DISSERTATION

REEVALUATING THE FUNCTIONAL ROLE OF THE C_2A DOMAIN OF SYNAPTOTAGMIN IN NEUROTRANSMITTER RELEASE

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Matthew Robert Bowers

Department of Biomedical Sciences

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Doctoral Committee:

Advisor: Noreen Reist

Susan Tsunoda
Santiago DiPietro
Michael Tamkun

ABSTRACT

REEVLAUATING THE FUNCTIONAL ROLE OF THE C_2A DOMAIN OF SYNAPTOTAGMIN IN NEUROTRANSMITTER RELEASE

Efficient cell-to-cell communication is critical for nervous system function. Fast, synchronous neurotransmission underlies this communication. Following depolarization of the nerve terminal, Ca^{2+} enters the presynaptic cell and drives fusion of vesicles with the membrane, releasing neurotransmitter. The synaptic vesicle protein, synaptotagmin, was identified as the Ca^{2+} sensor for this fast, synchronous neurotransmitter release.

It is hypothesized that Ca^{2+} binding by synaptotagmin acts as an electrostatic switch. At rest, vesicles are in a state of variable priming with repulsion between negatively charged residues in synaptotagmin and the negatively charged presynaptic membrane acting as a brake to prevent fusion of the vesicles. Following binding of positively charged Ca^{2+} ions, this electrostatic repulsion is switched to attraction, allowing hydrophobic residues in synaptotagmin to insert into the presynaptic membrane. This insertion is thought to lower the energy barrier for fusion, resulting in the synchronous fusion of many vesicles and the chemical propagation of a signal to the postsynaptic cell.

Synaptotagmin is composed primarily of two C_2 domains that have negatively charged Ca^{2+} binding pockets, C_2A and C_2B. The C_2B domain is thought to be the primary functional domain, with C_2A playing a supporting role. While using point mutations to the C_2A domain to investigate the functional roles of specific residues of the protein, I discovered that the C_2A domain, may, in fact, be much more important than anticipated.

In chapter 2, I created mutations disrupting the membrane penetrating hydrophobic residues of the C_2A domain. Mutation of these residues was hypothesized to only partially disrupt evoked release. Surprisingly, mutation of both residues in tandem resulted in the most dramatic phenotype of a C_2A domain mutation to date. This dramatic decrease in synaptic transmission is the first instance of a C_2A domain mutation resulting in a phenotype worse than the synaptotagmin null mutant.

In chapter 3, I generated mutations to various combinations of Ca^{2+}-binding aspartate residues in the Ca^{2+} binding pocket of C_2A. These mutations are hypothesized to prevent Ca^{2+} binding, while simultaneously neutralizing the charge of the pocket, essentially mimicking constitutive Ca^{2+} binding. Again surprisingly, evoked release was dramatically decreased in some of the mutants, suggesting C_2A Ca^{2+} binding mutants disrupt Ca^{2+} dependent synchronous release, a finding that increases our understanding of Ca^{2+} binding by the domain and contradicts some interpretations of previous reports.

My findings provide key mechanistic insights into the function of this critical protein. For one, investigation of the role of the C_2A hydrophobic residues revealed that the downstream effector interactions mediated by these hydrophobic residues are critical to drive synchronous vesicle fusion. Also, investigation of the role of the critical Ca^{2+}-binding residues in the C_2A domain revealed that these residues each play a distinct role in driving vesicle fusion, while further suggesting Ca^{2+} binding by C_2A is more important than originally posited. Most interestingly though, I believe the sum of my findings disproves the long-held belief that C_2A is purely a facilitatory domain, prompting many questions about how these two C_2 domains may work together to promote neurotransmitter release in tandem.

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CHAPTER 1. SYNAPTIC TRANSMISSION AND SYNAPTOTAGMIN[1]

1.1 Overview

The nervous system relies on fast, efficient communication between neurons. While neurons can rapidly transmit an internal signal in the form of an electrical impulse known as an action potential, these electrical signals are only able to propagate directly to neighboring cells in certain cases. The vast majority of neurons instead rely on small diffusible molecules, known as neurotransmitters, to transmit signals to neighboring cells. The release, and subsequent detection, of these neurotransmitters requires a specialized cell-to-cell contact site known as a synapse. The majority of synaptic transmission is unidirectional, with a signal passing from a presynaptic cell to a postsynaptic cell, each with their own unique local specializations to release and detect neurotransmitter respectively.

Pioneering studies in the 1950s by Bernard Katz and his colleagues first identified synaptic neurotransmitter release. Using the frog neuromuscular junction, they observed release of neurotransmitter in quantal packets [1-3], providing the first evidence of discrete unitary release, which we now know to be the fusion of neurotransmitter-filled vesicles fusing with the presynaptic membrane. Since then, the structure and function of the synapse has been slowly characterized across countless model systems. The current understanding is that action potential induced increases in Ca^{2+} concentration at the synapse leads to Ca^{2+} binding by the protein synaptotagmin. This Ca^{2+} binding triggers the fast, synchronous fusion of multiple neurotransmitter-filled vesicles with the presynaptic membrane. However, the mechanisms underlying synaptotagmin's role as the Ca^{2+} sensor are still being elucidated.

[1] Bowers MR

This dissertation investigates the mechanisms by which synaptotagmin is able to transduce a Ca^{2+} signal into the mechanical action of neurotransmitter release. Synaptotagmin has two primary functional domains, C_2A and C_2B. As the presynaptic release machinery has been better characterized, the hypothesized relative importance of these two domains has fluctuated. When I began my dissertation work, the understanding in the field was that the C_2B domain was the primary functional domain of synaptotagmin, while C_2A played only a supporting role. Here I present evidence that this understanding is incorrect, and that C_2A is playing and essential role in triggering neurotransmitter release. The recent experimental focus on the C_2B domain may be missing important roles mediated by the C_2A domain and synergistic actions between the domains.

1.2 Neurotransmitter release

The mystery of interneuronal communication was a foundational question in neuroscience. How could electrical signals propagate between spatially distinct neurons? The identification of synaptic vesicles as the source of neurotransmitter release was a revolutionary contribution to the understanding of synaptic transmission. In the 1950s, the proposal of the quantal release hypothesis by Bernard Katz and colleagues represented a paradigm shift in the field [1-3]. Electrophysiological recording at the frog neuromuscular junction identified small, spontaneous events that occurred in the absence of neural activity. They found that these events resembled the normal endplate potentials (EPPs) that follow an action potential, but at a much smaller scale. They termed these events mini endplate potentials (mEPPs). The random spacing and similar size led them to conclude that the neurotransmitter was being released in discrete quanta. Moreover, evoked EPPs in the presence of Mg^{2+}, which blocks Ca^{2+} entry into the cell,

decreased the amplitude of the EPPs and made them highly variable. When these EPPs were clustered by amplitude, they found that they fell into even steps, with the difference between steps being roughly equal to the amplitude of the observed mEPPs. These findings led to the hypothesis that neurotransmitter was being released in multimolecular quantized packets, and that an action potential caused the synchronous release of a large number of these packets, generating a large EPP in the postsynaptic cell. These findings were reinforced by electron microscopy of the synapse that revealed small membranous vesicles clustered at nerve terminals [4, 5]. This led to the vesicular release hypothesis: an action potential results in the synchronous fusion of synaptic vesicles that each contains a quantized amount of neurotransmitter.

This hypothesis allows for neurotransmitter release at the synapse to be understood in terms of the relatively simple equation $m=np$, where m is the average number of quanta released following a single nerve impulse (referred to as quantal content). The parameter m depends on the number of vesicles immediately available for release (n) and the probability (p) that any given vesicle will fuse with the membrane after a single impulse. This probability depends on multiple factors, including how "ready" the vesicle is to fuse, which we will refer to as its degree of priming, and the amount of Ca^{2+} influx into the presynaptic terminal. Statistical analysis of neurotransmitter release extends beyond this foundational concept (reviewed in [6]). This vesicular neurotransmitter release, however, requires a very tightly regulated series of steps. These steps are referred to as the synaptic vesicle cycle, and small changes at any given point within this cycle can alter the kinetics of release, altering the signaling between the pre- and postsynaptic cells.

1.1 The synaptic vesicle cycle

The vesicle cycle can be broken down into several general phases. First, vesicles must be loaded with neurotransmitter (Fig 1.1 a), transported to the active zone, and docked to the presynaptic membrane by way of specific molecular interactions (Fig 1.1 b). The population of docked vesicles at a synapse is thought to constitute a readily releasable pool of neurotransmitter. However, only a subset of docked vesicles is fusion competent at any given time, namely, those that are maximally primed (Fig 1.1 d). In a resting terminal, individual maximally-primed vesicles can spontaneously fuse with the presynaptic membrane, resulting in the release of a single quantum of neurotransmitter. In an activated neuron, the influx of Ca^{2+} through voltage-gated Ca^{2+} channels (Fig 1.1 e) triggers the fusion of multiple maximally-primed vesicles with the presynaptic membrane and results in a larger release of neurotransmitter (Fig 1.1 f, g). This is accomplished by Ca^{2+}-sensing proteins activating the fusion machinery. Neurotransmitter release occurs in two temporally distinct Ca^{2+}-dependent phases: synchronous and asynchronous. After neurotransmitter release, the fusion machinery is disassembled and recycled for subsequent use. Concurrently, the synaptic vesicle membrane and associated proteins are retrieved through endocytosis (Fig 1.1 h) to form new synaptic vesicles. These vesicles are reloaded with neurotransmitter and transported back to active zones, completing the cycle. Each step in this cycle is mediated by its own suite of proteins and is a dynamic process. This dissertation will focus specifically on the machinery underlying the Ca^{2+}-dependent fusion step. However, it is important to note, that, as the process is cyclical, changes in one phase of the vesicle cycle may have profound effects elsewhere in the cycle.

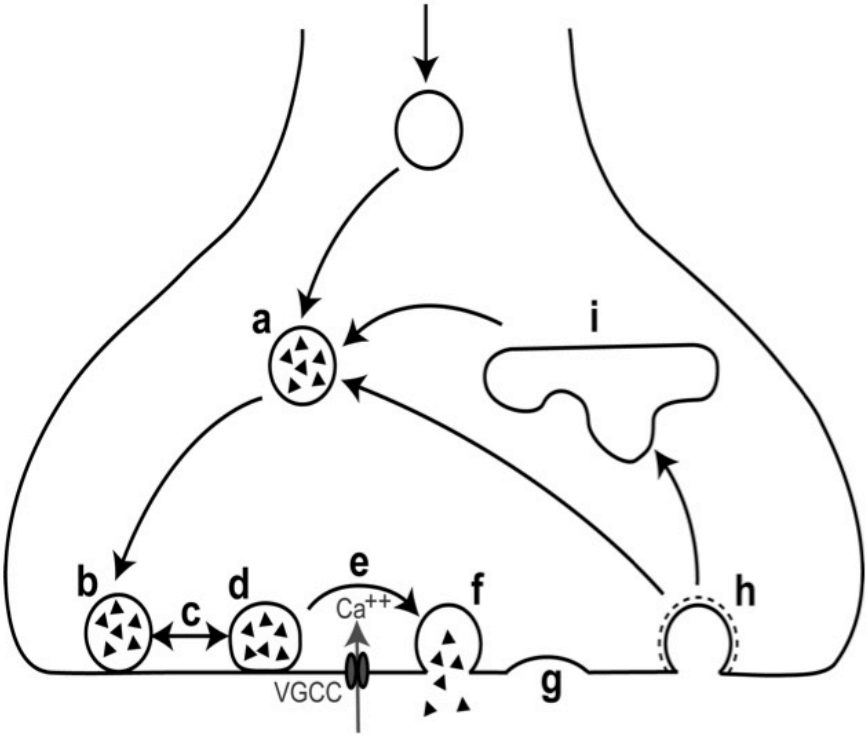

Figure 1.1 Schematic of the vesicle cycle. Vesicles are loaded with neurotransmitter (a) and docked to the presynaptic membrane (b), where they undergo variable priming (c). They oscillate between minimally (b) and maximally primed (d) states. Following Ca^{2+} influx via voltage gated Ca^{2+} channels (e), the vesicles fuse with the membrane, releasing neurotransmitter (f). After complete collapse into the presynaptic membrane (g), vesicles are endocytosed (h) and either refilled directly, or trafficked to endosomes (i).

1.3 Synaptic architecture

The synapse is highly organized and much of the basic structure is consistent across synapse types and organisms. At the core, presynaptic vesicular fusion is reliant upon the active zone material (AZM), critical protein machinery that organizes vesicles and drives fusion. Historically, the AZM was first defined as electron dense material near docked vesicles in electron micrographs at the frog NMJ [7]. Individual structural components of this material were subsequently resolved in ultrastructural studies and the functions of the constituent proteins of these macromolecular complexes are currently being elucidated.

The structure of the active zone material (AZM) varies among different types of synapses but must include the requisite protein machinery for vesicle docking, priming, and fusion. The major constituents of active zones include: active zone core proteins [8] [9, 10], essential fusion machinery [11], Sec1/Munc18-like (SM) proteins [12], Ca^{2+} channels, and Ca^{2+} sensors [13-16]. The main core proteins are Rab3-interacting molecules (RIM), Munc13, RIM binding protein (RIM-BP), alpha-liprin, and ELKS/CAST [17]. The essential fusion machinery consists of the soluble N-ethlymaleimide sensitive factor adaptor protein receptors, or SNAREs [11]. There are several different Ca^{2+}-sensing proteins that trigger neurotransmitter release, but I will focus on the main sensor for synchronous neurotransmitter release, synaptotagmin. Numerous additional regulatory and adaptor molecules are present at or near active zones and vary between synapses. This dissertation will focus on the protein synaptotagmin and its role triggering vesicle fusion. For a complete review of synaptic architecture and function see Appendix 1.

Fusion machinery

The minimum machinery required to fuse a vesicle with its target membrane is the SNARE fusion complex, which is comprised of a vesicle-associated SNARE protein (vSNARE) and target-membrane associated SNARE proteins (tSNAREs). At the synapse, the vSNARE is synaptobrevin-2, also known as vesicle-associated membrane protein 2 (VAMP2) and the tSNAREs are syntaxin-1 and synaptosomal-associated protein of 25 kDa (SNAP-25).

Each SNARE protein contains at least one SNARE motif of ~65 residues with a propensity to form coiled coils [18]. Synaptotobrevin and syntaxin each contain one SNARE motif, and SNAP-25 contains two [11]. The SNARE motif of syntaxin interacts with the SNARE motifs of synaptobrevin and SNAP-25. This creates a *trans*-SNARE complex, which is associated with both the vesicular membrane and the presynaptic plasma membrane. On any

given vesicle, complete coiling of multiple SNARE complexes [19] provides the energy required to drive fusion of the vesicle and target membranes [20, 21]. This tight coiling straightens the SNARE complex, changing its conformation from a *trans*-SNARE complex to a *cis*-SNARE complex. Following complete fusion, all components are located in the presynaptic membrane.

SNARE complexes mediate both constitutive and regulated vesicle fusion events throughout cells. At the synapse, multiple regulatory elements suppress constitutive SNARE-mediated fusion events and facilitate the fast, synchronous fusion of multiple synaptic vesicles upon Ca^{2+} influx. Synchronous fusion of multiple vesicles is essential for neuronal signaling and relies on the docking of vesicles at the active zone and their priming to a fusion ready state.

The basic concept of the minimal machinery necessary to fuse a vesicle with its target membrane is as follows: SNARE proteins on both the vesicular and target membranes associate to form coiled-coil configurations. When maximally coiled, *trans*-SNARE complexes force the two membranes together, thereby destabilizing the membranes' natural curvature. The coiling of *trans*-SNARE complexes all the way into their transmembrane domains provides the energy to drive fusion of the membranes [20, 22, 23]. Following fusion, the vSNAREs and tSNARES are now in a *cis*-SNARE complex formation, and the vesicular contents are released.

1.4 Synaptic Function

Docking and Priming

To become fusion competent, synaptic vesicles must be precisely targeted to the active zone and held in direct contact with the presynaptic membrane. First, vesicles are tethered near the presynaptic membrane at active zones. When the vesicle membrane touches the presynaptic

membrane, the vesicle is considered docked at the release site (Fig 1b). Once docked, additional interactions, referred to as priming, increase the probability of vesicle fusion (Fig1 c, d).

Recent work in the McMahan lab suggests that vesicle priming is in a state of dynamic equilibrium, such that only docked vesicles with the largest area of contact with the presynaptic membrane are maximally primed and ready to fuse upon Ca^{2+} influx (Fig 1 b,c,d [24]). One mechanism of accomplishing variable priming would be a balance between the repulsive forces of the vesicular and presynaptic membranes that result in SNARE complex uncoiling to favor minimal priming and SNARE complex coiling to favor maximal priming [24]. At any given moment, only a subpopulation of docked synaptic vesicles has a sufficient number of maximally-coiled *trans*-SNARE complexes to produce maximal SV-PM contact area. These synaptic vesicles constitute the maximally-primed population that is most likely to fuse upon Ca^{2+} influx. Once triggered by Ca^{2+}, these maximally-primed synaptic vesicles complete the SNARE coiling process all the way into the transmembrane domains of syntaxin and synaptobrevin [25]. The synaptic vesicle membrane merges with the presynaptic membrane, resulting in *cis*-SNARE complex formation. Thus, the coiling of SNARE complexes provides the energy needed to induce membrane fusion.

Vesicle Fusion

Fusion events can occur spontaneously or can be triggered by Ca^{2+} influx. Spontaneous events are single vesicle fusion events that occur in the absence of Ca^{2+} or neuronal activity. Ca^{2+} triggered events are the result of an action potential leading to the opening of voltage gated Ca^{2+} channels. The subsequent influx of Ca^{2+} triggers a series of intermolecular interactions that

results in the fusion of multiple maximally-primed vesicles with the presynaptic membrane Fig 1.1 c). The Ca^{2+} sensor present determines the kinetics of this release.

Ca^{2+} independent neurotransmitter release

Ca^{2+}-independent neurotransmitter release is referred to as spontaneous release. This form of neurotransmitter release occurs in resting nerve terminals when a single vesicle fuses with the presynaptic membrane in the absence of presynaptic Ca^{2+} influx. Classically, spontaneous release is considered to be random fusion events caused by low-probability conformational changes that complete coiling of the SNARE proteins and result in fusion. Since these spontaneously fusing vesicles would be in a maximally-primed, fusion-competent state, little energy is required to overcome the fusion barrier.

Spontaneous and evoked fusion events can occur at distinct active zones [26, 27] from separate recycling pools [28, 29]. Thus, location may partially determine the probability of a vesicle participating in spontaneous versus evoked neurotransmitter release. These recent studies, together with the classic spontaneous release hypothesis, suggest multiple mechanisms occurring during spontaneous fusion, with a subpopulation originating from maximally-primed vesicles reacting to transient changes in their environment, and another subpopulation occurring at specialized, Ca^{2+}-independent fusion sites

Synchronous neurotransmitter release

Ca^{2+}-dependent neurotransmitter release is differentiated temporally into two phases: fast, synchronous release and a slower, asynchronous release [30, 31]. The identity of the local Ca^{2+} sensor seems to be the main determinant in these release kinetics. The asynchronous phase most likely relies on a high affinity Ca^{2+} sensor that promotes fusion of vesicles for 10 to 100s of

milliseconds [15]. This type of release is negligible at the *Drosophila* neuromuscular junction and will not be discussed in this thesis. The fast, synchronous phase is the large burst of neurotransmitter release that occurs within milliseconds of the arrival of the action potential and influx of Ca^{2+}. This constitutes the classical neurotransmitter release pathway typically described in textbooks. This fast synchronous phase relies on a lower affinity Ca^{2+} sensor that requires higher concentrations of Ca^{2+} to saturate binding sites. This limits synchronous fusion of multiple vesicles to only the brief moment when the local Ca^{2+} concentration is at its highest. The closely related proteins, synaptotagmin 1 and 2, mediate this fast synchronous release. In mammals, synaptotagmin 1 is found primarily at central synapses, while synaptotagmin 2 is located peripherally [32]. In *Drosophila*, there is only one fast, synchronous sensor, synaptotagmin 1, that fills the role both centrally and peripherally [33].

Postsynaptic responses

The postsynaptic membrane's ability to transduce the chemical signal into an electrical signal is dependent on the opening of ion channels as a result of neurotransmitter binding (either directly to ionotropic receptors, or as downstream effectors of binding to GPCRs) The opening of these channels allow ions to travel down their electrochemical gradients, thus the ion selectivity of the activated channels determines the net potential generated in the postsynaptic cell. At a typical excitatory synapse, the open ion channels allow positively-charged ions, such as Na^{+} and Ca^{2+}, to flow down their electrochemical gradients into the postsynaptic cell. The influx of positive charge depolarizes the post-synaptic cell, otherwise known as an excitatory postsynaptic potential (EPSP). The net change in membrane potential depends on the summation of all synaptic inputs. The *Drosophila* neuromuscular junction is exclusively excitatory. At these

synapses, sufficient glutamate (rather than the acetylcholine at mammalian neuromuscular junctions) is released, resulting in depolarization of the muscle cell. The *Drosophila* larval muscle fiber does not generate action potentials and is isopotential, allowing accurate electrophysiological recording of EPSPs and spontaneous mEPSPs [34].

1.5 Synaptotagmin as an electrostatic switch

The synaptotagmin family of genes is made up of 17 members in mammals [35], but *Drosophila* only have 7 synaptotagmin genes [33]. These myriad isoforms are responsible for Ca^{2+} dependent exocytosis beyond just synaptic transmission, from cell membrane maintenance in plants [36] and animals [37] to roles in reproduction [38], diabetes [39]. and bone remodeling [40]. Of the 7 isoforms in *Drosophila*, synaptotagmin 1 is the only isoform that mediates fast, synchronous vesicle fusion events at the synapse. The other isoforms do not play a role in synchronous neurotransmission at the synapse. Synaptotagmin 4 is found to be expressed at detectable levels only postsynaptically [33] and is proposed to play roles in retrograde signaling at the synapse [41]. Synaptotagmin 7 is found non-synaptically, expressed mainly in the muscle tissue, but has been proposed to play roles in vesicle pool replenishment, asynchronous neurotransmitter release, and short term facilitation in mammalian systems [42]. Synaptotagmins 12 and 14 are found to have very low expression and are lacking Ca^{2+}-binding residues [33]. Finally, Synaptotagmins alpha and beta are only found localized specifically in several neurosecretory cells [33]. Since I will essentially only be discussing synaptotagmin 1 for the remainder of this thesis, I will be using "synaptotagmin 1" and "synaptotagmin" interchangeably and will only specify isoform if talking about one of the other isoforms.

In the second chapter of this dissertation, I review the history of synaptotagmin research as it pertains to its role as the electrostatic switch triggering synchronous vesicle fusion. While it has been well established that synaptotagmin is the Ca^{2+} sensor for synchronous neurotransmitter release, the mechanism by which synaptotagmin triggers fusion is still being elucidated. The current understanding of synaptotagmin function is that it acts as an electrostatic switch (Fig 1.2 A). Effectively, in the absence of Ca^{2+}, synaptotagmin acts as a brake, arresting vesicle fusion. Following Ca^{2+} binding, neutralization of electrostatic repulsion between the protein and the presynaptic membrane, permits downstream effector interactions, driving SNARE mediated fusion. My thesis focuses on experiments to better understand the importance of these downstream effector interactions and investigate the *in vivo* importance of Ca^{2+}-binding residues in the C_2A domain. In the process, I found evidence demonstrating that the C_2A domain may actually play a more pivotal role in protein function than has been postulated.

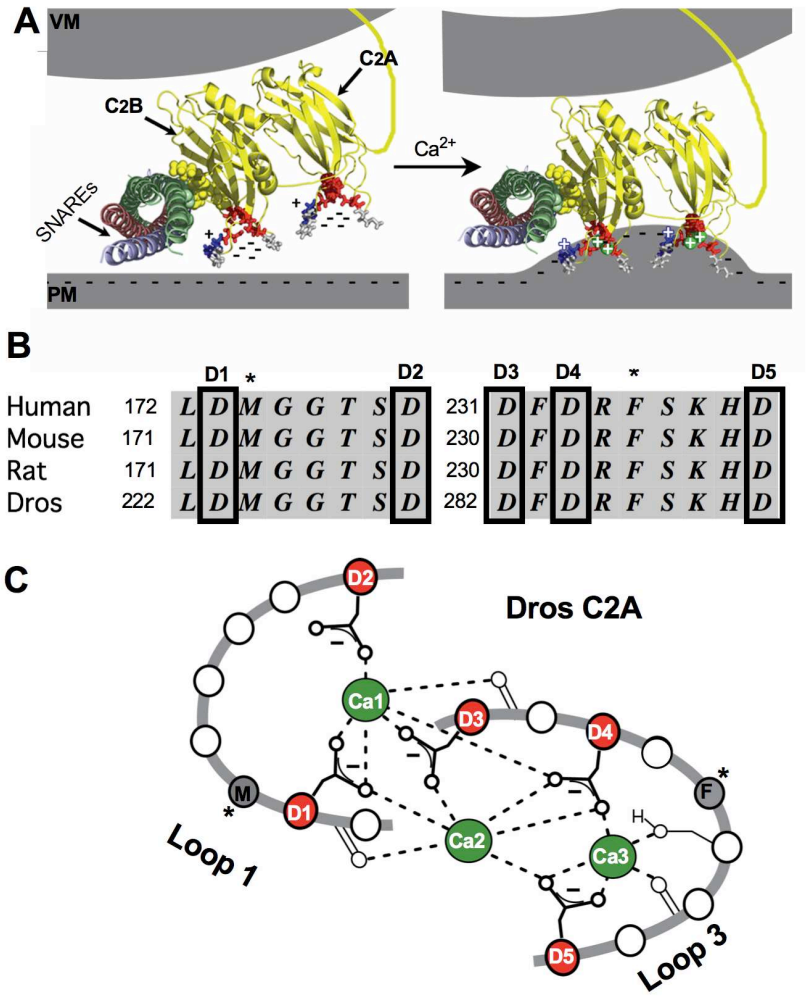

Figure 1.2- Synaptotagmin and the C_2A domain. A. Cartoon depiction of synaptotagmin-triggered fusion. LEFT- In the absence of Ca^{2+}, the negatively charged aspartate residues (red) repel the negatively charged phospholipid heads of the presynaptic membrane. This acts to clamp spurious fusion events. RIGHT- Following Ca^{2+} binding, the negative charge of the binding pockets is neutralized, permitting downstream effector interactions. Hydrophobic residues (grey) at the tips of the Ca^{2+} binding loops are thought to penetrate and warp membranes, potentially lowering the energy barrier for fusion and resulting in neurotransmitter release. B. A sequence alignment shows that synaptotagmin's C_2A Ca^{2+} binding pocket is highly conserved. Alignments of loops 1 and 3, which make up the Ca^{2+} binding pocket, are displayed. Critical Ca^{2+} binding aspartate residues, investigated in chapter four, are marked with black boxes and the hydrophobic residues that are the focus of chapter three are marked with asterisks. C. Schematic representation of the Ca^{2+} binding pocket of the C_2A domain residues essential to coordination of Ca^{2+} binding (adapted from [43]). Critical aspartates are noted (D1-D5, red) and the hydrophobic tip residues (M and F, grey residues) are marked with asterisks. Bound Ca^{2+} ions are indicated by Ca1, Ca2, and Ca3 (green). They are thought to bind sequentially in that order, with Ca1 being the highest affinity site [44].

1.6 The tandem C_2 domains of synaptotagmin

When I began my PhD studies, *in vivo* and in culture analyses of C_2A and C_2B domain function suggested that the C_2B domain was the principle functional domain of synaptotagmin, with the C_2A domain playing a supporting role. Mutations to the C_2B domain result in dominant negative effects and lethality, with analogous C_2A mutations having less detrimental effects. Mutations that alter a critical polylysine motif in the C_2B domain decreases evoked release ~40-50% [45-48], but they have no effect on evoked release when mutated in C_2A [49]. Mutation of a positively charged residue in loop 3 that mediates electrostatic interactions with the presynaptic membrane results in: a 60-80% decrease in evoked release in C_2B [50, 51], but only an ~45-55% decrease in C_2A [48, 50-52]. Similarly, mutations that disrupt Ca^{2+} binding in the C_2B domain via aspartate to asparagine substitutions are dominant negative or lethal C_2B [53, 54], but have little to no effect on evoked release in C_2A [55-57]. Taken together, these results led to the understanding that C_2B is critical, while C_2A only acts in a facilitatory manner. More recent evidence, however, suggested that the C_2A domain may contribute to synaptotagmin function more than the previously referenced studies suggested. While aspartate to asparagine substitutions that disrupt Ca^{2+} binding had dramatic effects in the C_2B domain [53], but no effect in the C_2A domain [55, 57], a glutamic acid (E) substitution in C_2A, that allowed the binding pocket to retain its negative charge, resulted in an 80% decrease in synchronous neurotransmitter release [58]. This was the first study to identify a role for Ca^{2+} binding by C_2A *in vivo*. This role, however, still seemed facilitatory, with Ca^{2+} binding disruption in the C_2B domain causing much more dramatic deficits [53]. These differences in mutations to the C_2 domains are more heavily reviewed in Table 1.1.

In chapters 3 and 4, I present my research demonstrating that the C_2A domain plays an essential, rather than facilitatory, role in neurotransmitter release. Chapter three focuses specifically on hydrophobic tip residues that were shown to penetrate membranes *in vitro* and *in silico* (Fig 1.2, A grey residues, B/C asterisks [59-61]). Mutation of these hydrophobic residues results in the most detrimental phenotype observed to date for the C_2A domain. The dramatic observed decrease in synchronous release was even lower than mutants lacking synaptotagmin. This is the first *in vivo* evidence that the C_2A domain is absolutely required for the function of the protein *in vivo*.

Table 1.1- In vivo functional effects of mutations to synaptotagmin's C_2 domains. Mutations are separated by domain. All experimental effects are on synchronous neurotransmitter release in the absence of WT synaptotagmin unless otherwise noted. Superscripts indicate model system: D = *Drosophila* NMJ, A = cultured hippocampal autapses (mouse), and N = cultured hippocampal neurons (mouse). (EL) denotes embryonic lethality and all other mutants were viable. N/D = no data. UP = unpublished data from the Reist lab. Mutants described in this dissertation are marked with an asterix. Aside from the C_2A mutations described in this dissertation and by Striegel et al. 2012 [58], mutations to the C_2B domain are more detrimental than their homologous C_2A counterparts.

C2A				C2B			
Interaction disrupted	Mutations Made	Experimental Effects	Ref	Interaction disrupted	Mutations Made	Experimental Effects	Ref
Membrane Attraction	R-Q^{D}	50% ↓	[50]	Membrane Attraction	R-Q^{D}	80% ↓	[50]
	R-Q^{N}	50% ↓	[62]		K-Q^{A}	none	[48]
	R-Q^{A}	~50% ↓	[48, 52]				
		none	[57]				
	K-Q^{A}	none	[52]				
Ca Binding	D2N^{D}	36% ↓	[55]	Ca Binding	D1N^{A}	70% ↓	[54]
		*46% ↓	UP		D2N^{D} (EL)	N/D	UP
	D2E^{D}	80% ↓	[58]		D2N^{A}	99% ↓	[54]
	D3N^{A}	none	[57]		D3N^{D} (EL)	N/D	UP
	D4N^{N}	40% ↓	[62]		D3N^{A}	99%↓	[54]
	D4N^{A}	none	[56]		D4N^{A}	none	[54]
		50% ↑	[57]		D5N^{A}	none	[54]
	D5N^{N}	50% ↓	[62]		D12N^{D} (EL)	90% ↓in hets	[53]
	D5N^{A}	none	[56]		D34N^{D}	99% ↓	[53]
	D12N^{A}	none	[57]			50%-60% ↓ in hets	[53, 63]
	D23N^{D}	*71% ↓	UP		D234A^{N}	99% ↓	[62, 65]
	D34N^{D}	none ,	[63, 64]				
		*23% ↑	UP				
	D34N^{A}	none	[57]				
	D234A^{N}	40%-50% ↓	[62, 65]				
	D345N^{A}	none	[57]				
	D2345N^{D}	*78% ↓	UP				
	D1-5N^{A}	none	[57]				
Membrane Penetration	F-E^{D}	50% ↓	[66]	Membrane Penetration	I-E^{D} (EL)	83% ↓, Het lethal	[66]
	F-Y^{D}	50% ↓	[66]				
	M-E^{D}	*50% ↓	[67]		3W^{A}	↑ Ca Affinity	[68]
	M-E, F-E^{D}	*98% ↓	[67]				
	3W^{A}	↑ Ca Affinity	[68]				
				SNARE/ Oligomerization	K-Q^{D}	35-65% ↓	[45]
						50% ↓	[46]
					K-A^{A}	55% ↓	[47]
					K-A, K-A^{A}	50% ↓	[48]
N/D	KA x4^{D}	none	[49]	N/D	Y-N^{A}	55% ↓	[47]

Furthermore, while investigating the role of the electrostatic repulsion of the C_2A Ca^{2+} binding pocket, I discovered additional evidence of C_2A's importance. Previous studies used aspartate to asparagine mutations in the Ca^{2+} binding pockets to prevent binding. While asparagine's neutral charge was chosen to disrupt Ca^{2+} binding, substitutions also neutralize the overall charge of the pocket. This decreases the electrostatic repulsion between the domain and presynaptic membrane, similar to the Ca^{2+} bound state. In order to investigate the role of decreasing electrostatic repulsion, I sequentially mutated aspartate residues in the C_2A domain to asparagines to block Ca^{2+} binding while decreasing electrostatic repulsion in a stepwise manner (Fig 1.2 A red residues, B/C D1-D5). Previous research suggested that these mutations would not decrease evoked release [55, 57, 64]. Surprisingly, however, mutation of specific aspartate residues resulted in a large decrease in evoked release. This agrees with previous research from the lab [58]. In addition to bolstering the case for C_2A's role being more equal to C_2B's, these results point towards certain aspartate residues in the binding pocket playing specific roles in coordinating Ca^{2+} binding and mediating electrostatic repulsion. Taken together, all of these findings challenge a long held understanding of the steps underlying synaptotagmin triggered neurotransmitter release, providing new mechanistic insights, and revealing that investigation of protein function *in vivo* needs to consider the greater contribution of the C_2A domain.

WORKS CITED

1. Fatt, P. and B. Katz, *An analysis of the end-plate potential recorded with an intra-cellular electrode.* J Physiol, 1951. **115**(3): p. 320-70.
2. Fatt, P. and B. Katz, *Spontaneous subthreshold activity at motor nerve endings.* J Physiol, 1952. **117**(1): p. 109-28.
3. Del Castillo, J. and B. Katz, *Quantal components of the end-plate potential.* J Physiol, 1954. **124**(3): p. 560-73.
4. De Robertis, E.D. and H.S. Bennett, *Some features of the submicroscopic morphology of synapses in frog and earthworm.* J Biophys Biochem Cytol, 1955. **1**(1): p. 47-58.
5. Palay, S.L., *Synapses in the central nervous system.* J Biophys Biochem Cytol, 1956. **2**(4 Suppl): p. 193-202.
6. Bennett, M.R. and J.L. Kearns, *Statistics of transmitter release at nerve terminals.* Prog Neurobiol, 2000. **60**(6): p. 545-606.
7. Couteaux R, P.-D.M., *Synaptic vesicles and pouches at the level of "active zones" of the neuromuscular junction.* C. R. Hebd. Seances Acad. Sci, 1970. **D271**: p. 2346-2349.
8. Zhen, M. and Y. Jin, *Presynaptic terminal differentiation: transport and assembly.* Curr Opin Neurobiol, 2004. **14**(3): p. 280-7.
9. Schoch, S., et al., *RIM1α forms a protein scaffold for regulating neurotransmitter release at the active zone.* Nature, 2002. **415**: p. 321.
10. Spangler, S.A., et al., *Liprin-alpha2 promotes the presynaptic recruitment and turnover of RIM1/CASK to facilitate synaptic transmission.* J Cell Biol, 2013. **201**(6): p. 915-28.
11. Sutton, R.B., et al., *Crystal structure of a SNARE complex involved in synaptic exocytosis at 2.4 A resolution.* Nature, 1998. **395**(6700): p. 347-53.
12. K.M.S. Misura, R.H.S., W.I Weiss, *Three-dimensional structure of the neuronal Sec1-syntaxin 1a complex.* Nature, 2000. **404**(6776): p. 355-62.
13. Hui, E., et al., *Three distinct kinetic groupings of the synaptotagmin family: candidate sensors for rapid and delayed exocytosis.* Proc Natl Acad Sci U S A, 2005. **102**(14): p. 5210-4.
14. Yao, J., et al., *Uncoupling the roles of synaptotagmin I during endo- and exocytosis of synaptic vesicles.* Nat Neurosci, 2011. **15**(2): p. 243-9.
15. Bacaj, T., et al., *Synaptotagmin-1 and synaptotagmin-7 trigger synchronous and asynchronous phases of neurotransmitter release.* Neuron, 2013. **80**(4): p. 947-59.
16. Brose, N., et al., *Synaptotagmin: a calcium sensor on the synaptic vesicle surface.* Science, 1992. **256**(5059): p. 1021-5.
17. Südhof, Thomas C., *The Presynaptic Active Zone.* Neuron, 2012. **75**: p. 11-25.
18. Lupas, A., *Prediction and analysis of coiled-coil structures.* Methods Enzymol, 1996. **266**: p. 513-25.
19. Yersin, A., et al., *Interactions between synaptic vesicle fusion proteins explored by atomic force microscopy.* Proc Natl Acad Sci U S A, 2003. **100**(15): p. 8736-41.
20. Sollner, T., et al., *A protein assembly-disassembly pathway in vitro that may correspond to sequential steps of synaptic vesicle docking, activation, and fusion.* Cell, 1993. **75**(3): p. 409-18.

21. Li, F., et al., *Energetics and dynamics of SNAREpin folding across lipid bilayers.* Nat Struct Mol Biol, 2007. **14**(10): p. 890-6.
22. Kameyama, K., et al., *Involvement of a postsynaptic protein kinase A substrate in the expression of homosynaptic long-term depression.* Neuron, 1998. **21**(5): p. 1163-75.
23. Castillo, P.E., *Presynaptic LTP and LTD of excitatory and inhibitory synapses.* Cold Spring Harb Perspect Biol, 2012. **4**(2).
24. Jung, J.H., et al., *Variable priming of a docked synaptic vesicle.* Proc Natl Acad Sci U S A, 2016. **113**(8): p. E1098-107.
25. Stein, A., et al., *Helical extension of the neuronal SNARE complex into the membrane.* Nature, 2009. **460**(7254): p. 525-8.
26. Melom, J.E., et al., *Spontaneous and Evoked Release Are Independently Regulated at Individual Active Zones.* The Journal of Neuroscience, 2013. **33**(44): p. 17253-17263.
27. Peled, E.S., Z.L. Newman, and E.Y. Isacoff, *Evoked and spontaneous transmission favored by distinct sets of synapses.* Curr Biol, 2014. **24**(5): p. 484-93.
28. Chung, C., et al., *Acute dynamin inhibition dissects synaptic vesicle recycling pathways that drive spontaneous and evoked neurotransmission.* J Neurosci, 2010. **30**(4): p. 1363-76.
29. Fawley, J.A., M.E. Hofmann, and M.C. Andresen, *Cannabinoid 1 and transient receptor potential vanilloid 1 receptors discretely modulate evoked glutamate separately from spontaneous glutamate transmission.* J Neurosci, 2014. **34**(24): p. 8324-32.
30. Goda, Y. and C.F. Stevens, *Two components of transmitter release at a central synapse.* Proc Natl Acad Sci U S A, 1994. **91**(26): p. 12942-6.
31. Atluri, P.P. and W.G. Regehr, *Delayed release of neurotransmitter from cerebellar granule cells.* J Neurosci, 1998. **18**(20): p. 8214-27.
32. Fox, M.A. and J.R. Sanes, *Synaptotagmin I and II are present in distinct subsets of central synapses.* J Comp Neurol, 2007. **503**(2): p. 280-96.
33. Adolfsen, B., et al., *Synaptotagmins are trafficked to distinct subcellular domains including the postsynaptic compartment.* J Cell Biol, 2004. **166**(2): p. 249-60.
34. Peron, S., et al., *From action potential to contraction: neural control and excitation-contraction coupling in larval muscles of Drosophila.* Comp Biochem Physiol A Mol Integr Physiol, 2009. **154**(2): p. 173-83.
35. Craxton, M., *Evolutionary genomics of plant genes encoding N-terminal-TM-C2 domain proteins and the similar FAM62 genes and synaptotagmin genes of metazoans.* BMC Genomics, 2007. **8**: p. 259.
36. Schapire, A.L., et al., *Arabidopsis synaptotagmin 1 is required for the maintenance of plasma membrane integrity and cell viability.* Plant Cell, 2008. **20**(12): p. 3374-88.
37. Shen, S.S., et al., *Molecular regulation of membrane resealing in 3T3 fibroblasts.* J Biol Chem, 2005. **280**(2): p. 1652-60.
38. Roggero, C.M., et al., *Complexin/synaptotagmin interplay controls acrosomal exocytosis.* J Biol Chem, 2007. **282**(36): p. 26335-43.
39. Mizuta, M., et al., *Localization and functional role of synaptotagmin III in insulin secretory vesicles in pancreatic beta-cells.* Diabetes, 1997. **46**(12): p. 2002-6.
40. Zhao, H., et al., *Synaptotagmin VII regulates bone remodeling by modulating osteoclast and osteoblast secretion.* Dev Cell, 2008. **14**(6): p. 914-25.
41. Harris, K.P., et al., *The postsynaptic t-SNARE Syntaxin 4 controls traffic of Neuroligin 1 and Synaptotagmin 4 to regulate retrograde signaling.* Elife, 2016. **5**.

42. Chen, C., et al., *Triple Function of Synaptotagmin 7 Ensures Efficiency of High-Frequency Transmission at Central GABAergic Synapses.* Cell Rep, 2017. **21**(8): p. 2082-2089.
43. Fernandez, I., et al., *Three-dimensional structure of the synaptotagmin 1 C2B-domain: synaptotagmin 1 as a phospholipid binding machine.* Neuron, 2001. **32**(6): p. 1057-69.
44. Ubach, J., et al., *Ca2+ binding to synaptotagmin: how many Ca2+ ions bind to the tip of a C2-domain?* Embo j, 1998. **17**(14): p. 3921-30.
45. Mackler, J.M. and N.E. Reist, *Mutations in the second C2 domain of synaptotagmin disrupt synaptic transmission at Drosophila neuromuscular junctions.* J Comp Neurol, 2001. **436**(1): p. 4-16.
46. Loewen, C.A., et al., *C2B polylysine motif of synaptotagmin facilitates a Ca2+-independent stage of synaptic vesicle priming in vivo.* Mol Biol Cell, 2006. **17**(12): p. 5211-26.
47. Borden, C.R., et al., *Synaptotagmin mutants Y311N and K326/327A alter the calcium dependence of neurotransmission.* Mol Cell Neurosci, 2005. **29**(3): p. 462-70.
48. Li, L., et al., *Phosphatidylinositol phosphates as co-activators of Ca2+ binding to C2 domains of synaptotagmin 1.* J Biol Chem, 2006. **281**(23): p. 15845-52.
49. Mace, K.E., et al., *Synaptotagmin I stabilizes synaptic vesicles via its C(2)A polylysine motif.* Genesis, 2009. **47**(5): p. 337-45.
50. Paddock, B.E., et al., *Ca2+-dependent, phospholipid-binding residues of synaptotagmin are critical for excitation-secretion coupling in vivo.* J Neurosci, 2008. **28**(30): p. 7458-66.
51. Wang, P., et al., *Mutations in the effector binding loops in the C2A and C2B domains of synaptotagmin I disrupt exocytosis in a nonadditive manner.* J Biol Chem, 2003. **278**(47): p. 47030-7.
52. Fernandez-Chacon, R., et al., *Synaptotagmin I functions as a calcium regulator of release probability.* Nature, 2001. **410**(6824): p. 41-9.
53. Mackler, J.M., et al., *The C(2)B Ca(2+)-binding motif of synaptotagmin is required for synaptic transmission in vivo.* Nature, 2002. **418**(6895): p. 340-4.
54. Nishiki, T. and G.J. Augustine, *Dual roles of the C2B domain of synaptotagmin I in synchronizing Ca2+-dependent neurotransmitter release.* J Neurosci, 2004. **24**(39): p. 8542-50.
55. Robinson, I.M., R. Ranjan, and T.L. Schwarz, *Synaptotagmins I and IV promote transmitter release independently of Ca(2+) binding in the C(2)A domain.* Nature, 2002. **418**(6895): p. 336-40.
56. Fernandez-Chacon, R., et al., *Structure/function analysis of Ca2+ binding to the C2A domain of synaptotagmin 1.* J Neurosci, 2002. **22**(19): p. 8438-46.
57. Stevens, C.F. and J.M. Sullivan, *The synaptotagmin C2A domain is part of the calcium sensor controlling fast synaptic transmission.* Neuron, 2003. **39**(2): p. 299-308.
58. Striegel, A.R., et al., *Calcium binding by synaptotagmin's C2A domain is an essential element of the electrostatic switch that triggers synchronous synaptic transmission.* J Neurosci, 2012. **32**(4): p. 1253-60.
59. Vermaas, J.V. and E. Tajkhorshid, *Differential Membrane Binding Mechanics of Synaptotagmin Isoforms Observed in Atomic Detail.* Biochemistry, 2017. **56**(1): p. 281-293.

60. Herrick, D.Z., et al., *Position of synaptotagmin I at the membrane interface: cooperative interactions of tandem C2 domains.* Biochemistry, 2006. **45**(32): p. 9668-74.
61. Bai, J., P. Wang, and E.R. Chapman, *C2A activates a cryptic Ca(2+)-triggered membrane penetration activity within the C2B domain of synaptotagmin I.* Proc Natl Acad Sci U S A, 2002. **99**(3): p. 1665-70.
62. Xu, J., et al., *Synaptotagmin-1 functions as a Ca2+ sensor for spontaneous release.* Nat Neurosci, 2009. **12**(6): p. 759-66.
63. Lee, J., et al., *Genetic analysis of synaptotagmin C2 domain specificity in regulating spontaneous and evoked neurotransmitter release.* J Neurosci, 2013. **33**(1): p. 187-200.
64. Yoshihara, M., Z. Guan, and J.T. Littleton, *Differential regulation of synchronous versus asynchronous neurotransmitter release by the C2 domains of synaptotagmin 1.* Proc Natl Acad Sci U S A, 2010. **107**(33): p. 14869-74.
65. Shin, O.H., et al., *Differential but convergent functions of Ca2+ binding to synaptotagmin-1 C2 domains mediate neurotransmitter release.* Proc Natl Acad Sci U S A, 2009. **106**(38): p. 16469-74.
66. Paddock, B.E., et al., *Membrane penetration by synaptotagmin is required for coupling calcium binding to vesicle fusion in vivo.* J Neurosci, 2011. **31**(6): p. 2248-57.
67. Bowers, M.R. and N.E. Reist, *The C2A domain of synaptotagmin is an essential component of the calcium sensor for synaptic transmission.* PLoS One, 2020. **15**(2): p. e0228348.
68. Rhee, J.S., et al., *Augmenting neurotransmitter release by enhancing the apparent Ca2+ affinity of synaptotagmin 1.* Proc Natl Acad Sci U S A, 2005. **102**(51): p. 18664-9.

CHAPTER 2. SYNAPTOTAGMIN: MECHANISMS OF AN ELECTROSTATIC SWITCH[2]

2.1 Summary

Synaptic transmission relies on the fast, synchronous fusion of neurotransmitter filled vesicles with the presynaptic membrane. Synaptotagmin is the Ca^{2+} sensor that couples this fast, synchronous vesicle fusion with the Ca^{2+} influx into nerve terminals following an action potential. Over two decades of synaptotagmin research has provided many clues as to how Ca^{2+} binding by synaptotagmin may lead to vesicle fusion. *In vitro* studies of molecular binding interactions are essential for elucidating potential mechanisms. However, an *in vivo* system to evaluate the postulated mechanisms is required to determine functional significance. The neuromuscular junction (NMJ) has long been an invaluable tool for synaptic research and studies at the NMJ will undoubtedly continue to provide key insights into synaptotagmin function.

2.2 Introduction

The mechanisms of synaptic transmission are highly conserved both across synaptic types and across species. Regardless of the synapse, depolarization-induced Ca^{2+} influx leads to the fusion of synaptic vesicles with the presynaptic membrane and the resultant release of neurotransmitter. Due to its unique accessibility and relative simplicity, the NMJ has played a pivotal role in advancing our understanding of synaptic transmission. From the pioneering studies of quantal release at the frog NMJ by Bernard Katz and colleagues in the 1950s [1, 2]

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through modern molecular work, analysis of synaptic transmission at the NMJ from multiple organisms has been essential for advancing the field.

A long-standing question for synaptic researchers is: what is the mechanism whereby a local increase in Ca^{2+} triggers the synchronous, mechanical fusion of neurotransmitter-filled vesicles with the presynaptic membrane? A large body of research, including biochemical and genetic studies, led to the discovery and characterization of many critical molecules, including: 1) the SNARE (soluble N-ethlymaleimide sensitive factor adaptor protein receptors) complex, the minimal membrane fusion machinery, and 2) synaptotagmin, the Ca^{2+} sensor that triggers fast, synchronous neurotransmitter release [3-5]. Analysis of synaptic function at the *Drosophila* NMJ was pivotal in demonstrating that synaptotagmin is indeed the Ca^{2+} sensor [6]. Since then, synaptotagmin research has focused on uncovering this critical protein's mechanism of action. The combination of *in vitro* biochemical studies with *in vivo* electrophysiological recordings at the NMJ has been a powerful approach. *In vitro* biochemical studies led to new insights into how synaptotagmin may interact with other components of the synaptic machinery. But the resultant hypotheses could only be tested for functional relevance by measuring synaptic transmission *in vivo*. The NMJ is an ideal model system for this *in vivo* analysis to determine *how* the synaptic machinery at an intact synapse actually works at the molecular level. While it is clear that multiple other proteins are essential for the precise regulation observed during neurotransmitter release [5, 7-9], here we will focus on synaptotagmin. There are also several isoforms of synaptotagmin, yet in drosophila, only synaptotagmin 1 mediates synchronous neurotransmitter release [10]. While we will discuss interactions with the SNARE proteins, immense bodies of research exist on SNARE interactions and mechanisms and are beyond the scope of this review (see [3, 11]). Here, we specifically review some of the highly conserved intermolecular

interactions of synaptotagmin 1 and emphasize the importance of the NMJ in demonstrating, or refuting, the importance of such interactions for synaptic transmission *in vivo*.

2.3 Synaptotagmin as the Ca^{2+} Sensor

In early studies, Matthew et al purified a synaptic vesicle protein of 65 kDa that had a wide distribution in neural tissues and that was highly conserved in vertebrates [12]. Sequencing of this protein, synaptotagmin, demonstrated that it contained two C_2 domain Ca^{2+}-binding motifs [13]. Later, it was found that the protein was highly conserved from humans down to *Drosophila* [14]. Coupled with biochemical studies demonstrating Ca^{2+}-dependent interactions with negatively charged phospholipids *in vitro* [15, 16] synaptotagmin was put forward as an excellent candidate for the Ca^{2+} sensor that triggers neurotransmitter release. But it was electrophysiological recordings at the *Drosophila* NMJ from synaptotagmin knock outs, showing a dramatic decrease in neurotransmitter release, that established the importance of synaptotagmin *in vivo* [17-20]. Conservation of synaptotagmin function at mammalian synapses was later confirmed by recordings of CNS neurons cultured from synaptotagmin knockout mice [21]. These seminal studies provided the first strong evidence that synaptotagmin is indeed critical for synaptic transmission.

But the question remained, was synaptotagmin the Ca^{2+} sensor? The protein is an integral membrane protein located on synaptic vesicles with tandem C_2 domains [13], which each contain a Ca^{2+}-binding pocket comprised of negatively charged aspartate residues [D, Fig. 2.1, red stick residues, [22, 23]]. Naturally, the function of these Ca^{2+} binding residues within each C_2 domain were the focus of intense investigation while trying to assess whether synaptotagmin was indeed the Ca^{2+} sensor for synaptic transmission. Mutation of these aspartates to polar, but neutral,

asparagines (N) blocked Ca^{2+} binding, yet had differential effects on evoked transmitter release *in vivo*. The DN mutations in the C_2A domain had little impact on evoked transmitter release, neither at the NMJ nor at cultured mammalian CNS synapses [24, 25]. This surprising result raised the concern that synaptotagmin may not be the Ca^{2+} sensor for synaptic transmission. However, similar DN mutations in the C_2B domain abolished evoked transmitter release at the NMJ. Indeed, C_2B-DN mutations had a dominant negative effect, inhibiting release even in the presence of the wild type protein. Thus, Ca^{2+} binding by the C_2B domain of synaptotagmin is required for triggering any synchronous vesicle fusion at an intact synapse [6]. Some of the DN mutations even resulted in embryonic lethality in the absence of the wild type protein (unpublished observation and [6]). This total block of synaptotagmin-triggered release in C_2B-DN mutants was later confirmed at CNS synapses in culture [26]. This combination of effects in C_2A and C_2B suggested that Ca^{2+} binding by the C_2B domain was essential while Ca^{2+} binding by the C_2A domain played little to no role in triggering neurotransmitter release. That interpretation, however, was later shown to be incorrect. Since the role of Ca^{2+} binding is to mask the negative charge of the pocket (see electrostatic switch below), the DN mutations in the C_2A domain [24, 25, 27] actually mimicked constitutive Ca^{2+} binding. An aspartate to glutamate (DE) mutation in the C_2A domain, which blocked Ca^{2+} binding by steric hindrance while maintaining the negative charge of the pocket, decreased evoked transmitter release at the NMJ by 80% [28]. Thus, Ca^{2+} binding by C_2B is required to synchronize fast vesicle fusion with Ca^{2+} influx but the negative charge of both Ca^{2+} binding pockets must be neutralized to effectively stimulate the fusion reaction. Importantly, it was the finding at the *Drosophila* NMJ, that mutation of C_2B Ca^{2+} binding residues blocked fast, synchronous neurotransmitter release [6],

which finally demonstrated that synaptotagmin was indeed the Ca^{2+} sensor required for synaptic transmission.

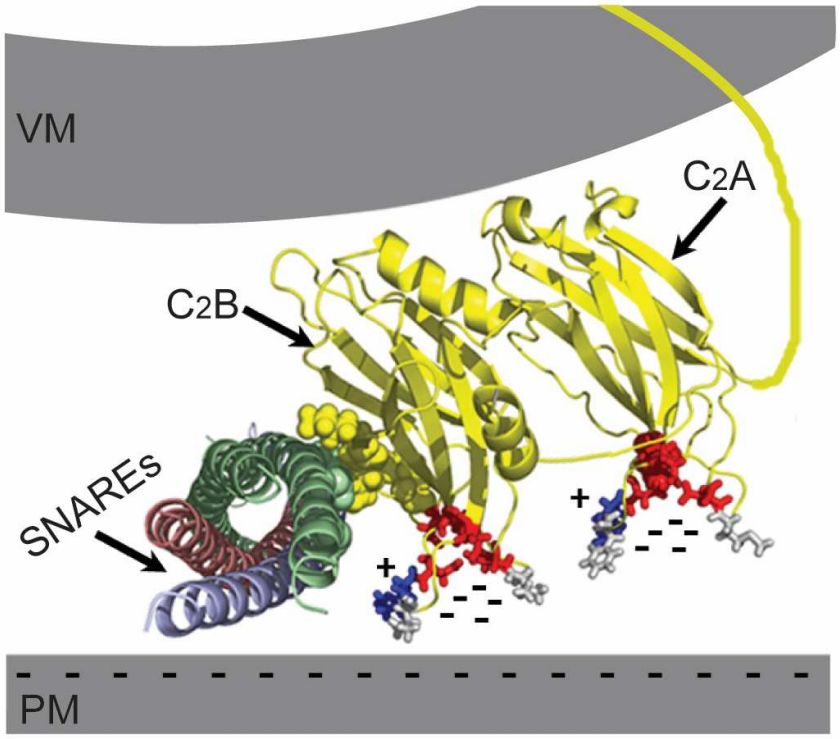

Figure 2.1. Cartoon representation of synaptotagmin and the SNARE complex. Synaptotagmin (yellow) is composed of tandem C_2 domains, C_2A [Protein Data Bank (PDB) file 1BYN] and C_2B (PDB 1K5W). The polylysine motif of C_2B (yellow ball residues) is shown interacting with the SNARE complex (PDB 1SFC). Each Ca^{2+} binding pocket has negatively-charged aspartates (red stick residues) that coordinate Ca^{2+} binding and provide electrostatic repulsion of the negatively-charged presynaptic membrane in the absence of Ca^{2+}. Basic residues at the tips of the Ca^{2+} binding pockets (blue stick residues) contribute to the electrostatic attraction of the presynaptic membrane following Ca^{2+} binding. Hydrophobic residues (grey stick residues) penetrate into the membrane(s) following Ca^{2+} binding. The vesicle membrane (VM) and negatively-charged presynaptic membrane (PM) are shown.

2.4 Highly conserved proposed effector domains of synaptotagmin

With synaptotagmin established as the Ca^{2+} sensor, research to elucidate the mechanism(s) whereby Ca^{2+} binding by synaptotagmin results in fusion of synaptic vesicles with the presynaptic membrane intensified. After decades of *in vitro* analyses to demonstrate its crystal and NMR structure [22, 29, 30] and biochemical assays to assess protein interactions,

coupled to electrophysiological recordings at the NMJ *in vivo* and at cultured synapses following induction of site-directed point mutations to disrupt specific interactions, significant progress has been made in our understanding of synaptotagmin function.

Here we'll review work on some of the highly conserved residues within the C_2 domains that early studies implicated as playing a functional role during the synaptic vesicle cycle: a polylysine motif in the C_2B domain (Fig. 2.1, yellow space-filled residues), a basic residue located at the tip of the C_2A and C_2B Ca^{2+} binding pockets (Fig. 2.1, blue stick residues), and hydrophobic residues also located at the tip of the pockets (Fig. 2.1, grey stick residues). Later studies have confirmed these sites have functional significance, although the precise mechanisms are still under debate.

The C_2B polylysine motif

In vivo recordings at the NMJ documented a functional role as C_2B polylysine motif mutants exhibited an ~40% decrease in evoked synaptic transmission [31]. Various biochemical analyses over the years suggested that the C_2B polylysine motif might play a role in vesicle fusion via interactions with negatively charged phospholipids [32-34], SNARE proteins [35-37] or other molecules of synaptotagmin [35]. Alternatively, interactions with SNARE proteins or Ca^{2+} channels could stabilize vesicle docking or priming [35, 38] while an interaction with the clathrin-adapter protein AP-2 implied a function in synaptic vesicle recycling [35, 39], Although *in vivo* recording at the *Drosophila* NMJ [40, 41] as well as in cultured CNS neurons [42] support a role for synaptotagmin in synaptic vesicle recycling, NMJ recordings also demonstrated that the C_2B polylysine motif does *not* impact the rate of vesicle endocytosis [40, 43]. Interactions between the polylysine domain and synaptic vesicle protein 2A may play a role

in co-trafficking synaptotagmin during vesicle recycling [44]. Recordings at the NMJ showing slowed recovery of the maximal synaptic response following high-frequency stimulation coupled with electron microscopic studies at cultured central synapses now strongly implicate the C_2B polylysine motif in the synaptic vesicle docking and/or priming required prior to fusion, most likely through its interaction with the SNARE complex [38, 43, 45, 46]

Basic residue at the tip of each C_2 domain

A similar combinatorial *in vitro* and *in vivo* approach has been useful for investigating the mechanism of action of a highly-conserved, positively-charged residue (arginine, R, or lysine, K) at the tip of each C_2 domain Ca^{2+}-binding pocket (Fig. 2.1, blue stick residues). This residue was first implicated by a study demonstrating that this positive charge was important for interactions with negatively-charged membranes *in vitro* upon Ca^{2+} binding by the C_2A domain. Mutation to a polar, but neutral glutamine (RQ) resulted in an ~50% decrease in the Ca^{2+} affinity of interactions with negatively charged phospholipids. In addition, the C_2A-RQ mutation appeared to decrease synaptic transmission at cultured hippocampal autapses, suggesting that this was a functionally important site for evoke neurotransmitter release [47]. Subsequent biochemical analyses demonstrated interactions by both C_2A and C_2B with negatively-charged membranes [13, 47-49]. Functional analysis at the *Drosophila* NMJ demonstrated that neutralization of this positive charge, C_2A-RQ, resulted in a 50% decrease in Ca^{2+} evoked transmitter release while the homologous mutation in C_2B (C_2B-RQ) resulted in an 80% decrease [50]. In combination with the mutational studies of the Ca^{2+} binding aspartate residues reviewed above, these studies indicate that the net charge of the C_2A domain is facilitatory, while that of the C_2B domain is

critical for evoked transmitter release. Thus, the early proposal that synaptotagmin acts as an electrostatic switch [23, 51] is still strongly supported today ([28, 52], and see below).

Hydrophobic residues at the tips of each C_2 domain

The C_2 domain beta-sheet structural unit (Fig. 2.1, yellow ribbons) has three amino acid loops extending from one end. Two of these loops (loop 1 and loop 3) contain the five negatively-charged Ca^{2+} binding residues that comprise the Ca^{2+} binding pocket (Fig 2.1, red stick residues,). These two loops also each contain a highly conserved hydrophobic residue at the tip of the pocket (Fig 2.1, grey stick residues, [22, 29, 53]). Biochemical analysis of C_2A and C_2B interactions with negatively-charge phospholipids demonstrate that these hydrophobic residues penetrate into the hydrophobic core of liposomes in a Ca^{2+} dependent manner *in vitro* ([49, 54, 55]), a finding supported by *in silico* modeling [56]. Interactions with the SNARE proteins have also been reported [57-59], suggesting that these hydrophobic residues may bind to SNARE complexes. However, disruptions of SNARE interactions are only seen for membrane embedded SNAREs and they do not correlate with disruptions of evoked transmitter release *in vivo* [57, 58]. Membrane insertion of these hydrophobic tip residues seems to disorder lipids and warp membranes. Insertion of these residues causes local disordering of acyl chain order [60] and increasingly disordered lipid composition favors vesicle fusion, while highly ordered lipid composition has the opposite effect [61]. In addition, membrane insertion has been shown to tubulate liposomes *in vitro* indicating that insertion induces high positive curvature of membranes [57, 62]. Importantly, an *in vitro* fusion assay that used giant liposomes demonstrated that synaptotagmin's ability to induce positive curvature is required to trigger liposome fusion *in vitro* when both membranes are not already highly curved [57].

While the synaptic vesicle is already at the ideal curvature for fusion [57], the presynaptic membrane is not, so the ability of synaptotagmin to induce curvature in this membrane could reduce the energy barrier for fusion. This theory is supported by previous electron microscopy observations that a small dimple in the presynaptic membrane is present before formation of the fusion pore [63]. To determine whether these hydrophobic residues mediate interactions important for synaptic transmission *in vivo*, electrophysiological recordings were made at the *Drosophila* NMJ in mutants where these residues were each changed to polar and/or negatively-charged residues to prevent any membrane insertion. Mutation of the loop 3 hydrophobic reside in C_2B (isoleucine to glutamate, IE) resulted in the most severe synaptotagmin mutation *in vivo* to date [58]. This mutation had a dominant negative effect, resulting in embryonic lethality in the *presence or absence* of wild type synaptotagmin. In the absence of the wild type protein, evoked transmitter release in the C_2B-IE mutant was inhibited to a greater extent than in the complete absence of any synaptotagmin I. Since the severity of the disruption in evoked neurotransmitter release at the NMJ *in vivo* correlated with the decrease in membrane interactions, but not with decreased SNARE interactions *in vitro* [58], membrane penetration by the C_2B loop 3 hydrophobic residue is essential for synaptic transmission.

In the C_2A domain, mutation of either the loop 1 or loop 3 hydrophobic residue also inhibited evoked transmitter release at the *Drosophila* NMJ *in vivo* ([58, 64]. Mutation of the loop 3 phenylalanine residue to either a tyrosine (FY, polar but neutral) or a glutamate (FE, polar and negatively charged) resulted in the *same* deficit – an ~50% decrease in evoked transmitter release in the absence of wild type synaptotagmin [58]. Thus, the loss of hydrophobicity, not the net charge of the pocket, correlated with the decrease in evoked transmitter release. Mutation of the loop 1 methionine residue to a glutamate (C_2A-ME) also resulted in an ~50% decrease in

evoked transmitter release [64]. Similar results have been obtained using mammalian cultured CNS neurons. Interestingly in these cultures, increasing the size of the hydrophobic side chain for all 4 of these C_2A and C_2B residues (to tryptophan, 4W) increased neurotransmitter release, while decreasing the size of all 4 (to alanine, 4A) decreased release [42, 65]. This suggests that the depth of insertion strongly impacts the efficacy of release. The results from these hydrophobic mutants at the NMJ, together with the findings that the C_2A-DE and the C_2A-RQ mutations inhibited release by ~80% and ~50%, respectively, again support the long-standing assumption that the C_2A Ca^{2+} binding pocket merely facilitates efficient evoked transmitter release, while that of the C_2B domain is essential for any fast, synchronous release. However, recent studies disprove this assumption. Simultaneous mutation of both the loop 1 and loop 3 hydrophobic residues in C_2A (C_2A-ME,FE) resulted in a more severe decrease in evoked transmitter release than that seen in the complete absence of the wild type protein [64]. This is the first C_2A domain mutation to ever show more severe deficits than the synaptotagmin knock out. Thus, it now seems that the C_2A Ca^{2+} binding pocket is also absolutely essential for any synaptotagmin-triggered neurotransmitter release.

2.5 Possible mechanisms for transducing Ca^{2+} binding to vesicle fusion

There is a tremendous body of data, highlights of which are reviewed above, showing that multiple putative effector domains both: 1) are indeed required for efficient synaptic transmission *in vivo*, and 2) mediate intermolecular interactions *in vitro* that could reasonably participate in the transduction process. So how does Ca^{2+} binding to synaptotagmin result in fusion of the synaptic vesicle and presynaptic membranes? This question is still a matter of intense research and debate. Here, we attempt to incorporate most of the current findings for the

conserved residues reviewed above into a plausible mechanistic model whereby Ca^{2+} entry into the nerve terminal could result in the fast, synchronous fusion of multiple synaptic vesicles with the presynaptic membrane (Fig 2.2).

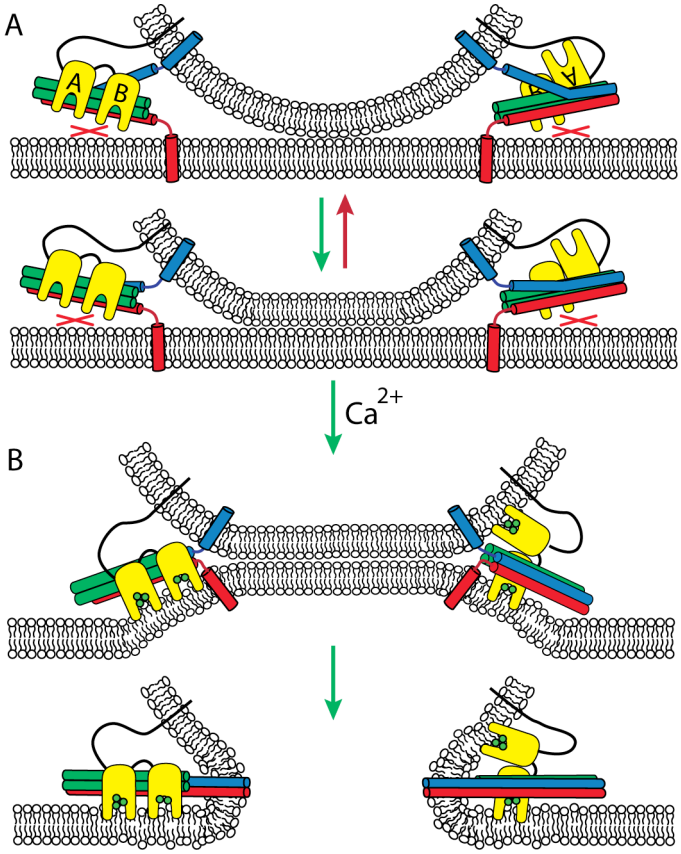

Figure 2.2. Proposed mechanism of synaptotagmin-triggered fusion. A) In the absence of Ca^{2+}, docked synaptic vesicles (direct VM/PM contact) are in a state of dynamic equilibrium (red and green arrows). The small area of contact indicates minimal priming (top panel) while the large area of contact indicates maximal priming (bottom panel). The closer association of the SNARE helices during zippering of the SNARE complex (blue, red, and green) provides force favoring maximal priming (green arrow). Electrostatic repulsion of the presynaptic membrane by synaptotagmin (red X) drives the priming reaction in the opposite direction (red arrow). B) Ca^{2+} binding (green spheres) by both C_2 domains flips the electrostatic switch: from repulsion to attraction of the presynaptic membrane by synaptotagmin. This switch both removes the clamp on the SNARE complex and allows the hydrophobic tip residues of the Ca^{2+} binding pockets to insert into the lipid membranes. The insertion induces local curvature of the presynaptic membrane, predicted to lower the energy barrier preventing fusion. In addition, the resultant disordering of the phospholipids favors complete coiling of SNARE proteins into their transmembrane domains which would help drive fusion. Whether the two C2 domains insert into and disorder both membranes (right side) or both insert into the presynaptic membrane (left side) is still a matter of debate. Importantly, only vesicles that happen to be in the maximally primed

state (which is predicted to pull Ca^{2+} channels closer to synaptotagmin associated with the SNAREs, not shown) would undergo the fast, synchronous fusion of synaptic.

Synaptotagmin as an electrostatic switch

The prevailing theory, which has stood the test of time, is that synaptotagmin's initial function in transducing the Ca^{2+} signal is to act as an electrostatic switch [28, 51, 52, 66, 67]. The SNARE proteins (Fig 2.1, blue, red, green helixes; Fig 2.2, blue, red, green cylinders) represent the minimal machinery required to fuse a vesicle with a target membrane [68, 69]. They mediate constitutive vesicle fusion events throughout cells by coiling around one another, progressing from the N-terminal end of their α-helical SNARE motifs toward the transmembrane domains of syntaxin and synaptobrevin (Fig 2.1, SNARES, coiled coil viewed end on [70]). Thus, the SNAREs are thought to act like a set of zippers providing force to pull vesicles closer to the target membrane (Fig 2.2A, blue/red/green cylinders progressing from top to bottom panel [71]) until they fuse. At the synapse this fusion capacity must be regulated. Prior to Ca^{2+} entry into the nerve terminal, this fusion capability must be clamped. Then, following Ca^{2+} influx, there must be fast, synchronous fusion of multiple synaptic vesicles with the presynaptic membrane. In other words, there must be a sufficient energy barrier preventing fusion prior to Ca^{2+} entry that is dramatically lowered immediately upon Ca^{2+} influx. The finding that synaptotagmin knockouts have an increased rate of spontaneous vesicle fusion events first implicated synaptotagmin as a clamp [17-20, 72]. A recent study now indicates that the C_2B domain is both necessary and sufficient to inhibit this elevated rate of spontaneous release seen in synaptotagmin knock out neurons in culture [38]. Several Ca^{2+}-independent interactions between synaptotagmin and the SNARE complex, one by the C_2B polylysine motif as reviewed above, could act to stabilize the docking and/or priming of synaptic vesicles at the active zone

prior to Ca2+ influx [43, 45, 46, 73, 74]. Such interactions could potentially help prevent full coiling of the SNARE complex. Importantly, the Ca^{2+} binding pockets of synaptotagmin's C_2 domains are thought to repel the negatively-charged presynaptic membrane due to their net-negative charge (see Fig 2.1, minus signs; Fig 2.2, red x). Data supporting this mechanism of clamping include: the C_2A-DN and C_2B-DN mutations, that block Ca^{2+} binding by neutralizing this negative charge, result in an increased rate of spontaneous fusion events both at the NMJ and at cultured CNS synapses [6, 72, 75]. Interestingly, recent work introducing additional positive or negative charges to one of the SNARE proteins immediately adjacent to the vesicle membrane/presynaptic membrane (VM/PM) interface increases or decreases, respectively, the rate of spontaneous vesicle fusion events [52]. Thus, the negative charge immediately surrounding the VM/PM interface is a critical component of the clamp preventing spontaneous SNARE-mediated fusion events.

Following Ca^{2+} influx into an activated nerve terminal, Ca^{2+} binding to synaptotagmin would rapidly mask the negative charge of the C_2A and C_2B Ca^{2+}-binding pockets, resulting in a net positive charge [23, 67]. This positive charge, along with the positive charge of the highly conserved basic residue located at the tip of each pocket, would then act like an instantaneous electrostatic switch that would now attract the negatively charged presynaptic membrane [28, 29, 51, 52, 66]. The sudden change in charge would both remove the clamp and favor closer apposition of the vesicle membrane and presynaptic membrane, promoting fusion. Mutational analyses support this hypothesis. Mutations that neutralize the positively-charged residue at the tip of the C_2A or C_2B Ca^{2+} binding pocket decrease evoked transmitter release [47, 50] due to decreased electrostatic attraction of the presynaptic membrane following Ca^{2+} binding. DN mutations in either C_2A or C_2B, that block Ca^{2+} binding by removing negative charge from the

pocket, increase spontaneous release, but only those in C_2B block evoked release [6, 24, 25, 27, 75]. Conversely, a C_2A-DE mutation, that blocks Ca^{2+} binding while maintaining the negative charge of the pocket, blocks evoked release while maintaining a normal rate of spontaneous release [28]. Together these results indicate that Ca^{2+} binding by both C_2A and C_2B are required to flip the electrostatic switch but only C_2B is required to synchronize vesicle fusion with Ca^{2+} influx.

What might determine which docked vesicles fuse and which do not? At amphibian NMJs for example, only about 1% of docked synaptic vesicles (Fig 2.2A, top and bottom panels, defined here as synaptic vesicles whose membranes are in *direct contact* with the presynaptic membrane with no discernable gap) fuse when an action potential invades the nerve terminal [76, 77]. Again, studies at the NMJ provide key insight into possible mechanisms. Recent electron tomographic studies at the frog neuromuscular junction [78] have revolutionized how the field thinks about synaptic vesicle priming, which is required prior to Ca^{2+} triggered fusion. Prior to this work, the transition from docking to maximal priming to fusion was generally considered to be a one way sequential series of steps where any direct contact between the VM and PM was considered docking and then variously defined molecular interactions must occur to maximally prime vesicles prior to fusion. However, Jung et al. demonstrated that all docked synaptic vesicles are likely in a state of dynamic equilibrium (Fig 2.2A double headed red arrow), continuously changing *back and forth* from minimally primed (top panel) to maximally primed (bottom panel). They measured the extent of the VM/PM contact area as well as the length of various active zone material macromolecular complexes and propose that changes in these measurements indicate the degree of vesicle priming. Minimally primed vesicles have a small VM/PM contact area (Fig 2.2A top panel) and longer macromolecular connections to the PM

(not shown). Maximally primed vesicles have a large VM/PM contact area (Fig 2.2A bottom panel) and shorter connections. Importantly, they found a normal distribution for all of these measurements indicating that priming is in a dynamic equilibrium. Mechanistically, the coiling of the SNARE proteins would provide force pulling the VM and PM together [71], increasing the VM/PM contact area and shortening the connections between the vesicle and presynaptic membrane [in which the SNAREs must be located [79-82]]. Opposing forces, to uncoil the SNAREs and push the vesicle away from the presynaptic membrane, could come from hydration repulsion of the membrane bilayers [83] and electrostatic repulsion, at least partially provided by synaptotagmin associated with the SNARE proteins within these connections. These repulsive forces would decrease the VM/PM contact area and lengthen the VM/PM macromolecular connections. Importantly, shortening of these connections also pulls PM macromolecules, thought to include voltage sensitive Ca^{2+} channels [77, 84, 85] closer to the VM/PM contact area [78]. Since small changes in distance between the Ca^{2+} channel and the Ca^{2+} sensor are predicted to have profound impacts on the fusion probability [86, 87], the finding that these PM macromolecules are pulled on average ~4 nm closer to the vesicles with the largest VM/PM contact area [78] could cause these vesicles to have a high fusion probability upon Ca^{2+} influx. Thus, it would only be the vesicles that happen to be maximally primed (largest VM/PM contact area and shortest connections) at the instant that the Ca^{2+} channels open that would experience a high enough $[Ca^{2+}]$ to saturate synaptotagmin and trigger fusion.

Synaptotagmin and disruption of membranes

The hypothesis that synaptotagmin flips an electrostatic switch to trigger fast, synchronous fusion as described above is consistent with the results from studies where charged

residues of synaptotagmin have been mutated. However, a simple switch from repulsion to attraction, in the absence of hydrophobic interactions with the membrane, is insufficient. Mutations to the hydrophobic tip residues in synaptotagmin are among the most potent inhibitors of evoked transmitter release *in vivo* and this inhibition is strongly correlated to disruptions in Ca^{2+}-dependent, synaptotagmin-membrane interactions *in vitro*. Since both the C_2B-IE mutation and the C_2A-MEFE mutation result in less transmitter release than that seen in the absence of the wild type protein [58, 64], the Ca^{2+}-dependent membrane penetration mediated by these residues in both C_2 domains [49, 55] is required for synaptotagmin-triggered vesicle fusion. Thus, a more complete picture requires that flipping the electrostatic switch leads to membrane penetration by these hydrophobic tip residues.

But how does membrane penetration contribute to vesicle fusion? As reviewed above, membrane penetration by synaptotagmin has been shown to induce positive curvature in the target membrane [57, 62]. Since high membrane curvature decreases the energy barrier for fusion [57], curvature induced by synaptotagmin insertion into the presynaptic membrane is thought to be necessary to stimulate fusion. This idea is supported by *in vitro* lipid fusion studies using either small (~65 nm) or giant (>1 μm diameter) liposomes. A membrane-penetration deficient synaptotagmin mutant could trigger the fusion of the small liposomes but fusion of the giant liposomes was significantly impaired. The giant liposome fusion could be rescued by addition of the curvature inducing peptide, N-BAR [57]. Since both C_2 domains insert into membranes and the ability of synaptotagmin to induce curvature is required, synaptotagmin is often diagramed with both C_2 domains inserting into the presynaptic membrane (Fig 2.2, left side).

However, alternative mechanisms have also been proposed. In recent work using site-directed fluorescence-interference contrast microscopy, Kiessling et al. were able to measure the tilt angle of the n-terminus of syntaxin relative to its transmembrane domain, which was located in a supported membrane. Thus, they could measure trans to cis conformational changes of the SNARE complex (Fig 2.2B, top panel to bottom panel, straightening of the SNARE complex) that occurred during an *in vitro* fusion assay. In the presence of synaptotagmin C_2AB domains and Ca^{2+}, the membrane embedded SNARE complex changed from the trans to the cis conformation and this change correlated with both vesicle fusion events and disordering of the lipids within the membrane [61]. These findings support an electrostatic switching mechanism triggering membrane insertion of synaptotagmin that then causes local disordering of lipids required for the trans to cis conformation change of the SNARE complex. If the tilt angle for synaptobrevin embedded in supported membranes were similarly impacted by C_2AB and Ca^{2+}, that would suggest one C_2 domain inserts into the PM while the other inserts into the VM. Although, that experiment has not been done, modeling based on site-directed spin labeling predicts that each C_2 domain inserts into opposite membranes [88]. Recently, electronic paramagnetic resonance (EPR) spectral analysis of full-length synaptotagmin in the presence of membranes (lacking SNARE proteins) identified the lipid preferences of the two domains [89]. This study confirmed the association of the C_2B domain with membranes whose composition mimicked the presynaptic membrane [33, 90]. Additionally, the C_2A domain favored binding to membranes that mimicked the lipid composition of synaptic vesicles. Thus, following Ca^{2+} influx, the C_2B domain almost certainly inserts into the presynaptic membrane. However, the C_2A domain may insert into the vesicle membrane (Fig 2.2, right side). Additional studies will be needed to settle this question. Regardless, *in vivo* studies at the NMJ clearly demonstrate that

the hydrophobic tip residues in both C_2A and C_2B are absolutely required for synaptotagmin-triggered vesicle fusion and this disruption in function correlates with the disruption of membrane interactions [58, 64].

2.6 The NMJ as a model system

The NMJ will be a critical model system, especially in genetically tractable organisms, both for future determinations of the molecular mechanisms underlying synaptic transmission and in studies of disease etiology. The ultrahigh resolution possible using electron tomography now permits the visualization of structures down to the molecular level. At the frog NMJ, macromolecular complexes have been resolved at 2-3 nm resolution, the highest to date [79, 91]. Thus, we now know both the structure of the active zone material as well as the identity of many proteins that must function there. The next key step will be the convergence of these fields such that each functional protein is mapped onto these macromolecular complexes. This could finally resolve questions regarding the functional organization of the fusion machinery at the synapse *in vivo*. In addition, recent work investigating the etiology of rare congenital myasthenic syndromes (CMS) highlights the utility of the *Drosophila* NMJ as a fast, genetic tool for elucidating the impact of individual amino acid substitutions in synaptotagmin. Two families with rare forms of CMS were found to have two different point mutations in synaptotagmin 2, the synaptotagmin isoform expressed at the human NMJ. Expression of the homologous mutations in the fruit fly recapitulated many of the physiological deficits seen in the patients, indicating a causal relationship [92, 93]. Additionally, the severe deficits seen in patients with individual amino acid substitutions of C_2B hydrophobic tip residues or Ca^{2+} coordinating aspartates in synaptotagmin 1 [94, 95], the isoform expressed at central synapses, can be largely explained by the transmitter

release deficits documented for homologous mutations at the NMJ reviewed above. As new intermolecular interactions are discovered via *in vitro* assays and new residues are implicated in disease etiology via whole exome sequencing, the NMJ will be invaluable for expanding our understanding of synaptotagmin and synaptic physiology *in vivo*.

WORKS CITED

1. Del Castillo, J. and B. Katz, *Quantal components of the end-plate potential.* J Physiol, 1954. **124**(3): p. 560-73.
2. Fatt, P. and B. Katz, *An analysis of the end-plate potential recorded with an intra-cellular electrode.* J Physiol, 1951. **115**(3): p. 320-70.
3. Sudhof, T.C., *Neurotransmitter release: the last millisecond in the life of a synaptic vesicle.* Neuron, 2013. **80**(3): p. 675-90.
4. Chapman, E.R., *A Ca(2+) Sensor for Exocytosis.* Trends Neurosci, 2018. **41**(6): p. 327-330.
5. Sudhof, T.C. and J.E. Rothman, *Membrane fusion: grappling with SNARE and SM proteins.* Science, 2009. **323**(5913): p. 474-7.
6. Mackler, J.M., et al., *The C(2)B Ca(2+)-binding motif of synaptotagmin is required for synaptic transmission in vivo.* Nature, 2002. **418**(6895): p. 340-4.
7. Rizo, J. and J. Xu, *The Synaptic Vesicle Release Machinery.* Annual Review of Biophysics, 2015. **44**(1): p. 339-367.
8. Jahn, R. and D. Fasshauer, *Molecular machines governing exocytosis of synaptic vesicles.* Nature, 2012. **490**(7419): p. 201-7.
9. Sudhof, T.C., *A molecular machine for neurotransmitter release: synaptotagmin and beyond.* Nat Med, 2013. **19**(10): p. 1227-31.
10. Adolfsen, B., et al., *Synaptotagmins are trafficked to distinct subcellular domains including the postsynaptic compartment.* J Cell Biol, 2004. **166**(2): p. 249-60.
11. Karmakar, S., et al., *Neuronal SNARE complex: A protein folding system with intricate protein-protein interactions, and its common neuropathological hallmark, SNAP25.* Neurochem Int, 2019. **122**: p. 196-207.
12. Matthew, W.D., L. Tsavaler, and L.F. Reichardt, *Identification of a synaptic vesicle-specific membrane protein with a wide distribution in neuronal and neurosecretory tissue.* J Cell Biol, 1981. **91**(1): p. 257-69.
13. Perin, M.S., et al., *Phospholipid binding by a synaptic vesicle protein homologous to the regulatory region of protein kinase C.* Nature, 1990. **345**(6272): p. 260-3.
14. Perin, M.S., et al., *Structural and functional conservation of synaptotagmin (p65) in Drosophila and humans.* J Biol Chem, 1991. **266**(1): p. 615-22.
15. Davletov, B.A. and T.C. Sudhof, *A single C2 domain from synaptotagmin I is sufficient for high affinity Ca2+/phospholipid binding.* J Biol Chem, 1993. **268**(35): p. 26386-90.
16. Brose, N., et al., *Synaptotagmin: a calcium sensor on the synaptic vesicle surface.* Science, 1992. **256**(5059): p. 1021-5.
17. DiAntonio, A. and T.L. Schwarz, *The effect on synaptic physiology of synaptotagmin mutations in Drosophila.* Neuron, 1994. **12**(4): p. 909-20.
18. Broadie, K., et al., *Absence of synaptotagmin disrupts excitation-secretion coupling during synaptic transmission.* Proc Natl Acad Sci U S A, 1994. **91**(22): p. 10727-31.
19. Littleton, J.T., H.J. Bellen, and M.S. Perin, *Expression of synaptotagmin in Drosophila reveals transport and localization of synaptic vesicles to the synapse.* Development, 1993. **118**(4): p. 1077-88.

20. DiAntonio, A., K.D. Parfitt, and T.L. Schwarz, *Synaptic transmission persists in synaptotagmin mutants of Drosophila.* Cell, 1993. **73**(7): p. 1281-90.
21. Geppert, M., et al., *Synaptotagmin I: a major Ca2+ sensor for transmitter release at a central synapse.* Cell, 1994. **79**(4): p. 717-27.
22. Sutton, R.B., et al., *Structure of the first C2 domain of synaptotagmin I: a novel Ca2+/phospholipid-binding fold.* Cell, 1995. **80**(6): p. 929-38.
23. Ubach, J., et al., *Ca2+ binding to synaptotagmin: how many Ca2+ ions bind to the tip of a C2-domain?* Embo j, 1998. **17**(14): p. 3921-30.
24. Robinson, I.M., R. Ranjan, and T.L. Schwarz, *Synaptotagmins I and IV promote transmitter release independently of Ca(2+) binding in the C(2)A domain.* Nature, 2002. **418**(6895): p. 336-40.
25. Fernandez-Chacon, R., et al., *Structure/function analysis of Ca2+ binding to the C2A domain of synaptotagmin 1.* J Neurosci, 2002. **22**(19): p. 8438-46.
26. Nishiki, T. and G.J. Augustine, *Dual roles of the C2B domain of synaptotagmin I in synchronizing Ca2+-dependent neurotransmitter release.* J Neurosci, 2004. **24**(39): p. 8542-50.
27. Stevens, C.F. and J.M. Sullivan, *The synaptotagmin C2A domain is part of the calcium sensor controlling fast synaptic transmission.* Neuron, 2003. **39**(2): p. 299-308.
28. Striegel, A.R., et al., *Calcium binding by synaptotagmin's C2A domain is an essential element of the electrostatic switch that triggers synchronous synaptic transmission.* J Neurosci, 2012. **32**(4): p. 1253-60.
29. Sutton, R.B., J.A. Ernst, and A.T. Brunger, *Crystal structure of the cytosolic C2A-C2B domains of synaptotagmin III. Implications for Ca(+2)-independent snare complex interaction.* J Cell Biol, 1999. **147**(3): p. 589-98.
30. Sutton, R.B., Fuson K.L., *High-resolution structure of Synaptotagmin 1 C2A.* To be published, 2014.
31. Mackler, J.M. and N.E. Reist, *Mutations in the second C2 domain of synaptotagmin disrupt synaptic transmission at Drosophila neuromuscular junctions.* J Comp Neurol, 2001. **436**(1): p. 4-16.
32. Fukuda, M., et al., *Functional diversity of C2 domains of synaptotagmin family. Mutational analysis of inositol high polyphosphate binding domain.* J Biol Chem, 1995. **270**(44): p. 26523-7.
33. Bai, J., W.C. Tucker, and E.R. Chapman, *PIP2 increases the speed of response of synaptotagmin and steers its membrane-penetration activity toward the plasma membrane.* Nat Struct Mol Biol, 2004. **11**(1): p. 36-44.
34. Arac, D., et al., *Close membrane-membrane proximity induced by Ca(2+)-dependent multivalent binding of synaptotagmin-1 to phospholipids.* Nat Struct Mol Biol, 2006. **13**(3): p. 209-17.
35. Chapman, E.R., et al., *Delineation of the oligomerization, AP-2 binding, and synprint binding region of the C2B domain of synaptotagmin.* J Biol Chem, 1998. **273**(49): p. 32966-72.
36. Rickman, C., et al., *Synaptotagmin interaction with the syntaxin/SNAP-25 dimer is mediated by an evolutionarily conserved motif and is sensitive to inositol hexakisphosphate.* J Biol Chem, 2004. **279**(13): p. 12574-9.
37. Rickman, C., et al., *Conserved prefusion protein assembly in regulated exocytosis.* Mol Biol Cell, 2006. **17**(1): p. 283-94.

38. Courtney, N.A., et al., *Synaptotagmin 1 clamps synaptic vesicle fusion in mammalian neurons independent of complexin.* Nat Commun, 2019. **10**(1): p. 4076.
39. Zhang, J.Z., et al., *Synaptotagmin I is a high affinity receptor for clathrin AP-2: implications for membrane recycling.* Cell, 1994. **78**(5): p. 751-60.
40. Poskanzer, K.E., R.D. Fetter, and G.W. Davis, *Discrete residues in the c(2)b domain of synaptotagmin I independently specify endocytic rate and synaptic vesicle size.* Neuron, 2006. **50**(1): p. 49-62.
41. Poskanzer, K.E., et al., *Synaptotagmin I is necessary for compensatory synaptic vesicle endocytosis in vivo.* Nature, 2003. **426**(6966): p. 559-63.
42. Yao, J., et al., *Uncoupling the roles of synaptotagmin I during endo- and exocytosis of synaptic vesicles.* Nat Neurosci, 2011. **15**(2): p. 243-9.
43. Loewen, C.A., et al., *C2B polylysine motif of synaptotagmin facilitates a Ca2+-independent stage of synaptic vesicle priming in vivo.* Mol Biol Cell, 2006. **17**(12): p. 5211-26.
44. Zhang, N., et al., *Phosphorylation of synaptic vesicle protein 2A at Thr84 by casein kinase 1 family kinases controls the specific retrieval of synaptotagmin-1.* J Neurosci, 2015. **35**(6): p. 2492-507.
45. Mohrmann, R., et al., *Synaptotagmin interaction with SNAP-25 governs vesicle docking, priming, and fusion triggering.* J Neurosci, 2013. **33**(36): p. 14417-30.
46. Chang, S., T. Trimbuch, and C. Rosenmund, *Synaptotagmin-1 drives synchronous Ca(2+)-triggered fusion by C2B-domain-mediated synaptic-vesicle-membrane attachment.* Nat Neurosci, 2018. **21**(1): p. 33-40.
47. Fernandez-Chacon, R., et al., *Synaptotagmin I functions as a calcium regulator of release probability.* Nature, 2001. **410**(6824): p. 41-9.
48. Earles, C.A., et al., *The tandem C2 domains of synaptotagmin contain redundant Ca2+ binding sites that cooperate to engage t-SNAREs and trigger exocytosis.* J Cell Biol, 2001. **154**(6): p. 1117-23.
49. Bai, J., P. Wang, and E.R. Chapman, *C2A activates a cryptic Ca(2+)-triggered membrane penetration activity within the C2B domain of synaptotagmin I.* Proc Natl Acad Sci U S A, 2002. **99**(3): p. 1665-70.
50. Paddock, B.E., et al., *Ca2+-dependent, phospholipid-binding residues of synaptotagmin are critical for excitation-secretion coupling in vivo.* J Neurosci, 2008. **28**(30): p. 7458-66.
51. Shao, X., et al., *Synaptotagmin-syntaxin interaction: the C2 domain as a Ca2+-dependent electrostatic switch.* Neuron, 1997. **18**(1): p. 133-42.
52. Ruiter, M., et al., *An Electrostatic Energy Barrier for SNARE-Dependent Spontaneous and Evoked Synaptic Transmission.* Cell Rep, 2019. **26**(9): p. 2340-2352.e5.
53. Fernandez, I., et al., *Three-dimensional structure of the synaptotagmin 1 C2B-domain: synaptotagmin 1 as a phospholipid binding machine.* Neuron, 2001. **32**(6): p. 1057-69.
54. Chapman, E.R. and A.F. Davis, *Direct interaction of a Ca2+-binding loop of synaptotagmin with lipid bilayers.* J Biol Chem, 1998. **273**(22): p. 13995-4001.
55. Herrick, D.Z., et al., *Position of synaptotagmin I at the membrane interface: cooperative interactions of tandem C2 domains.* Biochemistry, 2006. **45**(32): p. 9668-74.

56. Vermaas, J.V. and E. Tajkhorshid, *Differential Membrane Binding Mechanics of Synaptotagmin Isoforms Observed in Atomic Detail.* Biochemistry, 2017. **56**(1): p. 281-293.
57. Hui, E., et al., *Synaptotagmin-mediated bending of the target membrane is a critical step in Ca(2+)-regulated fusion.* Cell, 2009. **138**(4): p. 709-21.
58. Paddock, B.E., et al., *Membrane penetration by synaptotagmin is required for coupling calcium binding to vesicle fusion in vivo.* J Neurosci, 2011. **31**(6): p. 2248-57.
59. Lynch, K.L., et al., *Synaptotagmin-1 utilizes membrane bending and SNARE binding to drive fusion pore expansion.* Mol Biol Cell, 2008. **19**(12): p. 5093-103.
60. Lai, A.L., et al., *Synaptotagmin 1 modulates lipid acyl chain order in lipid bilayers by demixing phosphatidylserine.* J Biol Chem, 2011. **286**(28): p. 25291-300.
61. Kiessling, V., et al., *A molecular mechanism for calcium-mediated synaptotagmin-triggered exocytosis.* Nat Struct Mol Biol, 2018. **25**(10): p. 911-917.
62. Martens, S., M.M. Kozlov, and H.T. McMahon, *How synaptotagmin promotes membrane fusion.* Science, 2007. **316**(5828): p. 1205-8.
63. Chandler, D.E. and J.E. Heuser, *Arrest of membrane fusion events in mast cells by quick-freezing.* J Cell Biol, 1980. **86**(2): p. 666-74.
64. Bowers, M.R. and N.E. Reist, *The C2A Domain of Synaptotagmin: an Essential Component of the Calcium Sensor for Synaptic Transmission.* Plos One, in press.
65. Rhee, J.S., et al., *Augmenting neurotransmitter release by enhancing the apparent Ca2+ affinity of synaptotagmin 1.* Proc Natl Acad Sci U S A, 2005. **102**(51): p. 18664-9.
66. Davletov, B., O. Perisic, and R.L. Williams, *Calcium-dependent membrane penetration is a hallmark of the C2 domain of cytosolic phospholipase A2 whereas the C2A domain of synaptotagmin binds membranes electrostatically.* J Biol Chem, 1998. **273**(30): p. 19093-6.
67. Murray, D. and B. Honig, *Electrostatic control of the membrane targeting of C2 domains.* Mol Cell, 2002. **9**(1): p. 145-54.
68. Weber, T., et al., *SNAREpins: minimal machinery for membrane fusion.* Cell, 1998. **92**(6): p. 759-72.
69. Pobbati, A.V., A. Stein, and D. Fasshauer, *N- to C-terminal SNARE complex assembly promotes rapid membrane fusion.* Science, 2006. **313**(5787): p. 673-6.
70. Hanson, P.I., et al., *Structure and conformational changes in NSF and its membrane receptor complexes visualized by quick-freeze/deep-etch electron microscopy.* Cell, 1997. **90**(3): p. 523-35.
71. Min, D., et al., *Mechanical unzipping and rezipping of a single SNARE complex reveals hysteresis as a force-generating mechanism.* Nat Commun, 2013. **4**: p. 1705.
72. Liu, H., et al., *Autapses and networks of hippocampal neurons exhibit distinct synaptic transmission phenotypes in the absence of synaptotagmin I.* J Neurosci, 2009. **29**(23): p. 7395-403.
73. Zhou, Q., et al., *Architecture of the synaptotagmin-SNARE machinery for neuronal exocytosis.* Nature, 2015. **525**(7567): p. 62-7.
74. Grushin, K., et al., *Structural basis for the clamping and Ca(2+) activation of SNARE-mediated fusion by synaptotagmin.* Nat Commun, 2019. **10**(1): p. 2413.

75. Yoshihara, M., Z. Guan, and J.T. Littleton, *Differential regulation of synchronous versus asynchronous neurotransmitter release by the C2 domains of synaptotagmin 1.* Proc Natl Acad Sci U S A, 2010. **107**(33): p. 14869-74.
76. Rash, J.E., J.P. Walrond, and M. Morita, *Structural and functional correlates of synaptic transmission in the vertebrate neuromuscular junction.* J Electron Microsc Tech, 1988. **10**(2): p. 153-85.
77. Walrond, J.P. and T.S. Reese, *Structure of axon terminals and active zones at synapses on lizard twitch and tonic muscle fibers.* J Neurosci, 1985. **5**(5): p. 1118-31.
78. Jung, J.H., et al., *Variable priming of a docked synaptic vesicle.* Proc Natl Acad Sci U S A, 2016. **113**(8): p. E1098-107.
79. Szule, J.A., et al., *Regulation of synaptic vesicle docking by different classes of macromolecules in active zone material.* PLoS One, 2012. **7**(3): p. e33333.
80. Siksou, L., et al., *A common molecular basis for membrane docking and functional priming of synaptic vesicles.* Eur J Neurosci, 2009. **30**(1): p. 49-56.
81. Fernandez-Busnadiego, R., et al., *Cryo-electron tomography reveals a critical role of RIM1alpha in synaptic vesicle tethering.* J Cell Biol, 2013. **201**(5): p. 725-40.
82. Imig, C., et al., *The morphological and molecular nature of synaptic vesicle priming at presynaptic active zones.* Neuron, 2014. **84**(2): p. 416-31.
83. Leikin, S.L., et al., *Membrane fusion: overcoming of the hydration barrier and local restructuring.* J Theor Biol, 1987. **129**(4): p. 411-25.
84. Pumplin, D.W., T.S. Reese, and R. Llinas, *Are the presynaptic membrane particles the calcium channels?* Proc Natl Acad Sci U S A, 1981. **78**(11): p. 7210-3.
85. Robitaille, R., E.M. Adler, and M.P. Charlton, *Strategic location of calcium channels at transmitter release sites of frog neuromuscular synapses.* Neuron, 1990. **5**(6): p. 773-9.
86. Neher, E. and T. Sakaba, *Multiple roles of calcium ions in the regulation of neurotransmitter release.* Neuron, 2008. **59**(6): p. 861-72.
87. Shahrezaei, V. and K.R. Delaney, *Consequences of molecular-level Ca2+ channel and synaptic vesicle colocalization for the Ca2+ microdomain and neurotransmitter exocytosis: a monte carlo study.* Biophys J, 2004. **87**(4): p. 2352-64.
88. Herrick, D.Z., et al., *Solution and membrane-bound conformations of the tandem C2A and C2B domains of synaptotagmin 1: Evidence for bilayer bridging.* J Mol Biol, 2009. **390**(5): p. 913-23.
89. Nyenhuis, S.B., A. Thapa, and D.S. Cafiso, *Phosphatidylinositol 4,5 Bisphosphate Controls the cis and trans Interactions of Synaptotagmin 1.* Biophys J, 2019. **117**(2): p. 247-257.
90. Holz, R.W., et al., *A pleckstrin homology domain specific for phosphatidylinositol 4, 5-bisphosphate (PtdIns-4,5-P2) and fused to green fluorescent protein identifies plasma membrane PtdIns-4,5-P2 as being important in exocytosis.* J Biol Chem, 2000. **275**(23): p. 17878-85.
91. Harlow, M.L., et al., *The architecture of active zone material at the frog's neuromuscular junction.* Nature, 2001. **409**(6819): p. 479-84.
92. Shields, M.C., et al., *Drosophila studies support a role for a presynaptic synaptotagmin mutation in a human congenital myasthenic syndrome.* PLoS One, 2017. **12**(9): p. e0184817.

93. Herrmann, D.N., et al., *Synaptotagmin 2 mutations cause an autosomal-dominant form of lambert-eaton myasthenic syndrome and nonprogressive motor neuropathy.* Am J Hum Genet, 2014. **95**(3): p. 332-9.
94. Baker, K., et al., *Identification of a human synaptotagmin-1 mutation that perturbs synaptic vesicle cycling.* The Journal of Clinical Investigation, 2015. **125**(4): p. 1670-1678.
95. Baker, K., et al., *SYT1-associated neurodevelopmental disorder: a case series.* Brain, 2018. **141**(9): p. 2576-2591.

CHAPTER 3. THE C_2A DOMAIN OF SYNAPTOTAGMIN IS AN ESSENTIAL COMPONENT OF THE CALCIUM SENSOR FOR SYNAPTIC TRANSMISSION[3]

3.1 Summary

The synaptic vesicle protein, synaptotagmin, is the principle Ca^{2+} sensor for synaptic transmission. Ca^{2+} influx into active nerve terminals is translated into neurotransmitter release by Ca^{2+} binding to synaptotagmin's tandem C_2 domains, triggering the fast, synchronous fusion of multiple synaptic vesicles. Two hydrophobic residues, shown to mediate Ca^{2+}-dependent membrane insertion of these C_2 domains, are required for this process. Previous research suggested that one of its tandem C_2 domains (C_2B) is critical for fusion, while the other domain (C_2A) plays only a facilitatory role. However, the function of the two hydrophobic residues in C_2A has not been adequately tested *in vivo*. Here we show that these two hydrophobic residues are absolutely required for synaptotagmin to trigger vesicle fusion. Using *in vivo* electrophysiological recording at the *Drosophila* larval neuromuscular junction, we found that mutation of these two key C_2A hydrophobic residues almost completely abolished neurotransmitter release. Significantly, mutation of both hydrophobic residues resulted in more severe deficits than those seen in synaptotagmin null mutants. Thus, we report the most severe phenotype of a C_2A mutation to date, demonstrating that the C_2A domain is absolutely essential for synaptotagmin's function as the electrostatic switch.

[3] Reprinted with permission under the Creative Commons Attribution (CC-BY) copyright license. Bowers MR, Reist NE. The C2A domain of synaptotagmin is an essential component of the calcium sensor for synaptic transmission. PLoS One. 2020;15(2):e0228348.

3.2 Significance statement

The postulated role of synaptotagmin's C_2A domain in triggering neurotransmitter release has fluctuated wildly over the years. Early biochemical experiments suggested that the C_2A domain was essential, while the C_2B domain was superfluous. Then, functional experiments measuring neurotransmitter release *in vivo* following disruptions in Ca^{2+} binding suggested that C_2B was essential, while C_2A was superfluous. Subsequently, the use of more refined mutations to disrupt Ca^{2+} binding indicated that C_2A played a facilitatory role. Here we show two hydrophobic residues of the C_2A domain are *absolutely required* for synaptotagmin-triggered neurotransmitter release. Thus, after over twenty years of research, we now demonstrate that the C_2A domain of synaptotagmin is an essential component of the Ca^{2+} sensor for triggering synaptic transmission *in vivo*.

3.3 Introduction

Ca^{2+} binding by synaptotagmin triggers the fast, synchronous fusion of maximally-primed synaptic vesicles thereby releasing neurotransmitter onto the postsynaptic cell [1, 2]. In the absence of this Ca^{2+} sensor, evoked release of neurotransmitter is dramatically decreased [3-6]. Synaptotagmin is an integral membrane protein found on synaptic vesicles whose cytosolic domain is composed of two Ca^{2+}-binding C_2 domains, C_2A and C_2B [Fig 3.1A, B, [7]]. One end of each C_2 domain contains three loops of amino acids, two of which form a Ca^{2+}-binding pocket: loops 1 and 3 contain five negatively-charged aspartate residues that coordinate Ca^{2+}[8, 9]. In addition, there are two, highly-conserved, hydrophobic residues at the tips of each pocket: one in loop 1, adjacent to the first aspartate residue, and one in loop 3, between the 4^{th} and 5^{th} aspartate residues [Fig 3.1A,[8, 9]]. Prior to Ca^{2+} influx, the net negative charge of each Ca^{2+}-

binding pocket results in electrostatic repulsion of the negatively-charged presynaptic membrane, preventing fusion. Upon Ca^{2+} influx, Ca^{2+} binding to the pockets now results in a net positive charge. Accordingly, the electrostatic repulsion of the presynaptic membrane is changed to electrostatic attraction. Thus, synaptotagmin operates as an electrostatic switch [10-13]. Importantly, this electrostatic attraction now brings the hydrophobic residues located at the tips of the pockets into contact with the membrane. Modeling predicts, and *in vitro* studies confirm, that these hydrophobic residues insert into lipid bilayers in a Ca^{2+}-dependent manner [14-16], resulting in positive curvature of the membrane that is theorized to promote vesicle fusion [17, 18].

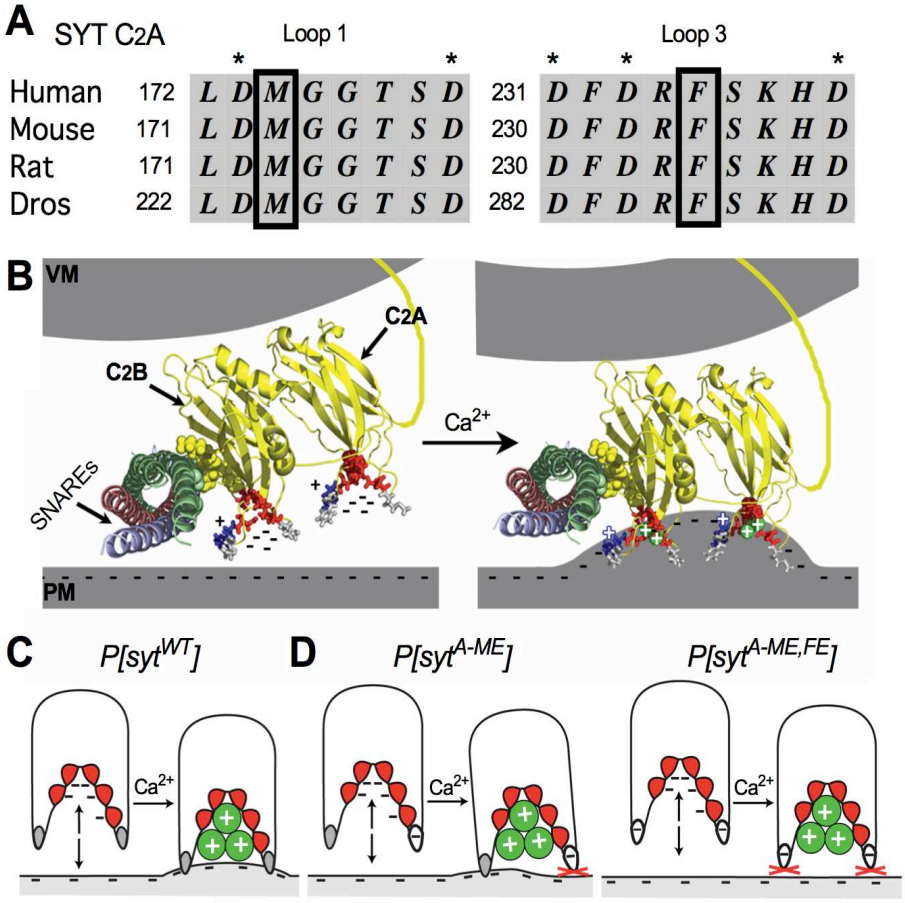

Figure 3.1. Synaptotagmin structure and C_2A mutations. **A,** Protein alignment of loops 1 and 3 of the C_2A domain of synaptotagmin 1 from Human, Mouse, Rat, and *Drosophila* (* = Ca^{2+}

binding aspartates, boxes = loop 1 and loop 3 hydrophobic tip residues) **B,** Crystal structure of synaptotagmin and the SNARE complex showing a postulated role of the C_2 domains in triggering fusion, adapted from [19]. Negatively charged residues of the Ca^{2+} binding pockets are shown as sticks in red, the hydrophobic residues at the tips of these pockets are shown as sticks in gray, and Ca^{2+} ions are shown as green spheres. VM=vesicle membrane and PM= presynaptic membrane. **C,** A cartoon depiction of the C_2A domain. Colors as in panel B. **D,** Hydrophilic glutamic acid substitutions are indicated in white. Sequential mutation of C_2A hydrophobic tip residues to hydrophilic residues is predicted to increasingly disrupt synaptotagmin's ability to penetrate, warp and disorder lipids of the presynaptic membrane.

C_2A is currently postulated to function as a secondary domain that is merely supportive of C_2B, the primary functional domain of synaptotagmin. Importantly, mutation of a hydrophobic tip residue in loop 3 of C_2B, which penetrates negatively-charged membranes, is embryonic lethal and causes a decrease in evoked release that is more severe than that seen in syt^{null} mutants. In comparison, mutating the analogous residue in the C_2A domain does not impact viability and only inhibited neurotransmitter release by 50% [19]. However, the functional impact of mutations of the C_2A hydrophobic residue in loop 1 has not been studied *in vivo*, nor has the impact of tandem mutations in both loops 1 and 3 simultaneously.

Since replacing the loop 3 hydrophobic residue in C_2B with a large, polar glutamate prevented phospholipid-binding in vitro [19], we made homologous mutations of the loop 1 and loop 3 residues in C_2A (Fig 3.1D). Electrophysiological recordings at the *Drosophila* neuromuscular junction revealed that mutation at either the loop 1 (this report) or loop 3 site [19] in isolation resulted in an ~50% reduction in evoked transmitter release, again suggesting C_2A plays only a facilitatory role. Surprisingly, mutation of both the loop 1 and loop 3 sites simultaneously resulted in an almost complete abolishment of evoked release. This reduction in transmission in the tandem mutation was actually more severe than that observed in synaptotagmin null mutants. The current study establishes that these two hydrophobic residues of C_2A, which have been shown to mediate Ca^{2+}-dependent effector interactions *in vitro* [17, 18,

20, 21], are absolutely required for evoked transmitter release *in vivo*. Thus, we report the most severe deficits caused by any C_2A domain mutation to date and demonstrate that the C_2A domain is an essential component for translating synaptotagmin's electrostatic switch function into vesicle fusion with the presynaptic membrane.

3.4 Materials and methods

Drosophila lines

We generated mutants with hydrophobic to hydrophilic substitutions of two residues. A transgenic wild type (*P[syt^{WT}]*) was used as a positive control across all experiments. For a direct comparison of the level of evoked transmitter release in the most severe mutation, a previously characterized synaptotagmin null line (*syt^{null}*) was used as a negative control [22]. Using the *Drosophila syt1* coding sequence [7, 23], a wild type control, a M224E, and a M224E/F286E mutant cDNA were synthesized by GeneWiz (South Plainfield, New Jersey) (Fig 3.1A, D). The cDNA was flanked by unique 5' EcoRI and 3' BglII restriction sites for directional subcloning into the pUAST-attB vector to place them under the control of the UAS promoter. The transgenes were injected into *Drosophila* embryos by BestGene (Chino Hills, California) where they were inserted into the attP2 landing site on the third chromosome using the PhiC31 targeted insertion system [24]. These *syt 1* transgenes were driven pan-neuronally by the UAS/Gal4 system [25] using the *elav* promoter [26]. All transgenes were expressed in the absence of endogenous synaptotagmin 1 by crossing them into a synaptotagmin 1 null mutant background, *syt^{AD4}* [22, 23]. As no sex selection was employed, both males and females were used across all experiments. This study used the following genotypes: *yw; syt^{AD4}elavGal4/ syt^{AD4}; P[UASsyt1^{WT}]/+* (referred to as *P[syt^{WT}]* or control), *yw; syt^{AD4}elavGal4/ syt^{AD4}; P[UASsyt1^{C2A-}*

$^{M224E]/+}$ (referred to as *P[syt$^{A\text{-}ME}$]*), *yw; syt^{AD4}elavGal4/ syt^{AD4}; P[UASsyt1 $^{C2A\text{-}M224E,F286E]/+}$* (referred to as *P[syt$^{A\text{-}ME,FE}$]*) and *yw; syt^{AD4}elavGal4/ syt^{AD4}* (referred to as *syt^{null}).* All experiments used 3rd instar larvae (L3).

Solutions

HL3.1 saline [70 mM NaCl, 5 mM KCl, 4 mM $MgCl_2$, 10 mM $NaHCO_3$, 5 mM Trehalose, 115 mM sucrose, 5 mM HEPES, pH 7.2 [27]], with the indicated Ca^{2+} concentrations, was used in all experiments. Phosphate buffered saline (PBS) consisted of [137 mM NaCl, 1.5 mM KH_2PO_4, 2.7 mM KCl, 8.1 mM Na_2HPO_4].

Immunoblotting

Synaptotagmin expression levels were determined using western blot analysis with actin levels serving as a loading control. The CNSs of L3s were dissected in HL3.1 saline where the Ca^{2+} was omitted to decrease vesicle fusion events during dissection. Individual CNSs were placed in Laemmli buffer (Bio-Rad, Hercules, CA) containing 5% β-mercaptoethanol, sonified with five 0.3 sec pulses at 1 Hz using a Branson Sonifier 450 (VWR Scientific, Winchester, PA), and separated by SDS-PAGE with 15% acrylamide. They were then transferred to Immobilon membranes (Millipore, Bedford, MA), and washed in blocking solution [5% milk, 4% normal goat serum (NGS, Fitzgerald Industries International, Acton, MA), 1% bovine serum albumin (BSA, Millipore-Sigma, Burlington, MA), and 0.02% NaN_3 in PBS containing 0.05% Tween 20 (PBS-Tween, Fisher BioReagents, Fair Lawn, NJ)]. The membranes were then incubated overnight at 4°C with a 1:2,500 dilution of anti-synaptotagmin antibody, Dsyt-CL1 [2] and 1:10,000 dilution of anti-actin antibody, (MAB 1501, Millipore Bioscience Research Reagents,

Billerica, MA) in PBS-Tween containing 10% NGS and 0.02% NaN_3, washed in PBS-Tween for 1-3 hours, and probed with secondary antibodies at a 1:5,000 dilution of peroxidase-conjugated AffiniPure Goat Anti-Rabbit IgG (Jackson ImmunoResearch, West Grove, PA) and a 1:5,000 dilution of peroxidase-conjugated AffiniPure Donkey Anti-Mouse IgG (Jackson ImmunoResearch, West Grove, PA) in PBS-Tween containing 10% NGS for 1 hour at room temperature, and washed in PBS-Tween for 30 min. Protein bands were visualized on an Epichemi^3 Darkroom with Labworks Imaging Software (UVP BioImaging, Upland, CA). To quantify expression levels within each blot, synaptotagmin/actin ratios were calculated and normalized to the mean synaptotagmin/actin ratio of the transgenic WT control lanes. This permitted comparison of synaptotagmin expression levels between blots. Outliers in loading amount, based on actin levels, were excluded from analysis. The analysis included at least 11 individual CNSs per genotype.

Immunolabeling

The localization of the transgenic synaptotagmin protein was visualized by immunohistochemistry. L3s were dissected in Ca^{2+}-free HL3.1, fixed in PBS containing 4% formaldehyde for 1 hour, incubated with a 1:400 dilution of Dsyt-CL1 in dilution media [PBS with 0.1% Triton (PBST), 1% BSA, and 1% NGS] overnight at 4°C, washed in PBST for 1-3 hours, incubated in dilution media containing a 1:400 dilution of Alexa Fluor 488 goat anti-rabbit antibody (Invitrogen, Carlsbad, CA) for 1 hour at room temperature, washed in PBST for one hour, and mounted on microscope slides in Citifluor (Ted Pella, Redding, CA). Confocal images of the neuromuscular junction on muscle fibers 6 and 7 were taken on a Zeiss 880 light-

scanning microscope (Zeiss, White Plains, NY), with a 40x objective and Zeiss Zen 2.1 acquisition software, version 11.0.3.190.

Electrophysiological recording and analyses

Electrophysiological recordings were made with an Axoclamp 2B amplifier (Molecular Devices, Sunnyvale, CA), a Powerlab 4/30 A/D converter (ADInstuments, Sydney, Australia), using LabChart software (ADInstruments, Sydney, Australia). L3s were dissected in Ca^{2+}-free HL3.1 saline to expose the body wall musculature and the CNSs were removed. The saline was then changed to HL3.1 with 1mM Ca^{2+}. Intracellular recordings were made from muscle fiber 6 of abdominal segments 3 and 4 using 10-20 MΩ intracellular electrodes that were pulled using a Sutter model P-97 micropipette puller (Novato, CA) and filled with 3 parts 2 M $K_3C_6H_5O_7$ to 1 part 3 M KCl. The resting potential was held at -65 mV by applying no more than ±1 nA of current. The nerve fiber was stimulated using an A360 stimulus isolator (World Precision Instruments, Sarasota, FL) through a glass suction electrode filled with HL3.1 containing 1mM Ca^{2+} and broken to have an ~1 micron tip.

Evoked release

Ten excitatory junction potentials (EJPs) were stimulated at 0.04 Hz and averaged for each muscle fiber. Mean responses are reported for 12-14 muscle fibers per genotype.

Spontaneous release

Spontaneous miniature EJPs (mEJPs) were recorded for 3 min prior to any external stimulation. Recordings were blinded and randomized and mEJPs were identified manually. mEJP frequency

was determined by counting the number of events that occurred during the second minute of recording. The average amplitude of the first 100 of this population of mEJPs was also calculated. Mean responses are reported for 12-14 muscle fibers per genotype.

Paired pulse

For each muscle fiber, the nerve was stimulated with pairs of pulses having interpulse intervals of 10ms, 20ms, 50ms, and 100ms. For each interpulse interval, five pairs of pulses separated by 5 sec were averaged. Each interpulse interval test was also separated by 5 sec. The amplitude of the first EJP was calculated from the baseline to the first peak. The amplitude of the second EJP was calculated from the trough following the first EJP to the peak of the second EJP. The amplitude of the second EJP was divided by the amplitude of the first EJP to yield a paired pulse ratio (PPR). The mean PPRs of 12-14 fibers per genotype are reported.

Ca^{2+} curves

Five EJPs evoked at 0.5 Hz were recorded from an individual muscle fiber across at least 3 different Ca^{2+} concentrations between 0.05 mM and 5 mM (0.05 mM, 0.25 mM, 0.5 mM, 1 mM, 1.5 mM, 2.5 mM, 5 mM) and averaged, yielding a mean EJP amplitude at each Ca^{2+} level. The first 5 EJPs were always recorded in HL3.1 with 1.5 mM Ca^{2+} and, following stimulation in at least 2 other Ca^{2+} levels, 5 more EJPs were recorded in HL3.1 with 1.5 mM Ca^{2+}. Recordings were only considered for analysis if the mean amplitude of the final EJPs in 1.5 mM Ca^{2+} was $\geq$ 90% of the initial mean EJP amplitude. Mean responses are reported for at least 12 muscle fibers per Ca^{2+} concentration. Lines of best fit were calculated using a nonlinear regression analysis, which provided EC50s and their 95% confidence intervals for each genotype. Each response

level was normalized to the maximum value predicted by the line of best fit. Additionally, the hillslope for each curve was calculated to compare cooperativity of Ca^{2+}-dependent release.

Experimental design and statistical analysis

Statistical analyses were performed using Prism 8 (GraphPad software, La Jolla, CA). All datasets included a minimum of 11 samples per genotype. In all electrophysiological experiments, recordings of mutants and controls were interspersed. Direct comparisons were only made between recordings done within the same time period, as absolute responses can be impacted by minor variations in solutions. All experiments requiring manual analysis of events had the genotypes blinded to the researcher. For comparison of two genotypes with Gaussian distributions of their datasets, we used unpaired student's t-tests. For comparisons of three genotypes with Gaussian distribution of their datasets, we used one-way ANOVAs with Tukey post hoc tests of multiple comparisons to determine significance between all three genotypes. In the paired pulse experiments, data were analyzed with a repeated measures two-way ANOVA with tukey post hoc tests. When datasets showed non-gaussian distributions, Kruskal-Wallis tests were used to compare the 3 genotypes, with Dunn's post hoc tests of multiple comparisons. An alpha p-value of 0.05 was considered significant for all of the above tests. To compare Ca^{2+} curves, a line of best fit was determined using a nonlinear regression model and the 95% confidence intervals of the EC50 of each genotype were compared. If the confidence intervals didn't overlap, the genotypes were considered significantly different.

3.5 Results

Synaptotagmin transgenes

P[syt^{WT}], *P[syt$^{A\text{-}ME}$]*, and *P[syt$^{A\text{-}ME,FE}$]*

Mutation of the hydrophobic residue at the tip of loop 3 of the C_2A Ca^{2+}-binding pocket inhibits evoked release by 50% [19], yet the *in vivo* function of the hydrophobic residue at the tip of loop 1 is unknown (see Fig 3.1A, Loop 1, M). Since both of these hydrophobic residues have been shown to mediate Ca^{2+}-dependent interactions *in vitro* [16, 18, 28], we tested whether the loop 1 hydrophobic residue is required for efficient neurotransmitter release. We generated two lines containing mutations of this loop 1 methionine. In the first, only the loop 1 methionine was mutated to a hydrophilic glutamic acid (Fig 3.1D, *P[syt$^{A\text{-}ME}$]*). In the second, both the loop 1 and loop 3 hydrophobic tip residues of C_2A were mutated to glutamic acids (Fig 3.1D, *P[syt$^{A\text{-}ME,FE}$]*). In all experiments, transgenic synaptotagmin was expressed in the *syt^{null}* background such that the only source of synaptotagmin 1 was from the transgene [22].

C_2A hydrophobic residues are required for synaptotagmin function

Ca^{2+}-evoked neurotransmitter release in the single *P[syt$^{A\text{-}ME}$]* mutant was decreased to a similar extent as that seen previously in the single *P[syt$^{A\text{-}FE}$]* mutant [19]. Electrophysiological recording of excitatory junction potentials (EJPs) from larval muscle fibers revealed an ~50% decrease in EJP amplitude in *P[syt$^{A\text{-}ME}$]* compared to *P[syt^{WT}]* (Fig 3.2A,B). EJP amplitude in *P[syt^{WT}]* was 30.8 ± 1.8 mV (mean ± SEM, n = 12). Whereas in *P[syt$^{A\text{-}ME}$]*, it was significantly reduced at only 14.6 ± 1.3 mV (mean ± SEM, n = 14; one-way ANOVA $F(2,37) = 137.7$, $p < 0.0001$, Tukey post hoc $p < 0.0001$). This partial block of fast, synchronous neurotransmitter

release is consistent with the idea that C_2A plays only a facilitatory role in synaptotagmin function [19, 29, 30].

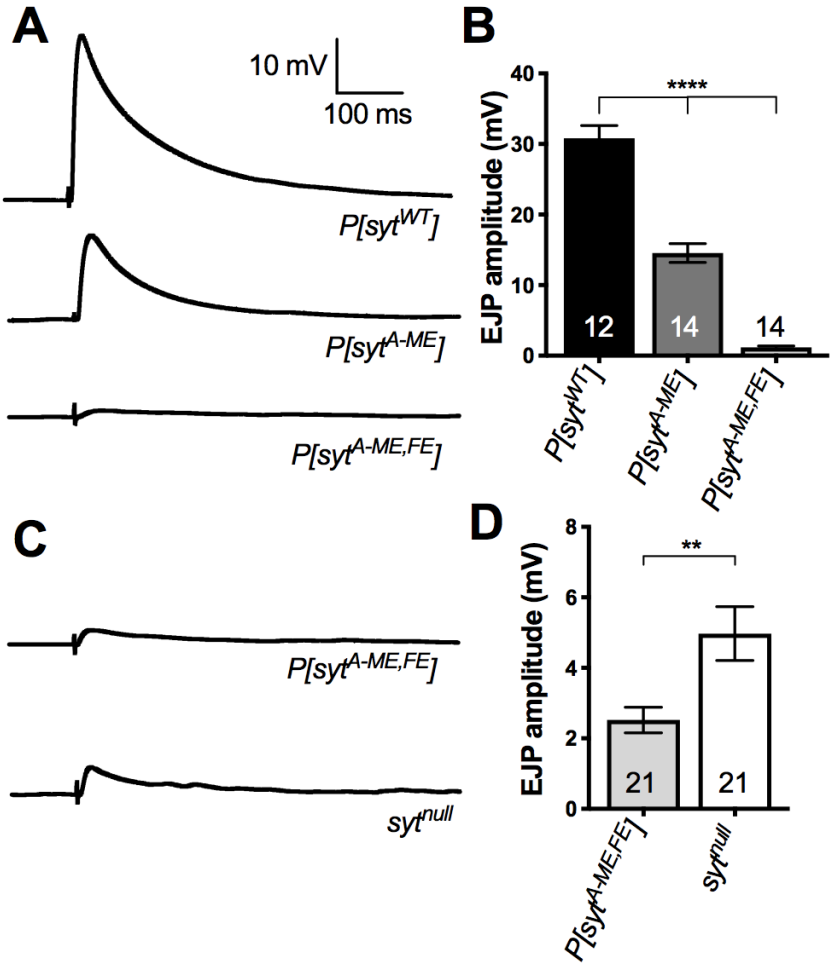

Figure 3.2. Mutation of the hydrophobic tip residues disrupts evoked transmitter release. The single hydrophobic mutation decreased neurotransmitter release by 50% while the double mutation inhibited release to a greater extent than that seen in *syt^{null}* mutants. **A,** Representative traces of EJPs for *P[syt^{WT}]*, *P[syt$^{A\text{-}ME}$]*, and *P[syt$^{A\text{-}ME,FE}$]*. **B,** Mean EJP amplitude ± SEM for *P[syt^{WT}]*, *P[syt$^{A\text{-}ME}$]*, and *P[syt$^{A\text{-}ME,FE}$]* (Tukey multiple comparisons, $p < 0.0001$= ****). **C,** Representative traces of EJPs for *P[syt$^{A\text{-}ME,FE}$]* and *syt^{null}*. **D,** Mean EJP amplitude ± SEM for *P[syt$^{A\text{-}ME,FE}$]* and *syt^{null}* (Tukey multiple comparisons, $p < 0.01$ = **).

Surprisingly, the double mutant, *P[syt$^{A\text{-}ME,FE}$]*, nearly abolished Ca^{2+}-evoked neurotransmitter release (Fig 3.2A,B). EJP amplitude in *P[syt$^{A\text{-}ME,FE}$]* mutants was only 1.2 ±

0.2 mV (mean ± SEM, n = 14). Since the double mutant showed such a dramatic decrease in EJP amplitude compared to both *P[syt^{WT}]* and *P[syt$^{A\text{-}ME}$]* (one-way ANOVA $F(2,37) = 137.7$, $p < 0.0001$, Tukey post hoc $p < 0.0001$), we also compared evoked transmitter release in *P[syt$^{A\text{-}ME,FE}$]* mutants and *syt^{null}* larvae, which express no synaptotagmin [22]. Importantly, we found that the *P[syt$^{A\text{-}ME,FE}$]* double mutant had a significantly decreased EJP amplitude even when compared to *syt^{null}* larvae (Fig 3.2C,D; mean ± SEM: *P[syt$^{A\text{-}ME,FE}$]* = 2.5 ± 0.4 mV, n = 21 and *syt^{null}* = 5 ± 0.8 mV, n = 21; unpaired t-test $t(40) = 2.896$, $p = 0.0061$). Thus, residues required for Ca^{2+}-dependent membrane penetration by both C_2 domains are absolutely required for synaptotagmin to function as the Ca^{2+} sensor for fast, synchronous neurotransmitter release. While we've known for many years the critical nature of the C_2B domain for triggering neurotransmitter release [2, 4, 19, 31, 32], no previous *C_2A mutation* has resulted in synaptic deficits more severe than those in *syt^{null}* mutants *in vivo.*

Expression and localization of transgenic synaptotagmin

The deficits in evoked release are not a result of mis-expression or mis-localization of the transgenic proteins. Western analysis was performed on single CNSs from larvae using our anti-synaptotagmin antibody [DsytCL1, [2]] and an anti-actin antibody (MAB 1501) as a loading control. Synaptotagmin expression in *P[syt$^{A\text{-}ME}$]* and in *P[syt$^{A\text{-}ME,FE}$]* was 114.4 ± 24.0% and 78.7 ± 17.6% (respectively, mean ± SEM, n = 13 and n = 11) of that in *P[syt^{WT}]* (n = 13). There were no significant differences in transgenic synaptotagmin expression levels between the control and either mutant line (Fig 3.3A,B, one-way ANOVA $F(2,34) = 0.8165$, $p = 0.4505$). Immunohistochemical labeling of the larval body wall musculature with anti-synaptotagmin antibody was used as a non-quantitative measure of protein localization, which demonstrated that

the transgenic synaptotagmin was highly concentrated in synaptic boutons at the neuromuscular junction in both transgenic mutants and the transgenic WT control (Fig 3.3C). Thus, the transgenic protein was appropriately targeted to synaptic sites.

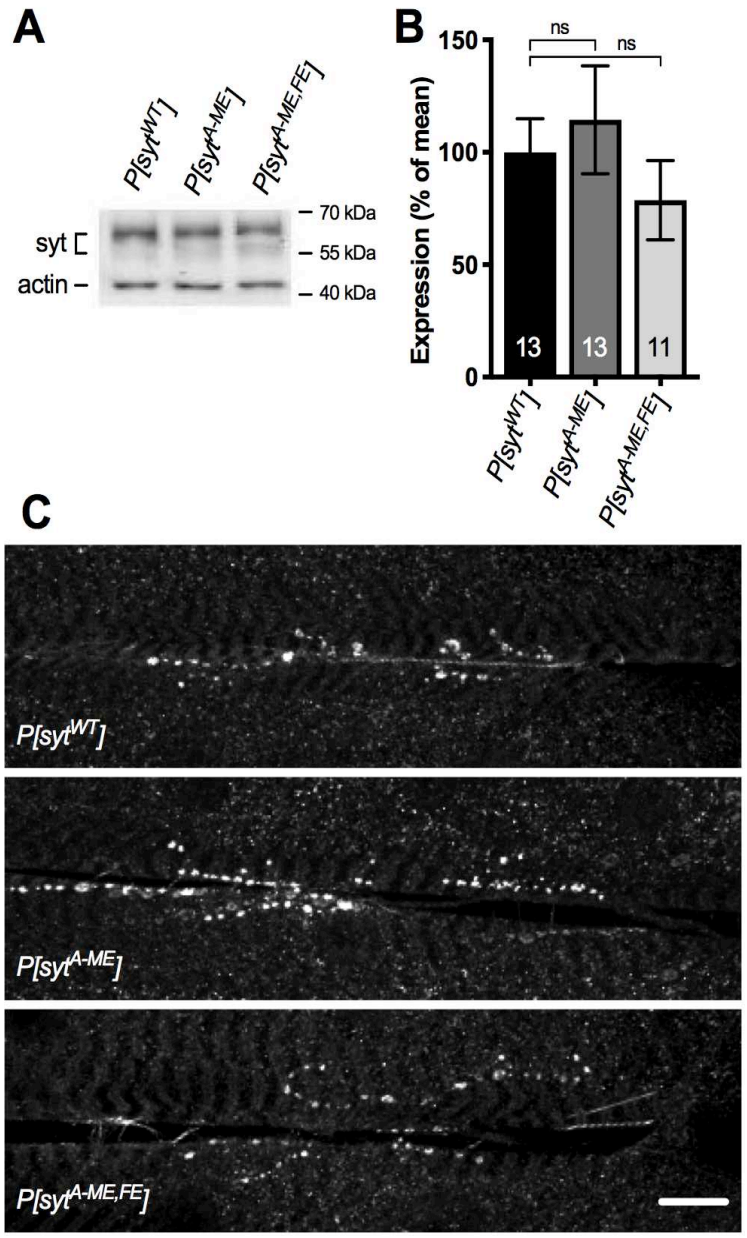

Figure 3.3. Expression and localization of synaptotagmin are unaffected by hydrophobic mutations. **A,** Representative western blots of transgenic synaptotagmin expression levels with actin as a loading control. **B,** Mean protein expression levels ± SEM, normalized to actin (One-

way ANOVA, no significant differences). **C,** Representative confocal images of larval neuromuscular junctions labeled with anti-synaptotagmin antibodies (scale bar = 20 μm). Synaptotagmin is appropriately concentrated at synaptic sites in all three genotypes.

C_2A hydrophobic mutations result in decreased release probability

A decrease in evoked transmitter release could result from a decrease in vesicular loading of neurotransmitter which would decrease quantal size and/or a decrease in release probability. The amplitude of spontaneous single vesicle fusion events, mEJPs, at the neuromuscular junction provides a convenient indication of quantal size, assuming there's no disruption in postsynaptic responsiveness. The amplitude of these single vesicle fusion events was unchanged between the mutants and the control (Fig 3.4A,B; mean ± SEM: $P[syt^{WT}]$ = 1.1 ± 0.06 mV n = 20, $P[syt^{A\text{-}ME}]$= 1.2 ± 0.08 mV n = 16, $P[syt^{A\text{-}ME,FE}]$ = 0.9 ± 0.05 mV n = 18; Kruskal-Wallis test H(2) = 7.61, p = 0.0222, Dunn's multiple comparisons test, $P[syt^{WT}]$ vs $P[syt^{A\text{-}ME}]$ p > 0.9999 , $P[syt^{WT}]$ vs $P[syt^{A\text{-}ME,FE}]$ p = 0.07). The lack of any difference between the control and either mutant is consistent with both vesicular loading and postsynaptic responsiveness being unchanged. Several synaptotagmin mutations result in an increase in the frequency of spontaneous fusion events [5, 33]. Therefore, we analyzed mEJP frequency and found no change between the mutants and the control (Fig 3.4A,C; mean ± SEM: $P[syt^{WT}]$ = 3.9 ± 0.5 Hz n = 20, $P[syt^{A\text{-}ME}]$ = 3.7 ± 0.3 Hz n = 16, $P[syt^{A\text{-}ME,FE}]$ = 3.9 ± 0.3 Hz n = 18; one-way ANOVA F(2, 51) = 0.1247, p = 0.8806). Thus, neither hydrophobic mutation caused a significant change in either the quantal size or the rate of spontaneous fusion events.

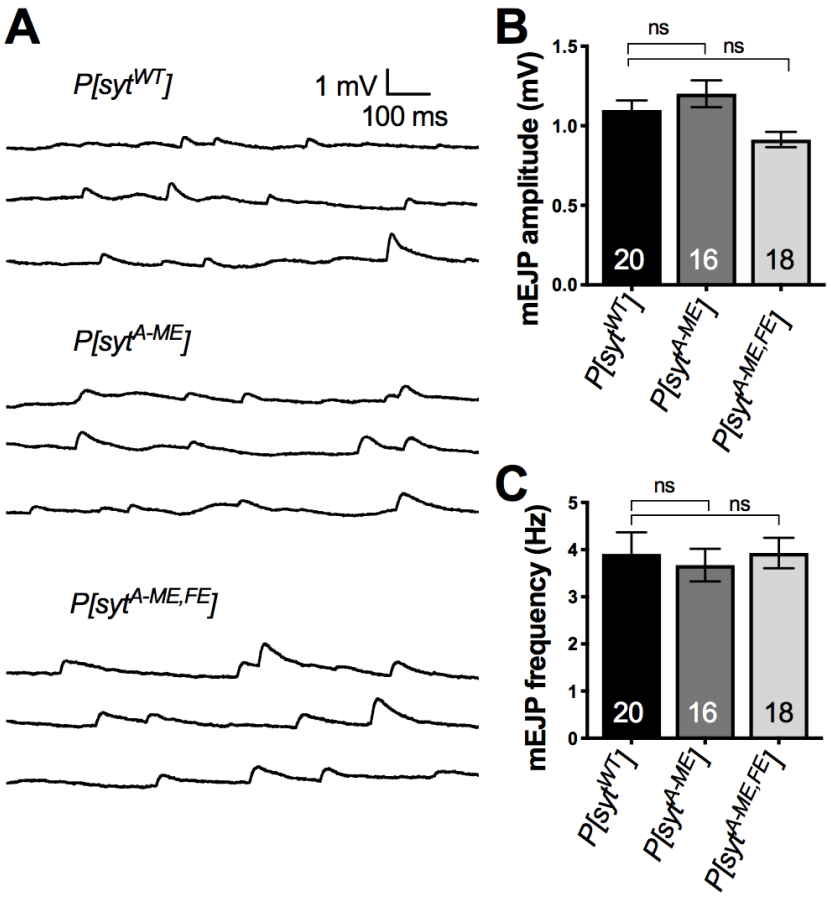

Figure 3.4. Spontaneous events are unaffected by hydrophobic mutations. **A,** Representative traces of mEJPs for *P[syt^{WT}]*, *P[syt$^{A\text{-}ME}$]*, and *P[syt$^{A\text{-}ME,FE}$]*. **B,** Mean mEJP amplitudes ± SEM (Kruskal Wallis test with Dunn's multiple comparison test, no significance = ns). **C,** Mean mEJP frequency ± SEM (Kruskal Wallis test with Dunn's multiple comparison test, no significance = ns).

While changes in quantal size cannot account for the differences in EJP amplitude we observed, another possibility is that the hydrophobic mutations decrease the probability of release. Since the ratio of two paired pulses (paired pulse ratio, PPR) is inversely correlated to release probability [34], we conducted a paired pulse analysis. The PPRs for *P[syt$^{A\text{-}ME}$]* and *P[syt$^{A\text{-}ME,FE}$]* were significantly different from control across all interpulse intervals (mean ± SEM. *P[syt^{WT}]* n = 13: 10 ms = 0.6 ± 0.05, 20 ms = 0.7 ± 0.04, 50 ms = 0.9 ± 0.03, 100 ms = 0.9 ± 0.01. *P[syt$^{A\text{-}ME}$]* n = 14: 10 ms = 1.1 ± 0.1, 20 ms = 1.0 ± 0.08, 50 ms = 1.1 ± 0.06, 100 ms =

1.1 ± 0.04. *P[syt$^{A\text{-}ME,FE}$]* n = 12: 10 ms = 2.7 ± 0.3, 20 ms = 1.8 ± 0.2, 50 ms = 1.6 ± 0.1, 100 ms = 1.5 ± 0.2. Two-way repeated measures ANOVA, F (36, 108) = 2.190 p = 0.001; Tukey post hoc – *P[syt^{WT}]* vs *P[syt$^{A\text{-}ME}$]:* 10 ms p = 0.0005, 20 ms p = 0.01, 50 ms p = 0.007, 100 ms p = 0.0003. *P[syt^{WT}]* vs *P[syt$^{A\text{-}ME,FE}$]*: 10 ms p < 0.0001, 20 ms p = 0.0001, 50 ms p = 0.0008, 100 ms p = 0.04). Furthermore, the PPR of the double mutant was significantly greater than that of the single mutant for every interpulse interval, except 100 ms (Fig 3.5A,B *P[syt$^{A\text{-}ME}$]* vs *P[syt$^{A\text{-}ME,FE}$]*, Tukey post hoc – *P[syt$^{A\text{-}ME}$]* vs *P[syt$^{A\text{-}ME,FE}$]*: 10 ms p = 0.0003, 20 ms p = 0.003, 50 ms p = 0.01, 100 ms p = 0.2). These results indicate that a decreased release probability could account for the decrease in neurotransmitter release, which is further bolstered by the finding that the release probability scales inversely with the severity of the mutation.

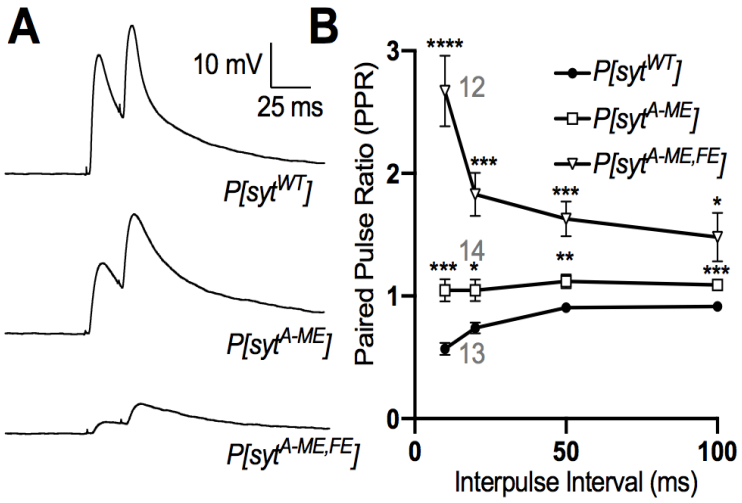

Figure 3.5. Probability of release is decreased by hydrophobic mutations. Probability of release was determined using a paired pulse protocol with interpulse intervals of 10, 20, 50, and 100ms. **A,** Representative traces of paired EJPs for *P[syt^{WT}]*, *P[syt$^{A\text{-}ME}$]*, and *P[syt$^{A\text{-}ME,FE}$]* with a 20ms interpulse interval. **B,** Mean paired pulse ratios ± SEM for *P[syt^{WT}]*, *P[syt$^{A\text{-}ME}$]*, and *P[syt$^{A\text{-}ME,FE}$]* (Two-way Repeated Measures ANOVA with Tukey post-hoc, p < 0.05 = *, p < 0.01 = **, p < 0.001 = ***, p < 0.0001 = ****). Indicated differences are between mutants and *P[syt^{WT}]*, though the paired pulse ratio was significantly different (p < 0.05) between *P[syt$^{A\text{-}ME}$]*, and *P[syt$^{A\text{-}ME,FE}$]*, for all interpulse intervals except 100ms.

C_2A hydrophobic mutations decrease the apparent Ca^{2+} affinity of release

Fast, synchronous neurotransmitter release is a Ca^{2+}-dependent, cooperative process [35]. In order to test whether the C_2A hydrophobic mutations affect the Ca^{2+} dependence of synaptotagmin-triggered release, we recorded EJPs at a variety of extracellular Ca^{2+} concentrations, ranging from 0.05 to 5 mM. In our dose-response curve, the intermediate decrease of EJP amplitude in *P[syt$^{A\text{-}ME}$]* mutants and the dramatic decrease of EJP amplitude in *P[syt$^{A\text{-}ME,FE}$]* mutants compared to control are evident at all Ca^{2+} concentrations (Fig 3.6A). We used nonlinear regression to fit curves to the data (Fig 3.6A solid curves). To determine whether these hydrophobic mutations impact the cooperativity of release, we compared the Hill coefficient, calculated from the equations of the curve for each genotype, and found no significant differences (Hill slope: *P[syt^{WT}]* = 1.8, *P[syt$^{A\text{-}ME}$]* = 1.8, *P[syt$^{A\text{-}ME,FE}$]* = 1.8). Thus, mutations of these C_2A hydrophobic residues do not impact the cooperativity of Ca^{2+}-dependent neurotransmitter release.

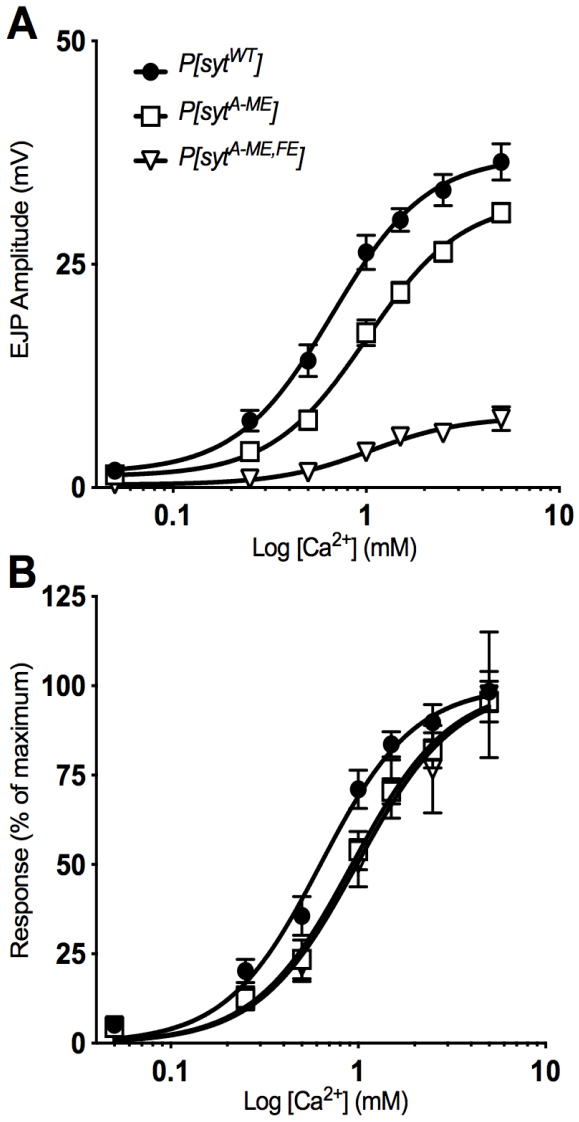

Figure 3.6. The apparent Ca^{2+} affinity of release is decreased by hydrophobic mutations. **A,** Mean EJP amplitude ± SEM across a range of Ca^{2+} concentrations fit with a nonlinear regression. **B,** Ca^{2+} curves normalized to maximum EJP amplitude predicted by the nonlinear regression. The significant rightward shift in the curve (EC50, non-overlapping confidence intervals) indicates a decrease in the apparent Ca^{2+} affinity of neurotransmitter release.

To assess the apparent Ca^{2+} affinity of release, the response at each Ca^{2+} level was normalized to the maximal response predicted by the non-linear regression equation for each genotype. Consistent with the decrease in Ca^{2+} affinity for phospholipid binding mutants reported previously [36], Fig 3.6B displays a similar shift in the curves for the Ca^{2+} dependence

of neurotransmitter release. (EC50, 95%CI: $P[syt^{WT}]$ = 0.6 mM, 0.5-0.7 mM, n = 13; $P[syt^{A\text{-}ME}]$ = 0.9 mM, 0.8-1.0 mM, n = 12; $P[syt^{A\text{-}ME,FE}]$ = 1.0 mM, 0.8-1.2 mM, n = 12; non-overlapping confidence intervals compared to control). The rightward shift of the Ca^{2+} curves demonstrates that both of the C_2A hydrophobic mutants caused a decrease in the apparent Ca^{2+} affinity of release.

3.6 Discussion

We investigated the role of two hydrophobic residues in the C_2A domain of synaptotagmin in neurotransmitter release. Mutation of the C_2A loop 1 hydrophobic residue ($P[syt^{A\text{-}ME}]$) resulted in a 50% reduction of evoked release (Fig 3.2B), consistent with previous findings for the loop 3 hydrophobic residue [$P[syt^{A\text{-}FE}]$, [19]]. Notably, mutation of both of these hydrophobic residues in tandem ($P[syt^{A\text{-}ME,FE}]$) nearly abolished the evoked response (Fig 3.2B). These deficits could result from the decreased release probability (Fig 3.5). Analysis of spontaneous release suggests that neither vesicle loading (mEJP amplitude) nor frequency of events (mEJP frequency) played a role in the observed deficits (Fig 3.4). Evoked responses at varying Ca^{2+} levels revealed that the apparent Ca^{2+} affinity of release was decreased by either our single or double hydrophobic residue mutations in C_2A, but the Ca^{2+} cooperativity of release was unaffected (Fig 3.6). Importantly, the double mutation decreased evoked release significantly more than the complete absence of synaptotagmin 1 (Fig 3.2D), making it the most severe mutation of the C_2A domain of synaptotagmin to date.

Previous *in vivo* and in culture analyses of C_2A and C_2B domain function suggested that the C_2B domain was essential, while C_2A played a secondary role. C_2B mutations resulted in dominant negative effects and lethality, while C_2A mutations were viable and only decreased

release by a maximum of 50-80%. Specifically, mutations disrupting Ca^{2+} binding resulted in: a dominant-negative effect or lethality in C_2B [2, 31], but only a 0-80% decrease in evoked release in C_2A [10, 29, 30, 37]. Mutations that altered the polylysine motif resulted in: an ~40-50% decrease in evoked transmitter release in C_2B [32, 38-40], yet did not impair evoked release in C_2A [41]. Similarly, mutation of a loop 3 positively-charged residue involved in electrostatic interactions with membranes resulted in: a 60-80% decrease in evoked release in C_2B [42, 43], see however [40], and only an ~45-55% decrease in C_2A [40, 42-44]. Importantly, mutation of the loop 3 hydrophobic residue resulted in: embryonic lethality in C_2B, but only a 50% decrease in evoked release in C_2A [19]. Taken together, these results led to the understanding that C_2B was critical, while C_2A only acted in a facilitatory manner.

The current study challenges this longstanding idea regarding the significance of the C_2A domain. The lethality caused by mutation of the C_2B loop 3 hydrophobic residue is still the most severe synaptotagmin phenotype to date [19] and demonstrates the predominant role of the C_2B domain *in vivo*. Here we report that simultaneous mutation of both the loop 1 and loop 3 hydrophobic tip residues to negatively-charged glutamates in C_2A resulted in the most dramatic deficit ever observed for a C_2A domain mutation. This could be the result of either removal of hydrophobicity or increased electrostatic repulsion of the negatively charged membrane. However, previous work found that mutation of the loop 3 hydrophobic tip residue in C_2A to *either* a hydrophilic, non-charged tyrosine *or* a hydrophilic, negatively-charged glutamate had an equal inhibitory impact on evoked transmitter release. Both decreased release by ~50% [19]. Thus, the net charge at the loop 3 hydrophobic site was inconsequential. Rather, the hydrophilic nature of the mutation correlated with the disruption of function [19]. As such, we chose to use glutamate substitutions at both hydrophobic sites in C_2A for the current study. While the C_2A

double glutamate mutant is still viable, this is the first C_2A mutation to result in less neurotransmitter release than that observed in *syt*^{null} larvae. Thus, the hydrophobic tip residues of *both* domains are essential for synaptotagmin-triggered vesicle fusion. This demonstrates for the first time that C_2A plays more than a facilitatory role; it is absolutely required for synaptotagmin to function as the Ca^{2+} sensor for fast synchronous neurotransmitter release.

The mechanism(s) by which these hydrophobic residues exert their effects has been extensively studied *in vitro*. These include SNARE [21] interactions with, and membrane penetration by, synaptotagmin. The impact of loop 1 and loop 3 hydrophobic residue mutations on SNARE interactions are controversial, with some studies indicating mutation of the hydrophobic residues has an impact on SNARE binding [18, 19] while others indicate that there is no impact on SNARE binding [21]. However, even the studies reporting decreased t-SNARE binding in mutants with decreased hydrophobicity demonstrated that the changes in synaptotagmin's membrane interactions, and NOT the changes in t-SNARE binding, correlated with the changes in synaptotagmin function *in vivo* [19]. Furthermore, the experiments that showed decreased Ca^{2+}-dependent interactions between t-SNAREs and hydrophobic synaptotagmin mutants required membrane-embedded t-SNAREs [18, 19]. Thus, the apparent decrease in Ca^{2+}-dependent SNARE interactions may actually be a result of disrupting membrane interactions.

There is, however, ample evidence supporting a role for the hydrophobic tip residues in membrane penetration. The loop 1 and loop 3 hydrophobic residues of both C_2 domains insert into membranes following Ca^{2+} binding, implicating membrane penetration, and resultant membrane bending and lipid disorder, as important downstream effector interactions of Ca^{2+} binding [15, 20]. *In vitro* membrane tubulation and liposome fusion assays required Ca^{2+}-

dependent synaptotagmin C_2 domain insertion into membranes [15, 18]. Mutations that prevented membrane penetration blocked liposome fusion and tubulation [17]. In cultured PC12 cells, these same mutations prevented vesicular cargo release [21]. Mutations that enhanced membrane penetration had the opposite effect [17, 21] and also increased the apparent Ca^{2+} affinity of neurotransmitter release at hippocampal autapses [45]. These findings indicate that the ability of the loop 1 and 3 hydrophobic residues to insert into the membrane is crucial to synaptotagmin's role in driving vesicle fusion.

Interestingly, each C_2 domain seemed to play equal roles *in vitro,* with C_2B even depending on the presence of C_2A to insert into membranes or bind liposomes [16]. Furthermore, in isolated C_2A domains, single residue substitutions of the loop 1 or loop 3 hydrophobic residues that blocked membrane penetration had summative effects in decreasing the Ca^{2+} dependence of liposome binding, while the double mutant effectively prevented liposome binding [36]. Thus, *in vitro* evidence suggested balanced roles for the C_2A and C_2B domains with regard to membrane penetration. Our current finding, that the hydrophobic residues in both domains are essential for synaptotagmin-triggered neurotransmitter release, is more consistent with the *in vitro* studies. However, disruption of C_2B function *in vivo* is still more severe than that of C_2A: 1) mutation of the loop 3 hydrophobic residue in C_2B results in dominant negative lethality, while mutation of both the loop 1 and loop 3 hydrophobic residues in C_2A does not ([19], and this study); and 2) mutations of Ca^{2+}-binding residues in C_2B also result in dominant negative phenotypes, while those in C_2A do not [2, 10, 30]. The predominance of the C_2B domain for synaptotagmin function *in vivo* could be due to its direct interactions with the SNARE complex [21, 32, 38, 41] or due to its greater ability to induce membrane bending [17, 18, 21].

For both C_2A and C_2B, mutation of the hydrophobic residues required for Ca^{2+}-dependent membrane penetration resulted in a more severe phenotype than mutation of the Ca^{2+} binding residues themselves [2, 10, 30]. This combination of findings indicates that: 1) the hydrophobic residues mediate a key effector interaction(s), and 2) the reported mutations of Ca^{2+} binding residues must not completely block these downstream interactions. For example, the *syt$^{A\text{-}ME,FE}$* hydrophobic mutation abolished all synaptotagmin-triggered neurotransmitter release (Fig 3.2D). Yet the mutation of the second of the five Ca^{2+} binding aspartates to a glutamate (*syt$^{A\text{-}D2E}$)*, which completely blocked Ca^{2+} binding by C_2A *in vitro*, supported some (20%) synaptotagmin-triggered Ca^{2+}-evoked release *in vivo* [10]. This combination of effects suggests that in the *P[syt$^{A\text{-}D2E}$]* mutant, the intact C_2B domain may be able to place the C_2A pocket close enough to membranes for some hydrophobic interactions to occur and facilitate a small amount of synaptotagmin-triggered fusion, despite the electrostatic repulsion by C_2A. While in the *P[syt$^{A\text{-}ME,FE}$]* mutant, the required downstream hydrophobic interactions by C_2A are completely blocked and no synaptotagmin-triggered release occurs in response to Ca^{2+} influx. For C_2B, the loop 3 hydrophobic mutant resulted in less Ca^{2+}-evoked release than seen in *syt^{null}* mutants, no change in spontaneous release, and embryonic lethality ([19] and unpublished observations). On the other hand, mutation of Ca^{2+} binding aspartates in C_2B to neutral asparagines (*syt$^{B\text{-}DN}$*) resulted in less evoked release than in *syt^{null}* mutants, but some could survive to larval or even adult stages, and there was an increase in spontaneous transmitter release [2]. No synaptotagmin-triggered, Ca^{2+}-evoked release was possible in either case. However, the decreased electrostatic repulsion of the presynaptic membrane in the *P[syt$^{B\text{-}DN}$]* mutants would permit membrane interactions by the hydrophobic residues resulting in increased spontaneous neurotransmitter release. Since these mutants were viable to a later stage than the loop 3 hydrophobic mutants,

this combination of effects suggests that the increase in spontaneous release in the *P[syt$^{B\text{-}DN}$]* mutants was beneficial to the organism. Thus, the increased severity of the hydrophobic mutations in both C_2 domains, compared to the Ca^{2+} binding mutations reported to date, suggests that the interactions mediated by the hydrophobic tip residues are absolutely essential for synaptotagmin function, are downstream of Ca^{2+} binding, and still occur to some degree in Ca^{2+} binding mutants.

Our results, coupled with previous *in vitro* and *in vivo* findings, are consistent with the idea that membrane insertion by both C_2 domains is a primary downstream effector interaction in synaptotagmin's transduction of Ca^{2+} influx into vesicle fusion. Prior to Ca^{2+} influx, the negative charge of both Ca^{2+}-binding pockets provides electrostatic repulsion of the negatively-charged presynaptic membrane (Fig 3.1). After Ca^{2+} entry, Ca^{2+} binding results in a net positive charge at the tip of each C_2 domain, which then attracts the presynaptic membrane – flipping the electrostatic switch [11, 12]. The hydrophobic residues at the tips of each C_2 domain are then able to insert into the hydrophobic core of the presynaptic membrane [14, 20, 46]. This insertion has been shown to result in positive curvature of the target membrane [17, 18] and disruption of phospholipid order [47], which could provide the final energy required to drive fusion of the vesicle membrane with the presynaptic membrane. A certain threshold of lipid destabilization could be required for fusion, and inhibiting penetration by C_2A may lower membrane destabilization below this threshold.

A recent study [48] suggests a mechanism whereby the membrane disruption resulting from synaptotagmin penetration could directly promote SNARE-mediated vesicle fusion. Synaptotagmin penetration is proposed to disorder lipids near the transmembrane domain of syntaxin allowing the bent linker in the juxtamembrane region to straighten, thereby facilitating

the transition from trans- to cis-SNAREs required for driving fusion. Synaptotagmin-triggered membrane curvature [17, 18] could augment this transition. Assuming a certain threshold of lipid disorder is necessary for SNAREs to alter their conformation, partial disruption of the membrane in our *P[syt$^{A\text{-}ME}$]* mutant or in the previously studied *P[syt$^{A\text{-}FE}$]* mutant [19] may allow only a subset of SNARE complexes to fully straighten leading to the observed 50% reduction in vesicle fusion. However, our double mutant (*P[syt$^{A\text{-}ME,FE}$]*), which should prevent any insertion by C_2A, would not contribute to the membrane disorder necessary for conformational change in the SNARE complex, thereby preventing fusion.

A remaining question is how do synaptotagmin 1 mutants result in less fast, synchronous fusion than that seen in the absence of the wild type protein (*syt^{null}*)? In these cases, the presence of the mutant protein must actively inhibit the residual neurotransmitter release remaining in *syt^{null}* mutants (Fig 3.2D and [2, 19]). The finding that the Ca^{2+} dependence of evoked release in *syt^{null}* mutants is the same as in wild type (no change in EC50 [2]) is consistent with the idea that another isoform of synaptotagmin could be the trigger. However, the only other synaptotagmin isoform known to be expressed at the neuromuscular junction in *Drosophila* is synaptotagmin IV and it is concentrated in the postsynaptic cell [49]. Whether there is any synaptotagmin IV in the presynaptic terminal or if its expression level is impacted in *syt^{null}* mutants is unknown. In short, the Ca^{2+} sensor that triggers the residual vesicle fusion in *syt^{null}* mutants is still unknown.

Our double mutant (*P[syt$^{A\text{-}ME,FE}$])* had less neurotransmitter release than the *syt^{null}* mutant (Fig 3.2 C,D). This is consistent with findings that the most debilitating C_2B mutations (to the hydrophobic residues [19] and Ca^{2+}-binding residues [2, 31]) also decrease release to levels worse than *syt^{null}*. The most likely explanation for this finding is that the mutant form of the protein is outcompeting whatever sensor is maintaining some Ca^{2+} sensing function in the

syt^{null}. There are several possibilities for the sensor that is maintaining minimal Ca^{2+} dependence in the absence of synaptotagmin 1. One candidate in mammals is Doc2B [50], however there is no *Drosophila* form of the protein. It could, however, be another synaptotagmin isoform. Synaptotagmins 12 and 14 are unlikely since they seem to have minimal expression and are thought to lack the ability to bind Ca^{2+}, and alpha and beta are only expressed in very specific neurosecretory cells [49]. Synaptotagmins 4 and 7 may rescue some release. Synaptotagmin 7 is a candidate for a presynaptic asynchronous Ca^{2+} sensor in mammalian systems [51], but overexpression in *Drosophila* doesn't rescue any release in a *syt*^{null} mutant [49]. Furthermore, a recent double synaptotagmin 1 and 7 knockout actually had more neurotransmitter release than a synaptotagmin 1 null knockout alone, suggesting that synaptotagmin 7 isn't the sensor rescuing release [52]. Synaptotagmin 4 is another possibility, as it is localized at the neuromuscular junction, though apparently only postsynaptically [49]. These experiments were done by light level immunohistochemistry though, so there may be presynaptic synaptotagmin 4 at low levels. Overexpression of synaptotagmin 4 has yielded conflicting results. One study found complete rescue of neurotransmitter release [30], while another found no rescue [49]. A double knockout of synaptotagmins 1 and 4, however, still observed a small amount of Ca^{2+} dependent release, suggesting that synaptotagmin 4 is also not the remaining sensor in the synaptotagmin 1 null [53]. The identity of the sensor rescuing the small amount of neurotransmitter release in the absence of synaptotagmin 1 is still very much under investigation. Our synaptotagmin 1 double hydrophobic mutant, though, must be able to outcompete whatever sensor is present in order to cause less neurotransmitter release than normally observed in a *syt*^{null} mutant.

Regardless of the mechanism of action, our characterization of the most severe C_2A domain mutation to date challenges the predominant model of synaptotagmin function. While all previous *in vivo* evidence suggested that C_2A acted only as a facilitatory domain, disrupting the hydrophobicity of the loop 1 and loop 3 Ca^{2+}-binding pocket residues of C_2A (Fig 3.1) decreased evoked neurotransmitter release to levels less than that in syt^{null} larvae (Fig 3.2). These findings are consistent with *in vitro* evidence indicating important roles for these hydrophobic tip residues in membrane penetration, and potential interactions with SNARE proteins. Our findings now demonstrate that, in contrast to the current view, the C_2A domain is absolutely required for synaptotagmin to trigger fast, synchronous vesicle fusion.

WORKS CITED

1. Brose, N., et al., *Synaptotagmin: a calcium sensor on the synaptic vesicle surface.* Science, 1992. **256**(5059): p. 1021-5.
2. Mackler, J.M., et al., *The C(2)B Ca(2+)-binding motif of synaptotagmin is required for synaptic transmission in vivo.* Nature, 2002. **418**(6895): p. 340-4.
3. Broadie, K., et al., *Absence of synaptotagmin disrupts excitation-secretion coupling during synaptic transmission.* Proc Natl Acad Sci U S A, 1994. **91**(22): p. 10727-31.
4. Geppert, M., et al., *Synaptotagmin I: a major Ca2+ sensor for transmitter release at a central synapse.* Cell, 1994. **79**(4): p. 717-27.
5. DiAntonio, A. and T.L. Schwarz, *The effect on synaptic physiology of synaptotagmin mutations in Drosophila.* Neuron, 1994. **12**(4): p. 909-20.
6. Littleton, J.T., et al., *Mutational analysis of Drosophila synaptotagmin demonstrates its essential role in Ca(2+)-activated neurotransmitter release.* Cell, 1993. **74**(6): p. 1125-34.
7. Perin, M.S., et al., *Structural and functional conservation of synaptotagmin (p65) in Drosophila and humans.* J Biol Chem, 1991. **266**(1): p. 615-22.
8. Fernandez, I., et al., *Three-dimensional structure of the synaptotagmin 1 C2B-domain: synaptotagmin 1 as a phospholipid binding machine.* Neuron, 2001. **32**(6): p. 1057-69.
9. Sutton, R.B., J.A. Ernst, and A.T. Brunger, *Crystal structure of the cytosolic C2A-C2B domains of synaptotagmin III. Implications for Ca(+2)-independent snare complex interaction.* J Cell Biol, 1999. **147**(3): p. 589-98.
10. Striegel, A.R., et al., *Calcium binding by synaptotagmin's C2A domain is an essential element of the electrostatic switch that triggers synchronous synaptic transmission.* J Neurosci, 2012. **32**(4): p. 1253-60.
11. Davletov, B., O. Perisic, and R.L. Williams, *Calcium-dependent membrane penetration is a hallmark of the C2 domain of cytosolic phospholipase A2 whereas the C2A domain of synaptotagmin binds membranes electrostatically.* J Biol Chem, 1998. **273**(30): p. 19093-6.
12. Ubach, J., et al., *Ca2+ binding to synaptotagmin: how many Ca2+ ions bind to the tip of a C2-domain?* Embo j, 1998. **17**(14): p. 3921-30.
13. Murray, D. and B. Honig, *Electrostatic control of the membrane targeting of C2 domains.* Mol Cell, 2002. **9**(1): p. 145-54.
14. Vermaas, J.V. and E. Tajkhorshid, *Differential Membrane Binding Mechanics of Synaptotagmin Isoforms Observed in Atomic Detail.* Biochemistry, 2017. **56**(1): p. 281-293.
15. Herrick, D.Z., et al., *Position of synaptotagmin I at the membrane interface: cooperative interactions of tandem C2 domains.* Biochemistry, 2006. **45**(32): p. 9668-74.
16. Bai, J., P. Wang, and E.R. Chapman, *C2A activates a cryptic Ca(2+)-triggered membrane penetration activity within the C2B domain of synaptotagmin I.* Proc Natl Acad Sci U S A, 2002. **99**(3): p. 1665-70.

17. Martens, S., M.M. Kozlov, and H.T. McMahon, *How synaptotagmin promotes membrane fusion.* Science, 2007. **316**(5828): p. 1205-8.
18. Hui, E., et al., *Synaptotagmin-mediated bending of the target membrane is a critical step in Ca(2+)-regulated fusion.* Cell, 2009. **138**(4): p. 709-21.
19. Paddock, B.E., et al., *Membrane penetration by synaptotagmin is required for coupling calcium binding to vesicle fusion in vivo.* J Neurosci, 2011. **31**(6): p. 2248-57.
20. Chapman, E.R. and A.F. Davis, *Direct interaction of a Ca2+-binding loop of synaptotagmin with lipid bilayers.* J Biol Chem, 1998. **273**(22): p. 13995-4001.
21. Lynch, K.L., et al., *Synaptotagmin-1 utilizes membrane bending and SNARE binding to drive fusion pore expansion.* Mol Biol Cell, 2008. **19**(12): p. 5093-103.
22. Loewen, C.A., J.M. Mackler, and N.E. Reist, *Drosophila synaptotagmin I null mutants survive to early adulthood.* Genesis, 2001. **31**(1): p. 30-6.
23. DiAntonio, A., K.D. Parfitt, and T.L. Schwarz, *Synaptic transmission persists in synaptotagmin mutants of Drosophila.* Cell, 1993. **73**(7): p. 1281-90.
24. Bischof, J., et al., *An optimized transgenesis system for Drosophila using germ-line-specific phiC31 integrases.* Proc Natl Acad Sci U S A, 2007. **104**(9): p. 3312-7.
25. Brand, A.H. and N. Perrimon, *Targeted gene expression as a means of altering cell fates and generating dominant phenotypes.* Development, 1993. **118**(2): p. 401-15.
26. Yao, K.M. and K. White, *Neural specificity of elav expression: defining a Drosophila promoter for directing expression to the nervous system.* J Neurochem, 1994. **63**(1): p. 41-51.
27. Feng, Y., A. Ueda, and C.F. Wu, *A modified minimal hemolymph-like solution, HL3.1, for physiological recordings at the neuromuscular junctions of normal and mutant Drosophila larvae.* J Neurogenet, 2004. **18**(2): p. 377-402.
28. Chapman, E.R., et al., *Delineation of the oligomerization, AP-2 binding, and synprint binding region of the C2B domain of synaptotagmin.* J Biol Chem, 1998. **273**(49): p. 32966-72.
29. Stevens, C.F. and J.M. Sullivan, *The synaptotagmin C2A domain is part of the calcium sensor controlling fast synaptic transmission.* Neuron, 2003. **39**(2): p. 299-308.
30. Robinson, I.M., R. Ranjan, and T.L. Schwarz, *Synaptotagmins I and IV promote transmitter release independently of Ca(2+) binding in the C(2)A domain.* Nature, 2002. **418**(6895): p. 336-40.
31. Nishiki, T. and G.J. Augustine, *Dual roles of the C2B domain of synaptotagmin I in synchronizing Ca2+-dependent neurotransmitter release.* J Neurosci, 2004. **24**(39): p. 8542-50.
32. Loewen, C.A., et al., *C2B polylysine motif of synaptotagmin facilitates a Ca2+-independent stage of synaptic vesicle priming in vivo.* Mol Biol Cell, 2006. **17**(12): p. 5211-26.
33. Liu, H., et al., *Autapses and networks of hippocampal neurons exhibit distinct synaptic transmission phenotypes in the absence of synaptotagmin I.* J Neurosci, 2009. **29**(23): p. 7395-403.
34. Zucker, R.S. and W.G. Regehr, *Short-term synaptic plasticity.* Annu Rev Physiol, 2002. **64**: p. 355-405.
35. Dodge, F.A., Jr. and R. Rahamimoff, *Co-operative action a calcium ions in transmitter release at the neuromuscular junction.* J Physiol, 1967. **193**(2): p. 419-32.

36. Gerber, S.H., J. Rizo, and T.C. Sudhof, *Role of electrostatic and hydrophobic interactions in Ca(2+)-dependent phospholipid binding by the C(2)A-domain from synaptotagmin I.* Diabetes, 2002. **51 Suppl 1**: p. S12-8.
37. Fernandez-Chacon, R., et al., *Structure/function analysis of Ca2+ binding to the C2A domain of synaptotagmin 1.* J Neurosci, 2002. **22**(19): p. 8438-46.
38. Mackler, J.M. and N.E. Reist, *Mutations in the second C2 domain of synaptotagmin disrupt synaptic transmission at Drosophila neuromuscular junctions.* J Comp Neurol, 2001. **436**(1): p. 4-16.
39. Borden, C.R., et al., *Synaptotagmin mutants Y311N and K326/327A alter the calcium dependence of neurotransmission.* Mol Cell Neurosci, 2005. **29**(3): p. 462-70.
40. Li, L., et al., *Phosphatidylinositol phosphates as co-activators of Ca2+ binding to C2 domains of synaptotagmin 1.* J Biol Chem, 2006. **281**(23): p. 15845-52.
41. Mace, K.E., et al., *Synaptotagmin I stabilizes synaptic vesicles via its C(2)A polylysine motif.* Genesis, 2009. **47**(5): p. 337-45.
42. Paddock, B.E., et al., *Ca2+-dependent, phospholipid-binding residues of synaptotagmin are critical for excitation-secretion coupling in vivo.* J Neurosci, 2008. **28**(30): p. 7458-66.
43. Wang, P., et al., *Mutations in the effector binding loops in the C2A and C2B domains of synaptotagmin I disrupt exocytosis in a nonadditive manner.* J Biol Chem, 2003. **278**(47): p. 47030-7.
44. Fernandez-Chacon, R., et al., *Synaptotagmin I functions as a calcium regulator of release probability.* Nature, 2001. **410**(6824): p. 41-9.
45. Rhee, J.S., et al., *Augmenting neurotransmitter release by enhancing the apparent Ca2+ affinity of synaptotagmin 1.* Proc Natl Acad Sci U S A, 2005. **102**(51): p. 18664-9.
46. Bai, J., W.C. Tucker, and E.R. Chapman, *PIP2 increases the speed of response of synaptotagmin and steers its membrane-penetration activity toward the plasma membrane.* Nat Struct Mol Biol, 2004. **11**(1): p. 36-44.
47. Lai, A.L., et al., *Synaptotagmin 1 modulates lipid acyl chain order in lipid bilayers by demixing phosphatidylserine.* J Biol Chem, 2011. **286**(28): p. 25291-300.
48. Kiessling, V., et al., *A molecular mechanism for calcium-mediated synaptotagmin-triggered exocytosis.* Nat Struct Mol Biol, 2018. **25**(10): p. 911-917.
49. Adolfsen, B., et al., *Synaptotagmins are trafficked to distinct subcellular domains including the postsynaptic compartment.* J Cell Biol, 2004. **166**(2): p. 249-60.
50. Yao, J., et al., *Doc2 is a Ca2+ sensor required for asynchronous neurotransmitter release.* Cell, 2011. **147**(3): p. 666-77.
51. Bacaj, T., et al., *Synaptotagmin-1 and synaptotagmin-7 trigger synchronous and asynchronous phases of neurotransmitter release.* Neuron, 2013. **80**(4): p. 947-59.
52. Guan, Z., et al., *Drosophila Synaptotagmin 7 negatively regulates synaptic vesicle release and replenishment in a dosage-dependent manner.* Elife, 2020. **9**.
53. Saraswati, S., B. Adolfsen, and J.T. Littleton, *Characterization of the role of the Synaptotagmin family as calcium sensors in facilitation and asynchronous neurotransmitter release.* Proc Natl Acad Sci U S A, 2007. **104**(35): p. 14122-7.

CHAPTER 4. CALCIUM BINDING BY DISTINCT RESIDUES IN SYNAPTOTAGMIN'S C_2A DOMAIN IS CRITICAL FOR SYNAPTIC TRANSMISSION[4]

4.1 Summary

Synaptotagmin, the Ca^{2+} sensor for synchronous neurotransmitter release, is hypothesized to function as an electrostatic switch. Prior to Ca^{2+} influx, the negatively charged Ca^{2+} binding pockets of the tandem C_2 domains electrostatically repel the negatively charged presynaptic membrane. This repulsion prevents SNARE-mediated vesicle fusion. Following depolarization-induced influx, Ca^{2+} binding to these negatively charged pockets results in a net positive charge that switches the electrostatic repulsion of the presynaptic membrane to electrostatic attraction. This switch permits both electrostatic and hydrophobic interactions that lead to SNARE-mediated fusion of synaptic vesicles with the presynaptic membrane. Here we use an assortment of novel and previously studied aspartate to asparagine mutations in the C_2A domain of synaptotagmin to investigate the relative contributions of the critical aspartates in driving fusion of synaptic vesicles. Using our novel mutations, we found that asparagine substitutions at some sites dramatically decreased synchronous neurotransmission, while substitutions at another site increased synchronous neurotransmission as previously documented. Our results suggest that the electrostatic charge, as well as Ca^{2+} binding abilities of distinct regions of the binding pocket result in different impacts on synaptic vesicle fusion. Furthermore, these results support my other recent findings that the C_2A domain plays a much larger role in the function of synaptotagmin than previously thought.

[4] Bowers MR and Reist NE

4.2 Introduction

Efficient, synchronous fusion of multiple neurotransmitter filled vesicles with the presynaptic membrane is critical for synaptic transmission. This fusion is triggered by depolarization-induced Ca^{2+} influx. The synaptic vesicle protein synaptotagmin 1 is responsible for detecting this increase in local Ca^{2+} concentration and triggering fast, synchronous fusion of vesicles at the synapse [1, 2]. When synaptotagmin is knocked out, this synchronous neurotransmitter release is almost completely abolished [3-6]. Synaptotagmin 1 is composed of tandem C_2 domains (C_2A and C_2B) tethered to synaptic vesicles via a flexible linker domain [7]. Each of these C_2 domains contains a Ca^{2+} binding pocket composed of 5 highly conserved negatively charged residues that coordinate Ca^{2+} binding (Fig 1 A,B-D1, D2, D3, D4, D5, red; C-red residues)[8-11]. Synaptotagmin has been shown to interact with the soluble n-ethlymaleimide-sensitive factor attachment protein receptor (SNARE) proteins [12-15] and the presynaptic membrane [16-18] in a Ca^{2+}-dependent manner. Taken together, these findings have led to the predominant hypothesis that synaptotagmin acts as the Ca^{2+} sensor to synchronize fusion of the synaptic vesicles with the presynaptic membrane.

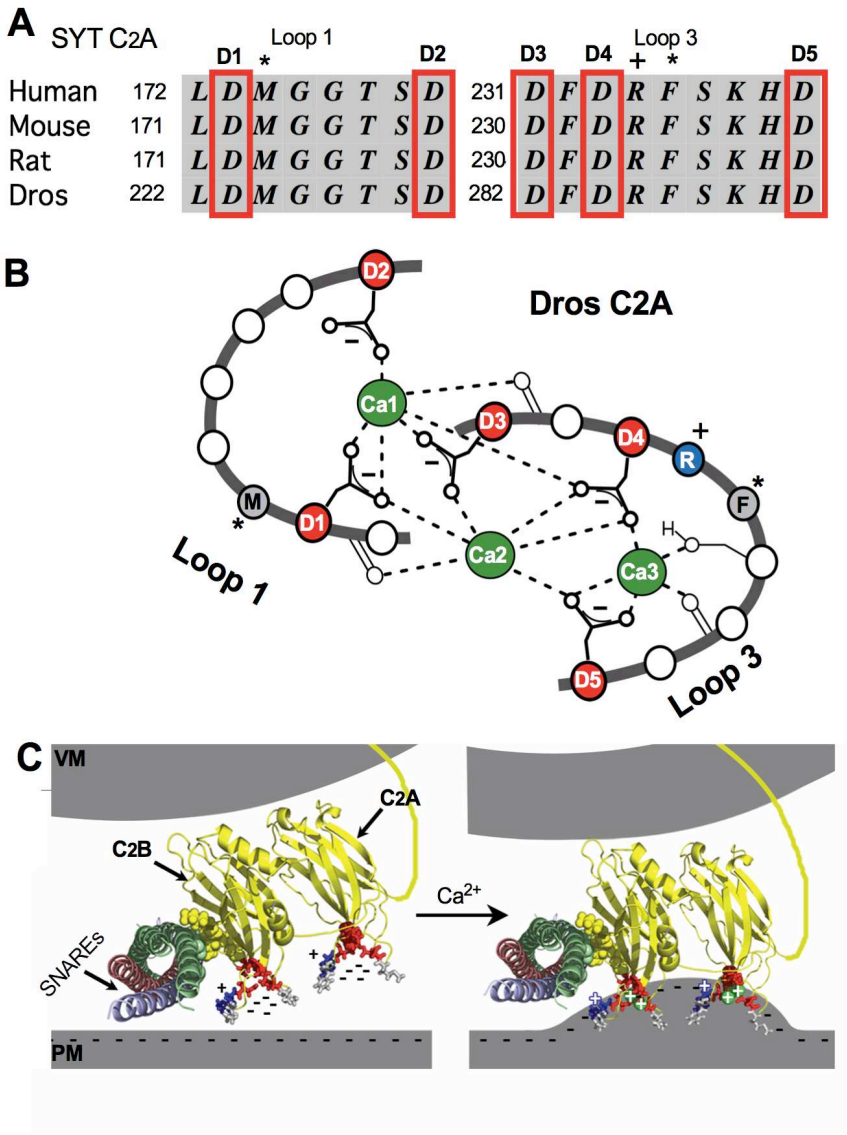

Figure 4.1. Synaptotagmin and Ca^{2+} binding residues of the C_2A domain. A, Protein alignment of loops 1 and 3 of the C_2A domain of synaptotagmin 1 from human, mouse, rat, and *Drosophila*. Critical, well-conserved Ca^{2+} binding aspartates are noted by red boxes (D1-D5). Hydrophobic tip residues are marked by an asterisk (M, F) and a critical basic arginine is marked by a plus sign (R). B, Schematic of the Ca^{2+}-binding loops of *Drosophila* synaptotagmin 1 (adapted from [11]). Ca^{2+}-binding aspartates are red (D1-D5), hydrophobic tip residues are light grey (M, F, *), and the basic arginine is blue (R, +). The three Ca^{2+} ions bind consecutively to sites Ca1, Ca2 and Ca3 (green, [8]). C, Mechanism of synaptotagmin function. Synaptotagmin (yellow) is composed of C_2A and C_2B and tethered to the vesicle membrane (VM). It also interacts with the SNARE complex (red, blue, and green helices). In the absence of Ca^{2+}, the negative aspartate residues (red) are electrostatically repulsed by the negative charge of the presynaptic membrane (PM), clamping SNARE mediated vesicle fusion. Following binding of Ca^{2+} (green), the net charge of the Ca^{2+}-binding pockets is switched from electrostatic repulsion to attraction. With

electrostatic repulsion neutralized, the basic residues in each domain (blue) help attract the PM, allowing the hydrophobic residues to insert into the membrane. This insertion is thought to disrupt lipid order, promote membrane bending, and ultimately trigger vesicle fusion.

Synaptotagmin has been proposed to function as an electrostatic switch [8, 19-22]. In the absence of Ca^{2+}, the negatively charged Ca^{2+} binding aspartates (red) are repelled by the negatively charged phospholipids of the presynaptic membrane (Fig 1 C, left). Depolarization by an action potential opens voltage gated Ca^{2+} channels. The increase in local Ca^{2+} concentration leads to Ca^{2+} ions (green) binding to these negatively charged pockets. This binding changes the electrostatic repulsion between synaptotagmin and the presynaptic membrane to electrostatic attraction. This electrostatic switching allows hydrophobic residues at the tips of these Ca^{2+} binding pockets to interact with the presynaptic membrane [23, 24]. It is thought that these interactions lower the energy barrier for SNARE-mediated vesicle fusion (Fig 1 C, right), leading to synchronous fusion of multiple vesicles with the membrane.

Previous investigation of Ca^{2+} binding by synaptotagmin *in vivo* and *in vitro* have used a variety of aspartate to asparagine (D-N) substitutions to block Ca^{2+} binding by neutralization of the residues' charges [1, 8, 25-31]. Interestingly, while these mutations disrupt Ca^{2+} dependent interactions by the isolated C_2A domain *in vitro*, these C_2A substitutions have little effect when both domains are present *in vitro* or *in vivo. In vitro*, D-N substitutions in the isolated C_2A domain decrease Ca^{2+} dependent phospholipid binding [26, 28], and prevent Ca^{2+} dependent interactions with the SNARE proteins [19]. When these C_2A mutations are expressed in a tandem domain construct, the presence of a functional C_2B domain rescues these deficits [16, 26, 29]. Similarly, *In vivo* substitutions of the 2nd aspartate residue deep in the Ca^{2+} binding pocket of the C_2A domain were reported to rescue evoked neurotransmitter release in synaptotagmin

null neurons [25, 26], although this rescue was not complete, resulting in a deficit in neurotransmitter release. Additionally, D-N substitutions in the C_2A domain that included the aspartate (D4) located immediately adjacent to a key positively charged residue and hydrophobic residue either had no effect on neurotransmitter release [27, 29] or stimulated increased release [25, 32]. An analogous mutation in the C_2B domain, however, resulted in embryonic lethality and was dominant negative when expressed in the heterozygous background [1]. The widely accepted interpretation of these results was that Ca^{2+} binding by the C_2B domain was both necessary and sufficient for evoked transmitter release, while Ca^{2+} binding by the C_2A domain appeared irrelevant [1, 26, 29]. Following these studies, much of the experimental focus has been trained on the function of the C_2B domain. However, the D-N substitutions were proposed to mimic Ca binding by neutralizing the Ca^{2+} binding pocket [25]. A study that mutated C_2A's D2 to a neutral glutamic acid (E), to prevent mimicking of the Ca^{2+} bound state, found that there was an 80% decrease in neurotransmitter release [1]. This displayed that Ca^{2+} binding by the C_2A domain was still functionally important. This suggests that we do not have a full understanding of the *in vivo* functional impact of mutations to the Ca^{2+} binding residues of the C_2A domain, and the relative functional roles of these critical aspartate residues require further investigation.

Aspartates 2 and 3 are located deep in the Ca^{2+} binding pocket and are the most well-ordered aspartates coordinating Ca^{2+} binding by the C_2A domain [9]. Single amino acid substitution studies suggest that they are the most critical aspartates in the C_2B domain for triggering Ca^{2+} dependent neurotransmitter release at hippocampal autapses [30] and Drosophila neuromuscular junctions (unpublished observations). However, the cooperative role in a tandem substitution had never been assayed. Here we have made novel D-N substitutions in the Ca^{2+} binding pocket of the C_2A domain of synaptotagmin to investigate the relative importance of

these residues for synaptic transmission. We have also generated mutants analogous to formerly investigated D-N substitution mutants to allow comparisons to previous data. Finally, we added mutations to hydrophobic residues [23, 24] that mediate membrane interactions downstream of Ca^{2+} binding to our D-N substitution mutants in order to assess the effects of the Ca^{2+} binding mutations on membrane interactions. Interestingly, we found that any combination of substitutions that altered the 2nd aspartate led to a decrease in neurotransmitter release, while a mutation to the 3rd and 4th aspartates actually stimulated increased release. The additional hydrophobic substitutions had opposite effects depending on the aspartate residues that had been mutated. Our results suggest that the aspartates in the C_2A domain Ca^{2+} binding pocket are playing unique roles in the coordination of Ca^{2+} binding and its downstream effector interactions.

4.3 Materials and methods

Drosophila lines

We generated a range of mutants with different combinations of aspartate to asparagine substitutions in loop 1 and loop 3 of the Ca^{2+} binding pocket of the C_2A domain of synaptotagmin. These aspartates are residues D229, D282, D284, D290; the 2nd, 3rd, 4th, and 5th Ca^{2+} coordinating aspartate residues in the binding pocket respectively. A transgenic WT synaptotagmin was used as a positive control. We used the *Drosophila syt 1* coding sequence [4, 7] to generate D229N, D229N/D282N, D282N/D284N, D229N/D282N/D284N/D290N, and wild type control cDNA flanked by EcoRI and Bgll2 restriction sites. This cDNA was synthesized by GeneWiz (South Plainfield, New Jersey), and was flanked by unique 5' EcoRI and 3' BglII restriction sites for directional subcloning into the pUAST-attB vector to place them

under the control of the UAS promoter. The transgenes were injected into *Drosophila* embryos by BestGene (Chino Hills, California) where they were inserted into the attP2 landing site on the third chromosome using the PhiC31 targeted insertion system [33]. Then, these *syt 1* transgenes expressed pan-neuronally by the UAS/Gal4 system [34] driven by the *elav* promoter [35]. All transgenes were expressed in the absence of endogenous synaptotagmin 1 by crossing them into a synaptotagmin 1 null mutant background, syt^{AD4} [4]. Both males and females were used across all experiments because no sex selection was used. This study used the following genotypes: *yw;* syt^{AD4}*elavGal4/* syt^{AD4}*; P[UASsyt1*^{WT}*]/+* (referred to as *P[*syt^{WT}*]* or control), *yw;* syt^{AD4}*elavGal4/* syt^{AD4}*; P[UASsyt1*$^{C2A\text{-}D229N}$*]/+* (referred to as *P[*$syt^{A\text{-}D2N}$*]*), *yw;* syt^{AD4}*elavGal4/* syt^{AD4}*; P[UASsyt1* $^{C2A\text{-}D229N,D282N}$*]/+* (referred to as *P[*$syt^{A\text{-}D2,3N}$*]*), *yw;* syt^{AD4}*elavGal4/* syt^{AD4}*; P[UASsyt1* $^{C2A\text{-}D282N,D284N}$*]/+* (referred to as *P[*$syt^{A\text{-}D3,4N}$*]*), and *yw;* syt^{AD4}*elavGal4/* syt^{AD4}*; P[UASsyt1* $^{C2A\text{-}D229N,D282N,D284N,D290N}$*]/+* (referred to as *P[*$syt^{A\text{-}D2,3,4,5N}$*]*).

In addition, we added hydrophobic to hydrophilic substitutions to two critical residues that mediate membrane interactions, similar to previous mutants that were generated [23, 24]. These specific mutations were M224S and F286Y, which alter the hydrophobicity of the residue without altering the charge, to prevent confounding alteration of the electrostatic potential of the binding pocket. Previous hydrophobic to hydrophilic substitutions of both of these residues blocked neurotransmitter release and had no effect on mEJP frequency compared to WT transgenic controls [23]. These substitutions were combined with *P[*$syt^{A\text{-}D3,4N}$*]* and *P[*$syt^{A\text{-}D2,3,4,5N}$*]* mutations. These genotypes are: *yw;* syt^{AD4}*elavGal4/* syt^{AD4}*; P[UASsyt1* $^{C2A\text{-}M224S,D282N,D284N,F286Y}$*]/+* (referred to as *P[*$syt^{A\text{-}MSD3,4NFY}$*]*), and *yw;* syt^{AD4}*elavGal4/* syt^{AD4}*; P[UASsyt1* $^{C2A\text{-}M224S,D229N,D282N,D284N,F286Y,D290N}$*]/+* (referred to as *P[*$syt^{A\text{-}MSD2,3,4,5NFY}$*]*).

Solutions

HL3.1 saline [70 mM NaCl, 5 mM KCl, 4 mM $MgCl_2$, 10 mM $NaHCO_3$, 5 mM Trehalose, 115 mM sucrose, 5 mM HEPES, pH 7.2 [36]], with varying Ca^{2+} concentrations(from addition of CaCl), was used in all experiments. Phosphate buffered saline (PBS) consisted of [137 mM NaCl, 1.5 mM KH_2PO_4, 2.7 mM KCl, 8.1 mM Na_2HPO_4].

Immunoblotting

Synaptotagmin expression levels were determined using western blot analysis with actin levels serving as a loading control. The CNSs of L3s were dissected in HL3.1 saline where the Ca^{2+} was omitted to decrease vesicle fusion events during dissection. Individual CNSs were placed in Laemmli buffer (Bio-Rad, Hercules, CA) containing 5% β-mercaptoethanol, sonified with five 0.3 sec pulses at 1 Hz using a Branson Sonifier 450 (VWR Scientific, Winchester, PA), and separated by SDS-PAGE with 15% acrylamide. They were then transferred to Immobilon membranes (Millipore, Bedford, MA), and washed in blocking solution [5% milk, 4% normal goat serum (NGS, Fitzgerald Industries International, Acton, MA), 1% bovine serum albumin (BSA, Millipore-Sigma, Burlington, MA), and 0.02% NaN_3 in PBS containing 0.05% Tween 20 (PBS-Tween, Fisher BioReagents, Fair Lawn, NJ)]. The membranes were then incubated overnight at 4°C with a 1:2,500 dilution of anti-synaptotagmin antibody, Dsyt-CL1 [1] and 1:10,000 dilution of anti-actin antibody, (MAB 1501, Millipore Bioscience Research Reagents, Billerica, MA) in PBS-Tween containing 10% NGS and 0.02% NaN_3, washed in PBS-Tween for 1-3 hours, and probed with secondary antibodies at a 1:5,000 dilution of peroxidase-conjugated AffiniPure Goat Anti-Rabbit IgG (Jackson ImmunoResearch, West Grove, PA) and a 1:5,000 dilution of peroxidase-conjugated AffiniPure Donkey Anti-Mouse IgG (Jackson

ImmunoResearch, West Grove, PA) in PBS-Tween containing 10% NGS for 1 hour at room temperature, and washed in PBS-Tween for 30 min. Protein bands were visualized on an Epichemi^3 Darkroom with Labworks Imaging Software (UVP BioImaging, Upland, CA).

Immunolabeling

The localization of the transgenic synaptotagmin protein was visualized by immunohistochemistry. L3s were dissected in Ca^{2+}-free HL3.1, fixed in PBS containing 4% formaldehyde for 1 hour, incubated with a 1:400 dilution of Dsyt-CL1 in dilution media [PBS with 0.1% Triton (PBST), 1% BSA, and 1% NGS] overnight at 4°C, washed in PBST for 1-3 hours, incubated in dilution media containing a 1:400 dilution of Alexa Fluor 488 goat anti-rabbit antibody (Invitrogen, Carlsbad, CA) for 1 hour at room temperature, washed in PBST for one hour, and mounted on microscope slides in Citifluor (Ted Pella, Redding, CA). Confocal images of the neuromuscular junction on muscle fibers 6 and 7 were taken on a Zeiss 880 light-scanning microscope (Zeiss, White Plains, NY), with a 40x objective and Zeiss Zen 2.1 acquisition software, version 11.0.3.190.

Electrophysiological recordings

All electrophysiological recordings were performed using an Axoclamp 2B amplifier (Molecular Devices, Sunnyvale, CA), a Powerlab 4/35 A/D converter (ADInstuments, Sydney, Australia), using LabChart software (ADInstruments, Sydney, Australia). L3s were dissected in Ca^{2+}-free HL3.1 saline to expose the body wall musculature and the central nervous systems were removed. The saline was changed to HL3.1 with 1mM Ca^{2+} prior to recording, unless otherwise noted. Intracellular recordings were made from muscle fiber 6 of abdominal segments

3 and 4 using 10-20 MΩ intracellular electrodes that were pulled using a Sutter model P-97 micropipette puller (Novato, CA) and filled with 3 parts 2 M $K_3C_6H_5O_7$ to 1 part 3 M KCl. The resting potential was held at -65 mV by applying no more than ±1 nA of current. The nerve fiber was stimulated using an A360 stimulus isolator (World Precision Instruments, Sarasota, FL) through a glass suction electrode that was broken to have an ~1 micron tip and filled with HL3.1 containing 1mM Ca^{2+}.

Evoked Release

Ten excitatory junction potentials (EJPs) were stimulated at 0.1 Hz in HL3.1 with 1mM Ca^{2+}. The ten responses were averaged for each muscle fiber. Mean responses are reported for 12-23 fibers per genotype.

Spontaneous release

Spontaneous miniature EJPs (mEJPs) were recorded for 3 min prior to any external stimulation. Recordings were blinded and randomized and mEJPs were identified manually. mEJP frequency was determined by counting the number of events that occurred during the second minute of recording. Mean responses are reported for 12-23 muscle fibers per genotype.

Experimental design and statistical analysis

Statistical analyses were performed using Prism 8 (GraphPad software, La Jolla, CA). All datasets included a minimum of 12 samples per genotype. In all electrophysiological experiments, recordings of mutants and controls were interspersed. Direct comparisons were

only made between recordings done within the same time period, as absolute responses can be impacted by variations in solutions. For comparisons of three or more genotypes with Gaussian distribution of their datasets but unequal standard deviations, we used a Welch's ANOVA with Dunnett's post hoc tests of multiple comparisons to determine significance between each genotype. An alpha p-value of 0.05 was considered significant for all of the above tests.

4.4 Results

Synaptotagmin transgenes

P[syt$^{A\text{-}D2N}$], P[syt$^{A\text{-}D2,3N}$], P[syt$^{A\text{-}D3,4N}$], P[syt$^{A\text{-}D2,3,4,5N}$], P[syt$^{A\text{-}MSD3,4NFY}$], and P[syt$^{A\text{-}MSD2,3,4,5NFY}$] Mutation of the negatively charged aspartates to neutral asparagines alters the electrostatic charge of the residue and prevents Ca^{2+} binding by that specific residue [8, 29]. We generated several different combinations of D-N substitutions to investigate the relative contributions of each aspartate to the electrostatic switching ability of synaptotagmin. We also designed the mutants to investigate how incremental decreases in the electrostatic repulsion of the pocket effect neurotransmitter release *in vivo*. Since the residue numbers of these aspartates vary between organism and isoform, we will refer to them as aspartates 1-5 (D1-5), depending on their order in the sequence. D1 and D2 are found in loop 1 of the Ca^{2+} binding pocket, and D3-5 are found in loop 3 (Fig 1A,B,C red residues). Additionally, we altered the hydrophobicity of critical residues at the tips of loops 1 and 3 in order to determine if changes in release were dependent on membrane interactions. We mutated these hydrophobic residues to be hydrophilic and neutral to avoid any sort of confounding change in net charge. A critical methionine was mutated to a serine, and a critical phenylalanine was mutated to a tyrosine (Fig 1A,B asterisks, C light grey residues).

D-N substitutions that affect the 2nd aspartate decrease neurotransmitter release

In all of our different permutations of aspartate to asparagine mutations, we found that any that included substitution of D2 resulted in a decrease in evoked neurotransmitter release. The single substitution mutant, *P[syt$^{A\text{-}D2N}$]*, led to an ~46% decrease in evoked neurotransmitter release compared to *P[syt^{WT}]* [Fig 2; mean ± SEM: *P[syt^{WT}]* = 26.36 ± 1.37 mV, n = 23; *P[syt$^{A\text{-}D2N}$]* = 14.14 ± 1.73 mV, n = 18; Welch's ANOVA, W = 91.35 (6.000, 44.38); Dunnett's post hoc: *P[syt^{WT}]* vs *P[syt$^{A\text{-}D2N}$]* $p < 0.0001$]. If the second aspartate was mutated in conjunction with any of the other aspartates, the results were even more dramatic. The *P[syt$^{A\text{-}D2,3N}$]* double mutant resulted in an ~71% decrease in evoked neurotransmitter release [Fig 2; mean ± SEM: *P[syt$^{A\text{-}D2,3N}$]* = 7.71 ± 1.06 mV, n = 15; Welch's ANOVA, W = 91.35 (6.000, 44.38); Dunnett's post hoc: *P[syt^{WT}]* vs *P[syt$^{A\text{-}D2,3N}$]* $p < 0.0001$], and the *P[syt$^{A\text{-}D2,3,4,5N}$]* quadruple mutant resulted in an ~78% decrease [Fig 2; mean ± SEM: *P[syt$^{A\text{-}D2,3,4,5N}$]* = 5.71 ± 0.56 mV, n = 17; Welch's ANOVA, W = 91.35 (6.000, 44.38); Dunnett's post hoc: *P[syt^{WT}]* vs *P[syt$^{A\text{-}D2,3,4,5N}$]* $p < 0.0001$].

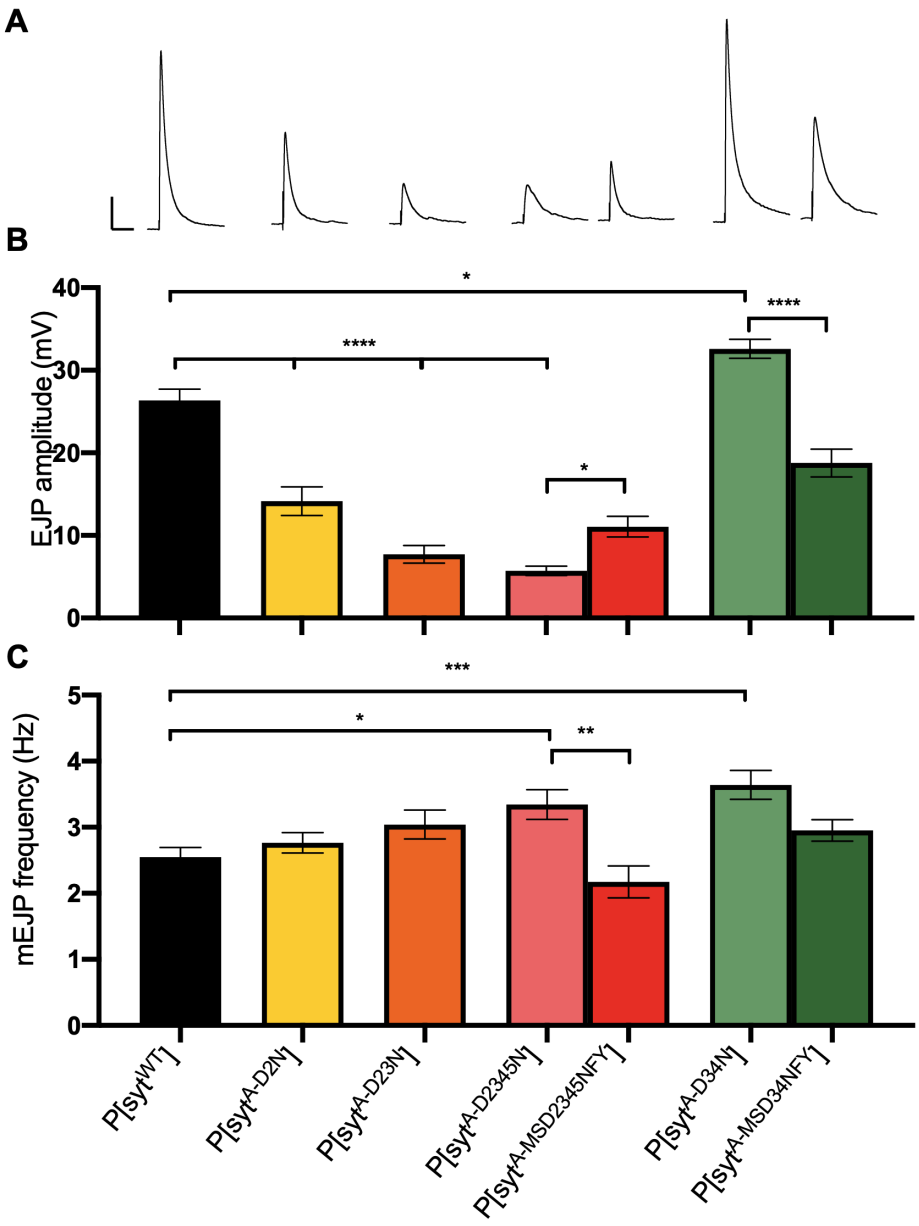

Figure 4.2. Different combinations of aspartate to asparagine substitutions in C_2A have differential effects on evoked release and increase spontaneous activity. A, representative average evoked traces for each genotype (scale bars: 5mV and 100ms). B, Mean evoked EJP amplitudes ± SEM for each genotype (Dunnett's test of multiple comparisons, $p \leq 0.05$ = *, $p \leq 0.0001$ = ****). C, Mean mEJP frequency ± SEM for each genotype (One-way ANOVA, Tukey's test of multiple comparisons, $p \leq 0.05$ = *, $p \leq 0.01$ = **, $p \leq 0.001$ = ***).

D-N substitution at positions 3 and 4 increased neurotransmitter release

While the above mutants decreased neurotransmitter release, the *P[syt$^{A\text{-}D3,4N}$]* mutant resulted in a small increase in neurotransmitter release (Fig 4.2 B; mean ± SEM: *P[syt$^{A\text{-}D3,4N}$]* = 32.59 ± 1.15 mV, n = 17). This increase in evoked neurotransmitter was significant relative to *P[syt^{WT}]* (Welch's ANOVA, W = 91.35 (6.000, 44.38); Dunnett's post hoc, p = 0.025]. This increase is consistent with previous reports of equal or elevated release in D-N substitutions of the 4th aspartate residue [25, 27, 29, 32].

Addition of hydrophobic mutations has differential effects on evoked release

Following the changes in evoked transmitter release stemming from substitution of different combinations of aspartates, we wanted to investigate whether these alterations were dependent on membrane interactions. To assay this, we blocked membrane interactions via mutation of the hydrophobic tip residues, as shown previously [23, 24]. It is important to note that, rather than glutamic acid substitutions [23] that alter the hydrophobicity *and* charge of the residues, we chose substitutions that altered the hydrophobicity without changing the net charge (methionine to serine, M-S and phenylalanine to tyrosine, F-Y) to avoid confounding the electrostatic effects of the included D-N substitutions. Interestingly, substitution of the hydrophobic residues that mediate membrane interactions to the D-N mutants resulted in opposite effects depending on which Ca^{2+} binding aspartates had been mutated. In the case of the D3,4N substitution, removal of the hydrophobicity at the tips of the Ca^{2+} binding pocket (*P[syt$^{A\text{-}MSD3,4NFY}$]*) resulted in an ~44% decrease in evoked neurotransmitter release relative to D3,4N alone [Fig 4.2 B; mean ± SEM: *P[syt$^{A\text{-}MSD3,4NFY}$]* = 18.77 ± 1.68 mV, n = 13; Welch's ANOVA,

W = 91.35 (6.000, 44.38); Dunnett's post hoc, *P[syt$^{A\text{-}D3,4N}$]* vs *P[syt$^{A\text{-}MSD3,4NFY}$]* $p < 0.0001$]. However, in the case of the D2,3,4,5N substitution, removal of the hydrophobicity at the tips of the pocket (*P[syt$^{A\text{-}MSD2,3,4,5NFY}$]*) resulted in an 48% increase in evoked neurotransmitter release relative to D2,3,4,5N alone [Fig 4.2 B; mean ± SEM: *P[syt$^{A\text{-}MSD2,3,4,5NFY}$]* = 11.06 ± 1.25 mV, n = 12; Welch's ANOVA, W = 91.35 (6.000, 44.38); Dunnett's post hoc: *P[syt$^{A\text{-}D2,3,4,5N}$]* vs *P[syt$^{A\text{-}MSD2,3,4,5NFY}$]* $p = 0.025$].

Decreased electrostatic repulsion increases spontaneous release

Aspartate to asparagine substitutions decrease the electrostatic charge of the Ca^{2+} binding pocket. This decreased repulsion has been hypothesized to increase the rate of Ca^{2+} independent spontaneous vesicle fusion [27, 37]. As the number of D-N substitutions increase, we observed increasing spontaneous vesicle fusion frequency, measured as the frequency of mEJPs. It is notable that this increase in frequency was only statistically significant when D3 and D4 were mutated to asparagines [Fig 4.2 C; mean ± SEM: *P[syt^{WT}]* = 2.55 ± 0.15 Hz, n = 23; *P[syt^{D2N}]* = 2.76 ± 0.16 Hz, n = 18; *P[syt^{D2,3N}]* = 3.04 ± 0.22 Hz, n = 15; *P[syt^{D2,3,4,5N}]* = 3.34 ± 0.22 Hz, n = 17; *P[syt^{D3,4N}]* = 3.64 ± 0.22 mV, n = 17; Ordinary One-Way ANOVA, F(6,108) = 6.007; Tukey's post hoc test: *P[syt^{WT}]* vs *P[syt^{D2,3,4,5N}]* $p = 0.03$; *P[syt^{WT}]* vs *P[syt^{D3,4N}]* $p = 0.0006$].

Hydrophobic mutations re-clamp spontaneous release

Mutation of the hydrophobic tip residues should prevent interactions by the C2A domain downstream of a loss of electrostatic repulsion [23, 24]. Introduction of hydrophobic to hydrophilic tip substitutions decreased the elevated spontaneous mEJP frequency observed for the *P[syt^{D3,4N}]* and *P[syt^{D2,3,4,5N}]*. This decrease was only significant for and *P[syt^{D2,3,4,5N}]*,

however, the frequency of and *P[syt^{MSD3,4NFY}]* was not significantly elevated above *P[syt^{WT}]* levels. [Fig 4.2 C; mean ± SEM: *P[syt^{MSD3,4NFY}]* = 2.95 ± 0.16 Hz, n = 13; *P[syt^{MSD2,3,4,5NFY}]* = 2.17 ± 0.24 Hz, n = 12; Ordinary One-Way ANOVA, F(6,108) = 6.007; Tukey's post hoc test: *P[syt^{D2,3,4,5N}]* vs *P[syt^{MSD2,3,4,5NFY}]* p = 0.03; *P[syt^{D3,4N}]* vs *P[syt^{MSD3,4NFY}]* p = 0.22; *P[syt^{WT}]* vs *P[syt^{MSD3,4NFY}]* p = 0.75].

All mutant transgenes were expressed and localized appropriately

In order to ascertain whether mutant transgenes were expressing appropriately, we performed western blot analyses. All mutants showed adequate expression of synaptotagmin (Fig 4.3). Localization of the mutant protein was confirmed using immunohistochemistry. All transgenic synaptotagmin proteins were found appropriately localized to the synaptic boutons at the neuromuscular junction (data not shown).

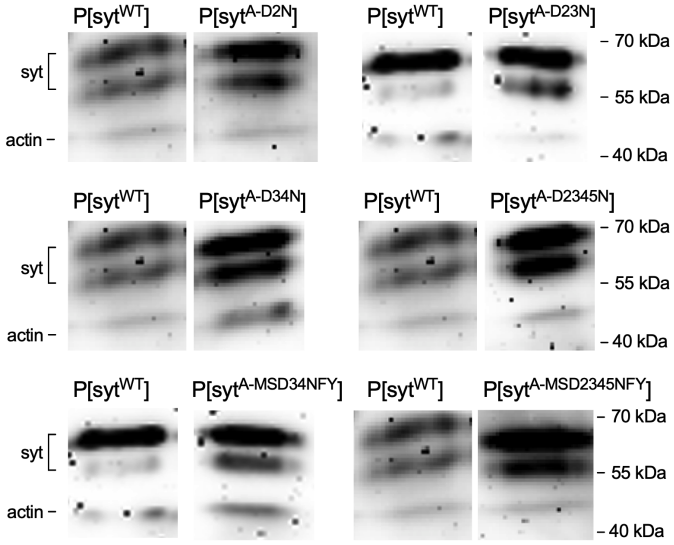

Figure 4.3 Mutant synaptotagmin is appropriately expressed. Representative western blots with antibodies labeling synaptotagmin and actin as a loading control. All mutant proteins are expressed in the null background and show protein levels comparable to the transgenic WT control.

4.5 Discussion

For this study, we generated sequential D-N substitutions in the Ca^{2+} binding pocket of synaptotagmin's C_2A domain. Similar D-N substitutions have previously been used to perturb Ca^{2+} binding [25-27, 29, 32]. While previous authors did not report significant decreases in evoked release in D-N mutants, we observed an ~45% decrease in evoked release in *P[syt$^{A\text{-}D2N}$]* mutants and ~71%-79% decrease in *P[syt$^{A\text{-}D2,3N}$]* and *P[syt$^{A\text{-}D2,3,4,5N}$]* mutants. Interestingly, previously published data did show an ~29-36% decrease in evoked release in a *P[syt$^{A\text{-}D2N}$]* mutant. However, the authors did not provide a statistical analysis; it was merely reported that evoked release was rescued by the mutant proteins compared to *syt^{null}* mutants [26]. Conversely, the *P[syt$^{A\text{-}D3,4N}$]* mutants showed an ~24% increase in evoked release. In addition, our suite of D-N substitutions also titrated the amount of electrostatic repulsion of the C_2A domain in a Ca^{2+} independent manner. This decreased electrostatic repulsion caused an increase in spontaneous neurotransmitter. Importantly, our results suggest that the various Ca^{2+} binding aspartate residues in the C_2A domain are playing differential roles in controlling the Ca^{2+} dependent fusion of synaptic vesicles

Previously reported aspartate to asparagine substitutions that disrupt Ca^{2+} binding showed dramatic reductions in vesicle fusion when the C_2B domain was mutated [1, 30], but mutations to the C_2A domain offer conflicting results [25, 26, 29, 32]. The only reported instance of a mutation to the Ca^{2+} binding domain of C_2A having a significant negative effect was a glutamic acid (E) substitution in C_2A that resulted in an 80% decrease in synchronous neurotransmitter release [22]. Our *in vivo* observations help to clarify the interpretations of these previous findings. A single A-D2N substitution decreased synchronous neurotransmitter release *in vivo* similar to decreases observed by Robinson et al. [26]. Two combinations of D-N

substitutions that included A-D2N dramatically decreased release (*P[syt$^{A\text{-}D2,3N}$]* and *P[syt$^{A\text{-}D2,3,4,5N}$]*). A previously investigated mutant increased release *in vivo* (*P[syt$^{A\text{-}D3,4N}$]*), mirroring previously observed increases when A-D4 was mutated [25, 32]. Our findings offer valuable insight into the individual roles each Ca^{2+} coordinating residue in the binding pocket may be playing and these insights are consistent with and build upon previous *in vitro* data.

Robinson et al. first reported that substitution of A-D2 did not affect synchronous neurotransmitter release at the *Drosophila* neuromuscular junction [26]. Our results, however, point to A-D2 as a critical C_2A residue for Ca^{2+} dependent neurotransmitter release. In the previous study, while the A-D2N mutant did rescue some neurotransmitter release compared to *syt^{null}*, Robinson et al. saw a decrease in release compared to wild-type, even at very high levels of Ca^{2+}. However, they did not state the magnitude of the decrease nor provide a statistical comparison. Importantly, their *in vitro* work found the mutation completely blocked Ca^{2+} dependent liposome binding in the isolated domain, but some binding remained with tandem domains [26]. This is consistent with the idea that the intact C_2B domain may still be able to compensate for some degree of C_2A dysfunction. Thus, although their interpretation is opposite from ours, the data are actually fairly consistent with our observations. The *P[syt$^{A\text{-}D2N}$]* mutant provides some rescue of evoked release compared to *syt^{null}* mutants, but this rescue is incomplete compared to the transgenic control (Fig 4.2, *P[syt$^{A\text{-}D2N}$]*).

Similar to the A-D2E substitution previously studied [22], mutation of the second aspartate to an asparagine (*P[syt$^{A\text{-}D2N}$]*) decreased evoked release. The 80% decrease observed in the A-D2E mutant would be expected to be greater than our observed ~45% decrease due to the fact that the D2E substitution blocked Ca^{2+} binding *and* maintained the negative charge of the binding pocket, while our *P[syt$^{A\text{-}D2N}$]* mutant blocked Ca^{2+} binding, but also neutralized some of

the electrostatic repulsion of the domain. This is consistent with analysis of Ca^{2+} binding kinetics by nuclear magnetic resonance (NMR) in the WT domain suggesting that A-D2 is critical for coordination of Ca^{2+} binding [8, 10]. Specifically, Of the three Ca^{2+} binding sites, A-D2 coordinates binding of Ca^{2+} to the highest affinity binding site, Ca1 [8, 10]. In addition, electrophysiology in heterozygous mutants with one copy of WT syt and one copy with D-N substitutions found that a B-D1,2N mutant reduced evoked release more than a B-D3,4N mutant [1]. Other electrophysiological evidence in the C_2B domain showed that a B-D2N mutant completely abolished evoked release [30]. The compounded evidence from both domains, suggests that the 2nd aspartate may be more critical than other residues for coordinating initial Ca^{2+} binding by C_2 domains.

On the opposite end of the spectrum, the electrostatic charge of A-D4 has been previously shown to be critically important. Mutation of this residue to an asparagine residue increased neurotransmitter release relative to transgenic controls at synaptotagmin null hippocampal autapses. Other combinations of D-N substitutions, however, rescued function only to the same level as a transgenic WT. In addition, any combination of D-N substitutions that included A-D4 increased the apparent Ca^{2+} affinity of release [25]. The A-D4N mutation in knock-in mouse neurons also exhibited increased release [32]. Accordingly, the neutralization of this 4th aspartate must play a critical electrostatic role since constitutive neutralization is able to increase evoked release. This makes sense given A-D4's position near an arginine known to play a critical role in the electrostatic switch [38] and a hydrophobic phenylalanine that mediates membrane interactions downstream of Ca^{2+} binding [23, 24]. If an A-D4N mutation neutralizes the negative charge near these sites, it may allow the downstream effector interactions to take place at a lower Ca^{2+} concentration. In fact, the apparent increase in Ca^{2+} affinity seen in

mutants with an A-D4N substitution, indicates that this mutation mimics constitutive Ca^{2+} binding, lowering the [Ca^{2+}] required to stimulate fusion. At the *Drosophila* neuromuscular junction, an A-D3,4N mutant displayed evoked response amplitudes equal to control and also increased the apparent Ca^{2+} affinity of release [27]. These findings are consistent with our results, where the *P[syt$^{A\text{-}D3,4N}$]* mutant resulted in a small, but significant, increase in neurotransmitter release compared to *P[syt^{WT}]*. This all points to a critical role for electrostatic repulsion by the 4th aspartate.

Our findings, coupled with previous analysis of D-N substitutions, suggest that the highly-ordered residues deep in the Ca^{2+} binding pocket, A-D2 and A-D3 [9], are critical for coordinating Ca^{2+} binding, while the charge at the more distal A-D4 site plays a critical role in the electrostatic switch. Previous NMR studies of Ca^{2+} binding by the C_2A domain suggested that the Ca^{2+} ions bind in a sequential fashion [8]. This concept is consistent with our findings. NMR spectral analysis found that Ca^{2+} binding site Ca1 is populated by metal ions at lower concentrations, and as the concentration increases, further sites begin binding metal ions (Fig 4.1 B, Ca1, Ca2 and Ca3, [8]). This Ca1 site is stabilized primarily by the 1st, 2nd and 3rd aspartate residues. Furthermore, mutation of the 2nd aspartate prevented ion binding to the Ca1 position and ion binding to subsequent sites was also impaired [8]. Models of the electrostatic change caused by Ca^{2+} binding predict that the distal ends of loops 1 and 3 (which contain the critical membrane interacting residues) don't see a large change in charge until biding of the 2nd and 3rd Ca^{2+} ions [8]. Accordingly, our *P[syt$^{A\text{-}D2N}$]* would be expected to disrupt binding of the primary Ca^{2+} ion, which would disrupt the downstream binding of Ca^{2+} ions to the secondary and tertiary sites [8]. The *P[syt$^{A\text{-}D2,3N}$]* mutation results in an even greater decrease in release. This suggests that the *P[syt$^{A\text{-}D2,3N}$]* likely abolishes Ca^{2+} binding to the primary site completely, impairing

binding to each subsequent site, though this mutant has not been specifically investigated via NMR. Yet, at the same time, the neutralization of charge from these asparagine substitutions are deeper in the pocket and may have minimal functional effects in terms of decreasing overall electrostatic repulsion of the presynaptic membrane by the distal tips of the domain. Notably, individual mutation of the two analogous residues in the C_2B domain (B-D2 and B-D3), each dramatically decrease neurotransmitter release (see Table 1.1, [30]). Since the Ca^{2+} binding pockets of both domains are highly homologous, D2 and D3 may be the most critical Ca^{2+}-binding residues.

Based on previous NMR data, the *P[syt$^{A\text{-}D3,4N}$]* mutation, on the other hand, may not affect Ca^{2+} binding to the primary site (Ca1). NMR data shows that D3 and D4 play minimal roles in coordinating Ca^{2+} binding at the Ca1 site [8]. In addition, mutation of the D4 residue altered binding to sites Ca2 and Ca3, but had no effect on the Ca^{2+} binding to Ca1 compared to WT [29]. Therefore, neutralization of D4 may leave Ca^{2+} binding at Ca1 intact. In addition, it is near critical residues mediating phospholipid binding (R285 [38], Fig 4.1 B, blue residue, +) and membrane penetration (F286 [23, 24], Fig 4.1 B, grey residue, *), so neutralization could decrease the local electrostatic repulsion, allowing these critical residues to interact with the presynaptic membrane and drive fusion. Mutating membrane interacting hydrophobic residues, in concert with the 3rd and 4th aspartates (*P[syt$^{A\text{-}MSD3,4NFY}$]*) was able to decrease evoked release, supporting this idea. In the case of *P[syt$^{A\text{-}D3,4N}$]*, neutralization of electrostatic repulsion essentially mimics Ca^{2+} binding by the C_2A domain. Coupled with an intact C_2B domain, this results in a decrease in the apparent Ca^{2+} affinity of release [25, 27] and explains the robust evoked release observed in this study and previously [25, 27, 32].

Finally, the quadruple mutant, *P[syt$^{A\text{-}D2,3,4,5N}$]* results in a large reduction of neurotransmitter release, on par with *P[syt$^{A\text{-}D2,3N}$]* (Fig 4.2, *P[syt$^{A\text{-}D2,3,4,5N}$]*). In this case, based on prior NMR data, the four substitutions would be expected to block all Ca^{2+} binding. However, the asparagine substitutions neutralize 4 of the 5 negatively charged aspartate residues, notably those more distal in the pocket, mimicking almost complete Ca^{2+} binding. In this case, with coupling to Ca^{2+} binding completely removed and the domain mimicking a constitutive Ca^{2+} bound state, the domain may just bind directly to lipids in the absence of Ca^{2+}, locking up the fusion machinery. Unlike the *P[syt$^{A\text{-}D3,4N}$]* mutant, which may retain some Ca^{2+} sensing ability, the *P[syt$^{A\text{-}D2,3,4,5N}$]* mutant's functional C_2B domain may be unable to rescue function since the protein would be essentially locked in one state, unable to respond to a Ca^{2+} signal. This would also explain why the addition of hydrophobic mutations(*P[syt$^{A\text{-}MSD2,3,4,5NFY}$]*) actually confers a slight benefit to function. By blocking membrane interactions by C2A, the hydrophobic mutants may prevent the electrostatically neutral C_2A D2,3,4,5N mutant domain from binding to lipids regardless of Ca^{2+} concentration, restoring some synchronous release via the intact C_2B domain.

It is important to note that our findings for the *P[syt$^{A\text{-}D2,3,4,5N}$]* mutant partially disagree with the findings of Stevens and Sullivan [25]. They observed no alteration in neurotransmitter release in an A-D1,2,3,4,5N mutant mouse autapses. This difference may come down to the fact that phenotypes at cultured hippocampal autapses do not match those at synapses between two cells, even displaying conserved release in the absence of synaptotagmin [39]. Another interpretation to that proposed above, is that, rather than locking C_2A into the membrane, *P[syt$^{A\text{-}D2,3,4,5N}$]* completely blocks Ca^{2+} binding by the domain and the intact C_2B domain is unable to overcome the deficit. The ability of the A-D1,2,3,4,5N mutation's [25] ability to rescue neurotransmitter release may then suggest that the A-D1 residue plays a similar role to A-D4.

Like A-D4, A-D1 is located near a critical hydrophobic residue [23, 24]. Neutralization of both of these aspartate residues in the A-D1,2,3,4,5N may be enough to allow the interactions of the critical hydrophobic tip residues of C_2A when the intact C_2B domain binds Ca^{2+}.

Taken together, our results suggest that, rather than an equally shared role in Ca^{2+} binding, each critical aspartate residue in the C_2A domain performs distinct functions. The second aspartate seems to be especially critical for initial Ca^{2+} binding at the Ca1 site. On the other hand, as the Ca^{2+} binding sites are sequentially filled, electrostatic neutralization of the more distal 4^{th} aspartate, and perhaps 1^{st} aspartate, seems to be critical for triggering the effector interactions downstream of Ca^{2+} binding that lead to vesicle fusion. Our study suggests that a more in depth analysis of these critical residues in isolation in C_2A, as was done in C_2B [30], could provide valuable insight into the function of C_2 domains. Additionally, this work adds to mounting evidence that the C_2A domain's role in triggering vesicle fusion may be more nuanced and important than previously suggested [23].

WORKS CITED

1. Mackler, J.M., et al., *The C(2)B Ca(2+)-binding motif of synaptotagmin is required for synaptic transmission in vivo.* Nature, 2002. **418**(6895): p. 340-4.
2. Brose, N., et al., *Synaptotagmin: a calcium sensor on the synaptic vesicle surface.* Science, 1992. **256**(5059): p. 1021-5.
3. Broadie, K., et al., *Absence of synaptotagmin disrupts excitation-secretion coupling during synaptic transmission.* Proc Natl Acad Sci U S A, 1994. **91**(22): p. 10727-31.
4. DiAntonio, A., K.D. Parfitt, and T.L. Schwarz, *Synaptic transmission persists in synaptotagmin mutants of Drosophila.* Cell, 1993. **73**(7): p. 1281-90.
5. DiAntonio, A. and T.L. Schwarz, *The effect on synaptic physiology of synaptotagmin mutations in Drosophila.* Neuron, 1994. **12**(4): p. 909-20.
6. Littleton, J.T., et al., *Mutational analysis of Drosophila synaptotagmin demonstrates its essential role in Ca(2+)-activated neurotransmitter release.* Cell, 1993. **74**(6): p. 1125-34.
7. Perin, M.S., et al., *Structural and functional conservation of synaptotagmin (p65) in Drosophila and humans.* J Biol Chem, 1991. **266**(1): p. 615-22.
8. Ubach, J., et al., *Ca2+ binding to synaptotagmin: how many Ca2+ ions bind to the tip of a C2-domain?* Embo j, 1998. **17**(14): p. 3921-30.
9. Sutton, R.B., et al., *Structure of the first C2 domain of synaptotagmin I: a novel Ca2+/phospholipid-binding fold.* Cell, 1995. **80**(6): p. 929-38.
10. Shao, X., et al., *Bipartite Ca2+-Binding Motif in C2 Domains of Synaptotagmin and Protein Kinase C.* Science, 1996. **273**(5272): p. 248-251.
11. Fernandez, I., et al., *Three-dimensional structure of the synaptotagmin 1 C2B-domain: synaptotagmin 1 as a phospholipid binding machine.* Neuron, 2001. **32**(6): p. 1057-69.
12. Chapman, E.R., et al., *Delineation of the oligomerization, AP-2 binding, and synprint binding region of the C2B domain of synaptotagmin.* J Biol Chem, 1998. **273**(49): p. 32966-72.
13. Chapman, E.R., et al., *Ca2+ regulates the interaction between synaptotagmin and syntaxin 1.* J Biol Chem, 1995. **270**(40): p. 23667-71.
14. Rickman, C., et al., *Synaptotagmin interaction with the syntaxin/SNAP-25 dimer is mediated by an evolutionarily conserved motif and is sensitive to inositol hexakisphosphate.* J Biol Chem, 2004. **279**(13): p. 12574-9.
15. Rickman, C., et al., *Conserved prefusion protein assembly in regulated exocytosis.* Mol Biol Cell, 2006. **17**(1): p. 283-94.
16. Bai, J., P. Wang, and E.R. Chapman, *C2A activates a cryptic Ca(2+)-triggered membrane penetration activity within the C2B domain of synaptotagmin I.* Proc Natl Acad Sci U S A, 2002. **99**(3): p. 1665-70.
17. Chapman, E.R. and A.F. Davis, *Direct interaction of a Ca2+-binding loop of synaptotagmin with lipid bilayers.* J Biol Chem, 1998. **273**(22): p. 13995-4001.
18. Herrick, D.Z., et al., *Position of synaptotagmin I at the membrane interface: cooperative interactions of tandem C2 domains.* Biochemistry, 2006. **45**(32): p. 9668-74.

19. Shao, X., et al., *Synaptotagmin-syntaxin interaction: the C2 domain as a Ca2+-dependent electrostatic switch.* Neuron, 1997. **18**(1): p. 133-42.
20. Davletov, B., O. Perisic, and R.L. Williams, *Calcium-dependent membrane penetration is a hallmark of the C2 domain of cytosolic phospholipase A2 whereas the C2A domain of synaptotagmin binds membranes electrostatically.* J Biol Chem, 1998. **273**(30): p. 19093-6.
21. Murray, D. and B. Honig, *Electrostatic control of the membrane targeting of C2 domains.* Mol Cell, 2002. **9**(1): p. 145-54.
22. Striegel, A.R., et al., *Calcium binding by synaptotagmin's C2A domain is an essential element of the electrostatic switch that triggers synchronous synaptic transmission.* J Neurosci, 2012. **32**(4): p. 1253-60.
23. Bowers, M.R. and N.E. Reist, *The C2A domain of synaptotagmin is an essential component of the calcium sensor for synaptic transmission.* PLoS One, 2020. **15**(2): p. e0228348.
24. Paddock, B.E., et al., *Membrane penetration by synaptotagmin is required for coupling calcium binding to vesicle fusion in vivo.* J Neurosci, 2011. **31**(6): p. 2248-57.
25. Stevens, C.F. and J.M. Sullivan, *The synaptotagmin C2A domain is part of the calcium sensor controlling fast synaptic transmission.* Neuron, 2003. **39**(2): p. 299-308.
26. Robinson, I.M., R. Ranjan, and T.L. Schwarz, *Synaptotagmins I and IV promote transmitter release independently of Ca(2+) binding in the C(2)A domain.* Nature, 2002. **418**(6895): p. 336-40.
27. Yoshihara, M., Z. Guan, and J.T. Littleton, *Differential regulation of synchronous versus asynchronous neurotransmitter release by the C2 domains of synaptotagmin 1.* Proc Natl Acad Sci U S A, 2010. **107**(33): p. 14869-74.
28. Zhang, X., J. Rizo, and T.C. Sudhof, *Mechanism of phospholipid binding by the C2A-domain of synaptotagmin I.* Biochemistry, 1998. **37**(36): p. 12395-403.
29. Fernandez-Chacon, R., et al., *Structure/function analysis of Ca2+ binding to the C2A domain of synaptotagmin 1.* J Neurosci, 2002. **22**(19): p. 8438-46.
30. Nishiki, T. and G.J. Augustine, *Dual roles of the C2B domain of synaptotagmin I in synchronizing Ca2+-dependent neurotransmitter release.* J Neurosci, 2004. **24**(39): p. 8542-50.
31. Earles, C.A., et al., *The tandem C2 domains of synaptotagmin contain redundant Ca2+ binding sites that cooperate to engage t-SNAREs and trigger exocytosis.* J Cell Biol, 2001. **154**(6): p. 1117-23.
32. Pang, Z.P., et al., *A gain-of-function mutation in synaptotagmin-1 reveals a critical role of Ca2+-dependent soluble N-ethylmaleimide-sensitive factor attachment protein receptor complex binding in synaptic exocytosis.* J Neurosci, 2006. **26**(48): p. 12556-65.
33. Bischof, J., et al., *An optimized transgenesis system for Drosophila using germ-line-specific phiC31 integrases.* Proc Natl Acad Sci U S A, 2007. **104**(9): p. 3312-7.
34. Brand, A.H. and N. Perrimon, *Targeted gene expression as a means of altering cell fates and generating dominant phenotypes.* Development, 1993. **118**(2): p. 401-15.
35. Yao, K.M. and K. White, *Neural specificity of elav expression: defining a Drosophila promoter for directing expression to the nervous system.* J Neurochem, 1994. **63**(1): p. 41-51.

36. Feng, Y., A. Ueda, and C.F. Wu, *A modified minimal hemolymph-like solution, HL3.1, for physiological recordings at the neuromuscular junctions of normal and mutant Drosophila larvae.* J Neurogenet, 2004. **18**(2): p. 377-402.
37. Shields, M.C., et al., *The role of the C2A domain of synaptotagmin 1 in asynchronous neurotransmitter release.* PLoS One, 2020. **15**(5): p. e0232991.
38. Paddock, B.E., et al., *Ca2+-dependent, phospholipid-binding residues of synaptotagmin are critical for excitation-secretion coupling in vivo.* J Neurosci, 2008. **28**(30): p. 7458-66.
39. Liu, H., et al., *Autapses and networks of hippocampal neurons exhibit distinct synaptic transmission phenotypes in the absence of synaptotagmin I.* J Neurosci, 2009. **29**(23): p. 7395-403.

CHAPTER 5. THE C_2A DOMAIN OF SYNAPTOTAGMIN PLAYS MORE OF A ROLE IN SYNAPTIC TRANSMISSION THAN ORIGINALLY PREDICTED[5]

5.1 Discussion

The relative importance of synaptotagmin's tandem C_2 domains has teetered and tottered throughout the study of the protein. Initial *in vitro* biochemical investigations of synaptotagmin 1 had suggested that the C_2B domain was largely unimportant for the function of the protein in driving neurotransmitter release [1, 2]. *In vivo* studies, however, began to find the opposite trend, with C_2B playing the dominant role [3, 4]. Concomitantly, it was discovered that the cloned sequence of the protein used for the biochemistry work had a mutation rendering the C_2B domain nonfunctional [5, 6]. With the correct sequence of the protein, researchers were surprised to find that C_2A mutations often had little effect *in vivo,* while those to the C_2B domain had deleterious results [3, 4, 7, 8]. These findings shifted the focus of synaptic research squarely towards the C_2B domain, potentially short-changing the role of the C_2A domain. Then, as more nuanced experiments and analysis became possible, the C_2A domain was found to contribute to the function of the protein, but not to the same extent as the C_2B domain [9]. In an attempt to investigate the balanced role of electrostatic residue repulsion and hydrophobic residue membrane insertion underlying electrostatic switching of synaptotagmin, I have uncovered a much more essential role for the C_2A domain in synaptotagmin function. While C_2B is still required to synchronize vesicle fusion to Ca^{2+} influx, the C_2A domain clearly plays an essential role in the electrostatic switching function of synaptotagmin that was missed by previous functional studies.

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Future studies will develop a more refined understanding of the cooperative interactions of the two C_2 domains of synaptotagmin and offer valuable insight into the function of C_2 domains in general. Synaptotagmin isoforms mediate all forms of Ca^{2+} dependent vesicle fusion beyond synaptic transmission. This includes everything from cell membrane maintenance in plants [10] and animals [11] to roles in reproduction [12] and diabetes [13]. Genetically heritable neurodevelopmental disorders have also been attributed to synaptotagmin point mutants [14-17]. Research continuing to elucidate the mechanistic underpinnings of synaptotagmin will shed light on its roles across organisms and systems and contribute to our understanding and treatment of synaptic pathologies.

5.2 C_2A's role in membrane penetration

In chapter 3, I identified a mutation to the C_2A domain that resulted in the most dramatic deficits observed to date following mutation of membrane penetrating residues [18]. Membrane penetration is one of the predominant effector interactions downstream of Ca^{2+} binding [19]. Mutation of these residues in our study (Chapter 3 [18]) decreased neurotransmitter release below levels observed in the synaptotagmin null mutant. These findings display that the C_2A domain is critical , specifically in regard to membrane interactions, but they also suggest that previous reports of C_2A function upstream of membrane interactions may need to be reevaluated. In other words, how can disruption of a downstream effector interaction result in a more serious phenotype than disruption of the upstream trigger, in this case, Ca^{2+} binding?

These results called into question some of the previous data surrounding Ca^{2+} binding mutations in the C_2A domain. This question, in part, prompted the experiments in Chapter 4 of this dissertation, along with opening several interesting lines of questioning that will be

discussed in more detail below. In regard to membrane penetration specifically, the disruptions that we observed suggest that membrane insertion is one of the more important effector interactions following Ca^{2+} binding. However, our results only show that the residues (which have been previously shown to interact with membranes [20-23]) are critically important. We did not perform experiments that directly tested whether the observed deficit was caused by a loss of membrane penetration. Directly assaying the ability of these mutants to bind lipids via lipid binding assays or their ability to insert into membranes via fluorescent reporters would help to increasingly establish membrane penetration as a critical part of synaptotagmin's function.

It is possible that these residues could also mediate interactions with the SNARE complex. To investigate this, SNARE binding assays of the WT and mutant proteins would be necessary. Previously, certain studies found that mutation to hydrophobic residues also decreased SNARE interactions [23, 24]. The decreased Ca^{2+} dependent SNARE interactions, however, were contingent on assays that included membranes, suggesting that the observed SNARE interactions could actually be the side effect of altered membrane interactions. While testing the SNARE binding abilities of the hydrophobic mutations from chapter 3, it would be important to take a membrane free approach to avoid convolution by altered membrane interactions.

Additionally, we observed that neurotransmitter release, although reduced, was still Ca^{2+} dependent in our hydrophobic tip mutants. This is consistent with the idea that the mutations alter downstream effector interactions without affecting Ca^{2+} binding. However, we did not directly assay the Ca^{2+} binding ability of these mutants. It is possible that the Ca^{2+} dependence we observed could be a result of the intact C_2B domain. Direct investigation of Ca^{2+} binding in these mutants, by either nuclear magnetic resonance (NMR) or isothermal titration calorimetry (ITC), would make it clear whether or not mutation to our hydrophobic tip residues affects Ca^{2+}

binding. The mutations used in chapter 3 were glutamic acid substitutions, which, in addition to being hydrophobic, also alter the charge of the residue from neutral to negative. This was done to prevent membrane insertion by both loss of hydrophobicity and increased electrostatic repulsion, but it is hard to say exactly how the altered charge could affect Ca^{2+} binding. The increased negative charge could result in spurious Ca^{2+} binding by altering the charge composition of the pocket (in ways that may either increase or decrease Ca^{2+} affinity). Previous electrophysiology showed equal amounts of neurotransmitter release between a neutral tyrosine substitution and a negative glutamic acid substitution [24], so this seems unlikely. However, in addition to NMR or ITC, a set of mutations that remove the hydrophobicity of the tip residues without changing the charge of the pocket would allow electrophysiological investigation of whether the alteration of charge at those residues is having an effect outside of membrane repulsion. If Ca^{2+} binding is not being affected, we would expect these "neutral" mutants to have an electrophysiological phenotype similar to our "negative" glutamic acid mutants. These experiments would help to determine the exact mechanisms underlying the large deficit we observe in our hydrophobic mutants.

Finally, our hydrophobic mutations could prove useful in investigating exactly why membrane penetration is critical for neurotransmitter release. Our results show that these residues are critical for function *in vivo*, but do not show what aspects of membrane penetration are affected. Does membrane insertion serve to bend lipids, lowering the energy barrier for fusion [23], or does it help to destabilize lipid order and facilitate SNARE complex straightening [25], or a combination of the two? Leveraging our mutations in vesicle fusion assays with large or small synaptic vesicles would allow investigation of whether or not our mutants prevent fusion by blocking Ca^{2+} dependent membrane warping. If, indeed, this is the case, our mutants

should allow fusion of small vesicles that already have highly curved membrane surfaces but prevent fusion of large vesicles due to a loss of membrane warping ability. Alternatively, site-directed fluorescence-interference contrast (sdFLIC) microscopy has been used to assess embedded SNARE complex conformational changes [25]. Conformational change in the SNARE complex was triggered by the presence of synaptotagmin and Ca^{2+}, and appeared normal even when the primary syt-SNARE interaction site (see chapter 2 section 4) was mutated. This suggested that the *cis-trans* conformational change observed in the SNARE complex is dependent on synaptotagmin membrane interactions. Our mutations (and the analogous mutants in the C_2B domain [24]), which disrupt this interaction, would allow direct testing of whether the loss of hydrophobicity in the C_2A domain prevents the lipid disorder necessary to permit SNARE coiling. If this were the case, hydrophobic mutants should prevent SNARE conformational change. If SNARE conformational changes remain intact, it could offer clues that some other unknown interaction is facilitating SNARE coiling. Either way, these experiments could offer valuable insight into the importance of membrane interactions.

Another approach to evaluate the role of membrane interactions, specifically in driving some of the above downstream effects in a way that doesn't rely on mutants is the use of Sr^{2+} instead of Ca^{2+}. Sr^{2+} binds to synaptotagmin and can drive evoked fusion, but mainly through interactions between C_2B and the presynaptic membrane, without promoting additional interactions with SNARE proteins [26]. Performing either vesicle fusion assays between large and small vesicles [23], or sdFLIC microscopy to analyze SNARE conformational change in the presence of Sr^{2+} would allow investigation of membrane interactions in isolation from synaptotagmin-SNARE interactions. If membrane warping or SNARE straightening are dependent on membrane interactions, they should still be present in the presence of Sr^{2+}, but if

the outcomes are altered, it would suggest that some other downstream effector interactions are mediating these effects.

My results in chapter 3 were the first to demonstrate that the C_2A domain is absolutely essential for synaptotagmin function *in vivo*. Mutation of the residues that have previously been shown to mediate membrane interactions resulted in a phenotype that was worse than a synaptotagmin null mutant. This is consistent with results from the C_2B domain that show mutation of even one of the analogous residues results in embryonic lethality [24]. This indicates that membrane penetration of the synaptotagmin C_2 domains is absolutely critical for synaptotagmin function. The exact mechanism whereby membrane insertion results in vesicle fusion is still under investigation. Following direct confirmation that our mutants do indeed prevent membrane interactions, our mutants could be leveraged to parse apart the effects of membrane insertion. This line of investigation could help to clarify how Ca^{2+} dependent interactions by synaptotagmin are driving fusion, while offering clues as to how C_2 domains in other proteins function.

5.3 C_2A's role in Ca^{2+} binding and electrostatic repulsion

Early investigation of Ca^{2+} binding abilities of the C_2A domain relied on D-N substitutions to block Ca^{2+}binding [4, 8, 27, 28]. Later, it was suggested that these substitutions actually mimicked Ca^{2+} binding by decreasing the electrostatic repulsion of the binding pocket [9, 27], leading to an increased spontaneous mEJP frequency with no effects on evoked release [29, 30]. We generated D-N mutants to look at whether the frequency of spontaneous fusion events could be incrementally increased by decreasing electrostatic repulsion of C_2A in a stepwise manner, offering insights into how much electrostatic neutralization is required for

vesicle fusion and whether Ca^{2+} binding is playing other roles beyond neutralization of the binding pocket. Strikingly, mutations to Ca^{2+} binding residues yielded surprising alterations in evoked transmitter release depending on which residues were mutated, prompting us to change focus. Mutations that altered A-D2 all led to decreased neurotransmitter release. Alternatively, the A-D3,4N mutant caused an increase in neurotransmitter release. These results indicate that, in regard to Ca^{2+} binding and electrostatic repulsion, all of the Ca^{2+} coordinating residues are not created equally. Furthermore, these findings support the idea that Ca^{2+} binding by the C_2A domain is actually an important factor in synaptotagmin function [9], but previous research may have overlooked this due to the fact that different Ca^{2+} binding residue mutations affect neurotransmitter release differently.

These unexpected results have led to numerous new questions about how Ca^{2+} binding is mediated on a residue-by-residue basis *in vivo.* I designed the mutants in our study to get the broadest picture of the importance of electrostatic repulsion by the C_2A domain, and to have some overlap with previously characterized mutations for comparison purposes. Now, given our unexpected findings, generation of single mutants of each of the critical aspartates could be very interesting to investigate. While our data suggest that the second aspartate is important for synaptotagmin function, which is corroborated by NMR findings [31], the double A-D2,3N mutant was much more detrimental in our study. This suggests that A-D3 may also play an important role. On the other hand, recordings from our A-D3,4N showed an increase in neurotransmitter release. Since both of these mutants (A-D2,3N and A-D3,4N) both contain a substitution at A-D3, but have opposing electrophysiological results, a single A-D3N mutant could help clarify the impact of this residue *in vivo*.

On the opposite end of the spectrum, our data, coupled with previous studies of A-D4 [8, 27, 28], suggest that the electrostatic charge at that residue is very important. The previous electrophysiological studies have all been in autaptic cultures though, so a single mutant at the *Drosophila* neuromuscular junction would be very interesting for comparison. Our A-D3,4N data suggests that these results would match previous findings at autapses, but a single A-D4 substitution may result in even greater stimulated release than we previously observed.

A-D1 and A-D5 would also be interesting. A-D1 was not part of our study, but, like A-D2, it participates in the coordination of Ca^{2+} binding to the first Ca1 site [31]. This suggests that it may result in similar evoked deficits when substituted for an asparagine. However, it is also located next to the critical hydrophobic methionine that was part of the focus of chapter 3. Similarly, to our hypothesis that neutralizing the charge of A-D4 near the hydrophobic phenylalanine and basic arginine maintains their ability to mediate downstream effector interactions when Ca^{2+} binding has been disrupted, this could be the case for A-D1. Neutralization here may actually allow the loop 1 hydrophobic tip residue to maintain function. Finally, results from this mutation could help clarify the findings we observed (and previous A-D4N mutants observed). If the conserved function in those mutants is a result of the functional C_2B domain and some conserved binding of Ca^{2+} to the Ca1 site in C_2A [8], coupled with constitutive neutralization near the tip of the residue, then an A-D1N mutant offers an interesting comparison. It should disrupt Ca^{2+} binding at the highest affinity Ca1 site (and subsequent sites assuming sequential binding [31]), but will neutralize loop 1 near the important hydrophobic tip residue. Normal or increased evoked release would suggest that neutralization near the important hydrophobic tip residues is enough to maintain function in the domain. Loss of function would

suggest that neutralization *and* some conserved Ca^{2+} binding are required for the increased release observed in our A-D3,4N and previous A-D4N mutants.

The single A-D5N mutant could also provide important insight, especially in comparison to our A-D2,3,4,5N mutant. It is interesting that we saw our greatest decrease in evoked release in this quadruple mutant, when it contains both a mutation to A-D2 (which decreases release) and A-D3,4 (which increases release). As mentioned in chapter 4, A-D3,4N may result in increased release due to some conserved Ca^{2+} binding and neutralization near key sites mediating downstream effector interactions. A-D5 is also near these key residues, so neutralization may confer some benefit that cannot be observed when in the presence of the 3 other D-N substitutions. Alternatively, an A-D5N substitution did impact the binding of the first Ca^{2+} ion while an A-D4N substitution did not [8], so perhaps decreased Ca^{2+} binding by the A-D5N substitution helps to explain the dramatic effects of our quadruple mutant. A single A-D5N substitution could help resolve the role of the fifth aspartate and offer insight into why our quadruple mutant had such a dramatic impact. Furthermore, previous investigation found that mutation of all 5 aspartates did not impact release in comparison to a transgenic WT at hippocampal autapses [27]. Mutation of all 5 aspartate residues simultaneously *in vivo* would be interesting to compare with these previous results. Judging by our data, a dramatic decrease in neurotransmitter release would be expected. It is possible however, that the addition of the A-D1N substitution could have some beneficial effect if it acts in a similar way in loop 1 to that observed for an A-D4N substitution in loop 3 (see above).

The interpretations of our findings in chapter 4 would be greatly enhanced by either NMR or ITC of the mutant proteins. This could help to determine if mutations to different residues are altering Ca^{2+} binding in different ways. While ITC provides the stoichiometry of

binding, NMR, with its ability to separate the binding components of each individual Ca^{2+} ion, would likely have the most utility it terms of helping to interpret the differences that we are observing between different mutations of Ca^{2+} binding residues. For instance, detection of conserved Ca1 site Ca^{2+} binding in our A-D3,4N mutant (similar to A-D4N [8]) would support our hypothesis that conserved binding at site Ca1 in combination with the constitutive decrease in electrostatic repulsion at site 4 leads to the increase in evoked release that we observe. Otherwise, loss of significant Ca^{2+} binding at site 1 would suggest that the increase in release we observe is solely due to charge neutralization near the distal portion of loop 3. Either way, we would know more about how Ca^{2+} binding by synaptotagmin is able to trigger release. In the proposed single aspartate substitution mutants, NMR would also play an important role. It would be critical in offering comparison to previous NMR studies [8, 31, 32] and helping to show how each aspartate residue participates in the Ca^{2+} sensing ability of synaptotagmin.

The mechanistic understanding of the mutants from chapter 4 and those proposed above would also benefit greatly from both SNARE and membrane binding assays. Pang et al. reported that their A-D4N mutation increased Ca^{2+} dependent SNARE binding without altering Ca^{2+} dependent phospholipid binding, while an A-D5N mutant had no effect on SNARE binding, but altered Ca^{2+} dependent phospholipid binding [28]. An A-D2N and an A-D3N mutant were also shown to decrease Ca^{2+}-dependent lipid binding [4, 33]. These findings suggest that Ca^{2+} binding to specific residues may mediate different downstream effector interactions of synaptotagmin. A complete investigation of synaptotagmin's effector interactions in the presence of our mutants in chapter 4 and the other mutants proposed above would help to clarify how binding of individual Ca^{2+} ions and neutralization of each aspartate leads to synaptotagmin's downstream effector interactions.

Our data provide evidence that individual Ca^{2+} binding aspartates of C_2A perform distinct functions during Ca^{2+} triggered neurotransmitter release. We observed opposing effects on release *in vivo* that were dependent on neutralization of different Ca^{2+}-binding aspartates. These interesting results are novel for the C_2A domain, but fit with experiments that identified different functional significance for each Ca^{2+}-binding aspartate in the C_2B domain in autaptic cultures [34]. In order to fully parse apart the unique roles of the Ca^{2+} binding aspartate residues, additional single aspartate mutants would be very beneficial. Additionally, characterization of the Ca^{2+}-binding properties and downstream effector interactions of proteins harboring these D-N substitutions would be enhance the mechanistic understanding of the roles played by each individual residue. Importantly, our results from chapter 4, coupled with those from chapter 3, show that the C_2A domain of synaptotagmin is playing an essential role in the electrostatic switch that was missed in previous C_2A studies. This, in turn, opens up new questions about the ways in which the tandem C_2 domains work in concert to allow synaptotagmin-triggered neurotransmitter release.

5.4 Cooperative roles of the C_2 domains

My results displaying that the C_2A domain is more important than previously thought is consistent with many different theories that require cooperative actions of the two domains. Previous research investigating whether the two domains were able to function in isolation or as doublets of the same domain (i.e. C_2AA or C_2BB) *in vivo* show that the presence of both domains is required for normal synaptotagmin function. The findings also suggested that the two domains must each mediate their own unique interactions [35]. Expression of mutant synaptotagmin with only single C_2 domains caused

a dramatic decrease in neurotransmitter release, suggesting the protein requires 2 C_2 domains. Mutations that made both C_2 domains copies of each other, a C_2AA and a C_2BB, also dramatically decreased neurotransmitter release, suggesting that each domain is critical to the function of the protein for independent reasons. In other words, C_2A cannot substitute for C_2B and vice versa. Furthermore, a mutant that had swapped order of the domains (C_2BA instead of C_2AB) also showed dramatic decreases in neurotransmitter release [36]. Therefore, the interactions mediated by each domain must also be reliant on their order in the protein.

These findings are consistent with data showing that each domain have certain interactions that are specific to that distinct domain. For instance, C_2B has been shown to interact with the SNARE complex, while C_2A does not appear to interact directly [37-39]. The polylysine motif of C2B also interacts with other molecules of synaptotagmin *in vitro* [37]. C_2B has been also shown to interact with membranes in the absence of Ca^{2+}, perhaps playing a role in vesicle docking [40]. While C_2A has less well-characterized individual interactions, the domain favors Ca^{2+}-dependent interaction with the vesicle membrane, while C_2B favors the lipid profile of the presynaptic membrane [41, 42]. Furthermore, it is thought that the domain may have a specific Ca^{2+}-dependent interaction with the SNARE protein syntaxin [43]. These unique interactions may explain why the domains cannot functionally replace each other.

Aside from any specific interactions of each domain, there is also evidence that there are cooperative effects between the two domains. An alternative to the model of synaptotagmin function described in the majority of this dissertation is the oligomerization model. As mentioned previously, synaptotagmin has been shown to interact with other

molecules of synaptotagmin *in vitro.* In the presence of membranes, electron microscopy has observed self-assembled ring-like oligomers of synaptotagmin [44]. The oligomerization model of synaptotagmin function is predicated on the idea that these synaptotagmin rings physically block vesicle fusion through Ca^{2+}-independent interaction with membranes and SNARE proteins by the C_2B domain. Then, following Ca^{2+} influx, both domains interact with the lipid membranes, and the oligomers break apart to permit vesicle fusion [45]. It is important to note that all evidence of this model is based on *in vitro* data, and there have been no *in vivo* functional assays to support this hypothesis. Synaptotagmin is also notoriously "sticky" in certain non-physiological conditions, so the formation of oligomers may not actually be physiologically relevant.

More likely, the cooperation between the two domains could underly some combination of a minimum requirement for lipid warping/disorder and potential bridging between membranes. In regards to lipid warping, *in vitro* data shows that both domains are required to drive membrane tubulation, while isolated domains are unable to warp membranes [23]. This suggests that some cooperation between the domains is required to warp the lipid membrane. The same may be true of disruption of the acyl chain order. Disorder of this acyl chain order was shown to permit straightening of the SNARE complex and also drive fusion of vesicles *in vitro* [25]. There may be a certain threshold for lipid disorder, requiring membrane insertion of both domains, though this has not been directly investigated. It is noteworthy, though, that a model based solely on a threshold for membrane warping or lipid disorder cannot fully account for the findings that C_2AA and C_2BB are unable to drive fusion *in vivo* [35].

One possibility is that the two unique domains are required to bridge between the vesicle and presynaptic membranes following Ca^{2+} influx. Modeling based on site-directed spin labeling predicts that each C_2 domain inserts into opposing membranes [46]. Recently, electronic paramagnetic resonance (EPR) spectral analysis of full-length synaptotagmin in the presence of membranes (lacking SNARE proteins) identified the lipid preferences of the two domains [40]. This study confirmed the association of the C_2B domain with membranes whose composition mimicked the presynaptic membrane [41, 42]. Additionally, the C_2A domain favored binding to membranes that mimicked the lipid composition of synaptic vesicles. Thus, following Ca^{2+} influx, the C_2B domain almost certainly inserts into the presynaptic membrane. However, the C_2A domain may insert into the vesicle membrane. Additional studies will be needed to settle this question. It is thought, though, that this bridging would help to shorten the distance between the vesicle and presynaptic membrane, bringing them into close apposition and helping to drive synchronous fusion.

Regardless of mechanism, my findings suggest that the function of both domains of synaptotagmin need to be considered in future models. While it was previously clear that each domain plays a unique role [35], the C_2A domain has long been considered only facilitatory. Now, we know that membrane penetrating hydrophobic residues in C_2A are absolutely critical (Chapter 3), and that the importance of Ca^{2+} binding by the domain seems to have been previously underestimated (Chapter 4). Future models of the function of synaptotagmin will need to consider the cooperative interactions of the two C_2 domains in the triggering of fast, synchronous vesicle fusion.

WORKS CITED

1. Davletov, B.A. and T.C. Sudhof, *A single C2 domain from synaptotagmin I is sufficient for high affinity Ca2+/phospholipid binding.* J Biol Chem, 1993. **268**(35): p. 26386-90.
2. Li, C., et al., *Ca(2+)-dependent and -independent activities of neural and non-neural synaptotagmins.* Nature, 1995. **375**(6532): p. 594-9.
3. Mackler, J.M., et al., *The C(2)B Ca(2+)-binding motif of synaptotagmin is required for synaptic transmission in vivo.* Nature, 2002. **418**(6895): p. 340-4.
4. Robinson, I.M., R. Ranjan, and T.L. Schwarz, *Synaptotagmins I and IV promote transmitter release independently of Ca(2+) binding in the C(2)A domain.* Nature, 2002. **418**(6895): p. 336-40.
5. Desai, R.C., et al., *The C2B domain of synaptotagmin is a Ca(2+)-sensing module essential for exocytosis.* J Cell Biol, 2000. **150**(5): p. 1125-36.
6. Earles, C.A., et al., *The tandem C2 domains of synaptotagmin contain redundant Ca2+ binding sites that cooperate to engage t-SNAREs and trigger exocytosis.* J Cell Biol, 2001. **154**(6): p. 1117-23.
7. Mackler, J.M. and N.E. Reist, *Mutations in the second C2 domain of synaptotagmin disrupt synaptic transmission at Drosophila neuromuscular junctions.* J Comp Neurol, 2001. **436**(1): p. 4-16.
8. Fernandez-Chacon, R., et al., *Structure/function analysis of Ca2+ binding to the C2A domain of synaptotagmin 1.* J Neurosci, 2002. **22**(19): p. 8438-46.
9. Striegel, A.R., et al., *Calcium binding by synaptotagmin's C2A domain is an essential element of the electrostatic switch that triggers synchronous synaptic transmission.* J Neurosci, 2012. **32**(4): p. 1253-60.
10. Schapire, A.L., et al., *Arabidopsis synaptotagmin 1 is required for the maintenance of plasma membrane integrity and cell viability.* Plant Cell, 2008. **20**(12): p. 3374-88.
11. Shen, S.S., et al., *Molecular regulation of membrane resealing in 3T3 fibroblasts.* J Biol Chem, 2005. **280**(2): p. 1652-60.
12. Roggero, C.M., et al., *Complexin/synaptotagmin interplay controls acrosomal exocytosis.* J Biol Chem, 2007. **282**(36): p. 26335-43.
13. Mizuta, M., et al., *Localization and functional role of synaptotagmin III in insulin secretory vesicles in pancreatic beta-cells.* Diabetes, 1997. **46**(12): p. 2002-6.
14. Shields, M.C., et al., *Drosophila studies support a role for a presynaptic synaptotagmin mutation in a human congenital myasthenic syndrome.* PLoS One, 2017. **12**(9): p. e0184817.
15. Bradberry, M.M., et al., *Molecular Basis for Synaptotagmin-1-Associated Neurodevelopmental Disorder.* Neuron, 2020.
16. Herrmann, D.N., et al., *Synaptotagmin 2 mutations cause an autosomal-dominant form of lambert-eaton myasthenic syndrome and nonprogressive motor neuropathy.* Am J Hum Genet, 2014. **95**(3): p. 332-9.
17. Baker, K., et al., *SYT1-associated neurodevelopmental disorder: a case series.* Brain, 2018. **141**(9): p. 2576-2591.

18. Bowers, M.R. and N.E. Reist, *The C2A domain of synaptotagmin is an essential component of the calcium sensor for synaptic transmission.* PLoS One, 2020. **15**(2): p. e0228348.
19. Chapman, E.R. and A.F. Davis, *Direct interaction of a Ca2+-binding loop of synaptotagmin with lipid bilayers.* J Biol Chem, 1998. **273**(22): p. 13995-4001.
20. Bai, J., P. Wang, and E.R. Chapman, *C2A activates a cryptic Ca(2+)-triggered membrane penetration activity within the C2B domain of synaptotagmin I.* Proc Natl Acad Sci U S A, 2002. **99**(3): p. 1665-70.
21. Herrick, D.Z., et al., *Position of synaptotagmin I at the membrane interface: cooperative interactions of tandem C2 domains.* Biochemistry, 2006. **45**(32): p. 9668-74.
22. Martens, S., M.M. Kozlov, and H.T. McMahon, *How synaptotagmin promotes membrane fusion.* Science, 2007. **316**(5828): p. 1205-8.
23. Hui, E., et al., *Synaptotagmin-mediated bending of the target membrane is a critical step in Ca(2+)-regulated fusion.* Cell, 2009. **138**(4): p. 709-21.
24. Paddock, B.E., et al., *Membrane penetration by synaptotagmin is required for coupling calcium binding to vesicle fusion in vivo.* J Neurosci, 2011. **31**(6): p. 2248-57.
25. Kiessling, V., et al., *A molecular mechanism for calcium-mediated synaptotagmin-triggered exocytosis.* Nat Struct Mol Biol, 2018. **25**(10): p. 911-917.
26. Shin, O.H., et al., *Sr2+ binding to the Ca2+ binding site of the synaptotagmin 1 C2B domain triggers fast exocytosis without stimulating SNARE interactions.* Neuron, 2003. **37**(1): p. 99-108.
27. Stevens, C.F. and J.M. Sullivan, *The synaptotagmin C2A domain is part of the calcium sensor controlling fast synaptic transmission.* Neuron, 2003. **39**(2): p. 299-308.
28. Pang, Z.P., et al., *A gain-of-function mutation in synaptotagmin-1 reveals a critical role of Ca2+-dependent soluble N-ethylmaleimide-sensitive factor attachment protein receptor complex binding in synaptic exocytosis.* J Neurosci, 2006. **26**(48): p. 12556-65.
29. Yoshihara, M., Z. Guan, and J.T. Littleton, *Differential regulation of synchronous versus asynchronous neurotransmitter release by the C2 domains of synaptotagmin 1.* Proc Natl Acad Sci U S A, 2010. **107**(33): p. 14869-74.
30. Shields, M.C., et al., *The role of the C2A domain of synaptotagmin 1 in asynchronous neurotransmitter release.* PLoS One, 2020. **15**(5): p. e0232991.
31. Ubach, J., et al., *Ca2+ binding to synaptotagmin: how many Ca2+ ions bind to the tip of a C2-domain?* Embo j, 1998. **17**(14): p. 3921-30.
32. Shao, X., et al., *Bipartite Ca2+-Binding Motif in C2 Domains of Synaptotagmin and Protein Kinase C.* Science, 1996. **273**(5272): p. 248-251.
33. Zhang, X., J. Rizo, and T.C. Sudhof, *Mechanism of phospholipid binding by the C2A-domain of synaptotagmin I.* Biochemistry, 1998. **37**(36): p. 12395-403.
34. Nishiki, T. and G.J. Augustine, *Dual roles of the C2B domain of synaptotagmin I in synchronizing Ca2+-dependent neurotransmitter release.* J Neurosci, 2004. **24**(39): p. 8542-50.
35. Lee, J., et al., *Genetic analysis of synaptotagmin C2 domain specificity in regulating spontaneous and evoked neurotransmitter release.* J Neurosci, 2013. **33**(1): p. 187-200.

36. Lee, J. and J.T. Littleton, *Transmembrane tethering of synaptotagmin to synaptic vesicles controls multiple modes of neurotransmitter release.* Proc Natl Acad Sci U S A, 2015. **112**(12): p. 3793-8.
37. Chapman, E.R., et al., *Delineation of the oligomerization, AP-2 binding, and synprint binding region of the C2B domain of synaptotagmin.* J Biol Chem, 1998. **273**(49): p. 32966-72.
38. Rickman, C., et al., *Synaptotagmin interaction with the syntaxin/SNAP-25 dimer is mediated by an evolutionarily conserved motif and is sensitive to inositol hexakisphosphate.* J Biol Chem, 2004. **279**(13): p. 12574-9.
39. Rickman, C., et al., *Conserved prefusion protein assembly in regulated exocytosis.* Mol Biol Cell, 2006. **17**(1): p. 283-94.
40. Nyenhuis, S.B., A. Thapa, and D.S. Cafiso, *Phosphatidylinositol 4,5 Bisphosphate Controls the cis and trans Interactions of Synaptotagmin 1.* Biophys J, 2019. **117**(2): p. 247-257.
41. Bai, J., W.C. Tucker, and E.R. Chapman, *PIP2 increases the speed of response of synaptotagmin and steers its membrane-penetration activity toward the plasma membrane.* Nat Struct Mol Biol, 2004. **11**(1): p. 36-44.
42. Holz, R.W., et al., *A pleckstrin homology domain specific for phosphatidylinositol 4, 5-bisphosphate (PtdIns-4,5-P2) and fused to green fluorescent protein identifies plasma membrane PtdIns-4,5-P2 as being important in exocytosis.* J Biol Chem, 2000. **275**(23): p. 17878-85.
43. Shao, X., et al., *Synaptotagmin-syntaxin interaction: the C2 domain as a Ca2+-dependent electrostatic switch.* Neuron, 1997. **18**(1): p. 133-42.
44. Zanetti, M.N., et al., *Ring-like oligomers of Synaptotagmins and related C2 domain proteins.* Elife, 2016. **5**.
45. Wang, J., et al., *Calcium sensitive ring-like oligomers formed by synaptotagmin.* Proc Natl Acad Sci U S A, 2014. **111**(38): p. 13966-71.
46. Herrick, D.Z., et al., *Solution and membrane-bound conformations of the tandem C2A and C2B domains of synaptotagmin 1: Evidence for bilayer bridging.* J Mol Biol, 2009. **390**(5): p. 913-23.

APPENDIX 1: SYNAPTIC TRANSMISSION CHAPTER IN CELL PHYSIOLOGY SOURCE BOOK, 5TH EDITION[6]

I. Summary

Most communication between neurons is mediated by release of chemical signals. This release takes place at specialized structures called synapses, consisting of a presynaptic element, a synaptic cleft, a postsynaptic element, and surrounding glial cells. Presynaptic cells are specialized to transduce an electrical signal into a chemical signal through the synthesis, packaging and release of neurotransmitter. The synaptic cleft contains molecules that direct the formation of synaptic specializations, enzymes that break down neurotransmitter, and scaffolding proteins that maintain the synaptic superstructure. The postsynaptic cell is responsible for transducing the chemical signal back into an electrical response. Chemical synapses can be modulated in an activity-dependent manner, resulting in physiological, morphological, and behavioral changes

II. Introduction

Nervous system function relies upon the transmission of signals between neurons. Neurons transmit information across long distances through the nervous system by sending an electrical impulse, known as an action potential, down its axon. However, these electrical signals generally do not propagate between spatially distinct cells. To cross the gap between nerve cells, the majority of cell-to-cell signaling relies on small diffusible chemicals known as neurotransmitters. These neurotransmitters are released from active neurons in a calcium

[6] Authors: Matthew Bowers*[,1,2], Mallory Shields*[,1,2], Noreen Reist[1,2] *equally contributing

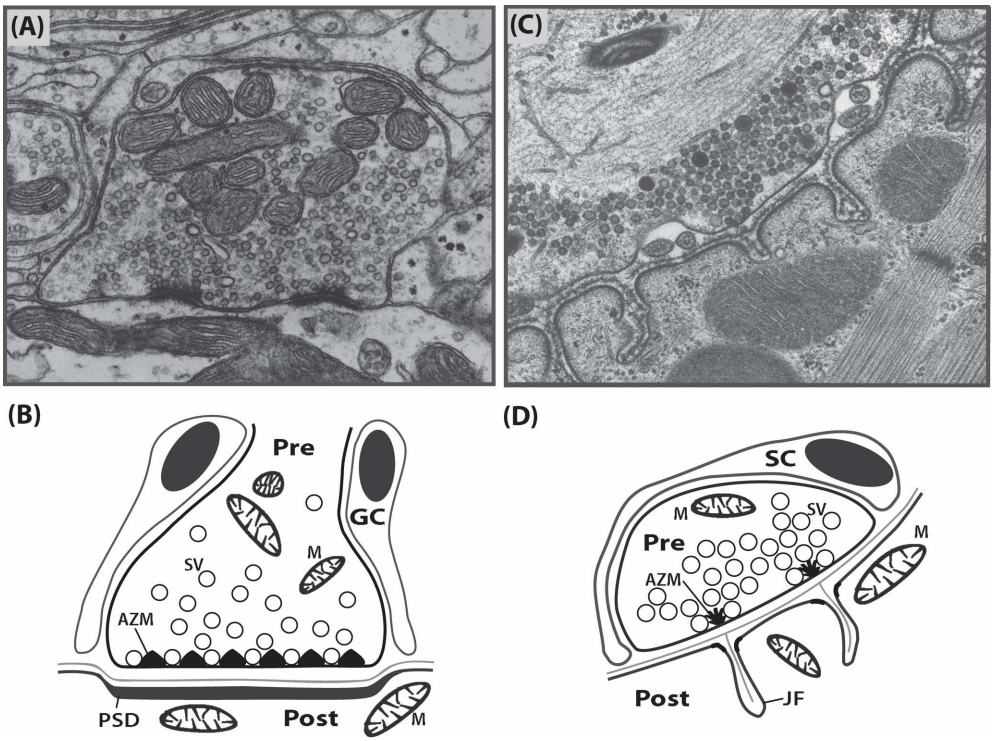
dependent manner at specialized cell-to-cell contact sites called synapses. It is important to note that direct electrical communication can occur between nerve cells through specialized structures called gap junctions. Chemical synaptic transmission is primarily unidirectional. Thus, the transmission of information required for canonical neuronal processes, such as sensation, movement, and thought, is primarily from the presynaptic cell to the postsynaptic cell. Retrograde signaling from the postsynaptic cell back to the presynaptic cell occurs but will not be addressed in this chapter. Slower and less-directed release of chemical transmitters occurs in the autonomic nervous system and neuroendocrine pathways, including release of proteinaceous signals from large dense core vesicles. These mechanisms will not be addressed here. This chapter is focused on the Ca^{2+}-dependent, release of classical chemical neurotransmitters from an activated presynaptic neuron and their effects on their postsynaptic target.

Pioneering studies by Bernard Katz and colleagues in the 1950s first identified that the release of neurotransmitters from nerve cells at the frog neuromuscular junction occurs via unitary packets [1-3]. These studies were foundational to the modern understanding of chemical synaptic transmission (section IV.b). In the 1960s and 1970s, experiments in rabbits and *Aplasia* demonstrated that synaptic transmission was highly plastic in response to activity by documenting changes in synaptic strength underlying changes in behavior [4-6] (section VIII). In the 1970s, extracellular molecules located in the synaptic cleft were discovered to direct the differentiation of synaptic contacts [7, 8]. Throughout the 1970-1980s, many postsynaptic receptor proteins were identified [9-15]. Essential players in the synaptic cleft began to be identified throughout the 1980s and 1990s. These include extracellular molecules such as agrin, the neural cadherins, and others [16-24]. Also in the 1980s and 1990s, the molecular identity of the presynaptic neurotransmitter release machinery was identified using a combination of

purification and cloning techniques in conjunction with clostridial neurotoxin studies [25-29]. Since then, these mechanisms have been found to be conserved at the majority of synapses.

III. Structure of the Chemical Synapse

While synaptic structure varies between cell types, many basic features are relatively conserved. This chapter will focus on generalized neuron-to-neuron synapses in the central nervous system (AF 1.1A,B) and neuron-to-muscle synapses peripherally (AF 1.1C,D). Chemical synapses possess a pool of small, clear synaptic vesicles clustered around electron dense synaptic machinery in the presynaptic cell. The pre- and postsynaptic cells are separated by a narrow space, the synaptic cleft, and are insulated by glial cells. Finally, the postsynaptic cells have defined, electron-dense domains containing receptors positioned opposite release sites.



AF 1.1. Synaptic structure. (A) Electron micrograph of a synapse in the central nervous system (CNS). (B) The presynaptic (PRE) and postsynaptic (POST) neurons of a CNS synapse mediating fast synaptic transmission showing mitochondria (M) and a population of small, clear

synaptic vesicles (SV) containing neurotransmitter. Some vesicles are docked on the presynaptic membrane adjacent to dense material containing the protein machinery for vesicle fusion, active zone material (AZM). Opposite the docked vesicles and AZM, the membrane of the postsynaptic neuron appears thickened due to the presence of neurotransmitter receptors and scaffolding proteins, referred to as the postsynaptic density (PSD). Glial cells (GC) envelop and insulate the synapse. (C) Electron micrograph of a neuromuscular junction. (D) The presynaptic neuron (PRE) and postsynaptic striated muscle fiber (POST) of a neuromuscular junction showing similar mitochondria and neurotransmitter filled SVs with a subset docked adjacent to AZM. Invaginations of the postsynaptic membrane, junctional folds (JF), are found immediately opposite the AZM. Receptors for neurotransmitter and scaffolding proteins are especially concentrated near the tops of the JFs. The insulating glial cell is a Schwann Cell (SC). (Micrographs courtesy of John Heuser).

III.a. Presynaptic Structure

The presynaptic terminal is generally a distinct bouton or varicosity filled with synaptic vesicles containing small, fast-acting chemical neurotransmitters (section IV.a). Synaptic vesicles must fuse with the presynaptic membrane to release their neurotransmitters into the synaptic cleft and initiate synaptic transmission. Thus, presynaptic terminals contain the machinery required to: 1) synthesize neurotransmitter, 2) package neurotransmitter into vesicles, 3) couple incoming electrical signals with an increase in cytosolic [Ca^{2+}], 4) fuse the vesicles with the presynaptic membrane in response to this Ca^{2+} influx, and 5) recycle the vesicle membrane and associated proteins back into the presynaptic terminal to make new synaptic vesicles.

Within presynaptic terminals, vesicles are clustered at specialized fusion sites along the presynaptic membrane, called active zones [30]. Active zones consist of specialized scaffolding proteins that organize the presynaptic machinery required for the Ca^{2+}-dependent, vesicular fusion events that release neurotransmitter. Electron microscopy has shed light onto the precise structural organization at the active zone (section V.a). This organization provides precise alignment of synaptic vesicles with the fusion machinery complex and Ca^{2+} channels. This

highly ordered structure is critical for proper triggering of neurotransmitter release in response to action potentials.

III.b. Synaptic Cleft Structure

The synaptic cleft contains structural proteins that control the surface area of the synaptic interface and the proximity between the pre- and postsynaptic membranes. Synaptic cleft width is related to the type of transmission at the synapse. Small synapses, with closer apposition of the pre- and post-synaptic cells (20-50 nm), are fast acting with localized effects. Larger synapses with wide synaptic clefts can have slower time scales and a wider range of postsynaptic targets [31]. Cell-adhesion molecules and scaffolding proteins control cleft width [32]. Regulatory proteins align domains on the pre- and postsynaptic membranes and direct the organization of the pre- and postsynaptic specializations required for efficient synaptic communications [18, 33].

Other synaptic cleft molecules provide clearance of neurotransmitter. Following release, neurotransmitters must be efficiently removed to terminate the signal from the pre- to the postsynaptic cell. Neurotransmitter may be enzymatically cleaved within the cleft or removed by uptake machinery on the surface of the surrounding glial cells, the presynaptic terminal, or the postsynaptic cell (section VI).

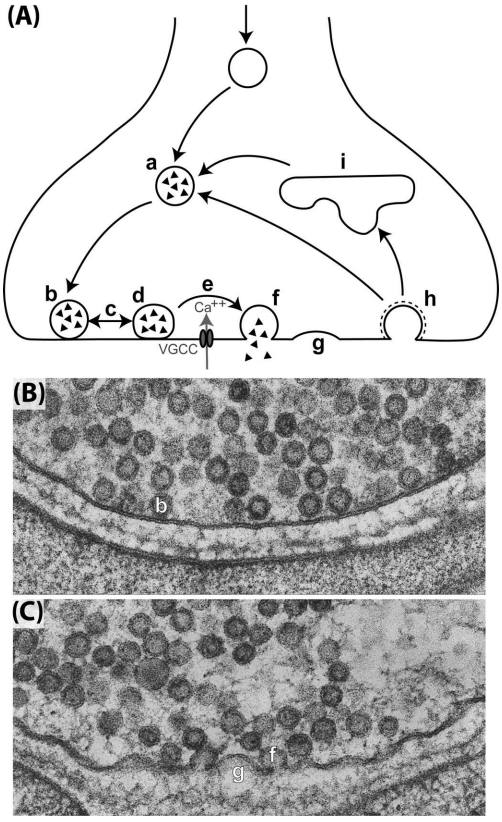
III.c. Postsynaptic Structure

The postsynaptic cell must transduce the chemical signal of the neurotransmitter back into an electrical signal. Required specializations within the postsynaptic membrane include a high concentration of neurotransmitter receptors, regulatory molecules, and ion channels to produce the electrical signal (reviewed in [34]). The postsynaptic response may be a fast, specific

response or a slower, more generalized response. It can be excitatory (depolarizing) or inhibitory (hyperpolarizing). The postsynaptic response depends on the specific neurotransmitter receptors present in the postsynaptic membrane, which are highly variable between cell populations, but generally fall into two main categories: ionotropic and metabotropic (section VII.a). Ionotropic receptors are ligand-gated ion channels; the binding of neurotransmitter opens the channel allowing specific ions to cross the membrane. The selective permeability for specific ions determines whether the cell is depolarized or hyperpolarized. Metabotropic receptors are integral membrane proteins that bind G-proteins, called G-protein coupled receptors (GPCRs). Neurotransmitter binding to GPCRs results in activation of the coupled G-protein, triggering second messenger cascades that cause downstream secondary effects. These include opening of ion channels, posttranslational modification of proteins, or transcriptional changes.

IV. Neurotransmitters

The presynaptic element is specialized to synthesize and release neurotransmitters. The type of neurotransmitter released varies between neuron types, with many neurons releasing more than one kind of neurotransmitter. During synaptic transmission, neurotransmitter-filled synaptic vesicles undergo a stereotypical cycle: docking at active zones, priming, Ca^{2+}-triggered fusion, vesicle reformation, refilling with neurotransmitter, and trafficking back to the active zone (AF 1.2). This continuous cycle provides a constantly replenished supply of neurotransmitter ready for release.



AF 1.2. The synaptic vesicle cycle. (A) Schematic of the synaptic vesicle cycle depicting vesicle loading with neurotransmitter (a), vesicle docking (b), variable vesicle priming (c), maximally-primed, docked vesicle (d), depolarization-triggered Ca^{2+} entry (e), vesicle fusion with presynaptic membrane (f), vesicle membrane collapse (g), endocytosis (h), and recycling endosome (i). (B) Electron micrograph showing docked vesicles (b) at a neuromuscular junction. (C) Electron micrograph showing fusing vesicles with omega morphology (f) and vesicle membrane collapse (g). (Micrographs courtesy of John Heuser).

IV.a. Neurotransmitter Synthesis, Loading and Clearance

To be classified as a neurotransmitter, a chemical must meet several criteria. There must be enzymatic machinery located at the synapse to synthesize the neurotransmitter. It must be released from the terminal in response to an electrical signal. Finally, there must be machinery to degrade or clear the neurotransmitter from the synaptic cleft.

The small, fast-acting neurotransmitters, shown in AT 1.1, can be grouped into a few general classes: cholinergic, amino acidergic, monoaminergic, and purinergic. While each has its

own specific synthesis, loading, and clearance machinery (reviewed in [35]), they share many common characteristics.

Vesicle loading for these neurotransmitters relies on a proton (H^+) gradient driving force. A proton pump, or vesicular-ATPase, hydrolyzes ATP to transport H^+ ions into the vesicles. Transporters for specific neurotransmitters rely on the resultant proton gradient to move neurotransmitter into the vesicle in exchange for allowing protons to exit (reviewed in [36, 37]).

AT 1.1. Neurotransmitter types, their receptors and notable examples.

Neurotransmitters and receptors			
Category	Neurotransmitter	Ionotropic receptors	Metabotropic receptors
Cholinergic	Acetylcholine	nAChR	mAChR
Amino Acidergic	Glutamate	Kainate, AMPA, NMDA	mGluR
	Glycine	Gly-R	
	GABA	GABAa, GABAc	GABAb
Monoaminergic	Dopamine		D_1, D_2
	Norepinepherine		α_1, α_2, β_1, β_2
	Serotonin	5-HT_3	5-HT_1, 5-HT_2
Purinergic	ATP, Adenosine	P_{2x}	P_1, P_{2y}

Cholinergic Neurons

Cholinergic neurons synthesize and release the neurotransmitter acetylcholine (ACh). In mammals, ACh is present in the central, peripheral and autonomic nervous systems. ACh is the neurotransmitter in motor neurons that innervate skeletal muscle, making it essential for movement. It is one of two predominant transmitters in the parasympathetic division of the autonomic nervous system and is increasingly thought to play an important role in cognition. ACh is synthesized by choline acetyltransferase, which attaches choline to acetyl coenzyme A.

Acetyl coenzyme A and choline acetyltransferase are synthesized in cholinergic neurons, but choline must be transported into these neurons by a Na^+-dependent carrier. Following synthesis of ACh, the neurotransmitter is loaded into vesicles by the vesicular acetylcholine transporter using the proton gradient mentioned above. Once released, ACh is cleared from the synaptic cleft by acetylcholinesterases that hydrolyze ACh. This produces acetate and choline, much of which is recaptured by the presynaptic terminal for synthesis of new ACh (reviewed in [38, 39]).

Amino Acidergic Neurons

Amino acidergic neurons release glycine, glutamate, or gamma-aminobutyric acid (GABA), which are common neurotransmitters released broadly throughout the CNS. Neurons synthesize glycine from serine using the enzyme serine hydroxymethyltransferase. Glycine can have excitatory or inhibitory effects on the postsynaptic cell depending on the receptor types present. Following release, specific transporters sequester it into glia or back into nerve terminals. Neurons are unable to synthesize glutamate or GABA *de novo* from glucose. They rely on glutamine production in surrounding glia as sources of the precursors for neurotransmitter production and reuptake of released neurotransmitter. In glia, alpha-ketoglutaric acid of the tricarboxylic acid cycle is converted to glutamate by the enzyme transaminase. This glutamate is then converted to glutamine, which is transported to the nerve terminal. Once there, the enzyme glutaminase catalyzes the conversion of glutamine back to glutamate. GABA is generated from glutamate by the enzyme glutamic acid dehydrogenase. Once synthesized, each is loaded into synaptic vesicles, again utilizing the proton gradient. Following release, they are transported from the cleft back into the nerve terminal for reuse or into surrounding glial cells. In glial cells, the neurotransmitter is converted back to glutamine for transport back to the

presynaptic cell. This glutamine-glutamate cycle between the neuron and glial cell provides the synapse with the necessary pool of neurotransmitter (reviewed in [40, 41]).

Monoaminergic Neurons

Monoaminergic neurons release a wide array of neurotransmitters (reviewed in [42, 43]). One class, known as catecholaminergic neurons, release dopamine, norepinephrine, or epinephrine. The catecholamines are synthesized from tyrosine through multiple intermediaries. Tyrosine is initially converted to L-dihydroxyphenylalanine (L-DOPA) by tyrosine hydroxylase, the rate-limiting step in catecholamine production. L-DOPA is decarboxylated to dopamine by l-amino acid decarboxylase. In dopaminergic neurons, dopamine is loaded into vesicles via its specific monoamine transporter. In neurons that release norepinephrine, dopamine is loaded into vesicles containing the enzyme dopamine-β-hydroxylase which converts the dopamine into norepinephrine. In epinephrine-releasing neurons, norepinephrine leaks out of vesicles, where it is converted to epinephrine by the cytosolic enzyme phenolethanolamide-N-methyltransferase. This epinepherine is then loaded into its own vesicle population. Following release, dopamine, norepinephrine and epinephrine are cleared from the cleft by Na^+- and Cl^--dependent monoamine transporters that move them back into nerve terminals. In the terminal, these monoamines are either broken down by mitochondrial monoamine oxidase (MAO) or reloaded into new synaptic vesicles.

Other monoaminergic neurons release the indoleamine, serotonin. In serotonergic terminals, tryptophan-hydroxylase converts the amino acid tryptophan into 5-hydroxytryptophan, which is decarboxylated by 5-hydroxytryptophan decarboxylase to serotonin (5-hydroxytryptamine or 5-HT). Serotonin is loaded into vesicles via vesicular monoamine

transporters. Following release, serotonin is cleared from the cleft and degraded in the cytosol of the presynaptic terminal by MAO or reloaded into synaptic vesicles.

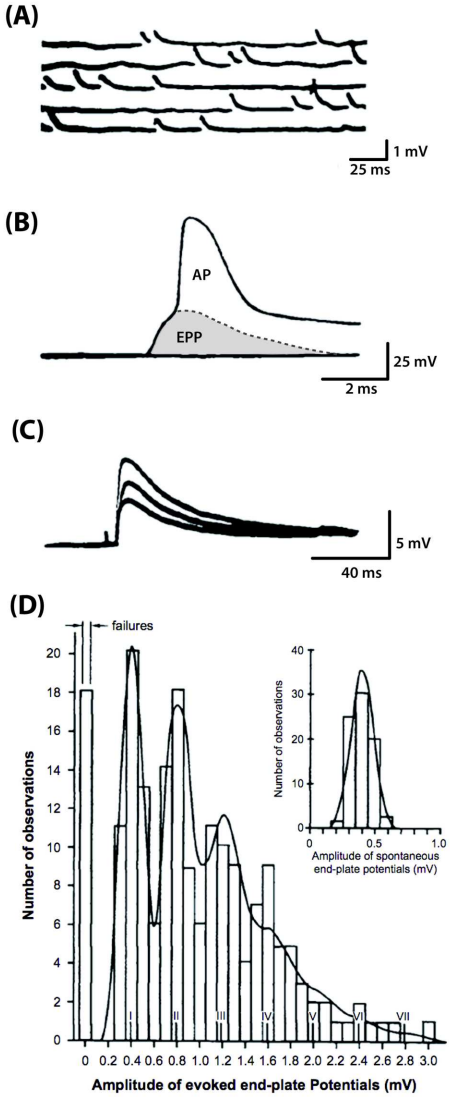
Purinergic Neurons

Purinergic neurons synthesize and release the nucleotides ATP and adenosine (reviewed in [44, 45]). ATP and adenosine are important for numerous cellular processes, and are therefore sequestered into different populations. At the synapse, they are loaded into vesicles to prevent other cellular processes from depleting the supply necessary for release. This class of neurotransmitter is often co-released with other neurotransmitters throughout the peripheral and central nervous systems. Once released, ATP and adenosine are degraded in the cleft by cell surface enzymes known as ectonucleotidases.

IV.b. Quantal Release Hypothesis

Identification of synaptic vesicles as the source of neurotransmitter release was a groundbreaking step in our understanding of synaptic transmission. Bernard Katz and his colleagues proposed the quantal release hypothesis in the early 1950s [1-3]. In pioneering electrophysiological studies at the frog neuromuscular junction, they identified spontaneous, small postsynaptic changes (AF 1.3A) that occurred in the absence of neural activity. These depolarizations resembled end plate potentials (EPPs) following an action potential (AF 1.3B), albeit on a much smaller scale. These small, spontaneous events are referred to as miniature end-plate potentials (mEPPs). Since mEPPs appeared to be random and similarly-sized (between 0.3 and 0.5 mV), this suggested neurotransmitter was being released in discrete packets, or quanta. When they evoked EPPs with presynaptic action potentials in the presence of excess Mg^{2+},

which decreases Ca^{2+} entry, the amplitude of the EPP was decreased and highly variable (AF 1.3C). Importantly, they noticed that the EPP amplitudes fell in roughly even steps, with the step height being multiples of the spontaneous mEPP amplitude. These findings led to the hypothesis that neurotransmitter was being released in multimolecular quantized packets. Furthermore, an action potential causes the synchronous release of a large number of these packets, generating a large EPP (AF 1.3B) in the postsynaptic cell. Later studies at the mammalian neuromuscular junction clearly demonstrated the step-wise distribution of EPP amplitudes under similar conditions (AF 1.3D).



AF 1.3. Intracellular recordings at the neuromuscular junction. (A) Spontaneous, mEPPs recorded from a resting (not stimulated) frog neuromuscular junction. Note that these small depolarizing potentials are less than 1 mV in amplitude and occur randomly. (B) Postsynaptic response to a presynaptic action potential. The initial hump on the recorded waveform is the EPP elicited by ACh released from the presynaptic terminal. Note that the EPP is a large depolarization (> 40 mV), sufficient to bring the end-plate to threshold and trigger a muscle action potential (AP) (A,B adapted with permission from [2], ©1952 The Physiological Society). (C) Fluctuations in EPPs when neurotransmitter output has been reduced by adding 10 mM Mg^{2+} to the bathing medium. (C adapted with permission from [3] ©1954 The Physiological Society). (D) Distribution of EPP amplitudes recorded from a mammalian end-plate under conditions of reduced transmitter release in high (12.5 mM) Mg^{2+}. The inset shows a histogram of spontaneous mEPPs recorded from a resting junction. Note that EPP amplitudes group around multiples of mean mEPP amplitude. The number of experimentally observed failures (0 quanta released) and single, double, triple, or more quantal responses, fit the theoretical distribution (solid curve) calculated from the Poisson equation. (Adapted with permission from [46] ©1956 The Physiological Society).

The concept that synaptic transmission could be explained by the quantized release of neurotransmitter was proposed around the same time that small membranous vesicles within nerve terminals were first identified by electron microscopy [47, 48]. Together, these findings led to the vesicular hypothesis for neurotransmitter release, which states that each synaptic vesicle contains a similar amount of neurotransmitter (1 quantum), and that the transmitter release following an action potential results from a discrete number of vesicles fusing synchronously with the plasma membrane.

This hypothesis allows for neurotransmitter release at the synapse to be understood in terms of the relatively simple equation $m=np$, where m is the average number of quanta released following a single nerve impulse (referred to as quantal content). The parameter m depends on the number of vesicles immediately available for release (n) and the probability (p) that any given vesicle will fuse with the membrane after a single impulse. This probability depends on multiple factors, including how “ready” the vesicle is to fuse, which we will refer to as its degree of priming, and the amount of Ca^{2+} influx into the presynaptic terminal (see sections V.a and

V.b). Statistical analysis of neurotransmitter release extends beyond this foundational concept (reviewed in [49]).

V. Presynaptic Vesicle Cycle

As introduced above, synaptic vesicles must undergo a distinct, tightly regulated series of steps. They must be loaded with neurotransmitter (AF 1.2A.a), transported to the active zone, and docked to the presynaptic membrane by way of specific molecular interactions (AF 1.2A.b and 1.2B.b). The population of docked vesicles at a synapse is thought to constitute a readily releasable pool of neurotransmitter. However, only a subset of docked vesicles are fusion competent at any given time, namely, those that are maximally primed (AF 1.2A.d). In a resting terminal, individual maximally-primed vesicles can spontaneously fuse with the presynaptic membrane, resulting in the release of a single quantum of neurotransmitter. In an activated neuron, the influx of Ca^{2+} through voltage-gated Ca^{2+} channels (AF 1.2A.e) triggers the fusion of multiple maximally-primed vesicles with the presynaptic membrane and results in a larger release of neurotransmitter (AF 1.2A.f,g and 1.2C.f,g). This is accomplished by Ca^{2+}-sensing proteins activating the fusion machinery. Neurotransmitter release occurs in two temporally distinct Ca^{2+}-dependent phases: synchronous and asynchronous. After neurotransmitter release, the fusion machinery is disassembled and recycled for subsequent use. Concurrently, the synaptic vesicle membrane and associated proteins are retrieved through endocytosis (AF 1.2A.h) to form new synaptic vesicles. These vesicles are reloaded with neurotransmitter and transported back to active zones, completing the cycle. The proteins covered in this and subsequent sections can be found in AT 1.2.

AT 1.2. Reference table for notable synaptic proteins organized by their synaptic localization

Compartment	Protein	Function
Presynaptic	RIM	Active zone material
	Munc13	Active zone material
	RIM-BP	Active zone material
	Alpha-liprin	Active zone material
	ELKS/CAST	Active zone material
	Synaptobrevin 2	Fusion machinery
	SNAP-25	Fusion machinery
	Syntaxin	Fusion machinery
	Munc18	SM protein
	Synaptotagmin 1/2	Ca^{2+} sensor, synchronous
	Synaptotagmin 7	Ca^{2+} sensor, asynchronous
	Doc2	Ca^{2+} sensor, asynchronous
	NSF	SNARE disassembly
	Clathrin	Endocytosis
	Dynamin	Endocytosis
Synaptic Cleft	Agrin	Synaptogenesis
	MuSK	Synaptogenesis
	LRP4	Synaptogenesis
	Neuroligins	Synaptogenesis/plasticity
	Neurexins	Synaptogenesis/plasticity
	Cadherins	Scaffolding/plasticity
	Eph Tyrosine Kinases	Scaffolding/plasticity
	Epherins	Scaffolding/plasticity
Postsynaptic	Ionotropic receptors	Changes in postsynaptic potential
	Metabotropic receptors	Changes in postsynaptic potential/ second messenger cascades

V.a. The Active Zone

Functionally, the role of the active zone is to transduce an electrical nerve terminal depolarization into neurotransmitter release. Couteaux and Pecot-Dechavassinein coined the term "active zone" in 1970 when they noticed synaptic vesicles at the frog neuromuscular junction docked to the presynaptic membrane adjacent to electron dense material in electron micrographs [30]. Subsequent ultrastructural studies revealed similar morphologies across organisms. The structure of this active zone material (AZM) varies among different types of synapses, but must

include the requisite protein machinery for vesicle docking, priming, and fusion. The major constituents of active zones include: active zone core proteins, essential fusion machinery, Sec1/Munc18-like (SM) proteins, Ca^{2+} channels, and Ca^{2+} sensors. The core proteins covered in this chapter are Rab3-interacting molecules (RIM), Munc13, RIM binding protein (RIM-BP), alpha-liprin, and ELKS/CAST [50]. The essential fusion machinery consists of the soluble N-ethlymaleimide sensitive factor adaptor protein receptors, or SNAREs. The Ca^{2+}-sensing proteins covered in this chapter that trigger evoked neurotransmitter release are synaptotagmin 1, 2, 7, and double C_2 containing protein (Doc2). Numerous additional regulatory and adaptor molecules are present at or near active zones, but are not covered.

The AZM holds a cluster of synaptic vesicles near release sites, including those docked against the presynaptic membrane. The precise structure of the AZM can vary in size and shape, depending on synaptic type and/or organism. In certain visual and auditory neurons, ribbon-like structures have been observed across several species. In contrast, neuromuscular junction ultrasturcture varies across organisms. In *Drosophila,* the neuromuscular AZM has a "T-bar" shape surrounded by synaptic vesicles. At mammalian neuromuscular junctions, the AZM contains short bars of electron-dense material located on either side of two adjacent docked vesicles with additional associated vesicles above. Frog neuromuscular junctions exhibit a long midline AZM with two rows of docked vesicles on either side (see AF 1.6) surrounded by additional synaptic vesicles. Despite their differences, *Drosophila*, frog, and mammalian neuromuscular junctions share common synaptic machinery. Recent electron tomography studies at the frog and mouse neuromuscular junctions reveal similar ultrastructural AZM components [51, 52]. The molecular identity of the individual proteins comprising these structures remains an active area of synaptic research.

Active Zone Core Proteins

Some of the proteins that are located in the core of the active zone include RIM, Munc13, RIM-BP, alpha-liprin, and ELKS/CAST. Together with additional adaptor proteins, they recruit synaptic vesicles, fusion machinery, and Ca^{2+} channels to the active zone. RIM and Munc13 work together to link synaptic vesicles to active zones, as discussed below. In addition, RIM binds Ca^{2+} channels, which are concurrently bound by RIM-BP and ELKS/CAST, creating a trimeric complex essential for recruiting Ca^{2+} channels to active zones. Munc13 is additionally involved in enhancing vesicle fusion competence (see section V.b). Alpha-liprins interact with multiple proteins that regulate active zone formation [53]. They are scaffold proteins that may act as anchors to recruit and stabilize molecules at the active zone, as they are critical for recruiting RIM [54, 55] and trafficking synaptic vesicles [56]. Studies in invertebrates suggest a role for alpha liprin in active zone morphogenesis as mutants exhibit larger, less dense active zones [57]. The invertebrate homolog of ELKS/CAST is implicated in active zone cytoskeletal formation [58] and Ca^{2+} channel recruitment [59].

Fusion Machinery

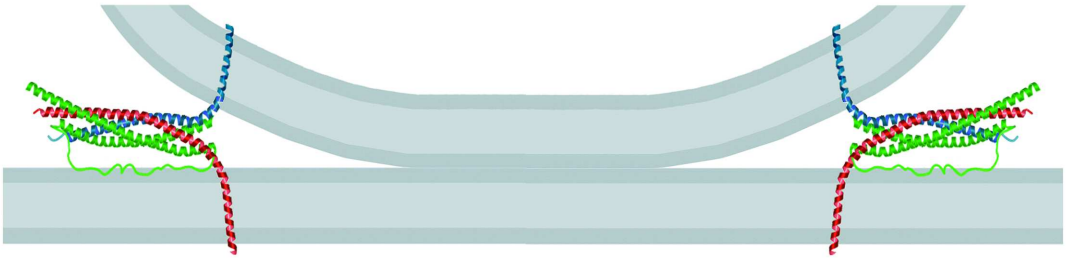
The minimum machinery required to fuse a vesicle with its target membrane is the SNARE fusion complex, which is comprised of a vesicle-associated SNARE protein (vSNARE) and target-membrane associated SNARE proteins (tSNAREs). At the synapse, the vSNARE is synaptobrevin-2, also known as vesicle-associated membrane protein 2 (VAMP2) and the tSNAREs are syntaxin-1 and synaptosomal-associated protein of 25 kDa (SNAP-25) (AF 1.4).

Each SNARE protein contains at least one SNARE motif of ~65 residues with a propensity to form coiled coils [60]. Synaptotobrevin and syntaxin each contain one SNARE

motif, and SNAP-25 contains two [61]. Prior to vesicle docking, syntaxin is found in a stable, closed conformation, with its SNARE motif bound to an internal three-helix-bundle regulatory domain, making it inaccessible for interactions with other SNARE proteins [62]. Syntaxin undergoes a conformational change triggered by Munc13 (see section V.b), which allows its SNARE motif to interact with the SNARE motifs of synaptobrevin and SNAP-25. This creates a *trans*-SNARE complex which is associated with both the vesicular membrane and the presynaptic plasma membrane. On any given vesicle, tight coiling of multiple SNARE complexes [63] provides the energy required to drive fusion of the vesicle and target membranes (AF 1.4) [64, 65]. Following membrane fusion, all members of the SNARE complex are located in the target membrane, and the complex is referred to as a *cis*-SNARE complex.

SNARE complexes mediate both constitutive and regulated vesicle fusion events throughout cells. At the synapse, multiple regulatory elements suppress constitutive SNARE-mediated fusion events and facilitate the fast, synchronous fusion of multiple synaptic vesicles upon Ca^{2+} influx. Synchronous fusion of multiple vesicles is essential for neuronal signaling and relies on the docking of vesicles at the active zone and their priming to a fusion ready state (section V.b).

The basic concept of the minimal machinery necessary to fuse a vesicular with its target membrane is as follows: SNARE proteins on both the vesicular and target membranes associate to form tight coiled-coil configurations. When maximally coiled, *trans*-SNARE complexes force the two membranes together, thereby destabilizing the membranes' natural curvature (AF 1.4). The coiling of *trans*-SNARE complexes all the way into their transmembrane domains provides the energy to drive fusion of the membranes [64, 66, 67]. Following fusion, the vSNAREs and tSNARES are now in a *cis*-SNARE complex, and the vesicular contents are released.



AF 1.4. Minimal fusion machinery. Schematic of a vesicle just prior to fusion showing two (of multiple) maximally-coiled, *trans*-SNARE complexes pulling the vesicle membrane flat against the presynaptic membrane. (Reprinted by permission from [61], Springer Nature: Springer, Nature, Crystal structure of a SNARE complex involved in synaptic exocytosis at 2.4 Å resolution. R Brian Sutton, Dirk Fasshauer, Reinhard Jahn, Axel T. Brunger, ©1998).

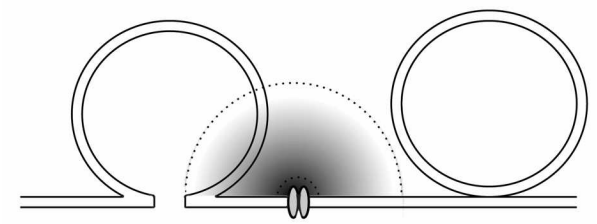
SM Proteins

SM proteins function during virtually all SNARE-dependent fusion reactions. These soluble proteins fold into general "clasp" shapes [68] with a propensity to bind four helix bundles. At least one SM protein is necessary for any SNARE-mediated fusion event to occur. SM proteins are believed to be essential to stabilize SNARE complexes spatially and support SNARE complex assembly. Munc18, a member of the SM protein family, binds the N-terminal region of syntaxin in its closed conformation [62, 68, 69]. This SM/SNARE interaction prevents SNARE complex assembly, thereby inhibiting fusion events. Once syntaxin undergoes a conformational change to its open configuration (see section V.b), Munc18 remains bound but alters its binding to interact directly with the four helix bundle of the assembling *trans*-SNARE complex, aiding in fusion [70, 71]. In this way, SM proteins are both negative and positive regulators of SNARE-mediated fusion.

Ca^{2+} Channels

The presence of voltage-gated Ca^{2+} channels at active zones is necessary to couple neuronal depolarization to vesicle fusion events. Not only do these channels need to be present at

active zones, their precise localization within an active zone can have immense effects on the efficacy of synaptic transmission. Ca^{2+} channels are located within 50 nm from docked vesicles [72-74]. When the cell is depolarized, the Ca^{2+} channels briefly open, creating a nanodomain of particularly high [Ca^{2+}] immediately adjacent to each channel. This [Ca^{2+}] reaches hundreds of μM at the mouth of the channel [75] and drops off rapidly with distance (AF 1.5) [76-79]. As the availability of Ca^{2+} is limited temporally and spatially, the closer the channel is located to the Ca^{2+} sensor, the greater the odds of saturating sensor binding and triggering vesicle fusion. Since the sensor for fast, synchronous neurotransmitter release is a low affinity sensor (see below), even a 5 nm change in the distance between the channel and the sensor has profound effects on vesicle release probability [80].



AF 1.5. Ca^{2+} nanodomains. Schematic depicting the rapid decay of [Ca^{2+}] with distance from the Ca^{2+} channel. The darker the gradient, the higher the [Ca^{2+}]. A Ca^{2+} sensor on the left vesicle would experience a very high [Ca^{2+}], which would maximize the probability of fusion. A Ca^{2+} sensor on the right vesicle would be exposed to a much lower [Ca^{2+}] and have a correspondingly lower probability of fusion.

Ca^{2+} Sensors

Synaptotagmins 1, 2, and 7, as well as Doc2 regulate two distinct forms of Ca^{2+}-dependent neurotransmitter release: fast, synchronous release and a longer lasting, asynchronous release (section V.c). While other Ca^{2+} sensors exist, only the previously mentioned proteins will be covered as they are currently the best understood.

Synaptotagmins are a large family of integral membrane proteins that contain two Ca^{2+}-binding motifs called C_2 domains. Synaptotagmin 1 and 2 are located on synaptic vesicles and play functionally homologous roles in different parts of the nervous system. In mammals, synaptotagmin 1 is expressed predominantly in the cerebral hemispheres, while synaptotagmin 2 is expressed predominantly in the brainstem, spinal cord, and at the neuromuscular junction [81, 82]. Synaptotagmins 1/2 are low-affinity Ca^{2+} sensors, meaning they only bind Ca^{2+} when intracellular [Ca^{2+}] is very high, as found in the Ca^{2+} nanodomains discussed above. In the Ca^{2+}-bound state, the C_2 domains of synaptotagmin interact with both SNARE proteins [83-88] and negatively-charged phospholipid membranes *in vitro* [89-91]. Upon Ca^{2+} binding *in vivo*, synaptotagmins 1/2 trigger the fast, synchronous phase of neurotransmitter release [92] primarily through membrane interactions [93, 94], although Ca^{2+}-dependent SNARE interactions likely contribute as well.

Synaptotagmin 7 is another member of the synaptotagmin family enriched at synapses. It is an integral membrane protein localized in the presynaptic membrane [95] that binds Ca^{2+} with a higher affinity than synaptotagmins 1/2. Doc2 is also enriched at synapses and contains two Ca^{2+}-binding C_2 domains with a high affinity for Ca^{2+} [96]. Unlike the synaptotagmins, Doc2 is a cytosolic protein. Both synaptotagmin 7 and Doc2 are attractive candidates for triggering asynchronous release (section V.c) due to their high Ca^{2+} affinities and slow Ca^{2+}-interaction kinetics [97].

V.b. Docking and Priming at the Active Zone

To become fusion competent, synaptic vesicles must be precisely targeted to the active zone and held in direct contact with the presynaptic membrane. First, vesicles are tethered near

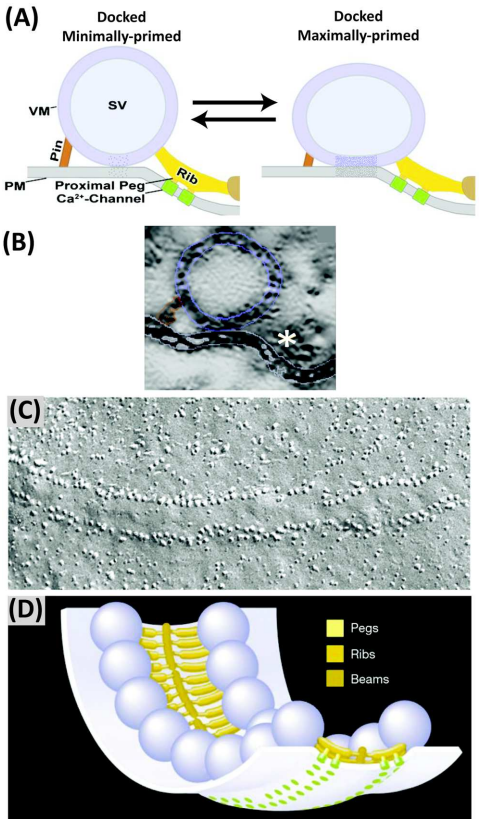
the presynaptic membrane at active zones. When the vesicle membrane touches the presynaptic membrane, the vesicle is considered docked at the release site. Once docked, additional interactions, referred to as priming, increase the probability of vesicle fusion.

Adaptor proteins piccolo/bassoon are thought to guide synaptic vesicles toward active zones in vertebrates [98, 99]. Once there, RIM and Munc13 contribute to vesicle docking [100]. RIM binds to Munc13, creating a RIM/Munc13 heterodimer. This heterodimer binds an accessory protein, either Rab3 or Rab27, and this complex provides the scaffold needed to precisely target and hold synaptic vesicles to active zones (AF 1.2A.b) [101]. These vesicles are now considered docked.

Additional intermolecular interactions are required before vesicles are maximally primed and fully fusion competent. Munc13, an essential active zone protein, is thought to orchestrate the first step in fusion machinery assembly (note: Munc13 should not to be confused with the SM protein, Munc18, discussed in section V.a). Munc13 binds to the closed conformation of syntaxin and facilitates a conformational change in syntaxin from its "closed" to its "open" state [62, 102]. Only then can syntaxin's SNARE motif interact with the SNARE motifs of SNAP-25 and synaptobrevin to form a *trans*-SNARE complex. Upon syntaxin's conformational change, the Munc18 interaction adjusts to facilitate the assembly of the *trans*-SNARE complex [70, 71], thereby contributing to the fusion-competency of the vesicle.

Recent work in the McMahan lab suggests that vesicle priming is in a state of dynamic equilibrium (AF 1.6A, arrows), such that only docked vesicles with the largest area of contact with the presynaptic membrane are maximally primed and ready to fuse upon Ca^{2+} influx [52]. One mechanism of accomplishing variable priming would be a balance between the repulsive forces of the vesicular and presynaptic membranes that result in SNARE complex uncoiling to

favor minimal priming (AF 1.6A, left) and SNARE complex coiling to favor maximal priming (AF 1.6A, right) [52]. At any given moment, only a subpopulation of docked synaptic vesicles have a sufficient number of maximally-coiled *trans*-SNARE complexes to produce maximal SV-PM contact area. These synaptic vesicles constitute the maximally-primed population that is most likely to fuse upon Ca^{2+} influx. Once triggered by Ca^{2+}, these maximally-primed synaptic vesicles complete the SNARE coiling process all the way into the transmembrane domains of syntaxin and synaptobrevin [103]. The synaptic vesicle membrane merges with the presynaptic membrane, resulting in *cis*-SNARE complex formation. Thus, the coiling of SNARE complexes provides the energy needed to induce membrane fusion.

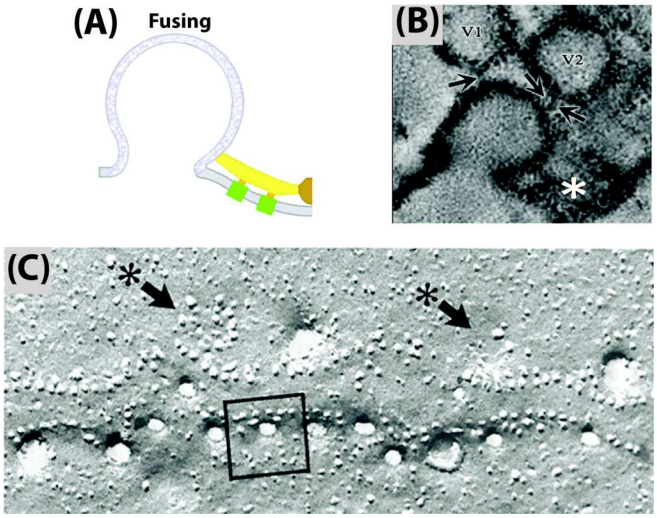


AF 1.6. Docking and variable priming of synaptic vesicles. (A) Left, schematic depicting a docked, minimally-primed vesicle at the presynaptic membrane, defined by the small area of contact between the vesicle and plasma membranes. Right, the fusion machinery located in the AZM (ribs, pegs) and in pins pulls the vesicle and presynaptic membranes together, increasing the area of contact, resulting in a maximally-primed vesicle that has the highest fusion probability. The degree of priming is in a state of dynamic equilibrium (arrows). Those vesicles

that happen to be maximally primed when Ca^{2+} influx occurs are thought to be the vesicles that fuse. (Adapted from [52]). (B) A docked synaptic vesicle viewed by electron tomography. White * represents AZM. (Adapted from [104]). (C) Freeze-fracture electron micrograph at the frog neuromuscular junction showing the characteristic parallel double-rows of particles that comprise the pegs of the AZM and include the voltage-gated Ca^{2+} channels required for triggering fusion. (Adapted from [72] ©1979 Journal of Cell Biology. 81:275-300. DOI:10.1083/jcb.81.2.275). (D) Schematic showing the relationship of docked synaptic vesicles, the AZM, and the double row of particles that include Ca^{2+} channels at the frog neuromuscular junction. (Reprinted with permission from [73], Springer Nature: Springer, Nature, The Architecture of active zone material at the frog's neuromuscular junction. Mark L Harlow, David Ress, Arne Stoschek, Robert M Marshall, Uel J McMahan, ©2001).

V.c. Vesicle Fusion

Fusion events can occur spontaneously or can be evoked. When an action potential depolarizes the presynaptic terminal, voltage-gated Ca^{2+} channels open. The subsequent influx of Ca^{2+} triggers a series of intermolecular interactions that results in the fusion of multiple maximally-primed vesicles with the presynaptic membrane (AF 1.2A,C and 1.7).



AF 1.7. Vesicle fusion. (A) Schematic depicting a vesicle that has just fused with the presynaptic membrane such that the vesicle membrane is now incorporated into the plasma membrane, forming an omega figure. (B) Electron tomograph of a fusing vesicle. V1 and V2 represent additional synaptic vesicles located near the active zone that are linked to the fusing vesicle (arrows). White * represents AZM. (A,B adapted from [104]). (C) Freeze-fracture electron micrograph showing the openings of fusing vesicles (box) just lateral to the double-rows of particles found at active zones. Cluster of particles characteristic of fully-collapsed vesicles (black * bold arrows) are also found lateral to the double-row of particles. (Adapted from [72] ©1979 Journal of Cell Biology. 81:275-300. DOI:10.1083/jcb.81.2.275).

Ca^{2+}-independent Neurotransmitter Release

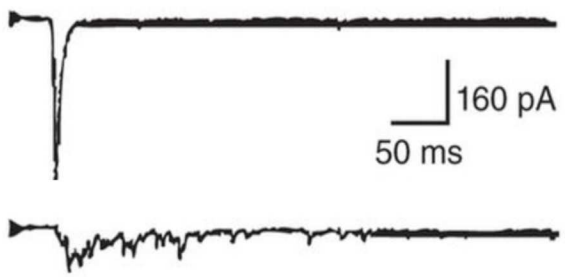
Ca^{2+}-independent neurotransmitter release is referred to as spontaneous release. This form of neurotransmitter release occurs in resting nerve terminals when a single vesicle fuses with the presynaptic membrane in the absence of presynaptic Ca^{2+} influx (AF 1.3A). Classically, spontaneous release is considered to be random fusion events caused by low-probability conformational changes that complete coiling of the SNARE proteins and result in fusion. Since these spontaneously fusing vesicles would be in a maximally-primed, fusion-competent state, little energy is required to overcome the fusion barrier.

Spontaneous and evoked fusion events can occur at distinct active zones [105, 106] from separate recycling pools [107, 108]. Thus, location may partially determine the probability of a vesicle participating in spontaneous versus evoked neurotransmitter release. These recent studies, together with the classic spontaneous release hypothesis, suggest multiple mechanisms occurring during spontaneous fusion, with a subpopulation originating from maximally-primed vesicles reacting to transient changes in their environment, and another subpopulation occurring at specialized, Ca^{2+}-independent fusion sites.

Ca^{2+}-dependent Neurotransmitter Release

Ca^{2+}-dependent neurotransmitter release is differentiated temporally into two phases: fast, synchronous release and a slower, asynchronous release (AF 1.8) [109, 110]. The fast, synchronous phase is the large burst of neurotransmitter release that occurs within milliseconds of the arrival of the action potential and constitutes the classical neurotransmitter release pathway typically described in textbooks (AF 1.8, upper trace). However, there is an additional slower, or asynchronous, phase of elevated release that is still Ca^{2+}-dependent and lasts for tens

to hundreds of milliseconds [109, 110]. This phase of release is most notable following periods of high frequency stimulation, at specialized synapses, and at synaptotagmin 1 knock out synapses (FA 1.8, lower trace) [110-114].



AF 1.8. Ca^{2+}-dependent neurotransmitter release at cultured hippocampal synapses. Upper, voltage trace depicts the fast, synchronous neurotransmitter release in cultures from wild type mice. Lower, voltage trace depicts the slower, asynchronous neurotransmitter release in cultures from synaptotagmin 1 knock out mice. (Reprinted from [115], Cell, 79/4, Martin Geppert, Yukiko Goda, Robert E Hammer, Cai Li, Thomas W Rosahl, Charles F Stevens, Thomas C Sudhof, Synaptotagmin I: a major Ca^{2+} sensor for transmitter release at a central synapse, p. 717-27. ©1994, with permission from Elsevier).

Fast, synchronous release of neurotransmitter occurs when multiple synaptic vesicles fuse within milliseconds of depolarization (AF 1.8, upper trace). The structure of the active zone provides optimal conditions to accomplish this task. Synaptotagmin 1/2 is a low-affinity Ca^{2+} sensor with fast binding/unbinding kinetics. Thus, saturated Ca^{2+} binding requires the exceptionally high [Ca^{2+}] that only occurs in nanodomains around open Ca^{2+} channels. Since this high [Ca^{2+}] drops off rapidly in time and space by diffusion [76-79], only synaptotagmin 1/2 located within such a nanodomain would be expected to trigger fusion; the rapid decrease in high [Ca^{2+}] ensures that the signal to fuse is terminated. Therefore, synaptotagmin's precise location relative to the presynaptic Ca^{2+} channels has profound effects on the release probability of the vesicle. As the SNAREs coil and pull the vesicle and plasma membranes together, not only does the membrane contact area increase, the distance between the Ca^{2+} channel and synaptotagmin is predicted to decrease [52]. According to this hypothesis, only the subpopulation of docked

synaptic vesicles that are maximally primed would situate synaptotagmin at its closest to Ca^{2+} channels. The closer synaptatogamin 1/2 is to the Ca^{2+} channel at the time of Ca^{2+} influx, the higher the [Ca^{2+}] that reaches its C_2B domains, favoring fusion.

Upon Ca^{2+} binding, the net charge of the Ca^{2+} binding pockets of synaptotagmin changes from negative to positive. This enhances interactions with the negatively charged presynaptic membrane and potentially with *trans*-SNARE complexes [83, 89, 116, 117]. Thus, Ca^{2+} binding allows synaptotagmin 1/2 to act as an "electrostatic switch" [118-121]. Switching from electrostatic repulsion to electrostatic attraction permits hydrophobic residues in the C_2 domains of synaptotagmin 1/2 to escape the hydrophilic cytosol by penetrating into the hydrophobic core of the presynaptic membrane. Therefore, synaptotagmin 1/2 can be thought of as providing the final push, much like popping a tightly-inflated balloon, to trigger fusion. This hypothesis can account for both the spatial and temporal restriction observed during fast, synchronous neurotransmitter release.

Asynchronous Release

Asynchronous release continues for several 100's of milliseconds up to a second (AF 1.8, lower trace). Asynchronous neurotransmitter release is observed in many neuronal types during and following extended high frequency activity [111]. Under these circumstances, the [Ca^{2+}] builds up since Ca^{2+} clearance is slower than Ca^{2+} entry. During the prolonged Ca^{2+} clearance phase, the residual [Ca^{2+}] is thought to drop below saturating binding levels for synaptotagmin 1/2 [109], but it remains high enough to activate a high-affinity Ca^{2+} sensor. While most synapses exhibit little to no asynchronous release following a single action potential,

asynchronous release is predominant in some specialized synapses [112-114]. Asynchronous release has been implicated in modulating postsynaptic responses [122].

The identity of the Ca^{2+} sensor(s) for asynchronous release is still debated. Like the fast, synchronous Ca^{2+} sensor, the asynchronous Ca^{2+} sensor needs be present at synapses and enriched at fusion sites. However, it must have a higher affinity for Ca^{2+} so that it can bind at lower [Ca^{2+}], permitting this protein to trigger release over a longer time course. Synaptotagmin 7 and Doc2 are current candidates for the Ca^{2+} sensor for triggering asynchronous neurotransmitter release, as they exhibit these characteristics [96, 97, 123].

V.d. SNARE and Vesicle Recycling

After a synaptic vesicle fuses, the SNARE complex is in a *cis* conformation since the vesicle membrane is incorporated into the presynaptic membrane. Cytosolic adaptor proteins specifically recognize and bind to *cis*-SNARE complexes and recruit N-ethylmaleimide-sensitive factor (NSF). NSF is an ATPase responsible for disassembling the SNARE complex [64]. This disassembly is required before the vSNAREs can be endocytosed for incorporation into new vesicles and before the tSNAREs can be reused in subsequent rounds of fusion events.

The synaptic vesicle membrane and all associated vesicle proteins must be rapidly recycled within nerve terminals to maintain the pool of vesicles available for fusion. There are multiple proposed mechanisms of synaptic vesicle recycling, but clathrin-mediated endocytosis (CME) is currently the best understood. CME was first described over 50 years ago [124] and occurs in many cell types (reviewed in [125]). It was first implicated in synaptic vesicle recycling in 1973 when it was shown to occur away from active zones following full collapse of the synaptic vesicle membrane into the presynaptic membrane (AF 1.2A,h) [124]. During CME,

a plethora of adaptor and accessory proteins facilitate appropriate membrane invagination and recruitment of clathrin to form spherical clathrin-coated membrane pits that project into the presynaptic cytoplasm [126]. The GTPase, dynamin, is then recruited to mediate a scission event that separates the clathrin-coated pit from the presynaptic membrane. The new vesicle is uncoated, targeted to endosomes or synapses, reloaded with neurotransmitter, and transported back to active zones. This completes the synaptic vesicle cycle, and can occur as fast as ~20-40 seconds [127].

Recently, a clathrin-independent endocytic mechanism was reported in both invertebrate [128] and vertebrate [129] neurons. This mechanism, called ultrafast endocytosis, occurs 200 times faster than CME. However, it must be stressed that this pathway still requires the action of clathrin for reformation of synaptic vesicles. The clathrin-independent bulk internalization of membrane produces large vesicles that form endosomes. The reformation of synaptic vesicles requires clathrin-mediated vesicle budding from these endosomes [130]. Another mechanism postulated to mediate vesicle recycling is known as “kiss and run”, where vesicles undergo a transient connection to the plasma membrane via a fusion pore, but the vesicle never collapses [131]. While this mechanism occurs in endocrine cells [132], its role in nerve terminals is questionable (reviewed in [133]).

VI. Synaptic Cleft

The synaptic cleft contains proteins and glycoproteins that support cell adhesion and regulate synaptic development and stability. Cleft proteins also clear neurotransmitter. The vast majority of proteins in the synaptic cleft provide structural support to the pre- and postsynaptic membranes from development through maturity. Many of these proteins are transmembrane cell

adhesion molecules (reviewed in [32]). Their cytosolic domains generally interact with intracellular synaptic proteins while their extracellular domains bind to transmembrane synaptic proteins of the opposing cell. Some extracellular and transmembrane synaptic proteins direct synaptogenesis, while others are important for neurotransmitter clearance, including enzymes that break them down and transporters that sequester them.

The protein machinery and organization varies by synapse type and location. A CNS synapse connects two neurons, and in some locations, it is functionally important for the strength of CNS synapses to be easily modified. At the neuromuscular junction, the postsynaptic cell is a muscle fiber where there is less need for rapid changes in synaptic strength. Nevertheless, many general processes are conserved across synapses. Machinery for synaptogenesis, scaffolding, and neurotransmitter clearance are universally required, but the individual molecular interactions will be synapse specific.

VI.a. Synaptogenesis

A specific role for an extracellular matrix molecule in regulating the formation and maintenance of synaptic connections has been best elucidated at the frog neuromuscular junction. Dr. U.J. McMahan and colleagues discovered that the synaptic basal lamina contains molecules that direct the formation of synaptic specializations in both regenerating nerve terminals [7] and regenerating muscle fibers [8]. They purified the basal lamina molecule responsible for directing postsynaptic specializations and named it agrin [19].

Agrin is a critical signal for synaptogenesis and synaptic maintenance [134, 135]. It is secreted from presynaptic nerve terminals and is stably incorporated into the synaptic basal lamina. It binds to and activates a receptor complex in the plasma membrane of the muscle fiber,

which then induces postsynaptic specializations [134]. This receptor complex is composed of the low-density lipoprotein receptor-related protein 4 (LRP4), which binds agrin, and a muscle-specific kinase (MuSK), which triggers a postsynaptic signaling cascade [136-138]. This cascade recruits acetylcholine receptors, acetylcholinesterase (AChE), and numerous other components of the postsynaptic apparatus to postsynaptic sites immediately opposite the nerve terminal [139]. Precise alignment is critical for the formation, organization, maintenance, and function of the neuromuscular junction.

CNS synaptogenesis is not well understood. However, the neuroligin and neurexin families of proteins may play a role. The neuroligins are postsynaptic cell adhesion molecules [140] that bind to the presynaptic cell adhesion molecules, the neurexins [141]. This heterotypic interaction across the cleft aggregates the machinery (both pre- and postsynaptic) necessary for a functional synapse [142, 143]. Neuroligin-1 is present exclusively in excitatory synapses [144], while neuroligin-2 is present exclusively in inhibitory synapses [145]. Knockdown of neuroligins [146] or interference with neuroligin-neurexin signaling [147] diminishes the number of fully developed synapses. This research has led to the hypothesis that in addition to offering the structural stability of cell adhesion molecules at developing synapses, the postsynaptic neuroligins and presynaptic neurexins are critical for aggregating synaptic components and assigning the valence of the synapse.

VI.b. Scaffolding

The stability of synaptic connections is tightly regulated based on the functional requirements of the particular synapse. Some synapses, like neuromuscular junctions, are relatively stable and provide reliable transmission for proper locomotion over long time periods.

While there is minor synaptic remodeling, the function at this synapse remains relatively constant in healthy individuals. Other synapses are short lived or exhibit synaptic strengths that vary widely within seconds. In the cerebral cortex, synaptic plasticity may strengthen some synapses in response to heavy activity while pruning others that are less active. Such alterations in synaptic strength are foundational to the mechanisms of learning and memory first posited by Donald Hebb [148]. Scaffolding molecules spanning the synaptic cleft can either maintain and support a synapse or promote retraction and weakening.

The cadherins are a family of transmembrane cell adhesion molecules (reviewed in [149]) present at most synapses. They are thought to provide critical support for synapse stability, particularly in dendritic spines [150, 151]. They are present both pre- and postsynaptically and bridge the synaptic cleft through homotypic binding interactions. Cadherins are generally concentrated around the edges of synapses [152]. This localization may form a diffusion barrier that helps sequester receptors and maintain the structural integrity of the synapse [153].

Other classes of transmembrane proteins have more varied affects. The Eph receptor tyrosine kinases and their binding partners, epherins, interact across the cleft through heterotypic interactions. Signals in both antero- and retrograde directions are postulated to promote remodeling of synaptic structure [154, 155]. Different subtypes of Eph receptors and epherins have alternate affects. Knockdown of EphB2 leads to decreased synapse number and loss of synaptic superstructure [156]. Therefore, EphB2 signaling encourages the stability of synapses [157]. Ephrin-A3 and EphA4 signaling, on the other hand, results in decreased synapse number and retraction of synaptic ultrastructure [158]. Thus, these scaffolding molecules are critical for both maintenance and alteration of existing synapses across time, and their trans-synaptic signaling is necessary during many neurological processes.

VI.c. Neurotransmitter Clearance

Removal and/or breakdown of neurotransmitter by cleft proteins is pivotal for regulating the temporal aspects of the signal strength between neurons. The dopamine transporter, a well-defined example of this role [159], is the primary regulator of dopamine concentration in the synaptic cleft in a key reward center in the brain. Inhibition of this transporter by cocaine prevents the removal of dopamine from the presynaptic cell into the cleft [160]. The resulting build up causes increased signaling to the postsynaptic cell and consequent over-activity of the reward circuitry, leading to addiction.

Enzymatic breakdown is the other way to clear neurotransmitter after release. A well-characterized example of this clearance mechanism is the enzyme AChE [161], which hydrolyzes ACh. The action of AChE is so fast that the rate-limiting step in the breakdown of ACh is diffusion of the transmitter. This rapid breakdown is critical for proper synaptic function as it regulates ACh concentration and thus the postsynaptic response. AChE inhibitors provide effective treatment for diseases involving deficits in ACh signaling. In myasthenia gravis, the immune system attacks ACh receptors such that normal ACh release from motor neurons cannot provide sufficient muscle stimulation. By slowing down the clearance of ACh, AChE inhibitors counteract the muscle's decreased ability to respond [162]. Presynaptic defects are similarly amenable to treatment. According to the cholinergic hypothesis of Alzheimer's disease [163], loss of cholinergic neurons may underlie some cognitive deficits. AChE inhibitors are again used to slow down clearance, resulting in an accumulation of the ACh released by the remaining cholinergic neurons. Thus, it is essential that proteins specific for clearance of each type of neurotransmitter(s) released are present in the synaptic cleft.

VII. Postsynaptic Function

The postsynaptic membrane is uniquely specialized to detect neurotransmitters and transduce this chemical signal back into an electrical signal. Binding of neurotransmitters to receptors can elicit either a fast, transient or slower, longer-lasting response which may activate or inhibit electrical signaling in the postsynaptic cell. The specific response depends on the neurotransmitter released as well as the postsynaptic receptor subtype(s) present. Ionotropic receptors are ion channels that open upon neurotransmitter binding. Metabotropic receptors are GPCRs that activate G-protein second messenger systems, leading to changes in ion channel opening. Activating either type of receptor can result in postsynaptic depolarization or hyperpolarization depending on the ion selectivity of the activated channels.

VII.a. Postsynaptic Receptors

Ionotropic Receptors

Ionotropic receptors are ligand-gated ion channels that form pores in the membrane allowing the passage of ions down their electrochemical gradients [164]. Ion channels are generally closed at rest. Binding of their specific neurotransmitter causes a conformational change, opening the pore and allowing ion influx. Early studies at the frog neuromuscular junction measured ACh-induced changes in ion conductances in muscle fibers demonstrating channel opening in response to a ligand [165]. This process is extremely rapid, allowing immediate ion influx that abruptly ceases when the ligand unbinds, and the channel closes. In addition, some channels inactivate, stopping ionic conductance even while a ligand remains bound. In this way, ionotropic channels generate fast and transient electrical signals in response to the presence of neurotransmitter.

Metabotropic Receptors

Metabotropic receptors have extracellular binding sites for specific neurotransmitters and bind to intracellular G-proteins [166]. Binding of neurotransmitter to these GPCRs causes activation of the intracellular G-protein, which dissociates from the receptor and may activate intracellular kinase cascades, phosphatases, and/or other second messengers, which then act on various downstream effectors, including opening of ion channels. The necessity of multiple protein interactions before the opening of an ion channel means that the time scale of metabotropic receptor effects will be much slower than those of ionotropic receptors. Second messenger cascades can also amplify the original signal. One neurotransmitter molecule can activate a G-protein, which can activate multiple kinases, which can activate multiple ion channels. In addition, the downstream second messengers may remain activated after the ligand comes unbound from the GPCR. Thus, the postsynaptic response generated by metabotropic receptors, though slower, tend to be larger and more persistent.

Second messenger systems can have additional wide-ranging affects independent of electrical changes in the postsynaptic cell. Some second messengers act as transcription factors, altering gene expression [167]. Others cause release of internal Ca^{2+} stores and lead to changes in synaptic strength [168]. The potential downstream effects of multiple, simultaneously activated signaling pathways can vary widely depending on the downstream machinery present in the postsynaptic cell.

VII.b. Generation of Postsynaptic Potentials

The postsynaptic membrane's ability to transduce the chemical signal into an electrical signal is dependent on the opening of ion channels (either ionotropic receptors, or downstream

effectors of GPCRs) that allow ions to travel down their electrochemical gradients. Thus, the ion selectivity of the activated channels determines the net potential generated in the postsynaptic cell. At a typical excitatory synapse, the open ion channels allow positively-charged ions, such as Na^+ and Ca^{2+}, to flow down their electrochemical gradients into the postsynaptic cell. The influx of positive charge depolarizes the post-synaptic cell, otherwise known as an excitatory postsynaptic potential (EPSP). If the activated channels are permeable to either Cl^- or K^+, the postsynaptic membrane will be hyperpolarized. The concentration gradient for each of these ions is great enough to drive them against the electrical gradient, so Cl^- will enter or K^+ will leave the cell, which drives the membrane potential to a more negative value. Such a hyperpolarization is called an inhibitory postsynaptic potential (IPSP).

The net change in membrane potential depends on the summation of all synaptic inputs. Mammalian neuromuscular junctions are exclusively excitatory. At these synapses, sufficient ACh is released to ensure that the postsynaptic muscle fiber is depolarized past threshold, resulting in muscle fiber contraction (AF 1.3B). In the CNS, all synaptic inputs to the postsynaptic neuron, which may include both EPSPs and IPSPs, are summated. If the postsynaptic neuron is sufficiently depolarized at its axon initial segment, its firing rate will increase. If the net effect at the axon initial segment is a hyperpolarization, its firing rate will decrease. Thus, it is the net activity that determines the overall response in the postsynaptic cell (reviewed in [169]).

VIII. Modulation of the Chemical Synapse

The response of a given postsynaptic cell to the same level of activity in a presynaptic cell can vary depending on the efficacy of the synapse. Synaptic efficacy is based on many

factors, including the quantal content (number of neurotransmitter-filled synaptic vesicles that fuse in response to a given stimulus, discussed in section IV.b) and the type and number of postsynaptic receptors. Importantly, these pre- and postsynaptic parameters can be regulated by activity patterns. The capacity to modulate synaptic efficacy is an important mechanism underlying learning and memory. These activity-dependent changes are known as synaptic plasticity.

Experimentally, synaptic plasticity is measured both pre- and postsynaptically. Presynaptically, the amount of neurotransmitter released can be decreased or increased, leading to depressed or facilitated responses, respectively. Specifically, the quantal content of a synapse (*m*) depends on the number of vesicles available to fuse (*n*), and the release probability of each vesicle (*p*) (section IV.b). The release probability is dependent on the magnitude and duration of Ca^{2+} influx, the speed of Ca^{2+} clearance, and the proximity of the Ca^{2+} sensor to the channel (section V.a). Higher quantal content causes larger responses in the postsynaptic cell, barring any postsynaptic adaptations. Synapses with a high quantal content result from some combination of a large readily releasable pool of vesicles, high vesicle release probability, high Ca^{2+} influx, and/or slow Ca^{2+} clearance. Alternatively, low quantal content synapses have the opposite characteristics and result in smaller postsynaptic responses.

Postsynaptically, changes in synaptic activity can lead to changes in the morphological makeup of the postsynaptic membrane through insertion or removal of receptors. Any change in receptor density results in a change in response strength to a given signal. Therefore, activity-dependent alteration of the postsynaptic machinery allows for flexible modulation of postsynaptic responses to neurotransmitter release.

VIII.a. Depression

Synaptic depression is defined as a reduction in the amplitude of the postsynaptic response following a given presynaptic stimulus. This can be observed at high quantal content synapses during paired pulse experiments, in which two closely spaced stimuli are administered to a cell [170, 171]. The first response, or EPSP, is robust, while the second response is reduced. At a high quantal content synapse, the first stimulation is sufficient to release a large fraction of the readily releasable vesicles. Accordingly, there are fewer vesicles available to fuse during the second stimulus, which results in a reduced EPSP. Another example of synaptic depression is seen during a high-frequency stimulus train to the presynaptic cell [172, 173]. Since exocytosis is more rapid than endocytosis, such stimulation depletes the readily releasable pool of synaptic vesicles, even at low quantal content synapses, and quantal content is decreased.

VIII.b. Facilitation

Facilitation is an incremental increase in the postsynaptic response to a given presynaptic stimulation observed in paired pulse experiments at low quantal content synapses. Since the first stimulus at such a synapse results in the fusion of only a small fraction of the readily releasable pool, the number of vesicles available for fusion following the second impulse is not significantly reduced. If the delay between the two stimuli is within tens of milliseconds, not all the Ca^{2+} that entered during the first pulse has been removed by the time of the second pulse. Therefore, the $[Ca^{2+}]$ experienced by vesicles during the second pulse is higher and triggers a greater number of synaptic vesicles to fuse. If the delay between stimuli is long enough to allow the Ca^{2+} from the first pulse to be removed, the second response is not facilitated. This is known

as the residual Ca^{2+} hypothesis [174-176], and this form of presynaptic facilitation is very short-lived.

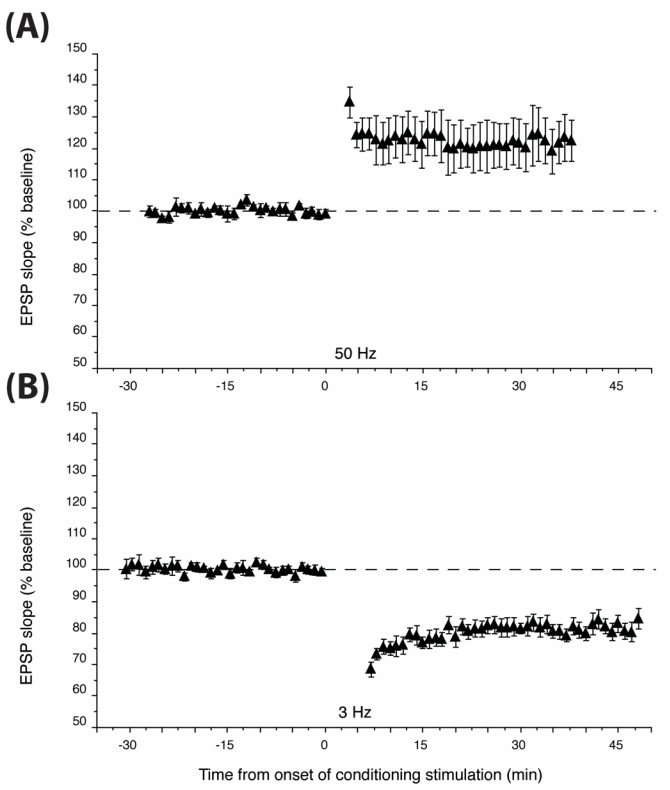
VIII.c. Long-term Potentiation

Long-term potentiation (LTP) is characterized by a long-lasting facilitated response in the postsynaptic cell following a brief tetanic stimulation of a strong input (AF 1.9A) or simultaneous stimulation of multiple weaker inputs. Since its discovery in the rabbit hippocampus in 1966 [4], the study of LTP has grown into an immense field. In the intervening 50 years, multiple forms of LTP have been detected in many different cells and brain regions. These changes can last from hours to days and rely on presynaptic and postsynaptic mechanisms (reviewed in [67, 177]). Here we describe a prototypic form of postsynaptic LTP, namely NMDA receptor-dependent LTP at excitatory synapses in the hippocampus (reviewed in [178]).

In this form of LTP, the postsynaptic neuron contains two classes of ionotropic glutamate receptors, AMPA receptors and NMDA receptors. NMDA receptors have extracellular Mg^{2+} bound in their pores at the resting membrane potential. Following a single stimulus, glutamate binds to both types of receptors. The AMPA receptors open, which allows Na^{+} influx and results in an EPSP. However, little to no current passes through the NMDA receptors due to the voltage-dependent Mg^{2+} block [179, 180].

During tetantic stimulation, the postsynaptic cell is sufficiently depolarized to remove the Mg^{2+} block from the NMDA receptor pore. With the block removed, glutamate binding results in a robust influx of Ca^{2+} through the NMDA receptor [181]. This is the essential step in the induction of LTP [182-184]. The increase in intracellular Ca^{2+} activates Ca^{2+}/calmodulin-dependent kinase II (CaMKII), resulting in the addition of more AMPA receptors in the

postsynaptic membrane [185-187]. This is an essential step in the expression of LTP [188-192], and AMPA receptor phosphorylation is likely involved [193-196]. The additional AMPA receptors may be locally inserted from intracellular pools and/or captured by the postsynaptic density from extrasynaptic receptors already in the membrane. While the precise mechanisms mediating the increase in AMPA receptors remain an active area of research, the presence of additional AMPA receptors in the postsynaptic membrane causes a given amount of glutamate release to elicit a potentiated response in the postsynaptic cell.



AF 1.9. Long-term potentiation and long-term depression. (A) Example of long-term potentiation induced by a brief high frequency stimulation (50 Hz for 900 pulses). (B) Example of long-term depression induced by a longer low frequency stimulation (3 Hz for 900 pulses). (Adapted from [197]).

VIII.d. Long-term Depression

Much like LTP, long-term depression (LTD) consists of multiple forms found in many different cell types and brain regions (reviewed in [198-200]). This chapter covers NMDA

receptor-dependent LTD in the hippocampus. LTD is a long-lasting, activity-dependent change in the efficacy of a synapse that results in a depressed postsynaptic response to a given presynaptic stimulus. It can be induced by prolonged low-frequency stimulation (AF 1.9B) [197]. As in LTP, this form of LTD requires both AMPA and NMDA receptors and is dependent on Ca^{2+} entry through unblocked NMDA receptors [197, 201-205]. Long duration, low-frequency synaptic stimulation results in a consistent, long-lasting, but smaller Ca^{2+} influx than the large, short-lived Ca^{2+} influx that causes LTP. This lower [Ca^{2+}] does not activate CaMKII [204]. Instead, it activates the phosphatases calcineurin and protein phosphatase 1 [66, 204, 206-208]. These phosphatases modify the phosphorylation state of AMPA receptors [196, 204, 209, 210] and tag them for internalization by clathrin and dynamin-dependent endocytosis [211-213]. The decrease in the number of AMPA receptors causes a depressed postsynaptic response. The structural changes in the number of postsynaptic AMPA receptors following either LTP or LTD induction allow the change in synaptic strength to last for a prolonged period of time.

WORKS CITED

1. Fatt, P. and B. Katz, *An analysis of the end-plate potential recorded with an intra-cellular electrode.* J Physiol, 1951. **115**(3): p. 320-70.
2. Fatt, P. and B. Katz, *Spontaneous subthreshold activity at motor nerve endings.* J Physiol, 1952. **117**(1): p. 109-28.
3. Del Castillo, J. and B. Katz, *Quantal components of the end-plate potential.* J Physiol, 1954. **124**(3): p. 560-73.
4. Lomo, T., *Frequency potentiation of excitatory synaptic activity in the dentate area of the hippocampal formation.* Acta Physiologica Scandinavia, 1966. **277**: p. 128.
5. Pinsker, H., et al., *Habituation and dishabituation of the gill-withdrawal reflex in Aplysia.* Science, 1970. **167**(3926): p. 1740-2.
6. Bliss, T.V. and T. Lomo, *Long-lasting potentiation of synaptic transmission in the dentate area of the anaesthetized rabbit following stimulation of the perforant path.* J Physiol, 1973. **232**(2): p. 331-56.
7. Sanes, J.R., L.M. Marshall, and U.J. McMahan, *Reinnervation of muscle fiber basal lamina after removal of myofibers. Differentiation of regenerating axons at original synaptic sites.* J Cell Biol, 1978. **78**(1): p. 176-98.
8. Burden, S.J., P.B. Sargent, and U.J. McMahan, *Acetylcholine receptors in regenerating muscle accumulate at original synaptic sites in the absence of the nerve.* J Cell Biol, 1979. **82**(2): p. 412-25.
9. Changeux, J.P., et al., *[Extraction from electric tissue of gymnotus of a protein presenting several typical properties characteristic of the physiological receptor of acetylcholine].* C R Acad Sci Hebd Seances Acad Sci D, 1970. **270**(23): p. 2864-7.
10. Takeuchi, A. and K. Onodera, *Effect of bicuculline on the GABA receptor of the crayfish neuromuscular junction.* Nat New Biol, 1972. **236**(63): p. 55-6.
11. Nickel, E. and L.T. Potter, *Ultrastructure of isolated membranes of Torpedo electric tissue.* Brain Res, 1973. **57**(2): p. 508-17.
12. Cartaud, J., et al., *Presence of a lattice structure in membrane fragments rich in nicotinic receptor protein from the electric organ of Torpedo marmorata.* FEBS Lett, 1973. **33**(1): p. 109-13.
13. Pfeiffer, F., D. Graham, and H. Betz, *Purification by affinity chromatography of the glycine receptor of rat spinal cord.* J Biol Chem, 1982. **257**(16): p. 9389-93.
14. Honore, T., J. Lauridsen, and P. Krogsgaard-Larsen, *The binding of [3H]AMPA, a structural analogue of glutamic acid, to rat brain membranes.* J Neurochem, 1982. **38**(1): p. 173-8.
15. Bradley, P.B., P.P. Humphrey, and R.H. Williams, *Evidence for the existence of 5-hydroxytryptamine receptors, which are not of the 5-HT2 type, mediating contraction of rabbit isolated basilar artery.* Br J Pharmacol, 1986. **87**(1): p. 3-4.
16. Hatta, K., T.S. Okada, and M. Takeichi, *A monoclonal antibody disrupting calcium-dependent cell-cell adhesion of brain tissues: possible role of its target antigen in animal pattern formation.* Proc Natl Acad Sci U S A, 1985. **82**(9): p. 2789-93.
17. Hirai, H., et al., *A novel putative tyrosine kinase receptor encoded by the eph gene.* Science, 1987. **238**(4834): p. 1717-20.

18. Magill, C., et al., *Agrin.* Prog Brain Res, 1987. **71**: p. 391-6.
19. Nitkin, R.M., et al., *Identification of agrin, a synaptic organizing protein from Torpedo electric organ.* J Cell Biol, 1987. **105**(6 Pt 1): p. 2471-8.
20. Ushkaryov, Y.A., et al., *Neurexins: synaptic cell surface proteins related to the alpha-latrotoxin receptor and laminin.* Science, 1992. **257**(5066): p. 50-6.
21. Bartley, T.D., et al., *B61 is a ligand for the ECK receptor protein-tyrosine kinase.* Nature, 1994. **368**(6471): p. 558-60.
22. Beckmann, M.P., et al., *Molecular characterization of a family of ligands for eph-related tyrosine kinase receptors.* Embo j, 1994. **13**(16): p. 3757-62.
23. Cheng, H.J. and J.G. Flanagan, *Identification and cloning of ELF-1, a developmentally expressed ligand for the Mek4 and Sek receptor tyrosine kinases.* Cell, 1994. **79**(1): p. 157-68.
24. Ichtchenko, K., et al., *Neuroligin 1: a splice site-specific ligand for beta-neurexins.* Cell, 1995. **81**(3): p. 435-43.
25. Block, M.R., et al., *Purification of an N-ethylmaleimide-sensitive protein catalyzing vesicular transport.* Proc Natl Acad Sci U S A, 1988. **85**(21): p. 7852-6.
26. Clary, D.O., I.C. Griff, and J.E. Rothman, *SNAPs, a family of NSF attachment proteins involved in intracellular membrane fusion in animals and yeast.* Cell, 1990. **61**(4): p. 709-21.
27. Clary, D.O. and J.E. Rothman, *Purification of three related peripheral membrane proteins needed for vesicular transport.* J Biol Chem, 1990. **265**(17): p. 10109-17.
28. Sollner, T., et al., *SNAP receptors implicated in vesicle targeting and fusion.* Nature, 1993. **362**(6418): p. 318-24.
29. Schiavo, G., et al., *Identification of the nerve terminal targets of botulinum neurotoxin serotypes A, D, and E.* J Biol Chem, 1993. **268**(32): p. 23784-7.
30. Couteaux R, P.-D.M., *Synaptic vesicles and pouches at the level of "active zones" of the neuromuscular junction.* C. R. Hebd. Seances Acad. Sci, 1970. **D271**: p. 2346-2349.
31. Hall, Z.W. and J.R. Sanes, *Synaptic structure and development: the neuromuscular junction.* Cell, 1993. **72 Suppl**: p. 99-121.
32. Missler, M., T.C. Sudhof, and T. Biederer, *Synaptic cell adhesion.* Cold Spring Harb Perspect Biol, 2012. **4**(4): p. a005694.
33. Specht, C.G. and A. Triller, *The dynamics of synaptic scaffolds.* Bioessays, 2008. **30**(11-12): p. 1062-74.
34. Sheng, M. and E. Kim, *The postsynaptic organization of synapses.* Cold Spring Harb Perspect Biol, 2011. **3**(12).
35. McMahon, H.T. and D.G. Nicholls, *The bioenergetics of neurotransmitter release.* Biochim Biophys Acta, 1991. **1059**(3): p. 243-64.
36. Marshansky, V. and M. Futai, *The V-type H+-ATPase in vesicular trafficking: targeting, regulation and function.* Curr Opin Cell Biol, 2008. **20**(4): p. 415-26.
37. Blakely, R.D. and R.H. Edwards, *Vesicular and Plasma Membrane Transporters for Neurotransmitters.* Cold Spring Harb Perspect Biol, 2012. **4**(2).
38. Sarter, M. and V. Parikh, *Choline transporters, cholinergic transmission and cognition.* Nat Rev Neurosci, 2005. **6**(1): p. 48-56.
39. Soreq, H., *Checks and balances on cholinergic signaling in brain and body function.* Trends Neurosci, 2015. **38**(7): p. 448-58.

40. Bak, L.K., A. Schousboe, and H.S. Waagepetersen, *The glutamate/GABA-glutamine cycle: aspects of transport, neurotransmitter homeostasis and ammonia transfer.* J Neurochem, 2006. **98**(3): p. 641-53.
41. Schousboe, A., et al., *Glutamate metabolism in the brain focusing on astrocytes.* Adv Neurobiol, 2014. **11**: p. 13-30.
42. Carlsson, A., *Perspectives on the discovery of central monoaminergic neurotransmission.* Annu Rev Neurosci, 1987. **10**: p. 19-40.
43. Ng, J., et al., *Monoamine neurotransmitter disorders--clinical advances and future perspectives.* Nat Rev Neurol, 2015. **11**(10): p. 567-84.
44. Abbracchio, M.P., et al., *Purinergic signalling in the nervous system: an overview.* Trends Neurosci, 2009. **32**(1): p. 19-29.
45. Burnstock, G., *Purinergic signalling: from discovery to current developments.* Exp Physiol, 2014. **99**(1): p. 16-34.
46. Boyd, I.A. and A.R. Martin, *The end-plate potential in mammalian muscle.* J Physiol, 1956. **132**(1): p. 74-91.
47. De Robertis, E.D. and H.S. Bennett, *Some features of the submicroscopic morphology of synapses in frog and earthworm.* J Biophys Biochem Cytol, 1955. **1**(1): p. 47-58.
48. Palay, S.L., *Synapses in the central nervous system.* J Biophys Biochem Cytol, 1956. **2**(4 Suppl): p. 193-202.
49. Bennett, M.R. and J.L. Kearns, *Statistics of transmitter release at nerve terminals.* Prog Neurobiol, 2000. **60**(6): p. 545-606.
50. Südhof, Thomas C., *The Presynaptic Active Zone.* Neuron, 2012. **75**: p. 11-25.
51. Nagwaney, S., et al., *Macromolecular connections of active zone material to docked synaptic vesicles and presynaptic membrane at neuromuscular junctions of mouse.* J Comp Neurol, 2009. **513**(5): p. 457-68.
52. Jung, J.H., et al., *Variable priming of a docked synaptic vesicle.* Proc Natl Acad Sci U S A, 2016. **113**(8): p. E1098-107.
53. Zhen, M. and Y. Jin, *Presynaptic terminal differentiation: transport and assembly.* Curr Opin Neurobiol, 2004. **14**(3): p. 280-7.
54. Schoch, S., et al., *RIM1α forms a protein scaffold for regulating neurotransmitter release at the active zone.* Nature, 2002. **415**: p. 321.
55. Spangler, S.A., et al., *Liprin-alpha2 promotes the presynaptic recruitment and turnover of RIM1/CASK to facilitate synaptic transmission.* J Cell Biol, 2013. **201**(6): p. 915-28.
56. Miller, K.E., et al., *Direct Observation Demonstrates that Liprin-α Is Required for Trafficking of Synaptic Vesicles.* Current Biology, 2005. **15**: p. 684-689.
57. Kaufmann, N., et al., *Drosophila Liprin-α and the Receptor Phosphatase Dlar Control Synapse Morphogenesis.* Neuron, 2002. **34**: p. 27-38.
58. Wagh, D.A., et al., *Bruchpilot, a protein with homology to ELKS/CAST, is required for structural integrity and function of synaptic active zones in Drosophila.* Neuron, 2006. **49**(6): p. 833-44.
59. Kittel, R.J., et al., *Bruchpilot promotes active zone assembly, Ca2+ channel clustering, and vesicle release.* Science, 2006. **312**(5776): p. 1051-4.
60. Lupas, A., *Prediction and analysis of coiled-coil structures.* Methods Enzymol, 1996. **266**: p. 513-25.

61. Sutton, R.B., et al., *Crystal structure of a SNARE complex involved in synaptic exocytosis at 2.4 A resolution.* Nature, 1998. **395**(6700): p. 347-53.
62. Dulubova, I., et al., *A conformational switch in syntaxin during exocytosis: role of munc18.* Embo j, 1999. **18**(16): p. 4372-82.
63. Yersin, A., et al., *Interactions between synaptic vesicle fusion proteins explored by atomic force microscopy.* Proc Natl Acad Sci U S A, 2003. **100**(15): p. 8736-41.
64. Sollner, T., et al., *A protein assembly-disassembly pathway in vitro that may correspond to sequential steps of synaptic vesicle docking, activation, and fusion.* Cell, 1993. **75**(3): p. 409-18.
65. Li, F., et al., *Energetics and dynamics of SNAREpin folding across lipid bilayers.* Nat Struct Mol Biol, 2007. **14**(10): p. 890-6.
66. Kameyama, K., et al., *Involvement of a postsynaptic protein kinase A substrate in the expression of homosynaptic long-term depression.* Neuron, 1998. **21**(5): p. 1163-75.
67. Castillo, P.E., *Presynaptic LTP and LTD of excitatory and inhibitory synapses.* Cold Spring Harb Perspect Biol, 2012. **4**(2).
68. K.M.S. Misura, R.H.S., W.I Weiss, *Three-dimensional structure of the neuronal Sec1-syntaxin 1a complex.* Nature, 2000. **404**(6776): p. 355-62.
69. Yang, B., et al., *nSec1 binds a closed conformation of syntaxin1A.* J Cell Biol, 2000. **148**(2): p. 247-52.
70. Dai, H., et al., *A quaternary SNARE-synaptotagmin-Ca2+-phospholipid complex in neurotransmitter release.* J Mol Biol, 2007. **367**(3): p. 848-63.
71. Dulubova, I., et al., *Munc18-1 binds directly to the neuronal SNARE complex.* Proceedings of the National Academy of Sciences, 2007. **104**(8): p. 2697-2702.
72. Heuser, J.E., et al., *Synaptic vesicle exocytosis captured by quick freezing and correlated with quantal transmitter release.* J Cell Biol, 1979. **81**(2): p. 275-300.
73. Harlow, M.L., et al., *The architecture of active zone material at the frog's neuromuscular junction.* Nature, 2001. **409**(6819): p. 479-84.
74. Eggermann, E., et al., *Nanodomain coupling between Ca(2)(+) channels and sensors of exocytosis at fast mammalian synapses.* Nat Rev Neurosci, 2011. **13**(1): p. 7-21.
75. Llinas, R., M. Sugimori, and R. Silver, *Microdomains of high calcium concentration in a presynaptic terminal.* Science, 1992. **256**(5057): p. 677-679.
76. Chad, J.E. and R. Eckert, *Calcium domains associated with individual channels can account for anomalous voltage relations of CA-dependent responses.* Biophys J, 1984. **45**(5): p. 993-9.
77. Fogelson, A.L. and R.S. Zucker, *Presynaptic calcium diffusion from various arrays of single channels. Implications for transmitter release and synaptic facilitation.* Biophys J, 1985. **48**(6): p. 1003-17.
78. Simon, S.M. and R.R. Llinas, *Compartmentalization of the submembrane calcium activity during calcium influx and its significance in transmitter release.* Biophys J, 1985. **48**(3): p. 485-98.
79. Naraghi, M. and E. Neher, *Linearized buffered Ca2+ diffusion in microdomains and its implications for calculation of [Ca2+] at the mouth of a calcium channel.* J Neurosci, 1997. **17**(18): p. 6961-73.
80. Shahrezaei, V. and K.R. Delaney, *Consequences of molecular-level Ca2+ channel and synaptic vesicle colocalization for the Ca2+ microdomain and neurotransmitter exocytosis: a monte carlo study.* Biophys J, 2004. **87**(4): p. 2352-64.

81. Ullrich, B., et al., *Functional properties of multiple synaptotagmins in brain.* Neuron, 1994. **13**(6): p. 1281-91.
82. Marqueze, B., et al., *Cellular localization of synaptotagmin I, II, and III mRNAs in the central nervous system and pituitary and adrenal glands of the rat.* J Neurosci, 1995. **15**(7 Pt 1): p. 4906-17.
83. Chapman, E.R., et al., *Ca2+ regulates the interaction between synaptotagmin and syntaxin 1.* J Biol Chem, 1995. **270**(40): p. 23667-71.
84. Lynch, K.L., et al., *Synaptotagmin C2A loop 2 mediates Ca2+-dependent SNARE interactions essential for Ca2+-triggered vesicle exocytosis.* Mol Biol Cell, 2007. **18**(12): p. 4957-68.
85. Vrljic, M., et al., *Molecular mechanism of the synaptotagmin-SNARE interaction in Ca2+-triggered vesicle fusion.* Nat Struct Mol Biol, 2010. **17**(3): p. 325-31.
86. Zhou, Q., et al., *Architecture of the synaptotagmin-SNARE machinery for neuronal exocytosis.* Nature, 2015. **525**(7567): p. 62-7.
87. Schupp, M., et al., *Interactions Between SNAP-25 and Synaptotagmin-1 Are Involved in Vesicle Priming, Clamping Spontaneous and Stimulating Evoked Neurotransmission.* J Neurosci, 2016. **36**(47): p. 11865-11880.
88. Wang, S., Y. Li, and C. Ma, *Synaptotagmin-1 C2B domain interacts simultaneously with SNAREs and membranes to promote membrane fusion.* Elife, 2016. **5**.
89. Bai, J., P. Wang, and E.R. Chapman, *C2A activates a cryptic Ca(2+)-triggered membrane penetration activity within the C2B domain of synaptotagmin I.* Proc Natl Acad Sci U S A, 2002. **99**(3): p. 1665-70.
90. Li, L., et al., *Phosphatidylinositol phosphates as co-activators of Ca2+ binding to C2 domains of synaptotagmin 1.* J Biol Chem, 2006. **281**(23): p. 15845-52.
91. Hui, E., et al., *Synaptotagmin-mediated bending of the target membrane is a critical step in Ca(2+)-regulated fusion.* Cell, 2009. **138**(4): p. 709-21.
92. Mackler, J.M., et al., *The C(2)B Ca(2+)-binding motif of synaptotagmin is required for synaptic transmission in vivo.* Nature, 2002. **418**(6895): p. 340-4.
93. Paddock, B.E., et al., *Ca2+-dependent, phospholipid-binding residues of synaptotagmin are critical for excitation-secretion coupling in vivo.* J Neurosci, 2008. **28**(30): p. 7458-66.
94. Paddock, B.E., et al., *Membrane penetration by synaptotagmin is required for coupling calcium binding to vesicle fusion in vivo.* J Neurosci, 2011. **31**(6): p. 2248-57.
95. Sugita, S., et al., *Synaptotagmin VII as a plasma membrane Ca(2+) sensor in exocytosis.* Neuron, 2001. **30**(2): p. 459-73.
96. Yao, J., et al., *Uncoupling the roles of synaptotagmin I during endo- and exocytosis of synaptic vesicles.* Nat Neurosci, 2011. **15**(2): p. 243-9.
97. Bacaj, T., et al., *Synaptotagmin-1 and synaptotagmin-7 trigger synchronous and asynchronous phases of neurotransmitter release.* Neuron, 2013. **80**(4): p. 947-59.
98. Mukherjee, K., et al., *Piccolo and bassoon maintain synaptic vesicle clustering without directly participating in vesicle exocytosis.* Proc Natl Acad Sci U S A, 2010. **107**(14): p. 6504-9.
99. Hallermann, S., et al., *Bassoon speeds vesicle reloading at a central excitatory synapse.* Neuron, 2010. **68**(4): p. 710-23.
100. Weimer, R.M., et al., *UNC-13 and UNC-10/Rim Localize Synaptic Vesicles to Specific Membrane Domains.* The Journal of Neuroscience, 2006. **26**(31): p. 8040-8047.

101. Dulubova, I., et al., *A Munc13/RIM/Rab3 tripartite complex: from priming to plasticity?* The EMBO Journal, 2005. **24**(16): p. 2839-2850.
102. Ma, C., et al., *Munc13 mediates the transition from the closed syntaxin–Munc18 complex to the SNARE complex.* Nature Structural &Amp; Molecular Biology, 2011. **18**: p. 542.
103. Stein, A., et al., *Helical extension of the neuronal SNARE complex into the membrane.* Nature, 2009. **460**(7254): p. 525-8.
104. Szule, J.A., et al., *Regulation of synaptic vesicle docking by different classes of macromolecules in active zone material.* PLoS One, 2012. **7**(3): p. e33333.
105. Melom, J.E., et al., *Spontaneous and Evoked Release Are Independently Regulated at Individual Active Zones.* The Journal of Neuroscience, 2013. **33**(44): p. 17253-17263.
106. Peled, E.S., Z.L. Newman, and E.Y. Isacoff, *Evoked and spontaneous transmission favored by distinct sets of synapses.* Curr Biol, 2014. **24**(5): p. 484-93.
107. Chung, C., et al., *Acute dynamin inhibition dissects synaptic vesicle recycling pathways that drive spontaneous and evoked neurotransmission.* J Neurosci, 2010. **30**(4): p. 1363-76.
108. Fawley, J.A., M.E. Hofmann, and M.C. Andresen, *Cannabinoid 1 and transient receptor potential vanilloid 1 receptors discretely modulate evoked glutamate separately from spontaneous glutamate transmission.* J Neurosci, 2014. **34**(24): p. 8324-32.
109. Goda, Y. and C.F. Stevens, *Two components of transmitter release at a central synapse.* Proc Natl Acad Sci U S A, 1994. **91**(26): p. 12942-6.
110. Atluri, P.P. and W.G. Regehr, *Delayed release of neurotransmitter from cerebellar granule cells.* J Neurosci, 1998. **18**(20): p. 8214-27.
111. Jensen, K., J.D. Lambert, and M.S. Jensen, *Tetanus-induced asynchronous GABA release in cultured hippocampal neurons.* Brain Res, 2000. **880**(1-2): p. 198-201.
112. Hefft, S. and P. Jonas, *Asynchronous GABA release generates long-lasting inhibition at a hippocampal interneuron-principal neuron synapse.* Nat Neurosci, 2005. **8**(10): p. 1319-28.
113. Daw, M.I., et al., *Asynchronous transmitter release from cholecystokinin-containing inhibitory interneurons is widespread and target-cell independent.* J Neurosci, 2009. **29**(36): p. 11112-22.
114. Best, A.R. and W.G. Regehr, *Inhibitory regulation of electrically coupled neurons in the inferior olive is mediated by asynchronous release of GABA.* Neuron, 2009. **62**(4): p. 555-65.
115. Geppert, M., et al., *Synaptotagmin I: a major Ca2+ sensor for transmitter release at a central synapse.* Cell, 1994. **79**(4): p. 717-27.
116. Chapman, E.R. and A.F. Davis, *Direct interaction of a Ca2+-binding loop of synaptotagmin with lipid bilayers.* J Biol Chem, 1998. **273**(22): p. 13995-4001.
117. Zhang, X., et al., *Ca2+-dependent synaptotagmin binding to SNAP-25 is essential for Ca2+-triggered exocytosis.* Neuron, 2002. **34**(4): p. 599-611.
118. Davletov, B., O. Perisic, and R.L. Williams, *Calcium-dependent membrane penetration is a hallmark of the C2 domain of cytosolic phospholipase A2 whereas the C2A domain of synaptotagmin binds membranes electrostatically.* J Biol Chem, 1998. **273**(30): p. 19093-6.
119. Ubach, J., et al., *Ca2+ binding to synaptotagmin: how many Ca2+ ions bind to the tip of a C2-domain?* Embo j, 1998. **17**(14): p. 3921-30.

120. Murray, D. and B. Honig, *Electrostatic control of the membrane targeting of C2 domains.* Mol Cell, 2002. **9**(1): p. 145-54.
121. Striegel, A.R., et al., *Calcium binding by synaptotagmin's C2A domain is an essential element of the electrostatic switch that triggers synchronous synaptic transmission.* J Neurosci, 2012. **32**(4): p. 1253-60.
122. Iremonger, K.J. and J.S. Bains, *Integration of asynchronously released quanta prolongs the postsynaptic spike window.* J Neurosci, 2007. **27**(25): p. 6684-91.
123. Hui, E., et al., *Three distinct kinetic groupings of the synaptotagmin family: candidate sensors for rapid and delayed exocytosis.* Proc Natl Acad Sci U S A, 2005. **102**(14): p. 5210-4.
124. Heuser, J.E. and T.S. Reese, *Evidence for recycling of synaptic vesicle membrane during transmitter release at the frog neuromuscular junction.* J Cell Biol, 1973. **57**(2): p. 315-44.
125. Kaksonen, M. and A. Roux, *Mechanisms of clathrin-mediated endocytosis.* Nat Rev Mol Cell Biol, 2018.
126. Reider, A. and B. Wendland, *Endocytic adaptors--social networking at the plasma membrane.* J Cell Sci, 2011. **124**(Pt 10): p. 1613-22.
127. Loerke, D., et al., *Cargo and dynamin regulate clathrin-coated pit maturation.* PLoS Biol, 2009. **7**(3): p. e57.
128. Watanabe, S., et al., *Ultrafast endocytosis at Caenorhabditis elegans neuromuscular junctions.* Elife, 2013. **2**: p. e00723.
129. Watanabe, S., et al., *Ultrafast endocytosis at mouse hippocampal synapses.* Nature, 2013. **504**(7479): p. 242-247.
130. Watanabe, S., et al., *Clathrin regenerates synaptic vesicles from endosomes.* Nature, 2014. **515**(7526): p. 228-33.
131. Ceccarelli, B., W.P. Hurlbut, and A. Mauro, *Depletion of vesicles from frog neuromuscular junctions by prolonged tetanic stimulation.* J Cell Biol, 1972. **54**(1): p. 30-8.
132. Neher, E. and A. Marty, *Discrete changes of cell membrane capacitance observed under conditions of enhanced secretion in bovine adrenal chromaffin cells.* Proc Natl Acad Sci U S A, 1982. **79**(21): p. 6712-6.
133. He, L. and L.G. Wu, *The debate on the kiss-and-run fusion at synapses.* Trends Neurosci, 2007. **30**(9): p. 447-55.
134. McMahan, U.J., *The agrin hypothesis.* Cold Spring Harb Symp Quant Biol, 1990. **55**: p. 407-18.
135. Tintignac, L.A., H.R. Brenner, and M.A. Ruegg, *Mechanisms Regulating Neuromuscular Junction Development and Function and Causes of Muscle Wasting.* Physiol Rev, 2015. **95**(3): p. 809-52.
136. Weatherbee, S.D., K.V. Anderson, and L.A. Niswander, *LDL-receptor-related protein 4 is crucial for formation of the neuromuscular junction.* Development, 2006. **133**(24): p. 4993-5000.
137. Zhang, B., et al., *LRP4 serves as a coreceptor of agrin.* Neuron, 2008. **60**(2): p. 285-97.
138. Kim, N., et al., *Lrp4 is a receptor for Agrin and forms a complex with MuSK.* Cell, 2008. **135**(2): p. 334-42.
139. Okada, K., et al., *The muscle protein Dok-7 is essential for neuromuscular synaptogenesis.* Science, 2006. **312**(5781): p. 1802-5.

140. Lise, M.F. and A. El-Husseini, *The neuroligin and neurexin families: from structure to function at the synapse.* Cell Mol Life Sci, 2006. **63**(16): p. 1833-49.
141. Missler, M. and T.C. Sudhof, *Neurexins: three genes and 1001 products.* Trends Genet, 1998. **14**(1): p. 20-6.
142. Scheiffele, P., et al., *Neuroligin expressed in nonneuronal cells triggers presynaptic development in contacting axons.* Cell, 2000. **101**(6): p. 657-69.
143. Graf, E.R., et al., *Neurexins induce differentiation of GABA and glutamate postsynaptic specializations via neuroligins.* Cell, 2004. **119**(7): p. 1013-26.
144. Song, J.Y., et al., *Neuroligin 1 is a postsynaptic cell-adhesion molecule of excitatory synapses.* Proc Natl Acad Sci U S A, 1999. **96**(3): p. 1100-5.
145. Varoqueaux, F., S. Jamain, and N. Brose, *Neuroligin 2 is exclusively localized to inhibitory synapses.* Eur J Cell Biol, 2004. **83**(9): p. 449-56.
146. Chih, B., H. Engelman, and P. Scheiffele, *Control of excitatory and inhibitory synapse formation by neuroligins.* Science, 2005. **307**(5713): p. 1324-8.
147. Levinson, J.N., et al., *Neuroligins mediate excitatory and inhibitory synapse formation: involvement of PSD-95 and neurexin-1beta in neuroligin-induced synaptic specificity.* J Biol Chem, 2005. **280**(17): p. 17312-9.
148. Hebb, D.O., *The Organization of Behavior*. 1949, New York: John Wiley and Sons, Inc.
149. Pokutta, S. and W.I. Weis, *Structure and mechanism of cadherins and catenins in cell-cell contacts.* Annu Rev Cell Dev Biol, 2007. **23**: p. 237-61.
150. Saglietti, L., et al., *Extracellular interactions between GluR2 and N-cadherin in spine regulation.* Neuron, 2007. **54**(3): p. 461-77.
151. Mendez, P., et al., *N-cadherin mediates plasticity-induced long-term spine stabilization.* J Cell Biol, 2010. **189**(3): p. 589-600.
152. Uchida, N., et al., *The catenin/cadherin adhesion system is localized in synaptic junctions bordering transmitter release zones.* J Cell Biol, 1996. **135**(3): p. 767-79.
153. Renner, M., et al., *Diffusion barriers constrain receptors at synapses.* PLoS One, 2012. **7**(8): p. e43032.
154. Arvanitis, D. and A. Davy, *Eph/ephrin signaling: networks.* Genes Dev, 2008. **22**(4): p. 416-29.
155. Lai, K.O. and N.Y. Ip, *Synapse development and plasticity: roles of ephrin/Eph receptor signaling.* Curr Opin Neurobiol, 2009. **19**(3): p. 275-83.
156. Henkemeyer, M., et al., *Multiple EphB receptor tyrosine kinases shape dendritic spines in the hippocampus.* J Cell Biol, 2003. **163**(6): p. 1313-26.
157. Kayser, M.S., et al., *Intracellular and trans-synaptic regulation of glutamatergic synaptogenesis by EphB receptors.* J Neurosci, 2006. **26**(47): p. 12152-64.
158. Murai, K.K., et al., *Control of hippocampal dendritic spine morphology through ephrin-A3/EphA4 signaling.* Nat Neurosci, 2003. **6**(2): p. 153-60.
159. Vaughan, R.A. and J.D. Foster, *Mechanisms of dopamine transporter regulation in normal and disease states.* Trends in Pharmacological Sciences. **34**(9): p. 489-496.
160. Huang, X., H.H. Gu, and C.G. Zhan, *Mechanism for Cocaine Blocking the Transport of Dopamine: Insights from Molecular Modeling and Dynamics Simulations.* J Phys Chem B, 2009. **113**(45): p. 15057-66.
161. Soreq, H. and S. Seidman, *Acetylcholinesterase--new roles for an old actor.* Nat Rev Neurosci, 2001. **2**(4): p. 294-302.

162. Pohanka, M., *Acetylcholinesterase inhibitors: a patent review (2008 - present).* Expert Opin Ther Pat, 2012. **22**(8): p. 871-86.
163. Francis, P.T., et al., *The cholinergic hypothesis of Alzheimer's disease: a review of progress.* Journal of Neurology, Neurosurgery & Psychiatry, 1999. **66**(2): p. 137-147.
164. Smart, T.G. and P. Paoletti, *Synaptic neurotransmitter-gated receptors.* Cold Spring Harb Perspect Biol, 2012. **4**(3).
165. Anderson, C.R. and C.F. Stevens, *Voltage clamp analysis of acetylcholine produced end-plate current fluctuations at frog neuromuscular junction.* J Physiol, 1973. **235**(3): p. 655-91.
166. Huang, Y. and A. Thathiah, *Regulation of neuronal communication by G protein-coupled receptors.* FEBS Lett, 2015. **589**(14): p. 1607-19.
167. Kvachnina, E., et al., *5-HT7 receptor is coupled to G alpha subunits of heterotrimeric G12-protein to regulate gene transcription and neuronal morphology.* J Neurosci, 2005. **25**(34): p. 7821-30.
168. Miyata, M., et al., *Local Calcium Release in Dendritic Spines Required for Long-Term Synaptic Depression.* Neuron, 2000. **28**(1): p. 233-244.
169. Blomfield, S., *Arithmetical operations performed by nerve cells.* Brain Res, 1974. **69**(1): p. 115-24.
170. Thomson, A.M., J. Deuchars, and D.C. West, *Large, deep layer pyramid-pyramid single axon EPSPs in slices of rat motor cortex display paired pulse and frequency-dependent depression, mediated presynaptically and self-facilitation, mediated postsynaptically.* J Neurophysiol, 1993. **70**(6): p. 2354-69.
171. Debanne, D., et al., *Paired-pulse facilitation and depression at unitary synapses in rat hippocampus: quantal fluctuation affects subsequent release.* J Physiol, 1996. **491 (Pt 1)**: p. 163-76.
172. Parnas, I., *Differential block at high frequency of branches of a single axon innervating two muscles.* J Neurophysiol, 1972. **35**(6): p. 903-14.
173. Parker, D., *Depression of synaptic connections between identified motor neurons in the locust.* J Neurophysiol, 1995. **74**(2): p. 529-38.
174. Katz, B. and R. Miledi, *THE EFFECT OF CALCIUM ON ACETYLCHOLINE RELEASE FROM MOTOR NERVE TERMINALS.* Proc R Soc Lond B Biol Sci, 1965. **161**: p. 496-503.
175. Katz, B. and R. Miledi, *The release of acetylcholine from nerve endings by graded electric pulses.* Proc R Soc Lond B Biol Sci, 1967. **167**(1006): p. 23-38.
176. Dodge, F.A., Jr. and R. Rahamimoff, *Co-operative action a calcium ions in transmitter release at the neuromuscular junction.* J Physiol, 1967. **193**(2): p. 419-32.
177. Malenka, R.C. and M.F. Bear, *LTP and LTD: an embarrassment of riches.* Neuron, 2004. **44**(1): p. 5-21.
178. Herring, B.E. and R.A. Nicoll, *Long-Term Potentiation: From CaMKII to AMPA Receptor Trafficking.* Annu Rev Physiol, 2016. **78**: p. 351-65.
179. Nowak, L., et al., *Magnesium gates glutamate-activated channels in mouse central neurones.* Nature, 1984. **307**(5950): p. 462-5.
180. Mayer, M.L., G.L. Westbrook, and P.B. Guthrie, *Voltage-dependent block by Mg2+ of NMDA responses in spinal cord neurones.* Nature, 1984. **309**(5965): p. 261-3.

181. Dingledine, R., *N-methyl aspartate activates voltage-dependent calcium conductance in rat hippocampal pyramidal cells.* J Physiol, 1983. **343**: p. 385-405.
182. Collingridge, G.L., S.J. Kehl, and H. McLennan, *Excitatory amino acids in synaptic transmission in the Schaffer collateral-commissural pathway of the rat hippocampus.* J Physiol, 1983. **334**: p. 33-46.
183. Wigstrom, H. and B. Gustafsson, *A possible correlate of the postsynaptic condition for long-lasting potentiation in the guinea pig hippocampus in vitro.* Neurosci Lett, 1984. **44**(3): p. 327-32.
184. Morris, R.G., et al., *Selective impairment of learning and blockade of long-term potentiation by an N-methyl-D-aspartate receptor antagonist, AP5.* Nature, 1986. **319**(6056): p. 774-6.
185. Liao, D., N.A. Hessler, and R. Malinow, *Activation of postsynaptically silent synapses during pairing-induced LTP in CA1 region of hippocampal slice.* Nature, 1995. **375**(6530): p. 400-4.
186. Durand, G.M., Y. Kovalchuk, and A. Konnerth, *Long-term potentiation and functional synapse induction in developing hippocampus.* Nature, 1996. **381**(6577): p. 71-5.
187. Shi, S.H., et al., *Rapid spine delivery and redistribution of AMPA receptors after synaptic NMDA receptor activation.* Science, 1999. **284**(5421): p. 1811-6.
188. Malinow, R., H. Schulman, and R.W. Tsien, *Inhibition of postsynaptic PKC or CaMKII blocks induction but not expression of LTP.* Science, 1989. **245**(4920): p. 862-6.
189. Malenka, R.C., et al., *An essential role for postsynaptic calmodulin and protein kinase activity in long-term potentiation.* Nature, 1989. **340**(6234): p. 554-7.
190. Silva, A.J., et al., *Alpha calcium/calmodulin kinase II mutant mice: deficient long-term potentiation and impaired spatial learning.* Cold Spring Harb Symp Quant Biol, 1992. **57**: p. 527-39.
191. Pettit, D.L., S. Perlman, and R. Malinow, *Potentiated transmission and prevention of further LTP by increased CaMKII activity in postsynaptic hippocampal slice neurons.* Science, 1994. **266**(5192): p. 1881-5.
192. Lledo, P.M., et al., *Calcium/calmodulin-dependent kinase II and long-term potentiation enhance synaptic transmission by the same mechanism.* Proc Natl Acad Sci U S A, 1995. **92**(24): p. 11175-9.
193. McGlade-McCulloh, E., et al., *Phosphorylation and regulation of glutamate receptors by calcium/calmodulin-dependent protein kinase II.* Nature, 1993. **362**(6421): p. 640-2.
194. Tan, S.E., R.J. Wenthold, and T.R. Soderling, *Phosphorylation of AMPA-type glutamate receptors by calcium/calmodulin-dependent protein kinase II and protein kinase C in cultured hippocampal neurons.* J Neurosci, 1994. **14**(3 Pt 1): p. 1123-9.
195. Barria, A., et al., *Regulatory phosphorylation of AMPA-type glutamate receptors by CaM-KII during long-term potentiation.* Science, 1997. **276**(5321): p. 2042-5.
196. Barria, A., V. Derkach, and T. Soderling, *Identification of the Ca2+/calmodulin-dependent protein kinase II regulatory phosphorylation site in the alpha-amino-3-hydroxyl-5-methyl-4-isoxazole-propionate-type glutamate receptor.* J Biol Chem, 1997. **272**(52): p. 32727-30.
197. Dudek, S.M. and M.F. Bear, *Homosynaptic long-term depression in area CA1 of hippocampus and effects of N-methyl-D-aspartate receptor blockade.* Proc Natl Acad Sci U S A, 1992. **89**(10): p. 4363-7.

198. Bellone, C., C. Luscher, and M. Mameli, *Mechanisms of synaptic depression triggered by metabotropic glutamate receptors.* Cell Mol Life Sci, 2008. **65**(18): p. 2913-23.
199. Gladding, C.M., S.M. Fitzjohn, and E. Molnar, *Metabotropic glutamate receptor-mediated long-term depression: molecular mechanisms.* Pharmacol Rev, 2009. **61**(4): p. 395-412.
200. Heifets, B.D. and P.E. Castillo, *Endocannabinoid signaling and long-term synaptic plasticity.* Annu Rev Physiol, 2009. **71**: p. 283-306.
201. Christie, B.R. and W.C. Abraham, *NMDA-dependent heterosynaptic long-term depression in the dentate gyrus of anaesthetized rats.* Synapse, 1992. **10**(1): p. 1-6.
202. Mulkey, R.M. and R.C. Malenka, *Mechanisms underlying induction of homosynaptic long-term depression in area CA1 of the hippocampus.* Neuron, 1992. **9**(5): p. 967-75.
203. Kandler, K., L.C. Katz, and J.A. Kauer, *Focal photolysis of caged glutamate produces long-term depression of hippocampal glutamate receptors.* Nat Neurosci, 1998. **1**(2): p. 119-23.
204. Lee, H.K., et al., *NMDA induces long-term synaptic depression and dephosphorylation of the GluR1 subunit of AMPA receptors in hippocampus.* Neuron, 1998. **21**(5): p. 1151-62.
205. Yang, S.N., Y.G. Tang, and R.S. Zucker, *Selective induction of LTP and LTD by postsynaptic [Ca2+]i elevation.* J Neurophysiol, 1999. **81**(2): p. 781-7.
206. Mulkey, R.M., C.E. Herron, and R.C. Malenka, *An essential role for protein phosphatases in hippocampal long-term depression.* Science, 1993. **261**(5124): p. 1051-5.
207. Mulkey, R.M., et al., *Involvement of a calcineurin/inhibitor-1 phosphatase cascade in hippocampal long-term depression.* Nature, 1994. **369**(6480): p. 486-8.
208. Kirkwood, A. and M.F. Bear, *Homosynaptic long-term depression in the visual cortex.* J Neurosci, 1994. **14**(5 Pt 2): p. 3404-12.
209. Roche, K.W., et al., *Characterization of multiple phosphorylation sites on the AMPA receptor GluR1 subunit.* Neuron, 1996. **16**(6): p. 1179-88.
210. Lee, H.K., et al., *Regulation of distinct AMPA receptor phosphorylation sites during bidirectional synaptic plasticity.* Nature, 2000. **405**(6789): p. 955-9.
211. Carroll, R.C., et al., *Rapid redistribution of glutamate receptors contributes to long-term depression in hippocampal cultures.* Nat Neurosci, 1999. **2**(5): p. 454-60.
212. Heynen, A.J., et al., *Bidirectional, activity-dependent regulation of glutamate receptors in the adult hippocampus in vivo.* Neuron, 2000. **28**(2): p. 527-36.
213. Man, H.Y., et al., *Regulation of AMPA receptor-mediated synaptic transmission by clathrin-dependent receptor internalization.* Neuron, 2000. **25**(3): p. 649-62.