Synchronous Fusion	Asynchronous Fusion	Spontaneous Fusion	References
		Mouse spiral ganglion-bushy cell auditory synapse	↓	↑	↑	(267)
		<i>Drosophila</i> NMJ; <i>Caenorhabditis elegans</i> NMJ	↓	↑	↑	(261, 266, 268, 269, 272, 282)
	SNARE motif mutations of complexin 1 or complexin	Autaptic mouse excitatory hippocampal neurons; cultured mouse cortical or olfactory bulb neurons; <i>Caenorhabditis elegans</i> NMJ	↓	ND	↑	(264, 268, 270, 274)
	Inhibitory peptide against complexin SNARE motif	<i>Drosophila</i> NMJ	↓	ND	=	(272)
	Accessory α -helix mutations of complexin 1 or complexin	Zebrafish retinal bipolar cells	=	ND	↑	(271)
		Autaptic mouse hippocampal neurons; cultured mouse cortical or olfactory bulb neurons; <i>Drosophila</i> NMJ	=	=	= or ↓ or ↑	(262, 263, 270, 272, 279)
	C-terminal truncation mutation of complexin 1 or complexin	Cultured mouse cortical neurons	=	=	= or ↑	(262, 265)
		<i>Drosophila</i> NMJ; <i>Caenorhabditis elegans</i> NMJ	↓	ND	↑	(261, 268)
	C-terminal point mutations of complexin 1 or complexin	Cultured mouse cortical neurons; <i>Drosophila</i> NMJ	=	=	= or ↑	(265, 282)
	C-terminal membrane attachment mutation of complexin 1	Cultured mouse cortical neurons	=	ND	↑	(262)
	N-terminal truncation mutation of complexin 1 or complexin	Autaptic mouse excitatory hippocampal neurons	↓	ND	ND	(274)
		Cultured mouse cortical neurons	↓	ND	=	(264)
		Cultured mouse olfactory bulb neurons	↓	ND	↑	(270)
		<i>Drosophila</i> NMJ	=	ND	↑	(272)
	N-terminal truncation + SNARE motif mutation of complexin 1	Autaptic mouse excitatory hippocampal neurons	↓	ND	ND	(274)
	N-terminal + accessory α -helix deletion mutations of complexin 1	Autaptic mouse excitatory hippocampal neurons	↓	ND	ND	(274)
		Cultured mouse cortical neurons	↓	ND	↑	(264)
		Cultured mouse olfactory bulb neurons	↓	ND	=	(270)
	KO/null mutation of complexin 2	Autaptic mouse excitatory hippocampal neurons	=	=	=	(273, 274)
	Double KO of complexins 1 and 2	Autaptic mouse excitatory hippocampal neurons	↓	=	= or ↓	(273, 275) (274)
		Autaptic mouse inhibitory striatal neurons	↓	ND	=	(275)
		Cultured mouse cortical neurons	↓	ND	↑	(262)
		Cultured mouse olfactory bulb neurons	↓	ND	↓	(262)

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Molecule	Manipulation	Experimental System	Synchronous Fusion	Asynchronous Fusion	Spontaneous Fusion	References
	Double KD of complexins 1 and 2	Cultured mouse cortical neurons	↑	= or ↓	↑	(262-265)
	KO of complexin 3	Cultured mouse olfactory bulb neurons	↑	ND	↑	(270) (262)
		Autaptic mouse excitatory hippocampal or inhibitory striatal neurons	=	ND	=	(275)
	Double KO of complexins 2 and 3	Autaptic mouse excitatory hippocampal or inhibitory striatal neurons	=	ND	=	(275)
	Triple KO of complexins 1-3	Autaptic mouse excitatory hippocampal neurons	↑	ND	= or ↓	(275, 279)
		Autaptic mouse inhibitory striatal neurons	↑	ND	=	(275)

Abbreviations: knockdown (KD), knockout (KO), neuromuscular junction (NMJ), not determined (ND), abolished or nearly abolished (↓), significantly reduced (↓↓), similar to control (=), significantly increased (↑).