EXAMINING DYNAMIC ASPECTS OF PRESYNAPTIC TERMINAL FORMATION VIA

LIVE CONFOCAL MICROSCOPY

By

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Examining Dynamic Aspects of Presynaptic Terminal Formation via Live Confocal Microscopy

Abstract

By

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To create a presynaptic terminal, molecular signaling events must be orchestrated within a number of subcellular compartments. In the soma, presynaptic proteins need to be synthesized, packaged together, and attached to microtubule motors for shipment through the axon. Within the axon, transport of presynaptic packages is regulated in order to ensure that developing synapses receive an adequate supply of components. At individual axonal sites, extracellular interactions must be translated into intracellular signals that can incorporate mobile transport vesicles into the nascent presynaptic terminal. Even once the initial recruitment process is complete, the components and subsequent functionality of presynaptic terminals need to constantly be remodeled. Perhaps most remarkably, all of these processes need to be coordinated in space and time. In this dissertation, I will discuss how these dynamic cellular processes occur in neurons of the central nervous system in order to generate presynaptic terminals in the brain, and describe experiments to further elucidate these mechanisms.
Chapter 1: General Introduction

Over 100 years ago, seminal work by Ramon y Cajal led to the development of the neuron doctrine, which states that the nervous system is comprised of individual cells (1, 2). Inspired by Cajal, Charles Sherrington proposed the existence of synapses that would allow communication between these cells (2, 3). From these early beginnings, it has become clear that the synaptic connections between neurons are indispensable for proper nervous system function and for life itself.

Synaptogenesis is important throughout life. Extensive synapse formation during neonatal and early postnatal development is responsible for initial establishment of the neural circuitry in the brain. However, synapse formation does not cease upon maturation. Synaptogenesis, in concert with synapse elimination, is a constant process that occurs throughout life as circuits in the brain are modified in response to varying external stimuli. The ability of the brain to constantly remodel its circuitry over time is critical in order to successfully adapt to and live within changing environments over the course of an organism’s life.

Studying how synapses form is necessary to understand both how the brain functions normally and how this goes awry in disease. Aberrations in synaptogenesis have been linked to a number of neurodevelopmental disorders, including autism and schizophrenia (4-7). In addition, poor synapse formation has been implicated in a wide variety of non-developmental neurological diseases, including addiction and depression (8-11). Furthermore, synapse loss is one of the hallmarks of neurodegenerative diseases such as Alzheimer’s disease, Parkinson’s disease, and Huntington’s disease (12-15). While it is not clear whether synapse formation is decreased or synapse elimination is
enhanced in these diseases (or both), enhancing synaptogenesis is an important therapeutic strategy to mitigate the effects of these devastating diseases (16).

**Basic synaptic physiology**

The basic purpose of a synapse in the brain is to enable transmission of electrical signals from one neuron to another. There are two types of synapses in the brain that accomplish this feat: electrical and chemical. Electrical synapses enable a direct connection between two neurons through the formation of gap junctions (17, 18). Gap junctions are specialized connections mediated by connexin proteins that result in a pore forming between two connected cells (19, 20). This pore not only physically links the cells, it also electrically links them by allowing rapid diffusion of ions from the cytoplasm in either direction across the junction. Therefore, unlike chemical synapses, electrical information can be transmitted by either cell to its connected partner. Because of this near-instantaneous bi-directional signaling capability, electrical synapses are often found between inhibitory neurons in the brain where they are utilized to synchronize inhibitory signals (17, 21).

While critical for normal nervous system function, electrical synapses make up only a small portion of synapses in the brain. Chemical synapses take the role as the major synaptic type in both the peripheral and central nervous systems. At chemical synapses, cells are physically separated by the synaptic cleft and electrical information is transferred between neurons via neurotransmitters. As action potentials reach presynaptic terminals, the resulting depolarization opens voltage-gated calcium channels within the presynaptic plasma membrane, leading to an influx of calcium. This, in turn, causes synaptic vesicles (SVs) loaded with neurotransmitter to fuse with the active zone
membrane, via exocytic SNARE proteins. As SVs fuse, neurotransmitters are released into the synaptic cleft, which stimulates the postsynaptic cell through the opening of neurotransmitter-gated ion channels and/or the activation of metabotropic neurotransmitter receptors. For more thorough reviews of presynaptic function please see the following (22-25).

The neurotransmitter and postsynaptic neurotransmitter receptors vary according to the neuronal cell type and location. In the brain, glutamate is the main excitatory neurotransmitter, while gamma-aminobutyric acid (GABA) functions as the main inhibitory neurotransmitter. However, many other neurotransmitters are produced and released in the brain, including dopamine, serotonin, acetylcholine, norepinephrine, ATP, cannabinoids, and various peptides (26-33). These other neurotransmitters can function in either an excitatory or inhibitory role, depending on the circuit they are utilized in and the type of receptor they activate.

Presynaptic distribution and composition

The location and number of presynaptic terminals within an axon varies. For motor neurons, presynaptic structures known as terminaux boutons are located at the distal end of the projecting axon where it makes contact with the muscle. In contrast, locally projecting neurons of the cerebral cortex typically form “en passant” presynaptic terminals along much of the length of unmyelinated axons. Strikingly, the number of presynaptic terminals per axon in the cortex can number in the thousands (34-36).

Recently, an elegant study showed that an “average” presynaptic terminal in the cortex or cerebellum is composed of approximately 300,000 individual molecules (37).
Although the number and diversity of proteins at the synapse is striking, many presynaptic proteins can be classified into two important groups: (i) synaptic vesicle (SV) associated proteins and (ii) proteins of the cytomatrix at the active zone (CAZ) (38, 39). SVs are 35-45nm spherical vesicles that fuse with the synaptic plasma membrane to release neurotransmitter (25, 40-43). SV-associated proteins control neurotransmitter uptake into SVs, trafficking of SVs within the synapse, and fusion of SVs with the plasma membrane. CAZ proteins are structural components of the presynaptic terminal, tethering SVs to the active zone and priming them for fusion with the plasma membrane. In addition, SNARE proteins within the CAZ interact with the v-SNARE synaptobrevin/VAMP2 on synaptic vesicles to enable SV fusion with the plasma membrane (44).

**STVs and PTVs – transport vesicles for efficient presynaptic protein delivery**

Building presynaptic terminals in the CNS poses unique challenges for the neuron. Most of the proteins that comprise the presynaptic side of the synapse are translated in the soma. Since some axons extend for millimeters, presynaptic components oftentimes need to be transported long distances from where they were made. Furthermore, presynaptic terminals form along an individual axon in an unsynchronized manner. This requires specific assembly of presynaptic proteins and structures at the right place at the right time.

To facilitate efficient delivery to developing synapses, groups of presynaptic components are sorted into precursor organelles (Figure 1.1). Specifically, SV proteins are incorporated into **Synaptic vesicle precursor protein Transport Vesicles** (STVs), while CAZ proteins are packaged into **Piccolo-bassoon Transport Vesicles** (PTVs) (45, 46).
STVs are tubulo-vesicular structures that are heterogeneous in size and shape (47-49). These golgi-derived vesicles can cycle with the axonal membrane and primarily contain proteins that are associated with SVs, including synapsin, synaptophysin, SV2, VGlut1, VAMP2, Rab3a, synaptotagmin and amphiphysin (Table 1) (47-58). PTVs primarily exist as 80nm dense-core vesicles (59, 60) that are derived from the trans-golgi network (61, 62), and they carry proteins of the CAZ, including piccolo, bassoon, syntaxin, RIM, Munc-18, ELKS2/CAST, SNAP-25, and n-cadherin (Table 1) (49, 59-62).
Table 1: STV and PTV proteins

<table>
<thead>
<tr>
<th>Synaptic proteins</th>
<th>Motors</th>
<th>Motor Linkage</th>
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<td><strong>STVs</strong></td>
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<tr>
<td>Synaptic vesicle proteins, including: synapsin, synaptophysin, SV2, VGlut1, VAMP2, Rab3a, synaptotagmin and amphiphysin</td>
<td>kinesin-3/KIF1A and KIF1B</td>
<td>syd-2/liprin-α</td>
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<tr>
<td></td>
<td>kinesin-1/KIF5</td>
<td>DENN/MADD, via Rab 3</td>
</tr>
<tr>
<td></td>
<td>dynein</td>
<td>?</td>
</tr>
<tr>
<td><strong>PTVs</strong></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Active zone cytomatrix proteins, including: piccolo, bassoon, syntaxin, RIM, Munc-18, ELKS2/CAST, SNAP-25, and n-cadherin</td>
<td>kinesin-1/KIF5</td>
<td>syntabulin, via syntaxin</td>
</tr>
<tr>
<td></td>
<td>Dynein</td>
<td>bassoon</td>
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Figure 1.1: Newly synthesized presynaptic proteins are packaged into STVs and PTVs and then transported along axonal microtubules for delivery to developing synapses. STVs and PTVs are derived from the trans-Golgi network. CAZ and SV-associated proteins are sorted into PTVs and STVs, respectively. STVs and PTVs then attach to kinesins and potentially dynein microtubule (MT) motors. Attachment can occur through direct binding of motors and their cargo or facilitated via a linking protein. STVs and PTVs are shipped through the axon on MTs. STV and PTV transport is coordinated, and these organelles can move together. Axonal MTs are arranged with their minus end toward the soma, suggesting that entry into the axon requires kinesin plus-end directed motors.
Motors

After STVs and PTVs are formed, they are tethered to microtubule (MT) motors and shipped through the axon (Figure 1.1). STVs and PTVs are associated with both kinesin and dynein motors, which move toward and away from MT plus ends, respectively (63). Since axonal MTs are oriented with their plus ends facing distally (64-67), kinesins drive vesicles anterogradely. STVs are transported via the kinesin-3/KIF1A and KIF1B motors and kinesin-1/KIF5, while PTVs are transported via kinesin-1/KIF5 (Table 1) (52, 68-79). Dynein drives both PTVs and STVs towards the soma (Table 1) (80-86). Because STVs and PTVs are likely linked to anterograde and retrograde motors simultaneously, they typically move in a bi-directional fashion (47, 48, 50, 53, 58, 60, 73, 87, 88). This movement is frequently interrupted by brief pauses. While the exact cause of these pauses is unknown, the sites in the axon where STVs pause are preferential sites of presynaptic terminal formation (51). Previously, it was not known whether STV and PTV transport is coordinated within the axon. The experiments described here in Chapter 2 attempt to determine the coordination of STV and PTV transport in order to identify a potential novel mechanism of rapid synapse formation via the instantaneous recruitment of SV and CAZ components to a developing synapse.

Linkage to motors

Both PTVs and STVs can attach to kinesins through linker proteins, and regulation of this interaction is critical for synapse development (Table 1). PTVs are primarily linked to KIF5B through syntabulin (73, 74). Syntabulin simultaneously binds to the KIF5B heavy chain and the CAZ protein syntaxin (74). Interrupting this interaction disrupts PTV transport in the axon and leads to a decrease in the number of functional
presynaptic terminals (73). A number of kinesin/cargo linking proteins enable transport of STVs. Invertebrate studies have identified syd-2/liprin-α as a link between STVs and kinesins (77, 89, 90). Disrupting this link leads to defective axonal transport and poor incorporation of presynaptic components into synapses (77, 89). In addition, the protein DENN/MADD (Differentially Expressed in Normal and Neoplastic cells/MAP kinase Activating Death Domain) mediates transport of STVs in mammalian neurons by simultaneously binding KIF1 and the STV-associated small GTPase Rab3 (91). DENN/MADD only binds to the GTP-associated form of Rab3 and can also function as a Rab3 GDP-GTP exchange factor (92-94). Consistent with a broader role for GTPases in regulation of the STV/KIF1 linkage and subsequent synapse formation, the GTPase Arl-8 is critical for proper presynaptic localization in C. elegans (95). Specifically, the GTP-bound form of Arl-8 directly interacts with UNC-104/KIF1A to ensure that aggregation of presynaptic components does not occur prematurely in proximal axonal sections (95, 96). Further work in C. elegans has revealed that the SV associated protein SAM-4 also interacts with the cargo-binding domain of unc-104/KIF1A and mediates STV transport (97). In addition, unc-104/KIF1A can directly bind to STVs through phosphatidylinositol-4,5-biphosphate (PI(4,5)P(2)), an integral lipid component of the STV membrane, and disrupting this interaction leads to aberrant STV trafficking (98, 99).

The physical links between presynaptic transport vesicles and dynein are less well characterized. Dynein consists of two large heavy chains that mediate ATP hydrolysis and MT binding. The heavy chains are dimerized by five different intermediate/light chains. These light chains also link dynein to adaptor complexes that mediate cargo binding (84, 100). One of these adaptor complexes, dynactin, links STVs to dynein, as
alterations to its p150Glued subunit leads to defective axonal transport of SV proteins (81, 85). In contrast, PTVs can directly interact with dynein light chains via bassoon, and disrupting this interaction leads to aberrant trafficking of PTVs and altered protein composition at synapses (83).

Additional mechanisms for regulation of STV and PTV transport

STV and PTV transport is regulated through a variety of dynamic mechanisms (Figure 1.2). For example, phosphorylation of kinesin reduces binding to STVs (75, 101). In addition, post-translational modification of MTs affects motor binding to MTs (102-105). Moreover, STV and PTV movement through the axon can be altered by physical impediments, such as reaching the end of a MT, binding of MT-associated proteins (MAPs), or a traffic jam caused by additional motors and cargo binding the same MT (106, 107). Neuronal activity can also affect axonal transport, possibly through calcium influx within the axon (51, 108). The number and types of motors on individual vesicles can vary as well, affecting transport (109). Finally, actin-based transport via myosins may contribute to trafficking of presynaptic components, especially within the axonal initial segment and at sites of synaptic protein recruitment (51, 103, 110-113).
Figure 1.2: Mechanisms of regulation of STV and PTV transport. Trafficking of STVs and PTVs within the axon can be modulated in a number of ways. Regulation of the following direct interactions between motor, cargo, and/or cytoskeletal elements can potentially alter STV and/or PTV transport: (1) kinesin attachment to MTs, (2) kinesin phosphorylation, (3) linker molecules between kinesin and cargo, (4) binding of myosin motors to cargo, (5) binding of myosin motors to actin and/or actin polymerization at specific sites in the axon, (6) direct physical interaction of kinesin or dynein with cargo, (7) dynein attachment to MTs. Indirect regulation involving a number of other factors might also affect STV and PTV trafficking: (1) motors and cargo reaching the ends of MTs, and/or switching to adjacent MTs, (2) MT-associated proteins (MAPs) that alter transport, (3) increased cytosolic Ca\(^{2+}\), via influx and/or intracellular sources, (4) traffic jams with other motors and cargo (synaptic or non-synaptic) on the same MT, (5) post-translational modifications of MTs, such as acetylation or de-tyrosination. It is not yet clear which of these mechanisms are employed to deposit synaptic material at sites of synapse formation.
For a presynaptic terminal to form, STVs and PTVs must be deposited at sites of axo-dendritic contact, requiring local cessation of transport. While many of the mechanisms described above regulate transport and are important for synapse formation, it remains unclear which of these mechanisms are used locally at sites of presynaptic terminal formation. Some of these mechanisms may contribute to synapse assembly by globally regulating transport within the axon, essentially controlling the supply of synaptic building materials. For instance, in *C. elegans*, disrupting axonal transport of presynaptic components leads to aberrant synapse formation (95, 96), and KIF1A is critical for synaptogenesis in the hippocampus (114). Conversely, other mechanisms may be engaged specifically at sites of synapse assembly in order to stop transport and deposit the building materials. It will be important to distinguish between these possibilities in the future. This will likely require local manipulation of each of these mechanisms at sites of presynaptic assembly combined with high-resolution time-lapse imaging.

**STV and PTV recruitment into developing synapses – a dynamic process**

Initial recruitment of presynaptic proteins into a developing synapse can be rapid. PTVs and STVs can be recruited to novel synaptogenic contacts within minutes (53, 115-117). STV and PTV co-recruitment suggests that a functional synapse can form within this time scale (118, 119). Indeed, both STVs and PTVs can instantly become trapped at sites of synaptogenic signaling as they are trafficked through the axon (88, 115, 116). Because STVs can cycle with the plasma membrane and likely release neurotransmitter prior to recruitment (47, 48, 50, 51, 54-58, 120), new sites of recruitment probably have at least some immediate functional capability. However, STV cycling is mechanistically and functionally different than SV cycling at mature synapses (54, 56). The time required
for functional maturation is unclear. Live imaging of developing synapses followed by retrospective electron microscopy indicated that the time required to accumulate a full complement of synaptic structures is variable and often takes hours (121, 122). In addition, it can take hours for axonal regions exposed to a postsynaptic synaptogenic signal to become saturated with newly formed synapses (88, 115).

Questions also remain about the order and potential coordination of STV and PTV recruitment. Since many CAZ proteins tether SVs at mature synapses, PTVs are typically thought to arrive at developing synapses first, where they can then recruit STVs (123). In support of this theory, the average time it takes for PTVs to be recruited to developing synapses tends to be shorter than the average time for STV recruitment, albeit within minutes of each other (116, 118). However, initial recruitment of STVs and PTVs has only been observed in separate cells (i.e. observing STV recruitment in one neuron and PTV recruitment in a different neuron). In both of the above studies, the time-course of recruitment for SV and CAZ proteins overlaps (116, 118). In addition, STVs and PTVs can co-localize with each other within the axon, suggesting that an entire complement of synaptic proteins might be recruited to a developing synapse simultaneously (49, 87). Furthermore, clustering of the trans-synaptic adhesion protein neurexin (see below) within the axon leads to the formation of presynaptic terminals (124, 125). Finally, live imaging of STVs and PTVs in separate axons of zebrafish embryos, followed by retrospective immunocytochemistry identified STVs as the first transport vesicle that arrives at the synapse (126). Therefore, live time-lapse imaging of both STVs and PTVs within the same axon is needed to fully understand this critical event in the formation of a presynaptic terminal.
Sites of Preferential Synapse Formation

Synapses form at discrete sites within neurons and are restricted in their overall size (41, 127). Even when synaptogenic signaling is initiated in a continuous axonal area tens of μm in length, presynaptic terminals appear at only certain points within this region (51, 88, 115, 128, 129). In addition, sites in the axon where STVs and PTVs pause are sites of preferential synapse development (51, 87). Pausing at these preferential sites might act as a mechanism for localizing presynaptic proteins to these areas, allowing rapid incorporation of STVs and PTVs into the developing synapse because these components are already present at the site once synaptogenic signaling commences. However, this hypothesis needs to be tested directly. Many dendritic filopodia make transient contact with neighboring axons, with only a portion of axo-dendritic contacts becoming stabilized (130-135). Therefore, dendritic filopodia may sample the axon to find the suitable synaptic sites.

The molecular mechanisms responsible for creating preferred sites of synapse formation within the axon are unknown. One possibility is that axonal synaptogenic adhesion proteins are involved. For example, distinct patterns or states of neurexin oligomerization might identify preferred sites of synapse formation. However, the interaction of neurexin with neuroligin is likely not required for initial formation of these sites, as they can be identified prior to axo-dendritic contact (51). Future studies directed at uncovering the molecular components responsible for these preferential sites will be critical to understand the initial processes behind presynaptic terminal formation.

Synaptogenic signaling through trans-synaptic adhesion proteins
For a presynaptic terminal to form, STVs and PTVs must be recruited to a specific site in the axon in response to a synaptogenic signal. This process is triggered by physical contact between two cells – in the brain, typically between an axon and dendrite. When axo-dendritic contact is established, binding occurs in trans between the extracellular domains of adhesion proteins that are localized to axons and dendrites. This, in turn, leads to synaptogenic signaling within the axon and dendrite, subsequent recruitment of synaptic components to both sides of the contact, and eventual formation of a functional synapse (136).

A number of synaptogenic trans-synaptic adhesion interactions have been identified (Table 2). Differential expression of these proteins between neuronal populations may help organize brain circuitry by controlling the specificity of synapse formation (136-138). For example, two isoforms of neuroligin - neuroligin-1 and neuroligin-2 - are specifically localized to excitatory and inhibitory synapses, respectively (139, 140). This specificity appears to be driven by the axon, arising from the interaction preferences of presynaptic neurexins found in excitatory and inhibitory axons (141, 142). Likewise, isoforms of SynCAM are differentially expressed in regions of the brain and display specific binding patterns between isoforms (143, 144). Binding specificity can be mediated by alternative splicing. The neuroligin-neurexin interaction is extensively regulated through this mechanism, and different binding pairs exhibit different functional properties, including differences in the rates of presynaptic terminal formation (115, 145-152).

Trans-synaptic adhesion proteins also display redundant functions during synapse formation. Mice lacking neuroligin-1, 2, and 3 have a normal number of synapses (153).
Likewise, synapse number is normal in mice lacking all isoforms of the α-neurexin gene (154). Although synaptic function is altered in these models, the ability to form synapses remains intact. However, the relative expression of neuroligin between neighboring cells in the brain does regulate synapse number (155). This suggests that the neuroligin/neurexin interaction is important for synapse development in a competitive, non-cell-autonomous manner. In contrast, increasing or knocking out SynCAM increases or decreases hippocampal synapse number, respectively (156). While alterations in synapse number point to a synaptogenic function of SynCAM, the magnitudes of the changes were approximately 10-20% (156), illustrating that other synaptogenic signals are involved in this process. In fact, there is no individual molecule that is absolutely critical for synapse formation in the brain. Therefore, determining how the multitude of trans-synaptic adhesion signals cooperate to control synapse formation will be critical for a full understanding of synapse and circuit formation in the brain.
Table 2: Trans-synaptic adhesion partners

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<td>Netrin-G Ligands (NGL-1,2,3)</td>
<td>(176-178)</td>
</tr>
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Signaling downstream of trans-synaptic adhesion

The downstream signaling pathways generated through trans-synaptic adhesion are less clear, especially presynaptically. The interaction between neurexin and neuroligin is the best characterized of the trans-synaptic proteins (Figure 1.3), and many of the presynaptic signaling mechanisms that act down-stream of neurexin are likely to be involved in synapse assembly induced by other trans-synaptic interactions.

The MAGUK protein CASK can bind to neurexin (179). CASK, in turn, binds to syndecan-2 and protein 4.1 (180, 181). Synaptic proteins might be recruited to this complex either through actin-mediated recruitment via filamentous-actin (F-actin) nucleation on protein 4.1 or through phosphorylation of various substrates by CASK itself (182, 183). Mint1 also binds to neurexin and can link neurexin, CASK, Veli/MALS, and the active zone protein Munc-18 into a single complex (184, 185). This CASK/Mint1/Veli complex may also play a role in SynCAM and APP mediated presynaptic development (129, 162, 186). Furthermore, the tandem-PDZ protein syntenin can directly interact with neurexin and is also linked to the CASK/Mint2/Veli complex through syndecan-2 (187, 188). Syntenin also interacts with the active zone protein CAST1/ERC2/ELKS via its PDZ domain, which in turn, binds RIM1, liprin-α, bassoon, and piccolo (188-192). Piccolo facilitates F-actin formation, which can lead to recruitment of additional synaptic components (193), while bassoon interacts directly with dynein to regulate recruitment of CAZ components (83).

Liprin-α interacts with a number of these complexes, and disrupting this interaction leads to aberrant synapse formation (194). Liprin-α (syd-2 in *C. elegans*) is
involved in active zone morphology and recruitment of active zone proteins (195-201). Interestingly, mutations that inhibit liprin-α binding to the CASK/Mint1/Veli complex have been linked to X-linked mental retardation (202). Because liprin-α also links STVs to KIF1, these interactions might be critical for initial recruitment of STVs into the synapse (77, 89, 97, 203). Liprin-α acts downstream of syd-1 during presynaptic development in *C. elegans* (196, 197). In *Drosophila*, Syd-1 binds to neurexin through its PDZ domain and mediates neurexin clustering within the axon (125), which appears to be necessary for neurexin’s synaptogenic function (88, 124, 204, 205). Recently, mammalian syd-1 was shown to organize synapses through binding with other active zone proteins, including liprin-α2 and munc18-1 (206). However, it is unclear if mammalian syd-1 binds to neurexin, as the PDZ domain that is critical for neurexin/syd-1 binding in Drosophila is absent in mammals.
Figure 1.3: Recruitment of presynaptic cargo downstream of neurexin-dependent synaptogenic adhesion. Neurexin interacts with a variety of presynaptic proteins; however, the mechanisms through which STVs and PTVs are incorporated into the
synapse remain unclear. This figure illustrates potential mechanisms that might be important for this process. (1) Bassoon directly interacts with dynein light chains 1 and 2, as well as myosin V. Blocking the bassoon/dynein interaction leads to aberrant bassoon and piccolo synaptic localization. (2) The PDZ-domains of Mint1, Veli/MALS, or CASK might interact with other synaptic proteins in STVs or PTVs in order to trap them at a developing synapse. (3) F-actin nucleation mediated by Protein 4.1 might enable the removal of STVs and/or PTVs from their MT highways via myosin motor binding. (4) Liprin-α can serve as a linker molecule between STVs and the KIF1 motor. Delivery of liprin-α to the synapse might serve to cluster and/or recruit other synaptic components. Alternatively, modulation of liprin-α to reduce its binding to KIF1 might play a role in cargo delivery. (5) CASK can directly phosphorylate substrates at presynaptic terminals. Therefore, it might phosphorylate a motor-binding protein integral to STVs or PTVs, a motor-cargo adaptor or the motor itself, which has been shown to reduce binding between kinesins and STVs. (6) Although neurexin interacts with a plethora of cytosolic targets, its cytoplasmic domain is completely dispensable for many of its synaptogenic properties. This suggests that cis interactions between the extracellular domain of neurexin and a yet-to-be-determined trans-membrane effector molecule(s) can induce recruitment of presynaptic proteins. The number and type of downstream recruitment mechanisms mediated by this potential interaction is unknown.
Many additional aspects of trans-synaptic signaling remain unclear. For example, trans-synaptic signaling does not affect all STVs and PTVs equally: many vesicles can pass directly through regions of trans-synaptic adhesion without becoming recruited into a synapse (88, 96). It was also previously not known whether STVs and PTVs were actively transported to sites of synaptogenic signaling or passively trapped at these sites. Furthermore it is currently unknown how trans-synaptic adhesion causes STVs and PTVs to dissociate from their MT motors and become incorporated into the synapse. Identifying these mechanisms and determining how they are coordinated during synaptogenesis will be critical to understanding how presynaptic terminals form. To that extent, experiments described here in Chapters 3 and 4 attempt to elucidate some of these mechanisms.

In this thesis, a number of major issues concerning presynaptic development will be addressed. In Chapter 2, the trafficking of two transport vesicles carrying distinct sets of presynaptic proteins was found to be coordinated prior to synapse formation, uncovering a potential mechanism for rapid recruitment of components into a developing synapse. The experiments in Chapter 3 determine the dynamics through which presynaptic proteins arrive at developing synapses and describe potential mechanisms through which these components become recruited. Finally, in Chapter 4, \textit{in vivo} imaging via two-photon microscopy through cranial windows is utilized to determine the dynamics of presynaptic terminals in the developing mouse brain.
Chapter 2: Coordinated trafficking of synaptic vesicle and active zone proteins prior to synapse formation

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Abstract

The proteins required for synaptic transmission are rapidly assembled at nascent synapses, but the mechanisms through which these proteins are delivered to developing presynaptic terminals are not understood. Prior to synapse formation, active zone proteins and synaptic vesicle proteins are transported along axons in distinct organelles referred to as piccolo-bassoon transport vesicles (PTVs) and synaptic vesicle protein transport vesicles (STVs), respectively. Although both PTVs and STVs are recruited to the same site in the axon, often within minutes of axo-dendritic contact, it is not known whether or how PTV and STV trafficking is coordinated before synapse formation. Here, using time-lapse confocal imaging of the dynamics of PTVs and STVs in the same axon, we show that vesicle trafficking is coordinated through at least two mechanisms. First, a significant proportion of STVs and PTVs are transported together before forming a stable terminal. Second, individual PTVs and STVs share pause sites within the axon. Importantly, for both STVs and PTVs, encountering the other type of vesicle increases their propensity to pause. To determine if PTV-STV interactions are important for pausing, PTV density was reduced in axons by expression of a dominant negative construct corresponding to the syntaxin binding domain of syntabulin, which links PTVs with their KIF5B motor. This reduction in PTVs had a minimal effect on STV pausing and movement, suggesting that an interaction between STVs and PTVs is not responsible for enhancing STV pausing. Our results indicate that trafficking of STVs and PTVs is coordinated even prior to synapse development. This novel coordination of transport and pausing might provide mechanisms through which all of the components of a presynaptic terminal can be rapidly accumulated at sites of synapse formation.
Introduction

In the cortex, the bulk of synapse formation occurs at high rates over a period of several weeks during early postnatal development. Formation of individual synapses is triggered when axons and dendrites contact one another (45, 46). For these axo-dendritic contacts to stabilize and form a synapse, synaptic vesicle and active zone proteins must be recruited to the site of contact very rapidly, within minutes to hours (51, 53, 115, 117, 118, 207-209). This rapid assembly is remarkable, considering that each presynaptic protein needs to be transported from the soma to individual sites of synapse formation along the axon.

Recent work using live imaging of developing neurons has revealed mechanisms that might facilitate rapid synapse assembly. For example, rather than transport each molecule individually, neurons package multiple presynaptic components into vesicles in the cell body and transport the entire group together. Two major groups of proteins are transported in this fashion: active zone proteins are transported in piccolo-bassoon transport vesicles (PTVs), while synaptic vesicle-associated proteins are transported in synaptic vesicle protein transport vesicles (STVs) (47-51, 53, 59, 60, 210). PTVs and STVs can be distinguished both morphologically and biochemically. PTVs have been described as dense-core vesicles or aggregates of vesicles and proteins that range in size from approximately 80nm in diameter for dense core vesicles to 130nm by 220nm in area for aggregates (49, 59). Proteins transported by PTVs include piccolo, bassoon, N-cadherin and syntaxin (49, 59, 60, 73, 74). STVs are heterogeneous in size and shape and are comprised of both tubulovesicular and clear core vesicles (47, 48, 53). Proteins carried by STVs include synaptophysin, synapsin Ia, synaptotagmin and
synaptobrevin-vesicle-associated membrane protein 2 (VAMP2) (47-49, 51, 53). Both types of vesicles are packaged via the trans-Golgi network before being transported through the axon (48, 49, 61).

Rapid assembly of presynaptic terminals can also be facilitated by having the source of synaptic proteins nearby and readily available. Indeed, many STVs and PTVs are mobile within axons well before synapse formation. STVs and PTVs travel in a saltatory fashion in both the anterograde and retrograde directions along the entire length of the axon [4, 8, 11, 13-15, 17, 19]. This provides a readily-available pool of synaptic vesicle and active zone proteins wherever and whenever axo-dendritic contact occurs.

Since both STVs and PTVs must be delivered to the same site during synapse assembly, we hypothesized that trafficking of STVs and PTVs might be coordinated even prior to synapse assembly, providing an additional mechanism to facilitate rapid accumulation of the full complement of proteins required for synaptic transmission. Such coordination could arise through co-transport of STVs and PTVs, through pausing of STVs and PTVs at the same sites, or both. Coordinated pausing presents a particularly intriguing possibility since sites along the axon where STVs pause their transport are preferential sites of synapse formation (51). It was previously suggested that cues that control pausing at these sites could promote rapid synapse assembly by increasing the probability that STVs are at or near any given site when axo-dendritic contact occurs. If this model is correct, then synapse assembly would be most efficient if PTVs are also attracted to these same sites. However, it is not yet known whether PTVs also pause at these sites and, if so, whether they do so simultaneous with STVs. A recent report demonstrated that PTVs and STVs (particularly those resembling small, clear vesicles)
can be observed tethered together in electron micrographs of developing axons (49). These aggregates could correspond to PTVs and STVs that are either being co-transported or pausing at the same site at the same time.

Here, we tested whether transport and pausing of PTVs and STVs is coordinated prior to synapse assembly using time-lapse confocal imaging of green and red fluorescent protein-tagged synaptic vesicle and active zone proteins within the same axon. We found that a significant portion of STVs and PTVs move together and share pause sites. Interestingly, both STVs and PTVs preferentially paused at these sites when another vesicle was present. These observations raised the question of whether a direct interaction between STVs and PTVs coordinates their transport and pausing. Reducing PTVs in the axon minimally affected the movement and pausing of STVs, arguing against this mechanism and suggesting that other unidentified signals are responsible for STV and PTV coordination. These findings represent novel mechanisms that can facilitate the rapid recruitment of presynaptic proteins to the same site within the axon and, therefore, promote synapse development.
Results

STVs and PTVs can move together

STVs and PTVs move in a similar saltatory fashion within the axon in both the anterograde and retrograde directions (47, 48, 51, 53, 58, 60, 73). However, because STVs and PTVs have almost always been imaged separately, it is not yet known if and how STV and PTV trafficking interrelate. To determine this, we used time-lapse imaging of neurons co-transfected with fluorescent STV and PTV markers. In this assay, 5-6 DIV rat cortical neurons were co-transfected with GFP-bassoon to label PTVs and either synaptophysin-mcherry or synaptophysin-mRFP to label STVs. Previous studies have indicated that fluorescent proteins attached to bassoon and synaptophysin effectively label PTVs and STVs, respectively (48, 50, 53, 60, 61, 115, 210, 211). 1-2 days after transfection, time-lapse images of the fluorescently labeled vesicles were obtained from individual axons. Images were taken every 10s for 7.5 minutes.

From the kymographs in Figure 2.1A, it is clear that PTVs and STVs moved together in both anterograde and retrograde directions. To quantify these correlated movements, the positions of individual STV and PTV puncta were tracked independently while blind to the other channel, and then their movements were compared. To determine whether these correlated movements could be accounted for by chance, the experimental data were ultimately compared to model axons where the initial positions of all of the puncta were randomized but the density of puncta and properties of their movements were derived from the experimental data (Figure 2.1B).
During the imaging period, 18.3% of PTVs moved with STVs (n = 224 vesicles), while 37.1% of STVs moved with PTVs (n = 97 vesicles; Figure 2.1C). STVs and PTVs that moved together also spent the majority of their time together (Figure 2.1D). The prevalence of correlated STV and PTV movements was over 10-fold higher for the experimental data than predicted by the same analysis of model axons (Figure 2.1C; model: n = 22400 PTVs and 9700 STVs). In addition, the percentage of time STVs and PTVs spent moving together was over 2.5-fold higher expected by chance (Figure 2.1D). These data indicate that a population of STVs and PTVs travel together within the axon, which could allow both sets of presynaptic proteins to be distributed together to sites of synapse formation.
Figure 2.1: STVs and PTVs move together. (A) Kymographs of an axon segment showing the movements of PTVs (GFP-bassoon; green) and STVs (synaptophysin-mcherry; magenta) in the same axon. Yellow lines underline the moving STVs and PTVs. Bottom panel, overlay of STV and PTV fluorescence. On the ordinate axis, one pixel corresponds to 10s. On the abscissa, the scale bar corresponds to 10µm. (B) Simulations of model axons were performed by randomizing initial positions of vesicles while maintaining movement and pausing characteristics of the original imaged vesicles. Diagrams illustrate kymographs of 3 simulations of model axons. Movements of two
vesicles are shown in each model axon. (C) Plot showing the percentage of PTVs that move with STVs (green) and STVs that move with PTVs (magenta). PTV and STV simulations (light green and magenta, respectively) correspond to the predicted values from the simulations. (D) Quantification of the percentage of time that PTVs and STVs spent together. Both the percentage of vesicles that moved together and the time spent together were substantially larger in the data than is predicted by chance (simulations).
**STVs and PTVs display similar pausing characteristics**

Although the movements of both STVs and PTVs have been extensively described individually, the pauses between movements have been less thoroughly analyzed and have not been compared. Comparing STV and PTV pausing behavior is important since STVs preferentially pause at sites of eventual synapse formation (51, 53). When PTV behavior was examined, it was clear that PTV pausing exhibited many of the properties previously described for STVs (Figure 2.2A-B and (51)). For example, the same PTV could be observed repeatedly pausing at a given site (Figure 2.2B, top panel). In addition, multiple PTVs paused at the same site, both sequentially and simultaneously (Figure 2.2B, middle and bottom panels, respectively). When STV movements were quantified, the mean STV pause frequency was 0.0081 +/- 0.0002 pauses/second (n = 262 vesicles, Figure 2.2C). The average STV pause duration was 107.4 +/- 3.4 seconds (n = 954 pauses, Figure 2.2D). Both measurements concur with previous observations (51).

As shown in Figure 2.2C-D, PTV pause frequency and duration in 7-8 DIV neurons were nearly identical to the STV data, with PTVs pausing at a frequency of 0.0082 +/- 0.0003 pauses/second and average duration of 108.3 +/- 3.5 seconds (n = 253 vesicles, n = 933 pauses). The similarities between the pausing behavior of STVs and PTVs raise the question of whether PTVs might pause with STVs at sites of eventual synapse formation. Like STVs (51), regulation of PTV pausing could be an important target of signals that control synapse assembly.
Figure 2.2: PTV pausing is qualitatively and quantitatively similar to STV pausing.

(A) Time-lapse images of PTVs labeled with GFP-bassoon. Individual PTVs are tracked with red, yellow or blue arrows. Most PTVs paused, and pauses were of varied durations. Frames were collected at the indicated times. Scale bars, 5 µm. (B) Kymographs
demonstrating the movements of PTVs in 3 axons. Individual PTVs paused repeatedly at the same site (*top panel*), and multiple PTVs paused at the same site (*middle and bottom panels*). Pausing of multiple PTVs at a given site occurred both sequentially (*middle panel*) and simultaneously (*bottom panel*). Individual vesicles are highlighted in different colors for visualization. Pause sites are outlined in *orange*. On the ordinate axis, one pixel corresponds to 10s. (*C-D*) STVs (*magenta*) and PTVs (*green*) paused at similar frequencies (*C*) and for similar mean durations (*D*). Data represent the mean + S.E.
STVs and PTVs pause simultaneously at the same sites in the axon

For a synapse to develop, both active zone and synaptic vesicle proteins need to be recruited to the same site. Sharing a pause site provides a potential mechanism for recruiting both STVs and PTVs to the same spot in the axon. Since places where STVs pause are preferred sites of synapse formation, it seemed likely that PTVs also pause at these sites. To test this hypothesis, STVs and PTVs were imaged and analyzed as described above. From the time-lapse panels and kymographs shown in Figure 2.3A-B, it is clear that both types of vesicles paused at sites where the other type of vesicle also paused.

To determine whether pausing of STVs and PTVs at the same sites was significantly greater than would be predicted by chance, pausing at the same sites was quantified in each axon then compared to simulations in model axons (Figure 2.3C). For STVs, 47.9% of pauses were at sites where a PTV also paused (n = 305 pauses, Figure 2.3D), compared to 24.5% using the same analysis of model axons (n = 33967 pauses, Figure 2.3D). This indicates that STVs preferentially pause at PTV pause sites. Likewise, 24.5% of all PTV pauses were at sites where an STV also paused (n = 620 pauses, Figure 2.3D), compared to 14.0% using the same analysis of model axons (n = 69263 pauses, Figure 2.3D), indicating that PTVs preferentially pause at STV pause sites.

Some STVs do not encounter PTV sites during our imaging time window and vice versa. To account for this, we also quantified pausing at the same site with the analysis limited to STVs and PTVs that had the opportunity to pause at PTV and STV sites, respectively (Figure 2.3E). 57.8% of STVs that encountered a PTV site paused (n = 135), and 84.4% of PTVs that encountered STV sites paused at those sites (n = 90). PTVs are slightly
more likely to pause at STV sites; however, this difference appears to be an inherent property of the vesicles and is accounted for in model axons (see Figure 2.4A,C). It has been shown previously that STV pause sites are preferred sites of synapse formation, and PTVs pause at these same sites; therefore, PTVs pause at sites of synapse formation.
Figure 2.3: STVs and PTVs pause at the same sites. (A) Time-lapse images of a segment of axon expressing both synaptophysin-mcherry (magenta) and GFP-bassoon
In each panel, fluorescence signals from the two channels are overlaid, and colocalization is indicated by white. Orange box, site where a PTV is paused. The arrow tracks a STV that then pauses at that site. Scale bars, 5 µm. (B) Kymographs showing two examples of STVs and PTVs that pause at the same sites. On the ordinate axis, one pixel corresponds to 10s. Bottom panel, overlay of STV and PTV fluorescence. Orange boxes outline 3 shared pause sites. (C) Simulations of model axons were performed by randomizing initial positions of vesicles while maintaining movement and pausing characteristics observed for the original experimental vesicles. Diagrams show the superimposed locations of all pause sites for individual STVs and PTVs in model axons. Three simulations of the same experimental data are shown. STV pause sites are indicated with a dot while PTV pause sites are indicated with a “+” sign. Each color represents an individual vesicle. Cyan and magenta boxes outline the pause sites of the same PTV and STV, respectively, in each model axon. For each axon imaged, 100 such simulations were performed, allowing estimation of the degree of co-transport and co-pausing expected from chance alone. (D) Plot illustrating the percentage of PTV (green) and STV (magenta) pause sites that are shared with STVs and PTVs, respectively. The fraction of shared sites is much higher than predicted by chance (via simulations, light green and light magenta). (E) A large majority of PTVs that encountered STV pause sites paused at those sites. Similarly, most STVs that encountered PTV pause sites then paused at those sites. Error bars display the 95% confidence interval.
Since both PTVs and STVs pause in the same places, the next question addressed was whether STVs and PTVs stop at these sites at the same time. Synapse assembly is a rapid process, and concurrent attraction of both STVs and PTVs could provide a mechanism for efficient assembly of presynaptic terminals. To determine whether PTVs and STVs are simultaneously attracted to sites of synapse formation, we quantified whether STVs preferentially pause at PTV pause sites when a PTV is present. As shown in Figure 2.4, STVs were more likely to pause at a site that contained a PTV than a site that did not. When STVs encountered pause sites with PTVs present, 76.4 +/- 11.2% of STVs paused with the PTV (n = 55; Figure 2.4A). In contrast, only 45.0 +/- 10.9% of STVs paused at PTV pause sites when a PTV was not there (n = 80). This 1.7-fold difference in the likelihood of pausing with a PTV could not be accounted for by chance because there was no interaction between STVs and PTVs when the same analysis was performed on data obtained from simulations with model axons (Figure 2.4A). In the model, 52.4 +/- 1.9% of STVs paused at PTV pause sites with a PTV present (n = 2334), and 51.6 +/- 1.3% of STVs paused at PTV pause sites without PTVs present (n = 5767).

The pause duration of an STV stopped at a PTV pause site was also measured. In contrast to the likelihood of pausing, the pause duration of STVs is not affected by the presence or absence of PTVs (Figure 2.4B). The average pause duration for STVs paused at sites with PTVs present was 101.0 +/- 13.4 seconds (n = 42 pauses), while the average pause duration for an STV paused at a site without a PTV present was 94.2 +/- 20.1 seconds (n = 36).

It is not yet known whether STVs and PTVs must be recruited to nascent presynaptic terminals in a defined order, so we also tested whether PTVs are more likely
to pause at sites that contain STVs. As shown in Figure 2.4C, PTVs paused at sites that contained STVs at a significantly higher rate (93.6 +/- 7.0%, n = 47) than at sites where STVs had previously paused but were no longer present (74.4 +/- 13.0%, n = 43). In contrast, this effect of STVs on PTV pausing behavior was not seen with analysis of the randomized model: in the simulations, 76.0 +/- 2.0% of PTVs paused at STV sites with an STV present (n = 1591), and 74.7 +/- 1.1% of PTVs paused at STV sites without STVs present (n = 4906). There was no difference in the probability that either vesicle arrived first at a shared pause site, based on 95% CI (data not shown). Similar to STVs pausing at PTV sites, the mean pause duration of PTVs pausing at STV pause sites is not affected by the presence of STVs (Figure 2.4D). The average pause duration for PTVs paused at sites with STVs present was 100.5 +/- 12.4 seconds (n = 44 pauses), while the average pause duration for PTVs paused at sites without STVs present was 80.9 +/- 15.3 seconds (n = 32 pauses). These data demonstrate that PTVs prefer to pause at sites that contain STVs and *vice versa*, suggesting that STVs and PTVs are attracted to the same sites at the same time.
Figure 2.4: STVs and PTVs preferentially pause at the same sites at the same time.

(A) STVs (magenta) are significantly more likely to stop at a PTV pause site when PTVs are at the site. This preference cannot be accounted for by chance since no dependence on the presence of PTVs was seen in simulations (light magenta). The data presented correspond to the mean + 95% confidence intervals. *, 95% confidence intervals do not
overlap. (B) STVs pause for similar lengths of time regardless of whether a PTV is present at the pause site. Data are presented as mean + S.E. (C) PTVs (*green*) are more likely to pause at a site when an STV is present. The same dependence was not observed in simulations (*light green*). (D) PTVs pause for similar lengths of time, regardless of whether STVs are present at the pause sites. Data are the mean + S.E.
Recruitment of a PTV enhances accumulation of additional PTVs at sites of synapse formation

Assembly of presynaptic terminals involves recruitment of multiple PTVs (60). Therefore, we wondered whether PTVs might also increase attraction of other PTVs to sites of synapse formation. To test this, we quantified the percentage of PTVs that paused at sites where another PTV was either present or had previously paused. When a PTV was already anchored at a particular pause site, there was high likelihood of an additional PTV pausing at that site: 83.3 +/- 13.3% of PTVs paused when they encountered sites with other PTVs (n = 30; Figure 2.5A). However, when a PTV was not present at the pause site, the likelihood that a PTV would pause was significantly lower (42.5 +/- 15.3%, n = 40). This nearly 2-fold increase in attraction of PTVs to sites containing other PTVs could not be explained by chance since the increase was not observed in our simulations. In model axons, PTVs paused at 67.1 +/- 1.6% of sites with other PTVs (n = 1267) and 68.1 +/- 1.2% of sites without other PTVs (n = 5827; Figure 2.5A). This indicates that the presence of a PTV at a pause site promotes recruitment of additional PTVs to that site.

Interestingly, the presence of an STV at a particular pause site did not significantly increase the probability of an additional STV pausing at that site (chance of pausing with another STV present = 71.4 +/- 23.7%, n = 14; without another STV present = 60.5 +/- 15.5%, n = 38; Figure 2.5B). As expected, no dependence of STV pausing on the presence of other STVs was observed in our randomized model axon (STV present = 56.8 +/- 2.4%, n = 1665; STV absent = 59.8 +/- 1.6%, n = 3443; Figure 2.5B). These data
suggest that STVs do not interact with one another in a way that promotes recruitment to sites of synapse formation.
Figure 2.5: Multiple PTVs are attracted to the same sites. (A) PTVs are more likely to pause when other PTVs are present (green). Spatially randomized simulations are shown in light green and cannot account for the tendency of PTVs to pause simultaneously with other PTVs. (B) STVs are not more likely to pause when other STVs are already present (magenta). Simulations are shown for comparison (light magenta). Error bars represent 95% confidence intervals. *, 95% confidence intervals do not overlap.
**STV pausing is only mildly dependent on PTVs**

The data presented above demonstrate that pausing of STVs at sites of synapse formation is enhanced at sites that contain PTVs and *vice versa*. This raises the question of whether a direct physical interaction between STVs and PTVs is important for recruitment of synaptic proteins to sites of synapse formation. If so, then STV pausing should be altered if PTVs are decreased or eliminated from the axon. Conveniently, a method for disrupting PTV transport – and, therefore, decreasing PTV density in the axon -- has recently been described (73, 74). This approach utilizes a dominant negative construct that disrupts the connection between the PTV-associated protein syntaxin and syntabulin, which links PTVs to the kinesin motor KIF5B. This construct mimics the syntaxin binding domain (SBD) of syntabulin and interferes with the syntaxin-syntabulin interaction. Expression of syntabulin-SBD has been shown to specifically prevent the majority of PTVs from being transported out of the cell body and into the axon without directly affecting STV transport or other KIF5 cargo (Figure 2.6A; (73, 74)). Imaging of our cortical cultures confirmed that the density of endogenous piccolo puncta is dramatically decreased in axons of neurons expressing syntabulin-SBD fused to GFP when compared to neurons expressing GFP alone (Figure 2.6B).

Consistent with the published data, transfection of neurons with the syntabulin-SBD construct did not interfere with STV transport in axons (Figure 2.6C): STVs still moved, and the mean distance moved over the course of imaging was unchanged (No SBD = 8.90 +/- 0.93 µm, n = 163 vesicles; SBD = 9.30 +/- 0.82 µm, n = 153 vesicles, p = 0.68). When STV pausing was quantified, expression of the syntabulin-SBD construct yielded no change in the pause frequency (No SBD = 0.0085 +/- 0.0003 pauses/second, n
= 163 vesicles; SBD = 0.0080 +/- 0.0004 pauses/second, n = 153 vesicles; p = 0.62;

Figure 2.6D) but did cause a slight decrease in the average duration of STV pauses (No
SBD = 109.9 +/- 4.4 s, n = 586 pauses; SBD = 102.4 +/- 4.3 s, n = 582 pauses; p = 0.03;
Figure 2.6E). In addition, the instantaneous velocities of STVs were slightly reduced in
SBD-transfected neurons (No SBD = 0.208 +/- 0.004 µm/s, n = 729 movements; SBD =
0.193 +/- 0.004 µm/s, n = 774 movements; p = 0.003; Figure 2.6F). Although there was a
decrease in STV pause duration when PTV transport was disrupted, the magnitude of the
effect and the lack of a change in the probability of pausing argue against PTVs
themselves controlling STV pausing. These data suggest that a direct interaction between
STVs and PTVs is not responsible for the increased attraction of STVs to sites that
contain PTVs.
Figure 2.6: A direct interaction between STVs and PTVs cannot account for the attraction of STVs to pause sites that contain PTVs. (A) PTV transport was inhibited using dominant-negative syntabulin (syntaxin binding domain, SBD) fused to GFP. (B) Expression of syntabulin-SBD-GFP decreases the density of PTV puncta in axons when compared to axons expressing GFP alone. PTVs were identified by immunofluorescent labeling for endogenous piccolo. (C) Images and kymograph (bottom) showing that STVs (magenta) move and pause in SBD-expressing axons (green). (D) The frequency of STV pausing was unchanged in the presence of SBD-GFP. (E) STVs pause durations are shorter when PTV localization in the axon is disrupted. p-values are from Wilcoxon rank-sum test. The change in pausing upon expression of SBD-GFP is not sufficient to account for the coordinated pausing of STVs and PTVs. (F) The instantaneous velocities of STVs are increased in SBD-expressing axons. Black arrow, mean instantaneous velocity.
Materials and Methods

All studies were conducted with an approved protocol from the Case Western Reserve University Institutional Animal Care and Use Committee, in compliance with the National Institutes of Health guidelines for the care and use of experimental animals.

Neuronal cultures and transfection

Primary neuronal cultures were prepared from postnatal rat visual cortex essentially as described previously [4, 5, 15], except neurons were maintained in Neurobasal-A medium supplemented with glutamax and B27 (Invitrogen). Neurons were transfected with Lipofectamine 2000 (Invitrogen, Carlsbad, CA) 24-48 h before live imaging. Excluding GFP-bassoon transfection, 1µg of DNA construct was combined with 1µg of Lipofectamine 2000 in 50ul of Optimem (Invitrogen) for each 18 mm coverslip. Transfection of GFP-bassoon was conducted in the same manner except 2µg of DNA were used to account for its large size. With double transfection, localization of each protein appeared similar when expressed alone. Also, the distribution and movement of STVs labeled with synaptophysin-mcherry, synaptophysin-mRFP, and synaptophysin-GFP all appeared similar to one another. GFP-bassoon (GFP-Bsn 95-3938), synaptophysin-mcherry, synaptophysin-mRFP, synaptophysin-GFP and syntaxin-SBD-GFP were generous gifts of Drs. Thomas Dresbach (University of Heidelberg, Heidelberg), Matthejs Verhage (Vrije Universiteit, Amsterdam), Jurgen Kingauf (University of Muenster, Muenster), Jane Sullivan (University of Washington, Seattle) and Zu-Hang Sheng (National Institute of Neurological Disorders and Stroke, Bethesda).
Each of these constructs has been shown previously to be functional and properly localized (50, 61, 73, 211-213).

**Live imaging**

Neurons were imaged at 7-8 DIV with a CI Plus confocal system with a Nikon Eclipse Ti-E microscope using a 40x Nikon Plan Apo 0.95 numerical aperture objective. Lasers were 488 nm argon and 543 nm helium-neon. Detection filters were 515/30 nm bandpass for GFP and 590/50 nm bandpass for mcherry/mRFP. Images were collected every 10s, with scan times no greater than 3.3s. This imaging interval was selected as a compromise between having a high temporal resolution and minimizing the time the neurons were exposed to the laser to avoid any toxicity and photobleaching. A total of 45 images were collected for each time-lapse series. Imaging was conducted with constant perfusion with artificial CSF (ACSF) (120 mM NaCl, 3 mM KCl, 2 mM CaCl$_2$, 2 mM MgCl$_2$, 30 mM D-glucose, 20 mM HEPES, and 0.2% sorbitol, pH 7.3). ACSF perfusion was performed at 25°C since STV transport and pausing are not significantly different at ambient and physiological temperatures (51). Axons were identified using morphological criteria, as described previously (50, 51). For dual-color imaging, channels were collected sequentially to eliminate bleed-through and neurons were imaged in which expression levels of both fusion proteins appeared comparable.

**Immunofluorescence**

Neurons were fixed for 15 minutes in 4% paraformaldehyde in PBS containing 4% sucrose, permeabilized for 5 minutes with 0.2% Triton X-100, and blocked with 10% horse serum. The primary antibody was piccolo (Synaptic Systems), and the secondary
antibody was Alexa 633-conjugated goat anti-rabbit (Invitrogen). Coverslips were mounted in Fluoromount (Fisher Scientific, Pittsburgh, PA) containing DABCO (1,4-diazabicyclo[2.2.2]octane) (Sigma, St. Louis, MO).

**Analysis and statistics**

STV and PTV movements were tracked using ImageJ (NIH, Bethesda, MD). To restrict the quantification of time-lapse movies to healthy neurons, axons were included in the analysis only if at least 1 vesicle moved within the field of view. Regions of axon with an intermediate STV or PTV density were imaged to allow us to track each vesicle reliably. STV and PTV movements were tracked independently while blind to the other channel.

For quantification of movement and pausing, positional data were imported into Matlab and analyzed with custom-written programs (Mathworks, Natick, MA). For pause analysis, a pause was defined as a period greater than or equal to 10s, during which the velocity of the vesicle went to 0+/- 0.1 µm/s. The cutoff zero velocity (0.1µm/s) was chosen based on the average diameter of vesicles, which was approximately 1µm. Vesicles that never moved were not included in the analysis. Therefore, vesicles which pause for very long durations might be underrepresented. Also, only vesicles that could be tracked for the entire imaging duration were included in the analysis. Vesicles that move at high velocities are more likely to leave the imaging area before the end of the movie. Therefore, these vesicles might also be underrepresented in the analysis.

Given the pixel density, scan speed and average vesicle size, a vesicle was typically imaged in approximately 10-50 ms, permitting reliable tracking of vesicles
based on their apparent size, shape, and intensity, with relatively low influence of vesicle movements on these parameters. The fastest STV movements, at a maximum approaching 1 µm/s (comparable with the fastest velocities that have been reported for STVs (51, 53, 210)), resulted in movements of 10 µm during the imaging interval and were easily measured. Maximal velocities of PTVs are lower than maximal STV velocities (60, 73) and were also easily recorded.

Data are presented cumulatively with 95% confidence intervals for binomially distributed data or as the mean +/- standard error of the mean (S.E.) where appropriate. Confidence intervals were calculated based on a normal approximation, and data sets were considered significantly different if their 95% confidence intervals were non-overlapping. For data presented as means, significance was evaluated using the Wilcoxon rank sum test.

**Randomized model**

In some cases, experimental data were compared to randomized simulations of vesicle movement. For each axon that was imaged, a model axon was generated with the same number of vesicles and axonal length as each experimental axon. In these models, the initial position of each vesicle was randomized. However, the number, timing and direction of movements, as well as the instantaneous velocity, and pause duration of each vesicle remained the same. The newly generated time-lapse series were then analyzed in the same manner as the experimental data. Each simulation was performed 100 times per axon to enhance statistical analysis.
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Chapter 3: Dynamic mechanisms of neuroligin-dependent presynaptic terminal assembly in living cortical neurons

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Abstract

Synapse formation occurs when synaptogenic signals trigger coordinated development of pre and postsynaptic structures. One of the best-characterized synaptogenic signals is trans-synaptic adhesion. However, it remains unclear how synaptic proteins are recruited to sites of adhesion. In particular, it is unknown whether synaptogenic signals attract synaptic vesicle (SV) and active zone (AZ) proteins to nascent synapses or instead predominantly function to create sites that are capable of forming synapses. It is also unclear how labile synaptic proteins are at developing synapses after their initial recruitment. To address these issues, we used long-term, live confocal imaging of presynaptic terminal formation in cultured cortical neurons after contact with the synaptogenic postsynaptic adhesion proteins neuroligin-1 or SynCAM-1. Surprisingly, we find that trans-synaptic adhesion does not attract SV or AZ proteins nor alter their transport. In addition, although neurexin (the presynaptic partner of neuroligin) typically accumulates over the entire region of contact between axons and neuroligin-1-expressing cells, SV proteins selectively assemble at spots of enhanced neurexin clustering. The arrival and maintenance of SV proteins at these sites is highly variable over the course of minutes to hours, and this variability correlates with neurexin levels at individual synapses. Together, our data support a model of synaptogenesis where presynaptic proteins are trapped at specific axonal sites, where they are stabilized by trans-synaptic adhesion signaling.
**Introduction**

Upon axon-dendrite contact, the extracellular domains of axonal and dendritic adhesion proteins interact, leading to recruitment of synaptic proteins and subsequent synapse formation (5, 136, 138). A variety of synaptogenic adhesion partners have been discovered, including neurexin/neuroligin, SynCAM/SynCAM, and neurexin/LRRTM2, among others (117, 128, 129, 143, 163-166, 176, 214-216). Of these, the most studied trans-synaptic pair is postsynaptic neuroligin and its interaction with presynaptic neurexin.

Although a number of studies have focused on understanding the functions and mechanisms of trans-synaptic adhesion molecules in recent years, it remains unclear how synaptogenic adhesion results in presynaptic protein recruitment and synaptogenesis (124, 217). Importantly, it is not known whether trans-synaptic adhesion actively attracts synaptic proteins to sites of signaling or acts primarily to stabilize proteins at nascent terminals. In addition, it is unknown whether synaptic protein recruitment proceeds continuously until the site is saturated, or if SV and AZ protein levels are modulated even at the earliest stages of development, as they are at mature synapses (218-225). Finally, it is unclear if trans-synaptic adhesion regulates synaptic protein levels at individual synapses or primarily creates sites in the axon that are capable of synapse formation while other cellular processes regulate levels of recruitment.

To answer these questions, we induced synaptogenesis via contact with neuroligin-1 and SynCAM-1, then employed short and long-term time-lapse confocal imaging of presynaptic protein recruitment to nascent sites of trans-synaptic signaling.
This paradigm allowed us to record recruitment from the initiation of trans-synaptic adhesion onward. We found that SV protein recruitment to individual sites of trans-synaptic adhesion fluctuated, as did protein recruitment at axon-dendrite synapses. These changes in synaptic protein levels strongly correlated with the amount of neurexin-1β at individual synapses. However, trans-synaptic adhesion did not attract SV or AZ proteins to contacts or significantly alter synaptic protein transport. Unexpectedly, neurexin alone was not the primary signal for recruitment since synaptic proteins were specifically recruited to sites of enhanced neurexin-1β clustering, even though neurexin was present throughout the contact region. Overall, our data support a model of synaptogenesis in which presynaptic proteins are trapped at, but not actively attracted to, specific axonal sites, where they are stabilized by trans-synaptic adhesion signaling.
Results

Synaptic protein recruitment fluctuates frequently and rapidly during assembly of individual presynaptic terminals

Contact between an axon and dendrite is one of the first steps of synapse formation; however, it is difficult to predict when and where axo-dendritic contact will occur and whether synapses will form at these sites. To overcome this challenge, we developed a strategy for long-term imaging of synaptic protein recruitment to nascent sites of presynaptic terminal formation, initiated by contact with neuroligin-1-expressing HEK293 cells as a proxy for post-synaptic dendrites. Specifically, 7-10DIV cortical neurons were sparsely transfected with synaptophysin-GFP, to label synaptic vesicle protein transport vesicles (STVs), which deliver SV proteins to developing presynaptic terminals (47-49, 51-53, 58, 68-71, 81, 87, 114, 115, 117, 210, 226, 227). We then imaged STVs in individual axons that contacted neuroligin-1-expressing HEK293 cells for up to 25h after contact (Figure 3.1A), collecting multiple “short sequences” of rapid imaging (every 10s for 7.5min) separated by 1-2.5h. The “short sequences” granted the ability to track rapid STV movements, observe recruitment of individual STVs, and distinguish between STVs that were stably recruited and those that were en route through the contact site while preserving neuron health. With this approach, it was possible to resolve a full time-course of presynaptic protein recruitment at individual sites of trans-synaptic signaling in individual axons.

To quantify STV recruitment within individual axons, the integrated densities of all STVs were summed in each axonal region that made contact with a neuroligin-1-
expressing HEK293 cell and compared to regions without contact in the same axon. Since integrated density corresponds to the sum of pixel intensities, STVs with higher integrated densities were brighter and/or larger, indicating more SV protein. Enrichment was calculated by determining the difference between fluorescence (integrated density per length of axon) inside and outside the contact region, divided by the sum of the fluorescence inside and outside. Overall, in regions that displayed recruitment (see Methods), synaptophysin-GFP was increasingly enriched at sites of trans-synaptic adhesion over the first 10h of imaging (Figure 3.1B; n = 12 contacts), similar to previous observations using immunocytochemistry to examine populations of axons (115). No enrichment was observed over the same time course when axons were contacted with HEK293 cells expressing only HcRed, with no neuroligin, indicating that the observed recruitment was a specific result of trans-synaptic adhesion (Figure 3.1C; n = 5 contacts). Enrichment at neuroligin contacts was due to increased SV protein recruitment to contacts without substantial changes in SV protein outside areas of contact (Figure 3.1D). Within contact areas, SV protein levels started to rise 2h after contact was initiated. This “rising/recruitment phase” progressed until reaching a plateau 10h after contact initiation (Figure 3.1D). However, recruitment at individual contacts was quite labile (Figure 3.1E). Throughout the imaging period, absolute levels of recruited synaptophysin-GFP increased or decreased from one hour to the next, even for axons that displayed enrichment for most or all of the 24h imaging period. These substantial fluctuations occurred during both rising and plateau phases. Importantly, variability in fluorescence was not caused by focal drift, since we employed Perfect Focus correction to maintain the focus (see Methods). Therefore, in individual axons, recruitment of proteins does not
consistently increase: rather, levels of recruited SV proteins fluctuate while remaining elevated compared to neighboring axonal areas without trans-synaptic signaling.
Figure 3.1: Levels of synaptic vesicle protein enrichment at individual trans-synaptic adhesion sites are modulated throughout recruitment. (A) Images from live, long-term, time-lapse confocal imaging of an axon expressing synaptophysin-GFP (green) and contacting an HEK293 cell (magenta and white outline) that expresses neuroligin-1 + HcRed. The top panel shows an axon and HEK293 cell at 1h after contact. In this panel, synaptophysin-GFP at the contact site appears white in the overlay. The remaining panels show only synaptophysin fluorescence for clarity. Images were collected 1h, 12.5h and 20h after contact was induced (scale bar = 5 μm). (B) Time course of STV enrichment in axonal regions that contact neuroligin-1-expressing cells. Enrichment corresponds to the difference between the total STV integrated density within the contact region and outside of the contact, normalized to the total STV integrated density throughout the axon and expressed as a percentage. Positive values indicate enrichment inside the contact area. Individual points (cyan) represent mean values from 45 images collected at 10s intervals, beginning at the time indicated on the x-axis. Dashed line, overall mean at each time point (n = 12 contacts). Overall, enrichment
increased gradually over the first 10h then remained elevated. Error bars = S.E.M. (C)

Over the same time course, STVs were not enriched at sites of contact between axons and HEK293 cells expressing HcRed but no neuroligin. (D) Integrated density of synaptophysin-GFP inside (solid line) and outside (dashed line) of contacts over time. Red bar, rising phase of enrichment (2-7.5h after contact). Synaptophysin increased at contacts while remaining stable outside contacts. (E) Enrichment for two axons. At individual contacts, enrichment was highly dynamic throughout the imaging period. Therefore, although STV enrichment increases at trans-synaptic adhesion sites overall, enrichment levels for individual axons vary throughout the first 24h of development.
Fluctuations in recruited SV protein levels could occur through at least two mechanisms (Figure 3.2A). Once formed, sites of STV recruitment could be stable while the level of protein at each stable site fluctuates. Alternatively, the sites themselves could form and disappear. These two mechanisms are not mutually exclusive, and our live imaging revealed both types of variability. Synaptophysin-GFP levels fluctuated at individual sites that were stable over several hours (Figure 3.2B). Fluctuations often occurred very rapidly, within minutes. In addition, sites of stable recruitment often persisted for multiple hours before completely, and sometimes immediately, disbanding (Figure 3.2C). Instant formation of stable sites was also observed (Figure 3.2C).

To assess the prevalence of fluctuations in synaptophysin recruitment levels at individual sites, we determined the number of sites of stable STV accumulation that displayed fluctuations in synaptophysin-GFP levels over time. Stable accumulation sites were defined as sites in the axon where synaptophysin-GFP signal was consistently present over the course of at least one short imaging sequence. At 24.9% of individual stable accumulation sites (n = 115), there were observable fluctuations of synaptophysin-GFP within short imaging sequences. For stable accumulation sites that persisted over multiple short imaging sequences, synaptophysin-GFP levels changed between short imaging sequences 18.4% of the time (n = 103). It should be noted that these are most likely conservative estimates of the actual synaptic vesicle protein level fluctuations at stable sites, as small fluctuations of protein at these sites are difficult to observe.

Additions and losses of entire stable sites of recruitment resulted in net changes in the density of discrete, stable recruitment sites from one short sequence to the next (i.e. over 1-2.5 hours) in 76.6% of imaging sequences during the rising phase and 73.8%
during the plateau phase. This indicates that sites of recruitment were highly labile throughout synapse assembly. During the rising phase of recruitment, the additions and losses resulted in net increases in the density of stable sites of recruitment in 54.7% of imaging sequences (Figure 3.2D; n = 64). In contrast, during the plateau phase, appearances and disappearances were more balanced, with net increases in discrete contact sites only 38.1% of the time (Figure 3.2D).
**Figure 3.2: Modulation of synaptic vesicle protein recruitment occurs through two distinct mechanisms.** (A) Model kymographs illustrating that variability in protein levels could arise from fluctuations in synaptic protein levels at individual recruitment sites (left) or through the addition/subtraction of entire recruitment sites (right). (B, C) Kymographs of STVs (green) in axons that contact neuroligin-1-expressing HEK-293 cells (magenta). (B, box) Synaptophysin-GFP levels at stable contacts are variable over the course of hours and even minutes (asterisks). (C, top box, top line) Sites of recruitment that are stable over the course of hours can be eliminated, sometimes rapidly. (C) To replace these sites, individual STVs can be captured (bottom line) and stabilized (bottom box) at contact sites. t = time after contact, scale bars = 5 μm. (D) Histograms of net changes in the densities of individual stable recruitment sites from one short imaging sequence to the next. Stable sites were defined as puncta that appeared at a given site throughout at least one short imaging sequence. The number of stable sites of recruitment varied at the majority of contacts during both the rising and plateau phases, with a bias toward puncta addition during the rising phase (left) but not the plateau phase (right).
Levels of synaptic vesicle proteins are highly labile at developing axo-dendritic contacts

To determine if the same types of fluctuations in SV protein recruitment occur at neuron-neuron synapses, we imaged regions of contact between axons expressing synaptophysin-GFP and morphologically identified dendrites and somas expressing RFP. Contacts were imaged for 12-24h at 7-10 DIV with the imaging protocol described above (Figure 3.3A). Axo-dendritic and axo-somatic synapses exhibited many of the same dynamics observed in axons contacting neuroligin-1-expressing HEK293 cells. Levels of synaptophysin-GFP at individual sites of stable recruitment varied over time (Figure 3.3B,C). Synaptophysin-GFP protein levels at contact sites fluctuated 10.9% of the time (n = 43) between short imaging sequences (over 1-2.5h) and 17.6% of the time (n = 49) within short sequences (over 7.5min). Furthermore, individual recruitment sites were established and dissolved over the course of hours (Figure 3.3B, C), with net changes in the density of stable sites of recruitment occurring in 55.5% of imaging sequences (Figure 3.3D; 31.1% additions and 24.4% losses; n = 45). These data indicate that the dynamics of synaptic vesicle protein recruitment at axo-dendritic synapses and hemi-synapses occurred over a similar time course. The similarities between recruitment dynamics at spontaneous neuron-neuron synapses and neuroligin-1-induced sites are especially striking since sites of recruitment may have been more mature in some neuron-neuron synapses, and synapse formation between neurons may involve additional signaling pathways. Together, the above data suggest that developing synapses undergo extensive fluctuations in presynaptic protein levels, even during initial formation.
Figure 3.3: Synaptic vesicle protein recruitment to axo-dendritic contacts is similar that at induced trans-synaptic signaling sites. (A) Image of an axon transfected with synaptophysin-GFP (green) contacting somato-dendritic region from an adjacent cell transfected with RFP (magenta). White box corresponds to contact region represented by
kymograph in (B). (B, C) Kymographs of STVs (*green*) in axons that contact RFP-expressing dendrites from different neurons (*red*). Boxes, as seen in induced axo-dendritic contacts, synaptophysin-GFP levels are variable at individual stable sites in neuron-neuron contacts over the course of hours and minutes (*asterisks*). \( t = \text{time after imaging begins} \), scale bars (A) = 10\( \mu \)m, (B, C) = 5\( \mu \)m. (D) Histogram of net changes in the densities of individual stable recruitment sites from one short imaging sequence to the next for neuron-neuron contacts. The number of stable sites of recruitment fluctuated between the majority of imaging sequences (corresponds to all non-zero bins), with a small bias toward addition of stable sites of recruitment.
Synaptophysin is preferentially stabilized at clustered neurexin

Neuroligin induces synaptogenesis through its presynaptic binding partner neurexin (5, 228). To ascertain the time course of neurexin clustering at contacts, neuroligin-1-expressing HEK293 cells were added to neuronal cultures transfected with neurexin-1β-GFP. Then contacts between neuroligin-1-expressing HEK293 cells and axons expressing neurexin-1β-GFP were imaged immediately. Prior to contact, neurexin-1β-GFP appeared dim and diffuse within the axon. Upon contact with neuroligin-1-expressing HEK293 cells, neurexin-1β-GFP began to be recruited to sites of contact within minutes, and showed strong recruitment within 1-2 hours (Figure 3.4A-C, n = 14 contacts), consistent with previous data (115). Because we have not found any neurexin-β selective antibodies for immunofluorescence, we could not determine the extent to which neurexin-1β-GFP was over-expressed in these experiments. However, similar fluorescently tagged neurexin-1β-GFP has been used in a number of studies to investigate the localization, trafficking and function of neurexin (229-232). Importantly, the distribution pattern of α-neurexin is not significantly influenced by the level of neurexin expressed (232). Therefore, although over-expression of neurexin in neurons increases synapse density (230), it not expected to change the dynamics or distribution of neurexin.

Neuroligin-1 recruited neurexin-1β-GFP to the entire contact region, where neurexin appeared in both punctate and diffuse forms, even within the same contacts (Figure 3.4B). Recent work has shown that the synaptogenic effect of neuroligin depends on the postsynaptic scaffolding protein S-SCAM, which works, in part, by clustering neuroligin (216). To determine if post-synaptic clustering of neuroligin facilitates presynaptic clustering of neurexin, HEK293 cells that co-expressed S-SCAM and
neuroligin-1 were dropped onto neurons that were transfected with neurexin-1β-GFP. This led to enhanced clustering of neurexin-1β-GFP in axons that contacted transfected HEK293 cells (Figure 3.4B), suggesting that the synaptogenic effects of S-SCAM-induced neuroligin clustering might be due to enhanced clustering of presynaptic neurexin. Indeed, artificial clustering of neurexin is sufficient to induce presynaptic protein recruitment (124), and recent work suggests that neurexin clustering is critical for the synaptogenic function of the neurexin-neuroligin interaction in Drosophila (125).

This raises the question of whether synaptic proteins are preferentially recruited to punctate clusters of neurexin or whether they can be recruited to any site with neurexin enrichment. To test this, neurons were co-transfected with neurexin-1β-GFP and synaptophysin-RFP. Neuroligin-1-expressing HEK293 cells were then added to these cultures, and contacts were imaged for up to 24h, as described above. Although there was a combination of diffuse and punctate recruitment of neurexin-1β-GFP to contacts (Figure 3.4A-C), stable sites of synaptophysin-RFP recruitment were almost exclusively co-localized with neurexin-1β-GFP puncta (Figure 3.4C). Surprisingly, neurexin-1β-GFP clustering and synaptophysin-RFP clustering occurred nearly simultaneously at most shared sites (Figure 3.4D, n = 13/18 clusters). Neurexin clustered concurrently with SV protein recruitment even when HEK293 cells expressed both neuroligin-1 and S-SCAM to enhance neurexin clustering. These data support a model in which discrete neurexin clustering is critical for stable, rapid, neuroligin-induced presynaptic protein recruitment in mammalian cortical neurons.
Figure 3.4: Synaptic vesicle protein accumulation occurs at clustered neurexin and correlates with levels of clustered neurexin. (A) Time-lapse images of an axon transfected with neurexin-1β-GFP (green) contacting a neuroligin-1-expressing HEK293 cell (white outline). Neurexin is recruited to contact sites within minutes and enhanced at these sites as imaging progresses. (B) Images of two axons that are transfected with neurexin-1β-GFP (green) and contact cells expressing neuroligin-1 (left, white outline) or neuroligin-1+S-SCAM (right, white outline). At neuroligin-1-only contacts, neurexin appears both punctate (arrows) and diffuse (bars). Addition of S-SCAM enhanced neurexin-1β-GFP clustering and reduced diffuse neurexin-1β-GFP labeling at contacts.
(arrow). (C) Persistent sites of synaptophysin-RFP (magenta) recruitment appeared at sites of punctate neurexin-1β-GFP (green) recruitment (arrows). t = time after contact established. (D) Kymographs of neurexin-1β-GFP and synaptophysin-RFP, starting immediately after contact with a neuroligin-1-expressing cell was established. Neurexin and synaptophysin clustered simultaneously at the same sites in the axon (arrows). (E) At an individual recruitment site, synaptophysin-RFP and neurexin-1β-GFP levels were positively (r = 0.98) and significantly (p = 2.0x10^{-5}) correlated over several hours of imaging. This correlation was significant for 11 of 14 co-recruitment sites that were present for at least 5h. (F) When combining data from all recruitment sites in the same axon, synaptophysin and neurexin integrated densities were also correlated (r = 0.91, p = 6.0x10^{-20}). Similar correlation was observed in 5 of 7 axons. (G) When the data from all axons were pooled, neurexin and synaptophysin were positively and significantly correlated (p = 1.0X10^{-74}, r = 0.82). (A-C) scale bars = 10μm, (D) scale bar = 5μm, (E-G) correlations made from non-transformed data and plotted in log-log form for display purposes.
Actin polymerization is occasionally co-localized with neurexin clustering

While the neuroligin-neurexin interaction by itself is capable of generating synaptogenic signaling, other signaling pathways can interact with and enhance neuroligin-neurexin-induced synapse formation. Specifically, n-cadherin can act in concert with neuroligin to enhance synaptic vesicle recruitment to developing presynaptic terminals (216, 233). N-cadherin can mediate its intracellular signaling through actin, and actin is known to play a role in the recruitment of presynaptic components to the developing synapse (234-236). Therefore, to determine whether actin plays a role in mediating the initial recruitment of presynaptic components to synapses, the time-course of actin polymerization to novel sites of neurexin signaling was determined. Specifically, HEK293 cells expressing neuroligin were dropped onto 6-8 DIV neuronal cultures that co-expressed neurexin-GFP and Lifeact-mcherry, which is a fluorescently-tagged small peptide that binds endogenous F-actin (237, 238). HEK293 cells that contacted neurexin-GFP and Lifeact-mcherry-expressing axons were then imaged immediately via live time-lapse confocal microscopy at a frame rate of one frame per minute. Neurexin recruitment, including neurexin clustering, was observed at multiple contact sites after 60 minutes. Approximately half of the contact sites (n = 4/9 contacts in 4 separate axons) displayed F-actin clustering 60 min. after the initiation of neurexin recruitment (data not shown).

The synaptic scaffolding molecule S-SCAM physically links n-cadherin with neuroligin via intracellular interactions on the postsynaptic side of the synapse (216). This linkage induces enhanced clustering of neurexin and enhanced synapse formation (216)(Figure 3.4B). Since n-cadherin binding can mediate downstream actin polymerization (235), we hypothesized that postsynaptically localizing n-cadherin with
neuroligin might enhance actin polymerization presynaptically, particularly at sites of neurexin clustering. Therefore, HEK293 cells expressing neuroligin + S-SCAM were dropped onto 7-8 DIV axons that co-expressed neurexin-GFP and Lifeact-mcherry and immediately imaged. Importantly, n-cadherin is endogenously expressed in HEK293 cells (239). Neurexin clustering was enhanced at these contact sites, compared to contacts with HEK293 cells that did not express S-SCAM. However, co-localized actin polymerization and neurexin clustering was observed in only 33% of contacts (data not shown, n = 4/12 contacts in 3 separate axons).

While it is tempting to infer that actin is not typically involved in the initial recruitment of synaptophysin, these experiments were performed in a limited number of neurons. In previous experiments, simultaneous co-clustering of neurexin and synaptophysin were not always observed (n = 5/18 contacts), suggesting that specific neurons or even specific sites within the same neuron are more or potentially less capable of this rapid co-clustering. Follow-up experiments involving additional neurons and HEK293 cell/axon contacts are needed to confirm these results both with and without S-SCAM as a postsynaptic organizer.

**Levels of recruited synaptophysin and neurexin are strongly correlated at developing presynaptic terminals**

Our observation that SV proteins and neurexin selectively and simultaneously co-cluster raises the question of whether synaptophysin recruitment and neurexin clustering are coordinated. To test this, we first determined whether synaptophysin and neurexin levels were correlated at individual sites of recruitment. Indeed, synaptophysin levels
were positively and significantly correlated with neurexin levels at individual co-clusters that were present for at least five consecutive short sequences of imaging (Figure 3.4E, p < 0.05 at 11/14 sites from 7 axons, r = 0.37-0.98). Neurexin-1β-GFP and synaptophysin-RFP levels were also significantly correlated when all clusters from individual axons were pooled (Figure 3.4F, p < 0.05 in 5/7 axons, r =0.52-0.96) or when all axons were combined (Figure 3.4G, n = 307 sites, p = 1.0X10^{-74}, r = 0.82). Therefore, recruitment of SV proteins and clustered neurexin are coordinated during presynaptic terminal assembly.

Next, we asked whether the long-term dynamics of synaptophysin and neurexin recruitment were similar. To test this, 7-10 DIV neurons were transfected with either synaptophysin-GFP and neurexin-tdTomato or the near-infrared fluorescent protein RFP670 (Figure 3.5) (240). Contacts between axons that expressed both synaptophysin-GFP and neurexin-tdTomato and dendrites that expressed RFP670 were then imaged over the course of 12-25h. Like synaptophysin, levels of neurexin fluctuated significantly over the course of long-term imaging at both spontaneously-formed axo-dendritic and axo-somatic neuron-neuron contacts (Figure 3.5A,B) as well as contacts formed with neuroligin-expressing HEK293 cells (Figure 3.5C). In addition, many neurexin-1β-GFP clusters formed and disappeared over the course of hours (Figure 3.4C and 3.5), resembling the formation and dissolution of stable sites of synaptophysin recruitment (Figure 3.2 and 3.5).
Figure 3.5: Neurexin and synaptophysin levels fluctuate over the course of minutes at axo-dendritic contact sites and neuroligin induced developing synapses. (A) Image of an axon co-expressing synaptophysin-GFP (green) and neurexin-tdTomato (red) and contacting the somato-dendritic region of a neuron expressing RFP670 (blue). White box corresponds to contact region represented by kymographs in (B). (B) Individual kymographs of an axon expressing synaptophysin-GFP (top) and neurexin-tdTomato (middle) that contacts a dendrite expressing RFP670 (bottom). Levels of both neurexin and synaptophysin fluctuate over the course of minutes to hours at sites that contact somato-dendritic regions of the adjacent neuron (arrows, top), including the formation (white box) and disappearance (white arrowhead) of co-clusters. (C) Kymographs of an axon expressing neurexin-GFP (left) and synaptophysin-RFP (right) contacting a neuroligin-expressing HEK293 cell. The amount of neurexin and synaptophysin at co-clustered sites (arrow, bottom) fluctuates extensively over hours and even within minutes (white arrowheads). Scale bars (A,B) = 10μm, scale bar (C) = 5μm.
Trans-synaptic signaling recruits synaptic proteins without substantially altering their transport

It is not known how STVs disengage from their microtubule tracks and become incorporated into synapses. Various signals that affect synaptogenesis also affect STV transport (51), and it has been suggested that neuroligin-neurexin signaling might recruit synaptic proteins by altering synaptic protein transport (241). Therefore, we next tested the hypothesis that trans-synaptic signaling recruits presynaptic proteins by altering STV transport. To test this hypothesis, synaptophysin-GFP was imaged every 10s for 7.5 min at 1-4 hours after contact was established between axons and neuroligin-1-expressing HEK293 cells (Figure 3.6A). Because STV transport is characterized by bursts of movement interrupted by pauses (47, 48, 51, 53, 58, 87, 210), we measured how fast STVs moved and how long they paused in the presence and absence of neuroligin-1. We found that STV pause duration increased at contacts with neuroligin-1-expressing cells when compared to contacts with HcRed-expressing control cells (Figure 3.6B; neuroligin = 145.3s, +/- 20.8, n = 40 pauses; no neuroligin = 85.7s, +/- 15.5, p = 0.0217, n = 42 pauses). However, there was no neuroligin-1-dependent change in instantaneous velocity (Figure 3.6B; neuroligin = 0.229µm/s, +/- 0.023, n = 40 movements; no neuroligin = 0.196µm/s, +/- 0.015, n = 56 movements, p = 0.391).

To investigate active zone protein transport, we looked at Piccolo-Bassoon transport vesicles (PTVs), which deliver active zone proteins to developing presynaptic terminals (49, 59-62, 73, 74, 81, 83, 87, 115, 118). STVs and PTVs can be transported together in the axon (49, 87, 96). Therefore, neuroligin-neurexin interactions could alter PTV transport, which in turn could recruit STVs. Similar to STVs, neuroligin-1 had no
effect on the instantaneous velocity of PTVs (Figure 3.6C; neuroligin = 0.140µm/s, +/- 0.008, n = 22 movements; no neuroligin = 0.158µm/s, +/- 0.009, n = 59 movements, p = 0.473). Unlike STVs, contact with a neuroligin-1-expressing cell had no effect on the pause duration of PTVs (Figure 3.6C; neuroligin = 173.1s, +/- 24.9s, n = 32 pauses; no neuroligin = 145.5s, +/- 14.0s, n = 53 pauses, p = 0.634). Therefore, neuroligin-neurexin signaling does not recruit STVs indirectly via regulation of PTV transport.

To determine if other forms of synaptogenic trans-synaptic signaling alter STV movement, we also performed the live-imaging assay described above using HEK293 cells transfected with SynCAM-1, a trans-synaptic adhesion molecule that possesses synaptogenic properties similar to neuroligin-1(129, 143, 156). Unlike neuroligin-neurexin signaling, SynCAM-1-mediated trans-synaptic adhesion did not alter STV pause duration (Figure 3.6D; SynCAM = 112.5s, +/- 14.0, n = 53 pauses; no SynCAM = 125.5s, +/- 13.4, n = 58 pauses, p = 0.332). However, SynCAM-1 signaling caused a small increase in the instantaneous velocity of STVs (Figure 3.6D; SynCAM = 0.235µm/s, +/- 0.021, n = 52 movements; no SynCAM = 0.183µm/s, +/- 0.012, n = 58 movements, p = 0.030). Since the time-course of presynaptic protein recruitment to SynCAM-1 adhesion sites has not been established, we also imaged STVs at 24h after contact with SynCAM-1. After 24h, SynCAM-1-expressing cells had no effect on STV movement (Figure 3.6E; pause duration: SynCAM = 140.3s, +/- 15.6, n = 62 pauses; no SynCAM = 116.5s, +/- 14.4, p = 0.197, n = 71 pauses, instantaneous velocity: SynCAM = 0.202µm/s, +/- 0.012, n = 71 movements; no SynCAM = 0.171µm/s, +/- 0.007, p = 0.060, n = 86 movements).
Neuroligin and SynCAM interact with different presynaptic adhesion molecules (5, 136, 242). Therefore, it might be expected that the effects of neuroligin and SynCAM would be additive or synergistic. However, contact with HEK293 cells that expressed both neuroligin-1 and SynCAM-1 had no effect on the movement of STVs (Figure 3.6F; pause duration: neuroligin/SynCAM = 124.6s, +/- 12.7, n = 61 pauses; no neuroligin/SynCAM = 116.7s, +/- 14.3, p = 0.482, n = 51 pauses; instantaneous velocity: neuroligin/SynCAM = 0.196μm/s, +/- 0.012, n = 63 movements; no neuroligin/SynCAM = 0.175μm/s, +/- 0.008, p = 0.187, n = 73 movements).
Figure 3.6: Trans-synaptic signaling has little effect on synaptic vesicle or active zone protein transport. (A) Time-lapse images of a neuroligin-1 + HcRed-expressing HEK293 cell (magenta) contacting an axon expressing synaptophysin-GFP (green).

**Arrows**, position of individual STV in each frame. Scale bar = 10μm. (B) Contact with neuroligin-1-expressing cells increases the mean pause duration but not instantaneous velocity of STVs inside the contact region. (C) For PTVs, neither the mean pause duration nor the instantaneous velocity was affected by neuroligin-1-induced trans-synaptic signaling. (D) Contact with SynCAM-1-expressing cells for 1-4h did not affect
STV pause duration but increased their instantaneous velocity. (E) Contact with SynCAM-1 for 24h had no effect on STV pause duration or instantaneous velocity. (F) Contact with both neuroligin-1 and SynCAM-1 had no effect on STV pause duration or instantaneous velocity. *, p < 0.05; t = time after imaging begins; error bars = S.E.M.
In addition to comparing the transport of STVs and PTVs in axonal regions that contact HEK293 that either did or did not express synaptogenic adhesion molecules, we also compared axonal transport inside and outside of contact regions within the same axon. Each combination of transport vesicle and non-neuronal cell treatment described above was tested. No significant (all p > 0.05) difference in average pause duration or instantaneous velocity was observed when comparing transport vesicles inside of contact regions vs. those outside of contact regions (data not shown), with one exception. The average pause duration of STVs was significantly increased inside axonal regions that contacted SynCAM-expressing cells for 24h (outside contact = 105.6s +/- 6.1, n = 339 pauses; inside contact = 140.3s +/- 15.6, n = 62 pauses).

Together, our data suggest that while trans-synaptic signaling might have some effect on STV movement, regulation of transport is not the main mechanism through which trans-synaptic adhesion mediates synaptic protein recruitment. It is unclear whether the distinct effects of neuroligin and SynCAM on STV trafficking represent mechanistic differences in how recruitment of STVs occurs down-stream of presynaptic neurexin and SynCAM. Since neuroligin plus SynCAM did not result in the same changes as either neuroligin or SynCAM, it is possible that either (i) the observed changes are not essential for STV recruitment, or (ii) when accumulated at the same sites, presynaptic neurexin and SynCAM compete with each other for some factor that is necessary for the observed effects on STV trafficking, essentially dampening their distinct effects.

**Synaptic vesicle and active zone protein transport vesicles are not attracted to sites of trans-synaptic signaling**
Synaptogenic adhesion could recruit presynaptic machinery by actively attracting synaptic proteins to sites of trans-synaptic signaling. To test this hypothesis, we determined whether STV movements are biased towards sites of neuroligin-1 contact (Figure 3.7). First, we quantified the net distance and direction moved for individual STVs. Movements toward the contact were assigned positive values, while movements away from the contact were negative. This analysis indicated that neuroligin-1 signaling did not bias net STV movement toward neuroligin-1 (Figure 3.7B; net movement = -0.078 +/- 0.857μm, n = 67 STVs), similar to contacts with HcRed-expressing HEK293 cells (Figure 3.7C; net movement = -1.39 +/- 1.22μm, n = 44 STVs; p = 0.60). Since trans-synaptic signaling might only attract vesicles near the contact, we then restricted the analysis to STVs with initial positions adjacent to the contact, within a region equal to the contact in length. Again, neuroligin-neurexin signaling did not bias STV transport (Figure 3.7D; Nlgn = 0.000 +/- 0.888μm, n = 17 STVs; HcRed = -3.08 +/- 1.69μm, n = 19 STVs; p = 0.23). Moreover, the distributions of the net movements were not altered by neuroligin-neurexin signaling (p > 0.15, Kolmogorov-Smirnov). In addition, neuroligin-1 did not change the fraction of STVs moving toward contacts (Figure 3.7E; # STVs moving toward/# STVs moving away: Nlgn = 0.89, n = 66 STVs; HcRed = 0.83, n = 44 STVs). Similarly, STVs adjacent to contacts were not attracted to contacts (Figure 3.7F; Nlgn = 0.89, n = 17 STVs; HcRed = 0.46, n = 19 STVs).

STVs also displayed no bias in movement towards HEK293 cells expressing SynCAM-1- or SynCAM-1+neuroligin-1. SynCAM-1 did not alter the net distance and direction moved by all imaged STVs (p = 0.33, n = 136 and 88 STVs for SynCAM and HcRed, respectively) or STVs adjacent to contacts (p = 0.23, n = 39 and 28 STVs for
SynCAM and HcRed). The same was true when contacts expressed both SynCAM-1 and neuroligin-1 (all STVs: \( p = 0.70, n = 136 \) and 69 STVs for SynCAM+neuroligin and HcRed, respectively; near contacts: \( p = 0.55, n = 39 \) and 18 STVs for SynCAM+neuroligin and HcRed, respectively). Likewise, STV movement was not biased toward contacts with SynCAM-1 after 24h of contact (\( p = 0.13, n = 39 \) and 36 STVs for SynCAM and HcRed, respectively). Again, the data were not significantly different when the distributions were compared using the Kolmogorov-Smirnov test (\( p > 0.11 \)). Furthermore, the ratio of STVs moving toward vs. away from contact regions (for SynCAM, SynCAM+neuroligin, or 24hr SynCAM) was less than or equal to 1.13, indicating a lack of attraction to sites of trans-synaptic adhesion. Taken together, the above data demonstrate that trans-synaptic signaling does not actively recruit STVs from outside regions of contact.

Although STVs were not actively attracted to contact sites, it remained possible that trans-synaptic signaling actively recruits active zone precursors to sites of contact, followed by passive capturing of STVs. To test this, we determined whether movements of PTVs were biased toward trans-synaptic adhesion sites. Similar to STVs, PTVs showed no bias in movement towards neuroligin-1 contact sites (Figure 3.7G), both when including all PTVs in the analysis (\( \text{Nlgn} = -0.35/\pm 0.56 \mu m, n = 79 \) PTVs; \( \text{HcRed} = 1.38/\pm 0.68 \mu m, n = 69 \) PTVs; \( p = 0.071 \)) and when limiting the analysis to PTVs with initial positions adjacent to contacts (\( \text{Nlgn} = -1.34/\pm 1.12 \mu m, n = 26 \) PTVs; \( \text{HcRed} = 0.72/\pm 0.86 \mu m, n = 28 \) PTVs; \( p = 0.046 \)). When the distributions were compared using the Kolmogorov-Smirnov test, no differences were seen (\( p > 0.084 \)). Collectively, our data
indicate that trans-synaptic adhesion signaling does not attract synaptic vesicle or active zone matrix proteins to developing synapses.
Figure 3.7: Trans-synaptic signaling does not attract synaptic vesicle or active zone proteins. (A) Schematics of HEK293 cell contact with an axon. Analysis was done on all STVs with initial positions either outside the contact area (gray area, left, center) or directly adjacent to the contact region (gray area, right). (B-D) Histograms of the net movement of STVs towards (positive) or away from (negative) contacts with HEK293 cells expressing either neuroligin-1 + HcRed (B, D) or HcRed only (C). STV movement was not biased towards sites of neurexin-neuroligin signaling, for either all STVs or only STVs adjacent to the contact. (E, F) Scatter plot of the number of STVs moving towards vs. away from contacts with cells expressing neuroligin-1 + HcRed (blue circles) or
HcRed only (red squares). Each small point represents the ratio for one axon, with larger points representing additional axons with the identical ratio. These ratios were approximately the same for axons that contact cells expressing neuroligin-1 or HcRed (black line = unity ratio line). (G) Similar to STVs, PTVs were not attracted to sites of neuroligin-1 contact. (B-D, G) Red arrows = mean.
**Initial arrival of STVs and PTVs to a developing synapse is likely uncoordinated**

Due to the variety of synaptic components transported by STVs and PTVs, it is possible one type of transport vesicle is initially recruited to a developing synapse and subsequently traps the other type of transport vesicle and/or additional transport vesicles of the same type. If this is the case, then one of these transport vesicles would always arrive at a developing synapse before the other. Therefore, to determine whether STVs or PTVs are recruited to novel presynaptic terminals first, 6-8 DIV neurons were transfected with either RFP670 or bassoon-GFP + synaptophysin-RFP. Axons that co-expressed bassoon-GFP and synaptophysin-RFP and made contact with RFP670-expressing dendrites were then imaged at a rate of 1 frame per 90s for up to 24 hours. During this time, newly stabilized presynaptic terminals were formed along the axo-dendritic contact. Synapse stabilization was defined as the co-recruitment of synaptophysin-RFP and bassoon-GFP to the same site in the axon where both components remained at that site continuously for at least one hour.

Overall, 51 newly stabilized presynaptic terminals were observed at 11 unique neuron-neuron contacts. Upon visual examination of the images, it appeared that synaptophysin-GFP arrived at the synapse first 31% of the time (n = 16/51 stabilized synapses), bassoon-GFP arrived at the synapse first 33% of the time (n =17/51 stabilized synapses), and simultaneous recruitment of synaptophysin-GFP and bassoon-GFP occurred 35% of the time (n = 18/51 stabilized synapses, data not shown). This implies that there is no defined order for when STVs or PTVs arrive at the synapse. In addition, the high proportion of simultaneous arrivals suggests that when synaptogenic signaling
occurs, both STVs and PTVs can be recruited to the same site rapidly, at least within 90 seconds of each other, as that was the length of time between imaging frames.

There are caveats to this analysis, however. The frame rate of 1 frame per 90s was utilized to maximize temporal resolution while maintaining cell health over long periods of time. Long-duration imaging was necessary because novel formation of presynaptic terminals is relatively rare on existing axo-dendritic contacts. It is possible that one type of transport vesicle is always stabilized first and then rapidly recruits the other type of vesicle into the synapse. In addition, although there were many instances of STVs or PTVs arriving to a new synaptic site first, it was often difficult to distinguish when one of these components was initially stabilized at a specific site because both STVs and PTVs appeared to pause at these sites before becoming more “permanently” fixed to the site. This poses a challenge for analysis, as it would be impossible to distinguish between a transport vesicle that pauses at the same site every 90s during the imaging of each frame and a truly stabilized synaptic component. Therefore, higher temporal resolution is needed to distinguish between these two phenomenon. Ongoing experiments will utilize the synapse induction and long-term imaging protocol described in Figures 3.1, 3.2, and 3.3 to address this issue.
Materials and Methods

All studies were conducted with an approved protocol from the Case Western Reserve University Institutional Animal Care and Use Committee, in compliance with the National Institutes of Health guidelines for the care and use of experimental animals.

Neuronal culture and transfection

Primary neuronal cultures were prepared from rat or mouse cortices on 18mm glass coverslips as described previously (50, 51, 87, 207) and maintained in Neurobasal A media with B27 Supplement (Invitrogen, Carlsbad, CA, USA). At 6-9 days in vitro (DIV) and 24-48h prior to imaging, neurons were transfected using Lipofectamine 2000, essentially according to the manufacturer’s instructions (Invitrogen). DNA was used at 1μg per 18mm coverslip with the exception of GFP-bassoon, which was transfected at 2μg per 18mm coverslip due to its large size. This amount of DNA results in efficient labeling of transport vesicles without significant over-expression within axons (50, 51, 53, 87). Even so, neurons were chosen for imaging that displayed moderate expression levels of the transfected constructs to avoid any possible effects of over-expression. For double transfections, the localization and movement of fluorescently-tagged proteins appeared similar to single transfections. In addition, synaptic vesicle protein transport vesicles (STVs) labeled with synaptophysin-RFP and synaptophysin-GFP were similar in size, movement, and localization.

Synaptophysin-GFP, GFP-bassoon (GFP-Bsn 95-3938), synaptophysin-mRFP, synaptophysin-mcherry, and neurexin-1β-GFP were generous gifts of Drs. Jane Sullivan (University of Washington, Seattle), Thomas Dresbach (Georg August University,
Gottingen), Jurgen Klingauf (University of Muenster), Matthijs Verhage (Vrije Universiteit, Amsterdam), and Camin Dean (The European Neuroscience Institute, Gottingen). These constructs have been shown to be functional and properly localized and have been used previously for studies of protein trafficking and localization (50, 61, 115, 211, 213, 243). Fluorescent proteins attached to bassoon and synaptophysin effectively label PTVs/active zones and STVs/synaptic vesicles, respectively (48, 50, 53, 60, 61, 87, 115, 210, 211). RFP670 was purchased from Addgene [plasmid # 45457]. Neurexin-tdTomato was generated by seamlessly replacing the GFP sequence in the neurexin-GFP construct with tdTomato via Gibson Assembly (New England Biosystems).

**HEK293 cell culture and transfection**

HEK293 cells were cultured and maintained in DMEM supplemented with 10% fetal calf serum and penicillin/streptomycin (Invitrogen, HyClone). Cells were transfected 24-48h prior to dissociation and dropping onto neuronal cultures. A 3:2 or 2:1 ratio of trans-synaptic adhesion DNA to HcRed DNA (Clontech) was used to ensure co-expression of both types of DNA constructs in the same HEK293 cell (51). To confirm co-expression, HEK293 cells were transfected with SynCAM-GFP, HA-neuroligin-1, and HcRed. After 48h, cells were fixed and labeled with anti-HA antibody to detect HA-neuroligin-1. Over 85% of HEK293 cells transfected with HcRed also expressed SynCAM-GFP and HA-neuroligin-1 (data not shown). HA-neuroligin-1 and HA-SynCAM-1 were generous gifts of Drs. Peter Scheiffele (University of Basel) and Philip Washbourne (University of Oregon, Eugene) and have previously been shown to recruit synaptic proteins in the hemi-synapse assay (128, 244). Myc-tagged S-SCAM was
provided by the labs of Yutaka Hata (Tokyo Medical and Dental University, Tokyo, Japan) and Yoshimi Takai (Kobe University, Kobe, Japan) (245) and purchased from Addgene (plasmid # 40213245). pDEST/Lifeact-mcherry-N1 was provided by Dr. Robin Shaw (Cedars-Sinai, Los Angeles, CA, USA) (238) and purchased from Addgene [plasmid # 40908].

Imaging

Imaging was performed with a C1 Plus confocal system on a Nikon Eclipse Ti-E microscope utilizing a 40x Nikon Plan Apo 0.95NA objective. Lasers used for excitation were 488nm argon and 543nm and 633 nm helium-neon, while detection filters were 515/30nm bandpass for GFP and 590/50nm bandpass for HcRed/mcherry/mRFP. For multi-color imaging, channels were imaged sequentially to avoid bleed-through. To avoid focal plane drift, the Perfect Focus System (Nikon) was used.

Long-term imaging

Transfected HEK293 cells were dissociated with Hank’s-based, enzyme-free cell dissociation buffer (Invitrogen). Cells were resuspended in neuronal media then $8 \times 10^4$ cells were dropped onto each coverslip of transfected neurons. Contacts between transfected axons and transfected HEK293 cells were imaged, with axons identified by their morphological characteristics (50, 51). Dual-color images were collected every 10s for 7.5min, with scan times for both frames not exceeding 3.3s. This interval yields high temporal resolution of STV movement with minimal phototoxicity (87). This imaging protocol was repeated every hour for the next 4h then every 2.5h for up to 25h. To ensure that cells remained healthy, imaging was performed in a custom made environmental
chamber held at 32°C. The pH was maintained at physiological levels by equilibration in 5% CO₂ for 15-30min prior to sealing with vacuum grease (Dow Corning).

Short-term imaging

Synaptogenesis was induced as described above. After 1-4h, contacts between transfected axons and transfected HEK293 cells were imaged every 10s for 7.5min with constant perfusion of artificial cerebrospinal fluid (ACSF: 120mM NaCl, 3mM KCl, 2mM CaCl₂, 30mM D-glucose, 20mM HEPES, and 0.2% sorbitol, pH 7.3) at 25°C. At this temperature, STV movement is not significantly altered compared to STV movement at physiological temperatures (51). In previous studies, the fastest velocities recorded for STV movements were approximately 1μm/s (51, 53, 210), while maximal PTV velocities were lower (60, 73). Vesicles moving at this maximal velocity would move 10μm between frames during of imaging, a small fraction of the total axonal area typically imaged. Therefore, the rapid imaging of STVs/PTVs performed here enabled reliable identification and tracking of vesicles.

Neurexin-GFP recruitment

Axons expressing neurexin-GFP were imaged for at least 5min before contact with HEK293 cells to identify neurexin-GFP that was previously clustered through endogenous mechanisms. HEK293 cells transfected with HA-neuroligin-1 plus HcRed were added to the imaged coverslip until at least one transfected HEK293 cell made contact with the neurexin-expressing axons. This contact was then imaged for up to 2h at a rate of 1 frame/min.

Analysis
STVs and PTVs were manually tracked using custom written macros for ImageJ (NIH, Bethesda, MD, USA), while blind to the locations of the HEK293 cells. Analysis was restricted to axons that did not move during imaging and where at least one vesicle moved within the imaged area. To facilitate tracking, imaging was conducted on axons with an intermediate to low density of STVs or PTVs. Movements were quantified by importing the positional data into Matlab and subjecting them to custom written analysis (Mathworks, Natick, MA, USA). Pausing was defined as a period of at least 10s where the velocity was lower than 0.1 μm/s, as previously described (51, 87). Vesicles that did not move during imaging were not included in this analysis, and analysis was restricted to vesicles that could be tracked throughout the imaging period. Therefore, very long pauses and vesicles that moved at high velocities for long periods of time might be underrepresented. Considering the scan speed, pixel density, and average size of transport vesicles, a typical STV or PTV was imaged within 10-50ms.

Integrated density analysis was performed using custom-written macros in ImageJ that determined the location and integrated density of each transport vesicle on the axon for each image in a sequence. Contact regions were defined as axonal regions that overlapped with fluorescence from a transfected HEK293 cell. ‘% Enrichment’ was determined by the following equation:

\[
\%\text{ Enrichment} = \left[ \frac{(\text{IntDen}_{\text{in}} - \text{IntDen}_{\text{out}})}{(\text{IntDen}_{\text{in}} + \text{IntDen}_{\text{out}})} \right] \times 100
\]

Where IntDen\text{in} corresponded to the mean integrated density per μm of axon length inside the contact, and IntDen\text{out} corresponded to the mean integrated density per μm outside the contact for the same axon. Contacts were considered to display recruitment when the %
enrichment was (i) positive for at least 70% of time points 4 hours after contact was initiated and (ii) greater than 10% for at least half of all time points 4+ hours after contact initiation. Data are shown as the mean +/- standard error when applicable. Significance was determined via Wilcoxon rank sum test unless otherwise indicated. For correlation data, r = Pearson’s linear correlation coefficient and p-values were determined using Student’s t-test.

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Chapter 4: Imaging presynaptic dynamics during mouse cortical development via two-photon confocal microscopy

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*For individual contributions, please see Materials and Methods
**Introduction**

The previous experiments described in Chapters 2 and 3 have determined how the transport of presynaptic components is coordinated prior to synapse formation and how recruitment of these components progresses in response to induced synaptogenic signaling. These experiments were done exclusively in living cultured cortical neurons, which allowed for easy manipulation and high spatial and temporal resolution when performing live, time-lapse confocal imaging. While this *in vitro* system offers many benefits, it lacks key characteristics that are present in the intact brain. Specifically, normal connectivity that develops between specific brain regions is not recapitulated in neuronal cultures. In addition, many developing cortical circuits often require environmental stimuli to refine their connectivity, which is also lacking in culture.

Understanding how the dynamic processes described above proceed within the intact mammalian brain would therefore yield critical insight into how cortical circuits are shaped. To achieve this insight, Cre-induced synaptophysin-tdtomato was exogenously expressed in the mouse brain by viral injection of Cre-GFP. Cranial windows were applied to these mice and synaptophysin-tdtomato was imaged within the brain via 2-photon microscopy over the course of minutes. To detect whether presynaptic dynamics change over the course of development, multiple imaging time-courses were taken from the same animals on separate dates ranging from postnatal day 13 (P13) to P22. These experiments are the first to detect fluorescently-tagged presynaptic proteins within axons of the intact mammalian brain and add insight into synaptic dynamics that occur during development.
**Results**

To analyze presynaptic dynamics during development *in vivo*, presynaptic terminals must be both labeled and detected within the brain. In order to fluorescently label presynaptic terminals, “stop-flox” synaptophysin-tdtomato transgenic mice were obtained. These mice possess an allele that contains a loxP-flanked stop codon that is upstream of a synaptophysin-tdtomato fusion gene (Figure 4.1A). This stop codon normally inhibits synaptophysin-tdtomato expression. However, with Cre expression, the stop codon is removed and synaptophysin-tdtomato is expressed (Figure 4.1A). To induce synaptophysin-tdtomato expression in the brain, an adeno-associated virus (AAV) encoding Cre-GFP was injected via Hamilton syringe into the right hemisphere of neonatal pups aged P2-P4. This limited expression to cell bodies located in the right hemisphere of the cortex (Figure 4.1B). In addition, the expression of Cre-GFP was driven by the synapsin promoter, which restricted expression to neurons.

In order to detect Cre-induced synaptophysin-tdtomato expression, cranial windows were applied at postnatal day 10 to mice which had previously been injected with Cre-GFP AAV. Windows were applied over the left hemisphere of the cortex in order to image synaptophysin-tdtomato expression on the contralateral side of the injection (Figure 4.1C). This restricted the observable synaptophysin-tdtomato signal to callosally projecting axons in the left hemisphere whose somas were located in the right hemisphere. This imaging strategy provided a number of distinct advantages compared to imaging the ipsilateral hemisphere. First, since only axons cross the midline and project to the contralateral side of the brain, the synaptophysin-tdtomato puncta that were observed were strictly axonal and not mislocalized to dendrites. In addition, only
excitatory neurons produce callosal projections, thereby limiting the synapses that are detected to a specific population of cortical neurons. This neuronal population is clinically relevant as well, as defects in callosal development have been linked to a number of neurological disorders (246, 247).
**Figure 4.1: Synaptophysin-tdtomato expression in transgenic knock-in mouse.** (A) Schematic for synaptophysin-tdtomato expression in stop-flox synaptophysin-tdtomato mice. Injection of AAV encoding Cre-GFP downstream of the synapsin promoter induces Cre expression. In stop-flox synaptophysin tdtomato mice, this Cre expression drives recombination of the loxP sites, thereby removing the stop codon and inducing synaptophysin-tdtomato expression in neurons. (B) Image of brain expressing Cre-GFP (green) in the right hemisphere (white circle). Cranial window application and subsequent imaging occurs on the contralateral side (white square, arrow). (C) Example of single image taken in living mouse via two-photon confocal imaging of synaptophysin-tdtomato (yellow) through a cranial window. Scale bar = 20μm.
To determine if synaptophysin-tdtomato effectively localized to presynaptic terminals \textit{in vivo}, stop-flox synaptophysin-tdtomato mice that were injected with Cre-GFP were perfused, sectioned, and labeled with antibodies against the presynaptic protein synapsin. As highlighted by the white circles in Figure 4.2A, synaptophysin-tdtomato colocalized frequently with synapsin labeling. To further confirm the synaptic localization of synaptophysin-tdtomato, stop-flox synaptophysin-tdtomato mice were cross-bred with Thy-1-YFP mice which express YFP in a sparse population of layer V pyramidal cells. After injection with the Cre-GFP virus, the brains of these mice were perfused and sectioned. As shown in Figure 4.2B, synaptophysin-tdtomato puncta frequently and closely apposed the apical dendrite of YFP+ cells, suggesting a synaptic localization. It should be noted that, although these data suggest a synaptic localization for many, if not most synaptophysin-tdtomato puncta, it does not rule out the possibility of some synaptophysin-tdtomato signal being non-synaptic and/or part of transport vesicles within the axon.
Figure 4.2: Synaptophysin-tdtomato is localized to presynaptic terminals. (A)

Synaptophysin-tdtomato (*magenta*) in the cortex co-localizes with antibody-labeled synapsin (*green*). (B, *left image*) Multiple synaptophysin-tdtomato puncta (*magenta*) are seen closely apposed to the apical dendrite of a YFP-filled cell (*green*). White circles and square highlight co-localization points. (B, *right images*) Z-projections of the area highlighted by the white square further indicate that synaptophysin-tdtomato and dendrites are closely apposed in three-dimensional space. Scale bar for left image in (B) = 10μm, scale bar for right images in (B) = 1μm.
In order to monitor presynaptic dynamics within the intact brain, synaptophysin-
tdtomato was imaged through cranial windows in living mice via two-photon time-lapse
imaging. The frame rate of all time-lapse movies was one frame per thirty seconds, and
images were collected for up to one hour. In between each imaging frame, 30 seconds of
rest without imaging was performed to prevent overheating of the brain tissue. In some
instances, multiple time-lapse images of the same animal over the course of days were
obtained. This was not possible in all cases, however, as bone growth under the cranial
window occasionally obscured synaptophysin-tdtomato detection. Upon imaging, it was
determined that the majority of synaptophysin-tdtomato was detected in superficial areas
near the pial membrane, likely in layer I of the cortex. Therefore, our analysis focused on
these synapses which were consistently and strongly labeled across multiple animals over
multiple days of imaging. In order to determine whether the dynamics of presynaptic
terminals in this cortical layer change during development, imaging was done in animals
ranging in age from P13-P22, a time period in which critical synaptic development takes
place (248, 249). Movies were then binned according to specific developmental age.

Both splitting and merging of individual synaptophysin-tdtomato puncta were
observed at all points in development (Figure 4.3A,B). Both the percent of puncta that
split per minute of imaging and the percent of puncta that merged per minute of imaging
increased during the P16-P19 time period (compared to P13-P15) before becoming
reduced back to near original levels during the P20-P22 time period (for all percentages,
value = (# split puncta in time-lapse series/ total puncta in time-lapse series / length of
time-lapse series): for P13-P15, % puncta split per minute = 0.038, +/- 0.013, n = 6 time-
lapse series; % puncta merged per minute = 0.067, +/- 0.027, n = 6 time-lapse series; for
P16-P19, % puncta split per minute = 0.14, +/- 0.032, n = 14 time-lapse series; % puncta merged per minute = 0.18, +/- 0.034, n = 14 time-lapse series; for P20-P22, % puncta split per minute = 0.066, +/- 0.018, n = 5 time-lapse series; % puncta merged per minute = 0.11, +/- 0.042, n = 5 time-lapse series). The rates for both processes were quite similar at each time period analyzed, suggesting that these processes likely do not contribute to any overall changes in synapse density that occur during development. However, splitting and merging of presynaptic components likely plays at least some role in refining circuits over time. The extent to which splitting and merging of existing synaptophysin-tdtomato structures contributes to this refining process, as well as the mechanisms responsible for these phenomena remain to be determined.

It should be noted that spatial resolution is limited in the axial (Z) dimension due to the point spread function of light detected through the objective. This could lead to false detection of merging events, if one synaptophysin-tdtomato puncta moves directly above or below another puncta and remains in close proximity to that puncta. It might also lead to lower detection of splitting events, if a single puncta splits into two distinct puncta that separate in the axial direction and do not move away from each other.

Resolution of the lateral (X, Y) dimensions is better than that of the axial dimension. However, small distinct puncta that are in close lateral proximity to each other might be detected as a single larger puncta or larger puncta that split but do not move away from each other would not be detected. Again, this would lead to an overrepresentation of merging events and an underrepresentation of splitting events in our data.
Figure 4.3: Synaptophysin-tdtomato puncta both split and merge *in vivo*. (A) An example of two synaptophysin-tdtomato puncta (*red*) merging over the course of minutes within the intact mouse brain. (B) The percent of synaptophysin-tdtomato puncta that merge and split is similar over the same developmental time-course, suggesting that these processes do not account for major changes in synapse number at this developmental stage. However, circuit refinement is a likely consequence of these dynamics.
In addition to splitting and merging, a small population of synaptophysin-tdtomato puncta was quite mobile within the cortex. These puncta were manually tracked via Imaris software and subjected to analysis via custom-written MATLAB programming (Figure 4.4A). Both instantaneous velocity and puncta displacement were measured for mobile synaptophysin-tdtomato at each developmental time point. At the earliest timepoint (P13-P15), the average instantaneous velocity was \(0.141 \, \mu\text{m/s}, +/- 6.2 \times 10^{-3} \text{mm/s}\) (Figure 4.4B, \(n = 35\) movements), while it was slightly but significantly lower during the two subsequent time points, indicating a reduction of rapid movement for the most mobile puncta (Figure 4.4B, for P16-P19, inst. vel. = \(0.129 \, \mu\text{m/s}, +/- 3.3 \times 10^{-3}\), \(n = 97\) movements, \(p = 0.040\) (compared to P13-15); for P20+, inst. vel. = \(0.124 \, \mu\text{m/s}, +/- 7.9 \times 10^{-3}\), \(n = 13\) movements, \(p = 0.044\) (compared to P13-15)). The average total distance moved per puncta (normalized for length of the individual time-lapse image) was also decreased with developmental age as this value was significantly lower at the latest time point (Figure 4.4C, at P20+ distance moved = \(0.15 \, \mu\text{m/puncta/min}, +/- 0.045\, \mu\text{m/puncta/min}, n = 8\) puncta), compared to the two earlier time periods (Figure 4.4C, P13-15 distance moved = \(0.70 \, \mu\text{m/puncta/min}, +/- 0.13 \, \mu\text{m/puncta/min}, n = 16\) puncta, \(p = 0.0016\) (compared to P20+); P16-19 distance moved = \(0.68 \, \mu\text{m/puncta/min}, +/- 0.063 \, \mu\text{m/puncta/min}, n = 63\) puncta, \(p = 4.8 \times 10^{-5}\) (compared to P20+)). These data taken together suggest that presynaptic terminals in the developing cortex become less dynamic as time progresses.
Figure 4.4: Instantaneous velocity and mean displacement decrease as development progresses. (A) Series of time-lapse images showing movement of two different synaptophysin-tdtomato puncta over time (white arrow, red arrowhead). (B) Instantaneous velocity of moving puncta decreases slightly as development progresses. (C) Normalized distance moved per minute also decreased at later developmental time points. Scale bar = 10μm, * = p-val < 0.05, ** = p-val < 0.005
Materials and Methods

Contributions

Time-lapse two-photon imaging was performed by TAE while tracking analysis with Imaris software and MATLAB programming was performed by LADB.

Virus injection

LSL-synaptophysin-ttdtomato homozygous mice were obtained from Jackson Laboratories (stock #: 012570 (tdtom), Bar Harbor, ME, USA) and bred to obtain pups with the flox-stop synaptophysin-ttdtomato gene. For some experiments, these mice were cross-bred with Thy-1-YFP mice (Jackson Laboratories, Bar Harbor, ME, USA). At postnatal day 2-4, pups were anesthetized via hypothermia, after which AAV1-hSyn1-Cre-IRES-GFP virus was injected into the right hemisphere of the cortex via Hamilton syringe. Virus was created by the Vector Core at the University of Pennsylvania (Philadelphia, PA, USA). Pups were then warmed by hand before being placed back into their original cage.

Cranial window

At P10, pups that previously had undergone viral injection were anesthetized via inhalation of approximately 1-3% isoflurane solution vaporized in pure O₂ dispensed at a rate of 1.5L/min. Pups were then given a subcutaneous injection of dexamethasone (0.1mg/kg) and carprofen (5mg/kg) to prevent post-surgery edema and pain, respectively. The head was then stabilized via stereotaxic instrument (Stoelting Co., Wood Dale, IL, USA) before removing the skin on top of the head to expose the skull. A surgical microdrill (Fine Science Tools, Foster City, CA, USA) was then utilized to gently cut
around and remove a section of the skull over the left hemisphere to expose the brain. During drilling and after removal of skull piece, the surgical region was constantly submerged in sterile saline in order to avoid inflammation during the procedure and to keep the skull cool during drilling. Any minor bleeding that occurred was remediated by gentle application of GelFoam (Baxter, Deerfield, IL, USA) to the affected area. Once any bleeding was stopped, GelFoam was removed and a 5mm glass coverslip was placed over the exposed region of the brain. The coverslip was attached to the remainder of the skull via Vetbond glue (3M, Saint Paul, MN, USA) and Ortho-Jet self-curing dental acrylic (Lang Dental Manufacturing, Wheeling, IL, USA). Acrylic was mounded around the outside of the coverslip to create a well for the water immersion objective that was used for imaging through the cranial window. In addition, a stabilization bar was mounted in the acrylic. After surgery, mice were removed from anesthesia to recover on a warm pad before being returned to their home cage.

**Two-photon confocal imaging**

To visualize synaptophysin-tdtomato, mice with cranial windows were anesthetized via isoflurane (dose) and attached to a stereotaxic instrument via the stabilization bar that was mounted in the dental acrylic upon cranial window surgery. These mice were then transferred to a custom built heated chamber kept at 37℃ that surrounded the objective of the microscope. Isoflurane anesthesia was maintained via dedicated lines installed in the environmental chamber. A Leica SP5 confocal microscope fitted with a 16W Ti/Sapphire IR laser (Chameleon, Coherent, Inc., Santa Clara, CA, USA) and a 20x Leica HCX-APO-L, N.A. 1.0 water immersion objective was used for excitation and imaging at wavelengths tuned between 840 and 977nm. The tip of the objective was submerged in
water that was pooled in the dental acrylic mound over the cranial window. The water
column was maintained by dedicated water lines included in the environmental chamber.
Time-lapse imaging was performed at a rate of 1 image per 30 seconds with 30 seconds
of rest in between images. Individual images consisted of z-projections with a step-size of
1.5μm. After imaging, mice were allowed to recover in a warmed area before being
returned to their home cage.

**Perfusion, sectioning, immunohistochemistry**

Mice were deeply anesthetized via isoflurane inhalation and then transcardially perfused
with 1x PBS followed by 4% paraformaldehyde. Brains were then removed and drop-
fixed in 4% paraformaldehyde for 1 hour before being transferred to 30% sucrose
solution for cryoprotection. After brains were sunk in the sucrose solution, they were
frozen in dry-ice cooled 2-methylbutane and stored at -80°C until sectioning. Prior to
sectioning, tissue was mounted in OCT Compound (Tissue-Tek, The Netherlands) and
frozen on dry ice. Brains were then sectioned via Leica CM1850 cryostat at thicknesses
of either 10-15um (for antibody labeling) or 40um (for sections without antibody
labeling). Immunohistochemistry was then performed on thin (10-15um) sections
utilizing rabbit anti-synapsin primary antibodies (Synaptic Systems, Goettingen,
Germany) with anti-rabbit secondary antibodies conjugated to Alexa Fluor 488. Sections
were then mounted with fluoromount containing DABCO (Sigma, St. Louis, MO, USA)
and imaged via a C1 Plus confocal system on a Nikon Eclipse Ti-E microscope utilizing
a 20x Nikon Plan Apo 0.75NA objective.

**Image analysis and statistics**
All synaptophysin-tdTomato puncta were manually tracked via Imaris software.

Movement was analyzed via custom written programs in MATLAB. Significance was determined via Wilcoxon rank sum test unless otherwise indicated. All error bars are S.E.M. unless otherwise noted.
Chapter 5: Discussion

In Chapter 2 we hypothesized that trafficking of STVs and PTVs is coordinated even prior to synapse assembly, which could facilitate rapid assembly. By performing live multi-channel fluorescence confocal imaging in neurons expressing both synaptophysin-mRFP and GFP-bassoon, we were able to record and analyze the movements of STVs and PTVs simultaneously in the same neuron. The results indicate that STVs and PTVs are coordinated during transport and before stabilization since PTVs and STVs move together within the axon. We also find that STVs and PTVs pause at the same sites within the axon, particularly when the other type of vesicle is also paused at that site. This attraction of STVs and PTVs to the same pause sites is not mediated by a direct interaction between STVs and PTVs since reducing the density of PTVs in the axon yielded only small changes in STV pausing. In summary, the data support a model of synapse formation in which STV and PTV trafficking is coordinated even prior to axo-dendritic adhesion. This coordination includes coincident stopping of STVs and PTVs at predefined sites of synapse formation, independent of a direct interaction between STVs and PTVs.

Is STV and PTV transport coordinated?

Although it is clear that both PTVs and STVs need to be recruited to the same sites in order for pre-synaptic terminals to develop, it is not immediately apparent as to how they both arrive at the same destinations. Previously, STV and PTV dynamics have been imaged only separately, leaving it unknown whether they are transported together (47, 48, 50, 51, 53, 58-60, 210, 250). Recent work showed that clear, synaptic vesicle
protein-containing vesicles and dense-core, PTV-like vesicles can be seen apparently tethered together in electron micrographs of young neurons (49). This was the first indication that PTVs and STVs might be transported together, but it remained unclear whether these vesicle aggregates correspond to either STVs and PTVs being transported together or paused together or a later stage in synapse development. Our analysis indicates that a sizable portion of STVs and PTVs move with each other and spend the majority of their time together. STVs and PTVs often moved separately before or after moving together, suggesting that STVs and PTVs can move with each other while also maintaining their separate identities. This observation indicates that, although it is possible that some STVs contained molecules of GFP-bassoon and/or a portion of PTVs contained molecules of synaptophysin-mRFP, missorting of STV and PTV marker proteins cannot account for the observed co-transport of STVs and PTVs. Coordinating STV and PTV movement could represent a mechanism for rapid synapse development, since a full complement of synaptic proteins could immediately be delivered to a potential synaptic site. It will be important in the future to determine whether co-transport is mediated by a direct interaction between STVs and PTVs or by other mechanisms.

Previously, it was shown that STV pause sites are preferred sites of synapse formation (51), raising the question of whether PTVs also tend to pause at these same sites of synapse formation. By labeling both STVs and PTVs within the same axon, we were also able to compare spatial and temporal properties of pausing for both types of vesicles. STV and PTV pausing were qualitatively and quantitatively similar. Like STVs, multiple PTVs paused at the same sites, and the same PTVs returned repeatedly to
a given site. The frequency of pausing and duration of pauses for STVs and PTVs were also similar. Importantly, STVs and PTVs paused at the same sites within the axon. This implies that PTVs also pause at predefined sites of synapse formation. The propensity of STVs and PTVs to preferentially pause at the same sites within the axon suggests that there is a mechanism that recruits both types of vesicles to the same site at the same time. These same mechanisms could be utilized by axons to recruit the necessary proteins during synapse formation.

**What causes STV and PTV pausing?**

Our data indicate that PTVs preferentially pause at sites where an STV is present. Likewise, STVs preferentially pause at sites where a PTV is present. There are at least three potential hypotheses which could account for this phenomenon (Figure 5.1). First, pausing of STVs and PTVs at the same sites could be a result of STVs and PTVs travelling together and consequently being recruited together. Second, it is possible that a paused vesicle can interact with and stabilize a moving vesicle. In this scenario, a local signal could cause one type of vesicle to pause at a specific site within the axon. That paused vesicle could then interact with other vesicles and cause them to pause at the same site. Third, STVs and PTVs could be independently attracted to a common signal within the axon. In this case, both STVs and PTVs would respond to that signal by preferentially pausing at the site of the signal, regardless of the presence of other vesicles. This, in turn, would increase the probability that either vesicle was present at the site of the signal and, therefore, the chance STVs and PTVs are simultaneously paused at the same site. Each hypothesis represents a viable mechanism through which both synaptic vesicle and active zone proteins could be recruited to the same site in the axon.
Our data are most consistent with the hypothesis that STVs and PTVs are recruited to the same sites at the same time as a consequence of responding to a common local signal. The first mechanism – simultaneous recruitment of STVs and PTVs that are transported together – cannot by itself explain coincident pausing since in many cases the STVs and PTVs that paused together paused sequentially rather than simultaneously (e.g. see Figure 2.3A, 2.3B-1 and 2.3B-3). To distinguish between the remaining two mechanisms, we reduced the density of PTVs within the axon, using a dominant negative construct corresponding to the syntaxin-binding domain of syntabulin. If PTVs interact with STVs to influence STV pausing and recruitment to a given site, then decreasing PTV density should substantially alter STV pausing. However, in SBD-transfected axons, STVs displayed only small differences in pause duration and no change in the frequency of pausing when compared to STVs in control axons. Although differences in pause duration and velocity were statistically significant, the relatively subtle change in STV pausing suggests that PTVs exert only a small influence on STV pausing. This argues against the second hypothesis, in which STV and PTV pausing is exclusively controlled by an interaction between STVs and PTVs. However, based on the data, it is still possible that STVs and PTVs respond to both local signals and one another.

It will be important in the future to identify the signals that recruit STVs and PTVs. It is not yet known which signals cause vesicles to pause. Potential signals include calcium, phosphorylation or small GTPase activity. Although it was previously shown that calcium could increase the duration of pausing, the probability of pausing was unchanged by reducing intracellular calcium (51), suggesting that calcium alone may not be responsible. Phosphorylation is known to regulate the activity of microtubule motor
proteins and their association with microtubules or vesicles (101, 251-254), raising the possibility that phosphorylation of motor proteins is the ultimate mechanism by which local signals cause STVs and PTVs to stop their transport. Small GTPases are well-known for their ability to control vesicle targeting (255-257), and recent work has shown that in *C. elegans*, arl-8, an Arf-like small G-protein, controls where along the axon synaptic proteins aggregate (95).

**Why don’t all vesicles pause at any given site?**

Although the majority of STVs and PTVs that encounter pause sites will pause at them, many vesicles ignore the pause sites and continue their movements. This is even true for stopping and recruitment that occurs in response to synaptogenic adhesion or axo-dendritic contact (51). It is not clear why. One possibility is that there could be a limited number of “docking sites” at each pause site. This seems unlikely since some vesicles pass by even while others can still pause. Alternatively, STVs and PTVs could exist in multiple states, at least one of which is unresponsive to local signals that cause pausing. PTVs and STVs display heterogeneity in apparent size, velocity, and pausing properties, which has been noted here and elsewhere (47, 48, 51, 53, 58, 60, 73). These apparent differences may correlate with structural differences (49). In general, smaller/dimmer vesicles appear more likely to move quickly and pass by pause sites without pausing. Perhaps these apparently smaller vesicles are unable to respond to the cues that induce pausing. Such a state could be a result of differences in their motors or in other proteins associated with the STVs or PTVs. The idea of STVs and PTVs as heterogeneous sub-populations will be an interesting topic for further exploration.
A model of coordinated transport

For synapses to form, all of the components of presynaptic terminals must be delivered rapidly to the site of synapse formation. Despite its importance, we are only beginning to understand how this occurs. Here we have proposed a model for presynaptic terminal assembly in which trafficking of synaptic vesicle and active zone proteins is coordinated even prior to axo-dendritic contact. This coordination occurs through a combination of co-transport, perhaps in aggregates of vesicles tethered together (49), and co-pausing in response to local signals. This coordination then can facilitate presynaptic terminal assembly and contribute to the rapid recruitment of synaptic components that has been consistently observed. The same signals that induce co-pausing prior to transport may act down-stream of synaptogenic adhesion to simultaneously attract STVs and PTVs to sites of synapse assembly.
Figure 5.1: Models for coordination of recruitment of STVs and PTVs to the same place at the same time. STVs and PTVs could be simultaneously attracted to a given site by (1) attraction of STVs and PTVs that are co-transported; (2) sequential recruitment via a physical interaction between STVs and PTVs; and (3) simultaneous but independent recruitment in response to a common signal. Our data are most consistent with the third model.
What are the dynamics of presynaptic protein recruitment at developing terminals?

In Chapter 3, live, time-lapse confocal imaging of fluorescently-tagged proteins was performed at developing presynaptic terminals which were artificially induced through the interaction of trans-synaptic adhesion molecules. By imaging presynaptic proteins at newly induced synapses over the course of minutes and hours, we were able to determine that presynaptic recruitment is much more labile than previously thought. In addition, live imaging of developing synapses in axons co-expressing synaptophysin-RFP and neurexin-GFP established the timecourse and dynamics through which neurexin mediates its synaptogenic signal. Furthermore, tracking STVs and PTVs outside of synaptogenic regions strongly suggested that these components are trapped at developing synapses, rather than actively recruited to them.

Presynaptic proteins are trapped at, but not attracted to, developing synapses

Presynaptic terminal formation is initiated by contact between axons and dendrites, which leads to interactions between trans-synaptic adhesion molecules, recruitment of presynaptic proteins and subsequent organization of presynaptic terminals (Figure 5.2). It remains unclear how trans-synaptic signaling recruits presynaptic proteins. On one hand, trans-synaptic adhesion could actively attract transport vesicles to sites of axo-dendritic contact. Alternatively, trans-synaptic adhesion could establish sites in the axon where synaptic proteins become trapped and organized into a presynaptic terminal, without actively attracting transport vesicles.

Our results favor the “trapping” model (Figure 5.2, step 2, top). STVs and PTVs did not preferentially move towards sites of trans-synaptic signaling, and their transport
was minimally altered by trans-synaptic adhesion. These findings suggest that trans-
synaptic adhesion does not actively attract transport vesicles into developing presynaptic
terminals. Instead, these data support the hypothesis that trans-synaptic adhesion
establishes sites in the axon that capture and stabilize available presynaptic protein.
Importantly, STVs were not attracted to SynCAM-1 or both neuroligin-1 and SynCAM-1, and STV movement was unaffected by this combination of trans-synaptic signaling.
Therefore, assembly of synaptic proteins at developing presynaptic terminals via
establishment of sites of stabilization (rather than active attraction) appears to represent a
general mechanism that is conserved across synaptogenic pathways.

SV proteins preferentially accumulate at predefined sites that are intrinsic to the
axon (51). Although it is not known what stabilizes synaptic proteins at these specific
sites, STVs frequently pause at these sites prior to synaptogenesis. Trans-synaptic
adhesion might facilitate recruitment by stabilizing paused STVs (5, 51, 136). It is
important to note that STVs carry a variety of synaptic vesicle and some active zone
proteins (53), so it remains unclear which proteins mediate any interactions (direct or
indirect) between STVs and neurexin or SynCAM. ARL-8, which is transported with
STVs in C. elegans, has recently been shown to control aggregation and delivery of SV
and AZ proteins to appropriate sites of synaptogenesis (95, 96). Synaptogenic adhesion
could act through regulation of ARL-8 or a similar pathway to stabilize paused STVs.

The trapping model predicts that the supply of synaptic proteins in the axon
would determine the probability that synaptic proteins encounter these sites and,
therefore, levels of trapping and recruitment. Consistent with this, synapse formation is
enhanced by increasing the number of mobile STVs in the axon (160, 258), and synaptic
protein recruitment to poly-D-lysine coated beads preferentially occurs in axons with high levels of protein expression and mobility (116). Interestingly, disruptions in transport have been linked to defects in synapse formation and function (95, 96, 102, 111, 259, 260) and associated with intellectual disability in humans (261).

**Synaptic proteins are labile at developing synapses**

While there have been studies of the stability of new synapses (215, 262), it remained unclear how stable synaptic proteins are at synapses after their initial recruitment. Here, we showed that enrichment of SV proteins at individual trans-synaptic signaling sites is highly labile: although SV proteins remained enriched at these sites, the degree of enrichment rapidly increased and decreased, oftentimes varying over the course of minutes. Similar fluctuations in synaptic protein levels have been observed at mature synapses, where changes in levels of synaptic vesicles and active zone proteins have been directly linked to changes in synapse function (263-265). At mature synapses, lability in presynaptic protein levels arises through sharing of presynaptic components between adjacent synapses (218-221, 223-225, 266, 267), and individual shared SVs can be functionally integrated into the synapse within minutes after their arrival (223, 267). Our results suggest that this dynamic arrival and departure (fluctuation) of SVs at synapses occurs at the earliest stages in synapse development. It remains to be seen whether SVs at developing synapses can rapidly become incorporated into the synapse and fuse with the plasma membrane in response to action potentials. However, STVs can fuse with the plasma membrane upon neuronal depolarization (47, 51, 58) and most likely release glutamate at these sites when they do (51). Since glutamatergic signaling regulates the accumulation of presynaptic proteins at developing synapses (268-270), varying the level
of presynaptic proteins at new excitatory synapses could alter glutamate release and contribute to feedback or auto-regulation of synapse maturation.

We observed both gradual and sudden, dramatic changes in the amount of synaptic proteins recruited. These two types of fluctuations in recruitment may represent distinct features of presynaptic growth and stabilization. The sudden appearance of synaptophysin at the site of trans-synaptic adhesion indicates that presynaptic proteins can be recruited and stabilized at contacts on the order of seconds. This is striking considering recent evidence that dendritic spines can form in seconds in response to glutamate uncaging (271). Together, these data suggest that complete synapses can form extremely rapidly. In addition, the immediate loss of synaptophysin puncta that we observed suggests that developing synapses can be eliminated equally quickly. Finally, the gradual accumulation or loss of synaptophysin that occurred at many contacts may correspond to changes in presynaptic maturation or strength (272).

**A model of neuroligin-neurexin mediated presynaptic protein recruitment**

It has been proposed that synaptogenesis occurs in a 2-step model: postsynaptic clusters of neuroligin induce neurexin clustering, then clustered neurexin seeds the recruitment of presynaptic proteins (124). Our data support the hypothesis that neurexin clustering is critical for stable synaptic protein recruitment. However, we also found that neurexin clustering and synaptophysin recruitment appeared simultaneously. Although the two steps of the model could occur sequentially but very rapidly, an intriguing alternative explanation is that a cue within the axon cooperatively contributes to both neurexin clustering and STV accumulation. Consistent with this idea, recent work in *Drosophila* showed that neurexin clustering is mediated by axonal syd-1, and that this
process is critical for the synaptogenic ability of neurexin (125). Syd-1 acts upstream of syd-2/liprin-α, a protein linked to transport of STVs and initiation of active zone formation (77, 196-198, 203, 273, 274). It will be important in the future to determine whether similar molecular mechanisms link neurexin clustering to STV stabilization in mammals. Although mammalian orthologs of syd-1 do not contain the requisite PDZ for neurexin organization, mammalian syd-1 (mSYD1A) has recently been shown to both regulate SV protein accumulation during synaptogenesis and interact with liprin-α2 (206). Interestingly, in Drosophila, feedback from the postsynaptic partner also contributes to presynaptic assembly and stabilization (125). We found that the presence of the postsynaptic scaffolding molecule S-SCAM in neuroligin-1-expressing HEK-293 cells increased the degree of neurexin clustering in the axon, consistent with analogous cooperative pre- and postsynaptic mechanisms existing in mammals.

While many proteins that interact with the cytoplasmic domain of neurexin have been thought to be attractive candidates for mediating neurexin’s synaptogenic signaling within the axon, recent work has revealed that the intracellular tail of neurexin is completely dispensable for its synaptogenic properties (217). This suggests that neurexin utilizes unidentified cis interactions with axonal transmembrane proteins to signal intracellularly. It will be important to identify the cis-interacting neurexin partner(s) and its cytosolic interactions in order to understand neuroligin/neurexin-mediated synapse formation.

Here, we have focused on synapse assembly down-stream of neuroligin-neurexin signaling, the prototypical synaptogenic adhesion pair. Additional factors also play a role in regulating protein levels at presynaptic terminals, including local actin polymerization
(117, 234, 235, 275), signaling through BDNF/TrkB (258, 276-279) and NMDA receptors (270), altering VGLUT1 expression (268, 269), and through other trans-synaptic adhesion molecules not addressed in our study (136). These signals could regulate neurexin clustering, function down-stream of neurexin clustering, or operate through parallel mechanisms. Either way, these mechanisms likely work in concert to dynamically regulate synapse development and function.

**A model for dynamic presynaptic protein recruitment to a developing synapse**

Our results strongly support a model of synapse formation where presynaptic proteins are trapped at developing presynaptic sites, rather than actively attracted to them (Figure 5.2, steps 1-2). Trapping tends to occur at sites of neurexin clustering, but not at sites of diffuse neurexin recruitment, supporting the hypothesis that the spatial arrangement of neurexin is critical for neurexin/neuroligin induced synaptogenesis. Neurexin clustering and SV protein recruitment occur simultaneously (Figure 5.2, step 3). Finally, at individual nascent presynaptic terminals, SV protein levels can vary within minutes to hours, and these levels correlate with the amount of clustered neurexin. These fluctuations might, in turn, affect later stages of synapse formation, maturation and function.
Figure 5.2: Model of synaptic vesicle protein transport vesicle (STV) recruitment to a developing presynaptic terminal. (1) Axo-dendritic contact induces diffuse neurexin recruitment to the contact site. (2) STVs are not attracted to this site (bottom). Instead, they are trapped as they encounter this site during normal axonal transport (top). (3) As STVs become trapped, neurexin simultaneously clusters, resulting in the formation of a stable site of synaptic protein accumulation.
In vivo synaptic dynamics

A number of non-mammalian experimental models have been developed to address presynaptic protein recruitment and terminal formation within intact organisms (96, 126, 280-289). In mice, two-photon confocal imaging has been used to analyze postsynaptic spine formation and elimination within the intact brain by tracking morphological changes of dendritic spines in GFP-filled cells (290-292). Presynaptic bouton dynamics have also been evaluated in the mouse brain by identifying and studying axon swellings in GFP/RFP/tdtomato-filled cells (293-296). However, the identification of real presynaptic boutons is somewhat arbitrary as they are difficult to distinguish from non-synaptic axonal swellings (297). Furthermore, smaller, more dynamic synapses might be undetectable as axonal swellings.

In Chapter 4, we address these concerns through two-photon imaging of synaptophysin-tdtomato in the brain of living mice through cranial windows. We find that these fluorescently-tagged presynaptic proteins properly localize to synapses in the brain, making it an excellent tool to study how presynaptic structures change over time. Live, time-lapse imaging indicated that components such as synaptophysin-tdtomato in many presynaptic terminals within the intact brain change over minutes via splitting, merging, or wholesale movement. In addition, these experiments have shown that the frequency and extent of these dynamic processes are altered over the course of development, suggesting a possible functional role in the formation of circuits within the intact brain.
A number of questions remain in regards to these dynamic presynaptic processes which can be addressed by the type of *in vivo* imaging described here. For example, what are the functional consequences of splitting, merging, or moving a presynaptic terminal for the circuit? These experiments also only addressed the dynamics of presynaptic terminals over approximately one week of development. Do these processes occur at earlier timepoints? Do they occur and/or are they important in the aging brain? In addition, the live imaging of synaptophysin-tdTomato was performed mainly in layer I of the cortex. Are the dynamics of presynaptic terminals different in different layers? Furthermore, are there any differences in presynaptic dynamics in different cell types and/or cortical areas? While mice in these experiments were anesthetized, it is most likely possible to perform similar experiments in awake behaving mice (298). It would, therefore, be interesting to determine whether environmental stimuli affect presynaptic dynamics. Finally, it will be critical to determine whether these dynamics are altered in disease states.

**Future outlooks – directions for future investigations**

*N-cadherin: trans-synaptic facilitator*

An informative example of how elucidating the interactions between different trans-synaptic adhesion systems can lead to a more complete understanding of downstream synaptogenic signaling is illustrated by n-cadherin. N-cadherin, a homophilic trans-synaptic adhesion protein, was one of the first trans-synaptic proteins identified and was immediately hypothesized to be important for synapse formation. It has since been shown that n-cadherin and its intracellular signaling through β-catenin, β-
pix, and scribble are important for localizing SVs to the presynaptic terminal through F-actin polymerization (160, 161, 235). N-cadherin is also recruited to newly formed synapses in zebrafish on the same time course as STVs (283). However, the importance of n-cadherin for synaptogenesis decreases as neurons mature (161). In addition, synapse number actually increases in β-catenin knockout mice (160), possibly due to enhanced SV distribution within the axon (258). Furthermore, n-cadherin by itself cannot induce presynaptic terminal formation, as axonal contact with a non-neuronal cell expressing n-cadherin does not lead to presynaptic terminal formation at the contact site as it does with other post-synaptic adhesion proteins such as neuroligin and SynCAM (128, 157).

Despite this, n-cadherin interacts with other trans-synaptic adhesion proteins to promote their function. For example, n-cadherin mediates the synaptogenic capability of neuroligin in dendrites, most likely by enhancing neuroligin clustering postsynaptically through intracellular interactions with β-catenin and the synaptic scaffolding molecule (S-SCAM) (216, 233, 299). In addition, the n-cadherin/β-catenin complex interacts with members of the leukocyte common antigen-related family receptor protein tyrosine phosphatase (LAR-RPTP) family of proteins, which are important for the development and organization of presynaptic terminals (300, 301). Also, n-cadherin is a substrate for PTPσ, which can form a trans-synaptic adhesion complex with postsynaptically localized TrkC to mediate synapse formation (169, 302). More work is necessary to characterize n-cadherin’s interactions with other axonal trans-synaptic adhesion molecules. However, by studying multiple trans-synaptic adhesion complexes in concert, we are beginning to understand the complex and intertwined set of instructions that underlie synapse formation.
Regulation of the axonal actin cytoskeleton may be an important step in presynaptic terminal assembly down-stream of synaptogenic adhesion. Several synaptic adhesion molecules control assembly of a localized network of F-actin at nascent synapses, and formation of this cytoskeletal structure is required for synapse formation (235, 236, 275, 303, 304). Actin depolymerization during early stages of development leads to reduced synapse size and number (234), and various F-actin regulators play a role in synapse development in mammals, *Drosophila*, and *C. elegans* (236, 305-309). Importantly, the precise role of the actin cytoskeleton in presynaptic development is beginning to be unraveled. It has long been known that F-actin within the presynaptic terminal organizes synaptic vesicles and may localize them near the active zone membrane. Prior to synapse assembly, depolymerization of actin alters the mobility of STVs and their pausing behavior at sites of eventual synapse formation (51). F-actin can also interact with active zone proteins and control active zone protein assembly at synapses (275). In turn, active zone proteins, such as piccolo, can promote the formation of presynaptic F-actin structures (193).

*New technologies for studying synaptogenesis*

It is clear that an important goal of developmental neuroscience is to understand the mechanisms of synaptogenesis, but it is equally clear that many large gaps remain in our understanding of the process of synapse formation. In many ways, the nature of synapse formation has made this a difficult problem to attack. For example, organisms need synapses to survive, limiting what can be discovered using traditional genetic screens. In addition, observing the events of synapse formation can be challenging
because of (1) the unpredictable nature of when and where en passant synapses form in the brain and (2) the rapid assembly of individual presynaptic terminals once the process is initiated at any given axo-dendritic contact. Moreover, synapse elimination occurs concurrently with synapse formation, making it impossible to determine whether manipulations have specifically altered synapse assembly (as opposed to elimination) in fixed tissue. Therefore, approaches such as fast, multi-channel time-lapse imaging are necessary, in order to examine the time course of changes at individual synapses.

Application of recent advances in time-lapse imaging to the study of synapse assembly should propel the field forward. For example, time-lapse imaging has traditionally relied on the use of exogenously expressed proteins bearing fluorescent tags, such as GFP, but exciting new methods have been developed that allow fluorescence imaging of endogenous proteins (237, 310). In addition, the vast majority of studies applying time-lapse imaging to study mammalian synaptogenesis have been performed in culture. While it is likely that the core mechanisms of synaptogenesis are conserved in vitro, it will be important to verify this with live in vivo imaging of presynaptic terminal formation. Pioneering studies have examined presynaptic development in non-mammalian models and at the mammalian neuromuscular junction (96, 126, 280-288); however, presynaptic terminal development and synaptic protein recruitment will need to continue to be investigated within the mammalian brain utilizing experiments like those described in Chapter 4.

Another technical advance with promise to dramatically improve our understanding of the mechanisms of synapse assembly is the development of high-throughput genetic screens in mammalian neurons. For decades, elegant genetic screens
in *Drosophila* and *C. elegans* have provided insight into the molecular mechanisms of synapse formation. Recently, screens have been used to identify molecules involved in mammalian synaptogenesis, as well (169, 214, 311-313). These screens have been particularly useful for identifying cell surface receptors that play a role in synaptogenesis. Future screens should be helpful in identifying intracellular signals that orchestrate synapse assembly. In addition, progress in cultivating neurons and organoids from human induced pluripotent stem cells suggests such screens could be performed on human neurons in the near future (314-316).

**Conclusion**

Synaptogenesis is a continuous process that occurs trillions of times over the course of a human life, and its disruption can lead to devastating neurological and psychiatric consequences. Although significant advancements into understanding presynaptic development have been made, much more work is required. Critical to this endeavor will be investigations into how all of these signaling mechanisms interact with one another and lead to the dynamics observed in presynaptic development. Additionally, insights into how disease-associated mechanisms affect the coordination of these processes are needed. Ultimately, this will lead to a better understanding of how circuits are established in the brain and how they are altered in disease.
Bibliography


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