Transmission, Development and Plasticity of Synapses

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Abstract

Chemical synapses are sites of contact and information transfer between a neuron and its partner cell. Each synapse is a specialized junction, where the presynaptic cell assembles machinery for the release of neurotransmitter, and the postsynaptic cell assembles components to receive and integrate this signal. Synapses also exhibit plasticity, during which synaptic function and/or structure are modified in response to activity. With a robust panel of genetic tools, potent imaging and electrophysiology approaches, and strong evolutionary conservation of molecular components, *Drosophila* has emerged as an essential model system for investigating the mechanisms underlying synaptic assembly, function and plasticity. We will discuss techniques for studying synapses in *Drosophila*, with a focus on the larval neuromuscular junction, a well-established model glutamatergic synapse. Vesicle fusion, which underlies synaptic release of neurotransmitters, has been well characterized at this synapse. In addition, studies of synaptic assembly and organization of active zones and postsynaptic densities have revealed pathways that coordinate those events across the synaptic cleft. We will also review modes of synaptic growth and plasticity at the fly NMJ, and discuss how pre- and postsynaptic cells communicate to regulate plasticity in response to activity.

Analyzing synaptic function at the *Drosophila* neuromuscular junction

Experimental approaches in *Drosophila* have provided fundamental advances in the fields of developmental biology, cell biology and neuroscience. By taking advantage of genetic techniques available in the fruit fly, Seymour Benzer and his lab identified and characterized individual mutations in neuronal genes that regulated various aspects of fly behavior (Benzer, 1967). With the initial characterization of neurological mutants such as *Shaker* (*Sh*) (Kaplan and Trout, 1969) and *shibire* (*shi*) (Ikeda *et al*., 1976), it was clear that *Drosophila* offered a powerful genetic system for dissecting the molecular machines underlying neuronal excitability and synaptic function. Coupled with the ease of performing electrophysiological recordings in *Drosophila* larvae (Jan and Jan, 1976), the glutamatergic neuromuscular junction (NMJ) has become an important model for characterizing synapse formation and function.

The developmental events underlying formation of the NMJ have been well characterized (Broadie and Bate 1993b). During mid to late embryogenesis, motor neurons select a specific target muscle(s) from a field of 30 potential targets. After axons leave the central nervous system (CNS) and contact their target, they undergo a morphological transition from a large flat growth cone to the formation of axonal

varicosities that occurs over several hours (Yoshihara *et al*., 1997). Following initial synapse formation, a second phase of synaptic development occurs characterized by the addition of new varicosities and synaptic branches (Schuster *et al*., 1996; Zito *et al*., 1999). Each varicosity (termed a synaptic bouton) contains spatially segregated active zones (AZs) that reside opposite a distinct cluster of glutamate receptors (GluRs) on the postsynaptic muscle surface (Fig. 1A). AZs are readily identifiable by electron microscopy (EM) as electron-dense T-bar structures (Feeney *et al*., 1998) (Fig. 1A). The precise matching of AZ to GluR cluster has allowed optical imaging of synaptic vesicle (SV) release at individual release sites at the fly NMJ (Peled and Isacoff, 2011; Melom *et al*., 2013), yielding an experimental system that offers unprecedented tools for analyzing how synaptic transmission is organized and regulated at individual AZs.

During the first week of the *Drosophila* lifecycle, the animal progresses through three larval stages during which muscle size expands significantly. With this change in postsynaptic surface area, the innervating axon adjusts its synaptic arbor by increasing varicosity number five- to ten-fold during development, depending upon the specific muscle. Synaptic function at the NMJ has been assayed primarily at two stages of development – the late embryonic/early 1st instar synapse and the more mature 3rd instar synapse (Fig. 1B). Of the 30 muscles present in each abdominal hemi-segment, muscles 6 and 7 have been studied most often due to their location on the ventral body wall musculature, making them easily accessible for physiology. Two motor neurons, MN6/7-Ib and MNSN b/d-Is, innervate muscles 6 and 7 (Hoang and Chiba, 2001). MN6/7 forms large type Ib (big) boutons, with physiological properties similar to tonic motor terminals described in crustaceans (Atwood *et al*., 1993). In contrast, the Is terminals are thought to be more similar to phasic nerve endings described in other invertebrates. Beyond muscles 6 and 7, muscle 4 has also been widely studied due to its smaller terminal arborization pattern.

For physiological analysis, embryos or larvae are dissected along the dorsal midline, exposing the ventral musculature. Motor neuron axons can then be cut from where they exit the ventral ganglion and placed into a suction electrode for direct control of action potential generation. Alternatively, the axons can be suctioned without severing them if one is interested in the endogenous bursting patterns normally generated by the CNS motor pattern generator. For studies at the embryonic NMJ, patch clamp recordings of muscle currents induced by axonal stimulation or endogenous firing patterns can be recorded (Broadie and Bate 1993b). The 3rd instar synapse can be analyzed with focal patch recordings from single boutons (Kurdyak *et al*., 1994), or through the use of two-electrode voltage clamp or single sharp electrode recordings (Jan and Jan, 1976). The embryonic synapse offers numerous advantages, but its small size makes the preparation difficult to work with, limiting its use to only a few labs with the required expertise. Recordings at the embryonic NMJ have been useful for examining the kinetics of SV release, as the small number of AZs at this stage of development (20-30 release sites) makes for highly sensitive measurements of the timing of vesicle fusion (Broadie *et al*., 1994; Yoshihara and Littleton, 2002; Yoshihara *et al*., 2010). In addition, the embryonic synapse provides the only opportunity to assay synaptic function for essential genes that result in early lethality (Broadie *et al*., 1995; Schulze *et al*., 1995; Sweeney *et al*., 1995; Yoshihara *et al*., 1999). Another advantage of the embryonic NMJ is the robust forms of synaptic plasticity it displays compared to the more mature 3rd instar counterpart

(Yoshihara *et al*., 2005; Barber *et al*., 2009). The embryonic synapse is also less susceptible to compensation mechanisms than the 3rd instar synapse. Despite these advantages, the difficulty in recording from embryonic terminals has made the 3rd instar synapse the more popular model preparation. It is far more accessible for physiological analysis, with hundreds of individual release sites along the axon at this stage of development. As such, the 3rd instar NMJ has become the favorite preparation for analyzing synaptic function in mutant animals that survive to this stage of development.

Molecular regulation of synaptic vesicle fusion

SNARE-mediated fusion

The core machinery for SV fusion and neurotransmitter release is the SNARE complex (Söllner *et al*., 1993; Littleton *et al*., 1998; Chen *et al*., 1999). Vesicle-anchored v-SNAREs (synaptobrevin/VAMP) engage target membrane t-SNAREs (syntaxin and SNAP-25) to zipper together to form a four helix coiled-coil structure that brings the synaptic vesicle and plasma membranes into close apposition (Fig. 2A) (Sutton *et al*., 1998; Weber *et al*., 1998). The rapid influx of Ca^{2+} through voltage-gated channels following an action potential triggers SV exocytosis within milliseconds (Katz and Miledi, 1967). Although the SNARE complex is a ubiquitous fusion machine present throughout the cell, neurons have evolved proteins that regulate SNARE dynamics to allow rapid Ca^{2+}-triggered fusion of the SV and plasma membrane bilayers specifically at the AZ (for review, see Südhof and Rothman 2009). Current models indicate that AZ components interact with SVs to cluster them near release sites, ultimately leading to morphological docking at the AZ membrane. Following docking, priming reactions occur that generate a vesicle-plasma membrane state that is ready for rapid fusion in response to Ca^{2+}. After priming, two molecular pathways are thought to be important for controlling the fusion reaction. First, a molecular "fusion clamp" is predicted to engage the SNARE proteins and prevent them from activating in the absence of Ca^{2+}. Second, upon Ca^{2+} influx, sensors in the vicinity of the AZ detect the rise in Ca^{2+} and trigger SNARE-mediated fusion. Although many of the candidate proteins in SV release were originally identified through biochemical approaches using vertebrate neurons, the ease of genetic manipulation in *Drosophila* has made the fly NMJ a model system for testing the function of these molecular players.

Studies of the SNARE fusion machine itself have been done in *Drosophila*, with null mutations in the loci encoding the v-SNARE n-Synpatobrevin (n-Syb) and the t-SNARE Syntaxin 1A (Syx1A) resulting in embryonic lethality (Broadie *et al*., 1995; Schulze *et al*., 1995; Yoshihara *et al*., 1999). This limits study of severe disruptions in these SNAREs to recordings at the embryonic NMJ. Loss of *syx1A* results in cell lethality due to its essential role in post-Golgi to plasma membrane fusion in all cells (Schulze and Bellen, 1996). Maternal RNA deposited into the egg allows *syx1A* null mutants to live to late embryogenesis, when synaptic function can be analyzed. At synapses, loss of *syx1A* results in an absence of both spontaneous and evoked neurotransmitter release, indicating this t-SNARE is absolutely required for SV fusion. In contrast, loss of the v-SNARE *n-syb* does not abolish spontaneous SV fusion, though it does eliminate evoked release. If the SNARE machinery were essential for all SV fusion events, it remains unclear why *n-syb* should maintain the ability to fuse vesicles spontaneously. One model is that v-

SNAREs primarily participate in evoked release, allowing coupling of the Ca^{2+} signal to the fusion reaction through unknown mechanisms. A more likely explanation is that other members of the v-SNARE family, of which five have been described in *Drosophila* (Littleton, 2000), can substitute for *n-syb* in spontaneous but not evoked release. In addition, some spontaneously fusing vesicles may normally use distinct v-SNAREs from those required for evoked release (Kavalali, 2015). A similar redundancy issue appears to be working for the other t-SNARE, SNAP-25. Null mutations in *Snap-25* have relatively normal neurotransmitter release at 3rd instar NMJs, with the similar SNAP-24 protein proposed to compensate for the loss (Vilinsky *et al*., 2002). These redundancy issues confound robust structure-function approaches that have been applied to other proteins like Synaptotagmin, although there have been some efforts in the field to examine hypomorphic mutations of the SNARE proteins in *Drosophila* (Littleton *et al*., 1998; Wu *et al*., 1999; Stewart *et al*., 2000; Rao, Stewart, *et al*., 2001; Bykhovskaia *et al*., 2013; Megighian *et al*., 2013; DeMill *et al*., 2014). Although the requirement for the SNARE complex in fusion is well accepted, there are still numerous questions. Is SNARE assembly required for vesicle docking and can SNAREs fully zipper without fusion of the vesicle? One popular model is that zippering of the SNAREs can be arrested at various points along the pathway to generate a partially zippered complex that promotes docking/priming of the SV. Full zippering driven by Ca^{2+} influx and downstream effectors would then lead to fusion. These are still models at this point in the field, however, and await future analysis.

SNARE complex disassembly

Following SV fusion, the SNARE complex must be disassembled to allow further rounds of fusion. The heximeric ATPase, NSF, and the SNAP family of adapter proteins that tether it to the SNARE complex, carry out this function. Mutations in NSF were originally identified as alleles of the *comatose* locus, a well-known temperature-sensitive behavioral mutant (Pallanck *et al*., 1995). Disruption of NSF function leads to the accumulation of SNARE complexes and a gradual reduction in synaptic transmission (Littleton *et al*., 1998; Tolar and Pallanck, 1998; Kawasaki and Ordway, 1999). By examining interactions with other synaptic mutants, NSF function has been linked to a post-exocytosis role at the synapse to disassemble cis-SNARE complexes (those residing in the same membrane) after fusion (Littleton, Barnard *et al*., 2001; Sanyal *et al*., 2001). NSF is also required at adult muscle synapses to replenish free t-SNAREs at the AZ for maintaining vesicle fusion during stimulation trains (Kawasaki and Ordway, 2009). Mutations in the α-SNAP protein that is required to tether NSF to the SNARE complex are embryonic lethal (Babcock *et al*., 2004). Recordings done in hypomorphic *α-SNAP* mutants have not yet revealed release defects, so further study is required to pinpoint how the disassembly machine is actually working. It may be that nerve terminals with large pools of SVs can function at a reduced level without SNARE disassembly being required until the SV pool has undergone multiple cycles and free SNAREs are depleted.

Synaptotagmin

To regulate the constitutive fusion machinery, an additional set of neuronal-specific proteins have been identified that serve to facilitate rapid Ca^{2+}-dependent SV fusion. Several proteins bind directly to assembled SNARE complexes, including

Synaptotagmin 1 (Syt1) and Complexin (Cpx). Syts are a family of vesicular Ca^{2+} sensors with two cytoplasmic Ca^{2+} binding C2 domains, termed C2A and C2B (Adolfsen and Littleton, 2001; Sudhof, 2004). Syt1 is one of the most well studied vesicle trafficking proteins and functions to sense Ca^{2+} influx and regulate fusion of SVs (Littleton *et al*., 1993, 1994; Broadie *et al*., 1994; DiAntonio and Schwarz, 1994; Reist *et al*., 1998; Littleton, Bai, *et al*., 2001; Yoshihara and Littleton, 2002; Mackler *et al*., 2002; Saraswati *et al*., 2007; Yoshihara *et al*., 2010; Striegel *et al*., 2012; Jorquera *et al*., 2012; J. Lee *et al*., 2013; Lee and Littleton, 2015). Neurotransmitter release is characterized by a synchronous phase of SV fusion that occurs within milliseconds, and a slower asynchronous component that can last for hundreds of milliseconds depending on the synapse (Kaeser and Regehr, 2014). *Drosophila syt1* null mutants lack the fast synchronous component of evoked fusion, and show enhanced asynchronous and spontaneous release as well (Fig. 2B) (Littleton *et al*., 1993; Yoshihara and Littleton, 2002; Yoshihara *et al*., 2010; Jorquera *et al*., 2012; J. Lee *et al*., 2013; Lee and Littleton, 2015). These data suggest that two kinetically distinct Ca^{2+} sensors exist, with Syt1 mediating the rapid, synchronous component of transmitter release (Yoshihara and Littleton, 2002), and a second unknown Ca^{2+} sensor underlying asynchronous fusion. Recent data indicate the subcellular localization of Syt1 may also differentially regulate these two mechanisms for SV fusion. Syt1 requires membrane tethering to SVs to activate synchronous SV release, although asynchronous release can be promoted by cytosolic versions of Syt1 lacking the transmembrane tether (Lee and Littleton, 2015). To examine how Syt1 controls SV fusion, the field has generated and performed electrophysiological analysis of transgenically expressed *Syt1* transgenes mutated at Ca^{2+} binding sites in C2A or C2B in the background of *Drosophila syt1* null mutants (Fig. 2B). Animals expressing mutations that disrupt Ca^{2+} binding to C2A have normal synchronous release, but do not suppress asynchronous release (Yoshihara *et al*., 2010). In contrast, rescue with Ca^{2+} binding mutants in C2B display little synchronous release, but suppress asynchronous fusion (Mackler *et al*., 2002; Yoshihara *et al*., 2010). These results indicate the tandem C2 domains of Syt1 play unique roles in neurotransmission, with Ca^{2+} binding to C2A suppressing asynchronous release and Ca^{2+} binding to C2B driving synchronous fusion. Based on this data, Syt1 may act during fusion similar to that of a rapidly inactivating ion channel, adopting a conformation that rapidly promotes release (linked to C2B Ca^{2+} binding), followed by a second conformational change (linked to C2A) that prevents further fusion. This would provide a robust synchronous SV fusion response following an action potential. Syt1 has also been implicated in SV docking and endocytosis, though these interactions appear to represent Ca^{2+}-independent properties of the protein (J. Lee *et al*., 2013). Beyond lipid penetration by the C2 domains following Ca^{2+} binding, little is known about how Syt actually drives the fusion process. Whether the two C2 domains engage only the plasma membrane, or rather bind both the SV and plasma membranes simultaneously is unknown. Likewise, although Syt-SNARE interactions are observed *in vitro*, if they are actually required *in vivo* is still unclear.

Complexin

Cpxs are cytosolic proteins that interact with fully assembled SNARE complexes with a 1:1 stoichiometry (McMahon *et al*., 1995). Loss of *cpx* function results in two distinct phenotypes at *Drosophila* NMJs: a dramatic increase in spontaneous release and

a reduction in evoked release (Huntwork and Littleton, 2007; Xue *et al*., 2009; Cho *et al*., 2010; Jorquera *et al*., 2012; Buhl *et al*., 2013; Bykhovskaia *et al*., 2013; Iyer *et al*., 2013; Choi *et al*., 2014). These findings suggest a dual role for Cpx at the synapse. First, the protein functions as a SV fusion clamp to inhibit spontaneous release that occurs in the absence of an action potential. An inhibitory role for Cpx in vesicle fusion has also been observed biochemically, where Cpx can reduce SNARE-mediated fusion *in vitro* in cell-cell (Giraudo *et al*., 2006) and liposome (Schaub *et al*., 2006) fusion assays. How Cpx inhibits release is still being elucidated. A popular model is that Cpx binds to SNARE complexes during zippering, preventing full assembly prior to fusion (Fig. 2C). There are computational studies to support such a role (Bykhovskaia *et al*., 2013), but it still remains speculative at this point. Another potential mechanism for Cpx as a clamp is suggested by the suppression of the dramatic increase in minis (>50-fold in *cpx* mutants alone) in double mutants with *syt1* (Fig. 2D) (Jorquera *et al*., 2012). This finding has several potential interpretations. One is that Cpx could function to modulate when Syt1 engages the SNARE complex. In the absence of Cpx, Syt1 would bind the SNARE complex in a distinct fashion that could trigger Ca^{2+}-independent release. An alternative model could be suggested by the loss of SVs outside of the AZ in *syt1* mutants due to Syt1's role in endocytosis. If Cpx were to function to clamp SVs from fusing specifically at non-AZ regions, this vesicle pool would be lost in the absence of Syt1, thus suppressing the increase in minis. A final model is known as the "zig-zag" hypothesis and is based on some structural evidence that Cpx can crosslink two SNARE complexes together. Although disruption of a zig-zag Cpx-SNARE complex interaction might alter clamping, the current data are not fully consistent with this hypothesis (Cho *et al*., 2014). In contrast to the uncertainty into how Cpx clamps release, there are some attractive models for how Cpx mediates its positive role in fusion to enhance evoked release. Studies in the fly have clearly indicated these two roles – clamping release and facilitating evoked fusion – can be genetically separated, indicating distinct effectors are likely (Cho *et al*., 2010, 2014). Although SNARE binding is required for both roles, Cpx's ability to regulate Syt1 function appears to drive its function in evoked release. Through overexpression and loss-of-function analysis, Jorquera *et al*. (2012) have shown that Cpx can regulate the Ca^{2+} cooperativity and speed of SV release. These properties suggest Cpx can help couple Syt1 to the SNARE fusion process to enhance its Ca^{2+} sensitivity and the speed of vesicle release.

Other regulators of synaptic release

In addition to SNAREs, Syt1 and Cpx, a few additional key regulators of release have been examined at the *Drosophila* NMJ. In particular, genetic approaches have indicated essential roles for the Unc13 and Unc18/Rop proteins in SV fusion. Unc13 interacts with Syntaxin and appears to control when the t-SNARE is allowed to form SNARE complexes (Richmond *et al*., 2001). Its loss leads to embryonic lethality and a complete absence of spontaneous and evoked fusion, indicating an absolutely required role (Aravamudan *et al*., 1999). Mutations in Unc18/Rop are also embryonic lethal, suggesting the protein is essential for SV release (Harrison *et al*., 1994). Partial loss-of-function alleles have fallen into two groups, one set with enhanced evoked release and another with reduced evoked release (Schulze *et al*., 1994; Wu *et al*., 1998). Although it is unclear how the protein regulates release, a popular model is that it chaperones the t-

SNARE Syx1A during the fusion cycle. Future studies of these two proteins are required to determine how they interface with Syt1 and Cpx to control SV trafficking. Beyond SNARE binding proteins, additional molecular processes, including ATP generation and Ca^{2+} buffering by mitochondria, have been implicated in the regulation of synaptic transmission, though it can be challenging to disentangle how these more global changes in energy production interface with the synaptic exocytotic and endocytotic machineries (Stowers *et al*., 2002; Wang *et al*., 2004; Guo *et al*., 2005; Verstreken *et al*., 2005; Chouhan *et al*., 2010). Given the emerging links between metabolic dysfunction and neurological disease, this is an area that is likely to see strong interest in future studies.

Synaptic vesicle recycling and recovery

Following fusion, SVs must be retrieved for additional rounds of release. There is a rich literature describing *Drosophila* endocytosis mutants (for review, see Zhang, 2003; Kidokoro, 2006; Rodal and Littleton, 2008). Beginning with the pioneering work on the *shibire* temperature-sensitive mutation (Ikeda *et al*., 1976), numerous studies have highlighted the key role of endocytotic proteins in vesicle retrieval. The general dogma is that clathrin-mediated endocytosis is the major route of SV retrieval, though there is debate over whether clathrin-independent kiss-and-run mechanisms for vesicle uptake are possible at the NMJ (Verstreken *et al*., 2002; Dickman *et al*., 2005). Clathrin is recruited to plasma membrane regions containing SV proteins through the actions of the adapter proteins of the AP2 complex (González-Gaitán and Jäckle, 1997) and AP180 (Zhang *et al*., 1998; Vanlandingham *et al*., 2014). Following SV budding from the plasma membrane driven by formation of clathrin cages, the dynamin GTPase protein encoded by *shibire* functions to complete the fission process (Koenig and Ikeda, 1989; van der Bliek and Meyerowitz, 1991; Ramaswami *et al*., 1994). A host of proteins have been identified that interact with dynamin, clathrin, the AP complexes or plasma membrane lipids to regulate the endocytosis process, including Endophilin (Guichet *et al*., 2002; Rikhy *et al*., 2002; Verstreken *et al*., 2002; Dickman *et al*., 2005), Synaptojanin (Verstreken *et al*., 2003; Dickman *et al*., 2005), Dap160 (Roos and Kelly, 1998; Koh *et al*., 2004; Marie *et al*., 2004), Eps15 (Koh *et al*., 2007), Stonin 2 (Martina *et al*., 2001) Stoned (Stimson *et al*., 1998, 2001; Fergestad *et al*., 1999), Tweek (Verstreken *et al*., 2009), and Rolling blackout (Vijayakrishnan *et al*., 2010).

Recent work in the field has also begun to highlight the distinct routes SVs can follow after fusion. Changes in Ca^{2+} influx following exocytosis may play important trigger roles in helping to initiate the endocytotic process (Yao *et al*., 2009; Haucke *et al*., 2011; Xue *et al*., 2012), which then can proceed through several distinct routes. Loss of the sole *Drosophila* synaptogyrin/synaptophysin homolog results in a defect in the ability to recycle vesicles from bulk cisternae that form following strong stimulation (Stevens *et al*., 2012). Bulk membrane retrieval is also triggered after acute disruption of dynamin and clathrin (Heerssen *et al*., 2008; Kasprowicz *et al*., 2014). As such, clathrin and dynamin function are required for vesicle budding to prevent bulk cisternae formation. Strong stimulation may outpace their activity, resulting in the endocytosis of larger plasma membrane compartments containing SV proteins. Recent findings also indicate an important role for endosomal trafficking of SVs at the *Drosophila* NMJ for protein quality control. Mutations in the GTPase activating protein Skywalker result in altered endosomal trafficking of SVs following fusion (Uytterhoeven *et al*., 2011; Fernandes *et*

al., 2014). Defects in this pathway indicate that endosomal trafficking results in SV protein turnover that serves to maintain neurotransmission by removing older and potentially damaged vesicle proteins.

Optical Imaging of Neurotransmitter Release at Single Release Sites

Following action potential propagation into the nerve terminal, voltage-gated Ca^{2+} channels open, allowing Ca^{2+} influx and the activation of the fusion machinery for SV release. *Drosophila* contains a single member of the Ca_V2 family of N/P/Q-type presynaptic Ca^{2+} channels that mediates neurotransmitter release (Littleton and Ganetzky, 2000). The N-type channel is encoded by the *cacophony* (*cac*) locus, and has been shown to mediate presynaptic Ca^{2+} entry that drives neurotransmitter release (Smith *et al*., 1996; Kawasaki *et al*., 2000; Rieckhof *et al*., 2003). Imaging of a transgenic GFP-tagged Cac reveals the protein is enriched at AZs within the presynaptic terminal (Kawasaki *et al*., 2004). Each AZ is spatially separated and opposed by a distinct cluster of ionotropic GluRs. The tight coupling of release sites and clustered receptors allows imaging of single SV fusion events at individual release sites by measuring Ca^{2+} influx through postsynaptic GluRs following neurotransmitter release (Desai and Lnenicka, 2011; Peled and Isacoff, 2011; Melom *et al*., 2013; Peled *et al*., 2014). This allows the generation of release probability maps for individual release sites (Fig. 3), a technique that has not been achieved at synaptic connections in other model systems.

Two genetically encoded Ca^{2+} sensors have proven useful in this area, including a myristoylated (membrane-tethered) GCaMP (Melom *et al*., 2013) and a transmembrane tethered GCaMP containing the Shaker K^+ channel PDZ interaction domain (Peled and Isacoff, 2011). By concentrating these sensors at the postsynaptic plasma membrane, one can observe robust and spatially segregated signals for Ca^{2+} influx through GluRs that occurs following SV fusion. Regions of interest (ROIs) defined by these spatially segregated Ca^{2+} signals can be identified and isolated over the entire axonal field, providing spatial and temporal information for vesicle fusion at individual AZs. Imaging synaptic transmission with myristoylated GCaMP5 results in fluorescent Ca^{2+} signals that peak in ~ 50 msec and decay to baseline by ~300 msec, with a change in baseline fluorescence ($\triangle F$) of 40-100%. Subsequent fusion events at the same site produce similar Ca^{2+} profiles, allowing vesicle fusion to be measured at defined ROIs until the sensor begins to bleach (~10 minutes at an 8 Hz acquisition rate). This approach has provided insights into the general rules for SV release at this model glutamatergic synapse. Vesicle fusion can occur through an evoked pathway following an action potential, or through spontaneous fusion (minis) in the absence of stimulation (Fatt and Katz, 1952; Katz and Miledi, 1969). One common hypothesis in the field is that individual AZs display similar release probabilities for both evoked and spontaneous fusion. This turns out to be incorrect, as release probability for the two modes of fusion is independently regulated at individual AZs (Fig. 3). In addition, subpopulations of AZs (~25%) are dedicated to spontaneous release, as they fail to show evidence of evoked fusion (Melom *et al*., 2013). Although the molecular nature of these spontaneous-only release sites is unknown, they may lack presynaptic Cac channels that would allow Ca^{2+} influx. Indeed, release sites with high levels of spontaneous fusion correlate with lower AZ levels of Bruchpilot (Brp) (Peled *et al*., 2014), a presynaptic component of the AZ involved in clustering Ca^{2+} channels. Other features of spontaneous release that have been

characterized through this optical imaging approach include the identification of non-uniform release probability rates across individual AZs, with an average rate of ~1 mini event per 80 seconds (Melom *et al*., 2013). In addition, AZs with similar release probabilities show some clustering within individual boutons, suggesting a potential role for the bouton as compartment for isolated signaling events that could alter release rates at nearby sites.

Although spontaneous release is largely Ca^{2+}-independent at *Drosophila* NMJs (Jorquera *et al*., 2012; J. Lee *et al*., 2013), evoked fusion is triggered by the influx of Ca^{2+} at AZs. Using GCaMP to image release, synchronous fusion of SVs across many AZs gives rise to the evoked response. At normal extracellular Ca^{2+} concentrations, multi-vesicular release at individual AZs was not commonly observed, as ΔF signals for spontaneous and evoked release at mixed mode AZs showed similar peak responses (Melom *et al*., 2013). These observations indicate single vesicle fusion at individual AZs is the primary mechanism for evoked release for isolated action potentials. Similar to the non-uniform nature of release probability per AZ for spontaneous fusion, evoked release probabilities were highly variable across the population as well (Fig. 3) (Peled and Isacoff, 2011; Melom *et al*., 2013). Evoked release probabilities varied from 0.01 to 0.55 across individual AZs, with an average probability of 0.04. What controls release probability at individual AZs, and whether release probability can be modulated during forms of synaptic plasticity, will be important questions for the future. Peled and Isacoff (2011) found that paired-pulse stimulation depressed high probability release sties, while facilitation enhanced release from low probability release sites. As such, short-term forms of plasticity are likely to be modulated by the basal release probability of individual AZs.

There are several mechanisms that have been hypothesized to control release probability that can be tested in future studies. One hypothesis is that release probability is determined by the density of presynaptic N-type Ca^{2+} channels at a single AZ, thereby controlling the level of presynaptic Ca^{2+} entry. A higher density of Ca^{2+} channels would lead to increased Ca^{2+} levels at an AZ, and thereby increase the likelihood that the presynaptic release machinery would be activated. An alternative model is that presynaptic Ca^{2+} channel density and Ca^{2+} entry is fairly constant over all AZs, and that evoked release probability is directly correlated with SV proteins and their number and/or state (i.e. phosphorylation status). It is well known that SVs contain from 10-100 copies of many SV proteins (Takamori *et al*., 2006), and that several of these can undergo post-translational modifications such as phosphorylation that may alter their function. In addition, altered transcription/translation may change their overall levels on SVs. Indeed, overexpression of either of the fusion regulators Syt1 or Cpx can increase neurotransmitter release (Jorquera *et al*., 2012). As such, it is possible that individual AZs maintain a preferred population of unique SVs that mediate release and that might coordinately receive local signals to regulate their functional state (for example through local activation of kinases/phosphatases that regulate their fusion promoting/inhibiting properties). Little is known about how individual AZs regulate their local SV pool. Beyond these two models, it is possible that another determinant besides SV protein content/phosphorylation or Ca^{2+} channel number/function regulates release probability. One such determinant could be the docking/priming state of SVs at single release sites. Perhaps high probability AZs have more docked SVs, or larger pools of attached vesicles to the T-bar itself. As such, more vesicles of identical “protein content and state” would

have access to a stochastic fusion process mediated by the same level of Ca^{2+} entry. A differential content or activity of the known family of AZ proteins could mediate such an effect. Current results indicate that the levels of the AZ protein Brp displays a positive correlation with evoked release probability (Peled *et al*., 2014). Identifying structural correlates for high and low probability AZs by EM following GCaMP imaging will be an exciting approach for further dissecting structural correlates of release probability at the ultrastructural level.

Dense Core Vesicle Release

In addition to SV fusion, synaptic terminals can release cargo from dense core vesicles (DCVs). The molecular machinery for DCV fusion is still being elucidated, but is thought to have some overlap with SV pathways. For example, SNAREs are thought to be required, as well as additional members of the Synaptotagmin family, including Sytα and Sytβ (Adolfsen *et al*., 2004; Park *et al*., 2014). In addition, one well-characterized DCV-specific regulator of release has been identified - the Ca^{2+}-activated protein for secretion (CAPS). Loss of the *Drosophila* CAPS homolog results in embryonic lethality and the accumulation of DCVs at synaptic terminals (Renden *et al*., 2001). Although the mechanism of CAPS function is unknown, it is thought to regulate DCV docking and fusion through Ca^{2+}-binding and SNARE interactions. In spite of some similarities to SVs, DCVs also have distinct features that likely require unique molecular regulators. DCVs bud from the Golgi and do not appear to undergo local recycling. They also are released in response to high frequency firing and must have mechanisms in place to ensure their delivery and availability at active nerve terminals. In addition, it is unclear if DCVs fuse at AZs directly, or prefer to fuse at extra-synaptic sites. Many of these mechanistic differences in DCV biology have been probed at *Drosophila* synapses using transgenic animals expressing an artificial neuropeptide - GFP-tagged rat atrial natriuretic factor peptide (ANF-GFP) (Rao, Lang, *et al*., 2001). This transgenic line has proved highly valuable for DCV biology, revealing mechanisms of activity-dependent recruitment of DCVs (Shakiryanova *et al*., 2005), DCV capture at active terminals (Wong *et al*., 2012; Bulgari *et al*., 2014), presynaptic ER-dependent Ca^{2+} release driving DCV fusion (Shakiryanova *et al*., 2007, 2011), and partial depletion of DCV contents upon release (Wong *et al*., 2015). In addition to aspects of DCV release and trafficking, studies have revealed the role of the basic-helix-loop-helix transcription factor Dimmed in driving neuroendocrine cell fate, including its role in regulating the expression of specific DCV proteins (Hewes *et al*., 2006; Hamanaka *et al*., 2010; Park *et al*., 2011, 2014). Although the identification of the mechanisms that control DCV trafficking and fusion have lagged behind those of SV release (largely due to the ease in physiologically measuring the consequences of SV fusion), new tools in the DCV field are beginning to fill in many of the missing gaps in DCV biology.

Synapse development: Coordinated assembly across the synaptic cleft

Synapse formation requires coordinated events on both sides of the synapse, allowing for precise apposition of the presynaptic AZ with the postsynaptic density (PSD). While this process is still not fully understood, advances in super resolution microscopy and long-term *in vivo* imaging have provided an increasingly detailed picture

of synaptic assembly at the NMJ. Furthermore, the identification of mutations affecting the spacing, size, density and apposition of AZs and GluR clusters provides insight into pathways regulating the assembly process.

The Active Zone Cytomatrix

The AZ is a region of the presynaptic membrane that is highly specialized to facilitate neurotransmitter release. The AZ contains docked SVs and clusters of voltage-gated Ca^{2+} channels to create local high concentrations of Ca^{2+} for synaptic release. The AZ also contains a proteinacious cytomatrix (cytomatrix of the active zone, or CAZ) that serves as an organizational platform for proteins involved in synapse assembly and regulation. Numerous vertebrate CAZ components have been identified (eg Bassoon, Piccolo, RIM, ELKS/CAST; see Hida and Ohtsuka 2010), and homologs of many of these are found at *Drosophila* synapses.

The CAZ of *Drosophila* synapses is readily identifiable by EM as a T-bar structure (Fig. 1A) (Feeney *et al*., 1998). EM tomography provides a detailed picture of CAZ ultrastructure. A T-bar consists of a central core that is approximately 200 nm wide and 50 nm high and is attached to the plasma membrane by a grid of peg-like connections. Numerous filaments extend from the core into the cytoplasm, and these extensions are decorated with SVs (Jiao *et al*., 2010). T-bars are identifiable at most synapse types in *Drosophila*, which makes it possible to investigate how synaptic ultrastructure is regulated, often in subtle ways, in parallel with electrophysiological studies that test synaptic function. The scaffolding protein Brp is considered the principal structural component of the T-bar. Brp is the single *Drosophila* homolog of the ELKS/CAST family, which are important CAZ scaffolding proteins across species (Hida and Ohtsuka, 2010). Loss of function of *brp* results in a loss of T-bar structures, loss of Ca^{2+} channel enrichment to AZs, defects in neurotransmitter release and defects in short-term plasticity (Kittel *et al*., 2006; Wagh *et al*., 2006). By conventional confocal microscopy, AZs can be identified as Brp-postitive puncta. Super resolution microscopy has further revealed how Brp molecules shape T-bar structures. When visualized using stimulated emission depletion (STED) fluorescence microscopy, Brp appears in donut structures likely analogous to single T-bars (Kittel *et al*., 2006). Brp molecules are oriented with the N-terminus in the core, directly overlying the Ca^{2+} channels, and the C-terminus extending away from the core (Fouquet *et al*., 2009). The Brp C-terminus is important for SV tethering, as an allele that lacks the C-terminal 17 amino acids (brp^{nude}) shows an impairment in SV tethering near T-bars (Hallermann *et al*., 2010). Two isoforms of Brp appear to be arranged in an alternating circular pattern that is important for normal T-bar structure and SV tethering (Matkovic *et al*., 2013). Furthermore, quantitative inferences about CAZ substructure and stoichiometry were made using data from stochastic optical reconstruction microscopy (dSTORM), which can provide information on the distribution of single molecules (Ehmann *et al*., 2014). The authors propose that Brp molecules are clustered into 10 nm filaments, consistent with structures defined by EM, and that an average CAZ contains ~137 molecules of Brp.

Several other proteins localize to the CAZ and are important for T-bar ultrastructure. As characterized by STED microscopy, *Drosophila* RIM-binding protein (DBRP) forms a ring at the base of the Brp core, where it surrounds a cluster of Ca^{2+} channels (Liu *et al*., 2011). Mutants for *drbp* lack regular T-bars, instead displaying

misshapen electron-dense material that is frequently detached from the plasma membrane. Consistent with a severe disruption of the CAZ, *drbp* mutants have a reduced density of SVs at the plasma membrane, reduced Ca^{2+} channel levels, and impairment of evoked release (Liu *et al*., 2011). Syd-1 and Liprin-α localize to the periphery of the CAZ (Fouquet *et al*., 2009; Owald *et al*., 2010). Mutants of *syd-1* and *liprin-α* have irregular T-bars, which may be small, misshapen, ectopic or enlarged multi-T-bars, consistent with a role in regulating CAZ assembly (Kaufmann *et al*., 2002; Owald *et al*., 2010). Syd-1 clusters Liprin-α at the CAZ along with the transsynaptic adhesion protein Neurexin (Nrx), which is important for transsynaptic signaling for PSD organization and apposition (Owald *et al*., 2010, 2012). A *Drosophila* ortholog of Piccolo, a core CAZ protein previously thought to exist only in vertebrates, was recently identified and named *fife*. Fife colocalizes with Brp at AZs, and loss of *fife* results in defects in AZ ultrastructure, including detachments of the presynaptic membrane and a reduction of SV clustering at T-bars (Bruckner *et al*., 2012). Another recently identified CAZ component is the presynaptic scaffolding protein Dyschronic (DYSC). DYSC localizes next to AZs and *dysc* mutants have abnormally large BRP-positive donuts when examined using STED (Jepson *et al*., 2014). DYSC binds to the K+ channel Slowpoke (SLO) and together they regulate the presynaptic cytoskeleton, AZ size and neurotransmission.

In summary, AZ components serve to cluster SVs, organize Ca^{2+} channels at release sites, and bind transsynaptic partners to facilitate clustering of GluRs (Fig. 4B). Although poorly studied, these interactions are likely to be dynamically regulated to alter release properties during synaptic plasticity.

The Postsynaptic Scaffold

Across the synaptic cleft, the postsynaptic cell also assembles a complement of proteins to bind neurotransmitters and facilitate both anterograde and retrograde signal transduction (Fig. 4B). In many synapses, this domain is recognizable as an electron density by EM, termed the postsynaptic density (PSD). At type-I NMJ boutons, the postsynaptic membrane is not appreciably electron dense, but instead forms numerous convoluted folds and invaginations, resulting in a complex membranous structure called the subsynaptic reticulum (SSR) (Fig. 4A) (Johansen *et al*. 1989). Numerous PSD proteins are found throughout SSR membranes, including GluRs, ion channels, scaffolding molecules, adhesion molecules and signaling complexes.

An important component of the postsynaptic scaffold is Discs large (Dlg), a member of the membrane-associated guanylate kinase (MAGUK) family. MAGUK proteins play essential roles in many cellular processes, including organizing the PSD and regulating ionotropic GluR trafficking in mammalian neurons (Elias and Nicoll, 2007; Zheng *et al*., 2011). At the NMJ, Dlg is enriched throughout the SSR and recruits several other PSD proteins to the synapse, including the K+ channel Sh and the adhesion protein Fasciclin II (FasII; Thomas *et al*. 1997; Zito *et al*. 1997). Dlg also plays an important role in the formation of the SSR, through recruitment of the t-SNARE Gtaxin (Gtx) to type-I boutons (Gorczyca *et al*., 2007). Gtx regulates membrane addition at the developing synapse and is essential for expansion of both the SSR and the extensive endomembrane system in the muscle.

Ionotropic glutamate receptors at the NMJ

Glutamate is the primary fast excitatory neurotransmitter at the *Drosophila* NMJ. The GluRs in the body wall muscles are excitatory ionotropic non-NMDA-type receptors composed of four subunits. A total of five GluRs have been found at NMJs: GluR-IIA, GluR-IIB, GluR-IIC/GluR-III, GluR-IID and GluR-IIE (Schuster *et al*., 1991; Petersen *et al*., 1997; Marrus *et al*., 2004; Featherstone *et al*., 2005; Qin *et al*., 2005). These assemble into two possible four-subunit configurations, which contain either subunit IIA ("A-type") or IIB ("B-type"), along with the obligate components IIC, IID and IIE. A-type and B-type GluRs are redundant in some respects. Deletion of either IIA or IIB does not prevent GluR formation or viability of the animal, in contrast to deletion of IIC, IID, IIE, or simultaneous deletion of IIA and IIB, all of which result in embryonic lethality (Petersen *et al*., 1997; DiAntonio *et al*., 1999; Marrus *et al*., 2004; Featherstone *et al*., 2005; Qin *et al*., 2005). However, A-type and B-type receptors display differences in their electrophysiological properties and distributions. For example, subunit composition affects quantal size and desensitization (DiAntonio *et al*., 1999), and the incorporation of A/B-type receptors within the PSD is activity-dependent and changes as synapses mature (Schmid *et al*., 2008). Furthermore, the IIA and IIB subunits compete to bind IIC (Marrus *et al*., 2004). Thus the ratio of A/B-type receptors is an important component of synaptic assembly and plasticity.

Formation and maturation of glutamate receptor clusters

Synapse formation begins in late embryogenesis when a motor neuron axonal growth cone reaches its target muscle (Hoang and Chiba, 2001; Ruiz-Canada *et al*., 2006). GluR subunit transcripts are already expressed in the muscles at this time (Currie *et al*., 1995; Petersen *et al*., 1997; Marrus *et al*., 2004; Qin *et al*., 2005) and functional receptors are distributed throughout the muscle surface (Broadie and Bate, 1993a; Nishikawa and Kidokoro, 1995). GluRs then begin to cluster at nascent synapses, which requires innervation from the motor neuron (Broadie and Bate, 1993a; Saitoe *et al*., 1997). This implies the action of a transsynaptic signal to initiate postsynaptic development, but interestingly such a signal has yet to be identified. Release of glutamate may not be required, as GluR clusters form even when SV release (both evoked and spontaneous) is blocked by loss of function of Syntaxin or Shibire (Featherstone *et al*., 2002; Schmid *et al*., 2006), or when vesicular loading of glutamate is blocked (Daniels *et al*., 2006).

GluRs are not required for synaptic assembly per se. Animals completely lacking GluRs (the GluRIIC/III null allele or IIA/IIB double mutants) accumulate other PSD components, such as PAK and Dlg, opposite presynaptic AZs (Chen and Featherstone, 2005; Schmid *et al*., 2006). Accumulation of FasII at nascent synapses appears to be one of the earliest events in postsynaptic differentiation, detectable shortly after formation of the nerve contact site (Kohsaka *et al*., 2007). FasII and Dlg are mutually dependent for their postsynaptic enrichment, and loss of *fasII* results in a significant reduction of both A-type and B-type receptors. GluRs nevertheless play an important role in synaptic development, as synapses with greatly reduced levels of GluRs form mostly immature synapses with poor apposition between pre- and postsynaptic markers (Yoshihara *et al*., 2005; Schmid *et al*., 2006).

An essential factor in GluR cluster formation is the auxiliary subunit Neto. Auxiliary subunits are emerging as important components regulating the localization and

function of ionotropic GluRs (Kim and Serpe, 2013). Neto is a multidomain transmembrane protein resembling vertebrate Neto1 and Neto2. Neto and GluRs colocalize and are mutually dependent for their clustering at the NMJ (Kim *et al*., 2012).

A detailed understanding of how GluR clustering proceeds comes from live imaging of fluorescently tagged GluR transgenes. Sigrist and colleagues (Rasse *et al*., 2005; Schmid *et al*., 2008) produced GFP- and mRFP-tagged GluRIIA and GluRIIB transgenes under the control of their own promoters. These transgenes expressed at physiological levels and were able to rescue *GluRIIA GluRIIB* double mutant animals to the same extent as untagged *GluRIIA/B* transgenes. Using these tools, the authors conducted long-term live imaging experiments, combined with fluorescence recovery after photobeaching (FRAP), to observe the formation of nascent PSDs and the dynamics of GluR subunits during larval development. New GluR fields form independently, deriving from diffuse extrasynaptic receptors rather than splitting off from existing receptor clusters. Over time, nascent clusters grow by incorporating additional receptors and GluRs tend to become far less mobile after they enter a cluster, as determined by FRAP (Rasse *et al*., 2005). Simultaneous imaging of GluRIIA-GFP and GluRIIB-mRFP revealed interesting dynamics of subunits over time. New PSDs tend to be enriched for GluR-IIA, while more mature PSDs tend to have a balanced composition of GluR-IIA and GluR-IIB (Schmid *et al*., 2008). GluR accumulations are detected prior to the appearance of adjacent accumulations of the AZ marker Brp, and increases in the Brp signal over time are accompanied by increased maturity of the GluR cluster, such as with a shift to a lower A-type/B-type receptor ratio (Rasse *et al*., 2005; Schmid *et al*., 2008)

Subunit composition is important because A-type and B-type receptors differ in their responses to stimulation. A-type receptors have slower desensitization kinetics than B-type receptors and a larger proportion of A-type receptors increases quantal size (DiAntonio *et al*., 1999). Consistent with the idea that subunit composition is a point of regulation of synaptic response, Schmid *et al* (2008) demonstrate the plasticity of GluRIIA and GluRIIB incorporation into GluRs by showing that suppressing evoked release with tetanus toxin light chain (TNT) results in a shift towards increased GluRIIA incorporation.

Modulation of GluR subunit composition

Several signaling pathways regulate GluR subunit composition and it appears that distinct modules support A-type versus B-type receptor cluster formation. A-type receptors bind directly to Coracle (Cora), the *Drosophila* homolog of the protein 4.1 family, members of which typically function as links between transmembrane proteins and the actin cytoskeleton. The abundance of A-type receptors depends on both Cora and the integrity of postsynaptic actin filaments (Chen *et al*., 2005). Several interactors of Cora and the actin cytoskeleton are also required for A-type receptor clustering, including Pak (Albin and Davis, 2004), CASK (calcium/calmodulin-dependent serine protein kinase; Chen and Featherstone 2011) and Dystroglycan (Dg; Bogdanik *et al*. 2008). Protein-O-mannosyl transferase 1 (POMT1), an enzyme that glycosylates Dg, is similarly required for A-type receptor clustering (Wairkar *et al*., 2008).

The Nrx/Neuroligin (Nlg) transsynaptic adhesion complex also modulates subunit composition. Nrx and Nlg are synaptic adhesion proteins that play important roles in the

formation, maturation and organization of synapses across species (Bang and Owczarek, 2013). There is a single *nrx-1* gene in *Drosophila* and four *nlg* genes. Typically Nrx is the presynaptic partner and Nlg the postsynaptic partner. However, Nrx has a postsynaptic role at some synapses, including the fly NMJ (Chen *et al*., 2010), and *Drosophila* Nlg-2 is required both pre- and postsynaptically (Chen *et al*., 2012). Each gene has a distinct set of phenotypes encompassing organization of AZs and GluR clusters and overall synaptic morphology. Two groups have reported that loss of *nrx-1* results in an increase in A-type receptors, suggesting that Nrx-1 normally inhibits A-type receptor clustering (Li *et al*., 2007; Chen *et al*., 2010). In contrast, live imaging of GluR incorporation revealed that mutants for *nrx-1*, as well as interacting partners *nlg-1* and *syd-1*, are deficient in early GluRIIA incorporation (Owald *et al*., 2012). While newly formed GluR clusters are typically rich in GluRIIA, these mutants have new clusters that are rich in GluRIIB. One reason for these different findings could be the dynamic nature of receptor cluster formation. It is possible that direct observation during development can reveal new aspects that cannot be appreciated from analyzing mature receptor clusters. Other Nlgs also regulate GluR subunit composition. Nlg-3 promotes A-type clustering (Xing *et al*., 2014). Nlg-2 regulates total GluR abundance, and may also affect subunit composition (Sun *et al*., 2011; Chen *et al*., 2012). Thus, several interactors of the cytoskeleton, as well as transsynaptic Nrx/Nlg signaling, modulate A-type receptor abundance. As interactions between Nrx/Nlg, CASK, Cora and actin have been reported in mammalian studies (Cohen *et al*., 1998; Biederer and Sudhof, 2001; Chao *et al*., 2008), cooperation of these factors to specifically modulate clustering of A-type receptors is an attractive model that will be interesting to test in future studies.

A-type receptor abundance is further regulated by components of the NF-κB signaling pathway. The transcription factor NF-κB/Dorsal is enriched at the PSD and appears to function locally at the synapse without any nuclear role. Dorsal acts along with inhibitory molecule IκB/Cactus and kinase IRAK/Pelle to regulate A-type receptor density (Cantera *et al*., 1999; Heckscher *et al*., 2007). The serine/threonine kinase Akt also promotes A-type receptor levels, and may do so by regulating the levels of Dorsal and Cactus at the synapse (H.-G. Lee *et al*., 2013). It is unclear how Dorsal, Cactus and Pelle affect GluRs. Observations from Hecksher *et al* (2007) are most consistent with a post-transcriptional mechanism. Given the synaptic enrichment of these proteins, it will be interesting to determine if they act in a complex at the PSD and if the kinase activity of Pelle is required for GluR regulation.

Separate mechanisms appear to govern the abundance of B-type receptor clusters. Mutants for *dlg* specifically lose B-type but not A-type receptors (Chen and Featherstone, 2005). Another membrane-associated scaffolding protein, Lethal (2) giant larvae (Lgl), has the opposite function, where loss of *lgl* leads to an increase in B-type, but not A-type receptors (Staples and Broadie, 2013). Dlg and Lgl are otherwise known to interact in epithelial cells where they cooperate to specify the basolateral membrane domain (Laprise and Tepass, 2011). Lgl also interacts with FMRP (fragile X mental retardation protein), an RNA-binding protein that regulates translation of synaptic proteins (Tessier and Broadie, 2012). In mutants of *dfmr1*, A-type receptors accumulate at the expense of B-type receptors, resulting in a shift of GluR composition (Pan and Broadie, 2007). Interestingly, Lgl has been shown to interact with FMRP in regulating NMJ architecture and to form a complex with FMRP and mRNAs (Zarnescu *et al*., 2005) Furthermore,

mammalian FMRP has been shown to bind the mRNA of Lgl and Dlg homologs (Darnell *et al*., 2011). It is possible that FMRP-Dlg-Lgl form a module to specifically regulate B-type receptors. Both Dlg and Lgl have the capacity to bind and recruit multiple interaction partners, but the mechanism by which they interact with GluR subunits is currently unknown.

Finally, two nuclear proteins regulate GluR subunit composition: Kismet, a chromatin remodeling enzyme (Ghosh *et al*., 2014), and Nesprin-1, a component of the LINC complex connecting the nucleoskeleton to the cytoskeleton (Morel *et al*., 2014). Loss of either of these genes results in a reduction in A-type receptor clustering, along with defects of synaptic transmission and NMJ morphology. Kismet does not affect GluR transcripts directly, but likely regulates targets that modulate signaling from the presynaptic motor neuron. The mechanism of Nesprin-1 action is unknown, but it could feasibly affect transcription or localization of transcripts involved in synaptic function.

In summary, several protein modules control the relative abundance of A-type and B-type receptors, which has important functional consequences for the synapse. However, it is still unclear how these signaling pathways interact to dynamically modulate GluR function.

Regulation of synaptic organization

In recent years a number of groups have screened for mutations affecting synaptic organization using antibodies that recognize Brp and GluRs (Graf *et al*., 2009; Wairkar *et al*., 2009; Valakh *et al*., 2012; Blunk *et al*., 2014). These and other studies have defined several molecular pathways that regulate distinct and separable features such as the size, density and apposition of individual synapses.

AZ size, density and spacing

One class of synaptic organization defects is characterized by enlarged, misshapen or multiple T-bars along with enlarged GluR clusters (Fig. 4C). These irregularities of synaptic ultrastructure are typically accompanied by defects in AZ spacing, where multiple Brp puncta cluster opposite single enlarged or fused GluRs. Mutations in several synaptic genes exhibit variations of this phenotype, including genes encoding the CAZ components *syd-1* and *Liprin-α* (Kaufmann *et al*., 2002; Fouquet *et al*., 2009; Owald *et al*., 2010), transsynaptic adhesion genes *nrx-1*, *nlg-1* and the teneurins *ten-m* and *ten-a* (Li *et al*., 2007; Banovic *et al*., 2010; Owald *et al*., 2012), and components of the postsynaptic actin cytoskeleton (Pielage *et al*., 2006; Blunk *et al*., 2014). There is evidence of cooperation between many of these genes, which begins to provide a picture of how synaptic organization is coordinated across the synaptic cleft.

Syd-1, Liprin-α, Nrx and Nlg appear to act together to form a transsynaptic link. Syd-1 forms a complex with Nrx-1 and is important for the clustering of Nrx-1 and postsynaptic Nlg-1. Nlg-1 is in turn involved in stabilizing Syd-1-Liprin-α clusters in the initial stages of AZ formation (Owald *et al*., 2010, 2012).

A second transsynaptic adhesion pair, the Teneurins, interact with Nrx-1 and Nlg-1 in synaptic organization. Teneurins are conserved EGF-repeat containing transmembrane proteins involved in synaptic development. The *Drosophila* Teneurins localize to the NMJ, where Ten-a is presynaptic and Tem-m is predominantly postsynaptic (Mosca *et al*., 2012). Loss of *teneurins* produces similar phenotypes to loss

of *nrx-1* or *nlg-1*. In addition to AZ/GluR size and spacing defects, mutations in all of these genes exhibit disruption of the postsynaptic spectrin cytoskeleton, disorganization of presynaptic microtubules and loss of SSR (Li *et al*., 2007; Banovic *et al*., 2010; Mosca *et al*., 2012).

Consistent with this observation, AZ/GluR size and spacing defects are also seen with disruption of the postsynaptic actin cytoskeleton, as revealed by knockdown of α- or β-spectrin in the muscle (Pielage *et al*., 2006), and analysis of a point mutation in the spectrin-binding domain of the muscle-specific actin gene Actin57B (Blunk *et al*., 2014). These mutants also have severely compromised SSRs. Genetic interaction experiments support cooperation between *actin57B*, *nrx-1* and *nlg-1* (Blunk *et al*., 2014). Other mutations affecting the postsynaptic actin cytoskeleton result in a mild increase in GluR cluster size without any reported effects on AZ size or spacing. These include the adaptor protein Ankyrin, which is recruited to the NMJ by β-spectrin (Pielage *et al*., 2006), as well as components of the Par complex, which organize the spectrin network (Ruiz-Canada *et al*., 2004; Ramachandran *et al*., 2009).

Loss of *dlg* also causes an expansion of synaptic contacts, measured as the size of presynaptic electron densities, or as the size of GluR fields (Thomas *et al*., 1997; Karunanithi *et al*., 2002; Chen and Featherstone, 2005). The *dlg* locus generates multiple isoforms, but the two major products at the NMJ are DlgA and DlgS97, which differ in the presence of an L27 domain in DLGS97 (Mendoza *et al*., 2003). Both of these isoforms are required to regulate synaptic size (Mendoza-Topaz *et al*., 2008), and DlgS97 specifically interacts with the PDZ domain protein DLin-7 and the MAGUK protein Metro in this process (Bachmann *et al*., 2010). Mutants for *fasII* similarly results in expanded synaptic size and multiple T-bars (Stewart *et al*., 1996).

Fascinating open questions remain about the molecular mechanisms that determine synaptic size and spacing on both sides of the synapse. It is unclear whether size and spacing are separable features, or whether irregular spacing is a consequence in mutants with the most severely enlarged and misshapen synaptic zones. One possible model is that AZ size could be constrained by presynaptic CAZ components and/or GluR size could be constrained by the postsynaptic actin cytoskeleton and perisynaptic proteins Dlg and FasII. Indeed, Pielage *et al*. (2006) propose that a hexagonal actin-spectrin lattice could provide an ideal template for synaptic spacing. Information about synaptic size and spacing could then be transmitted across the synaptic cleft by transsynaptic signaling. Notably, Dlg is a binding partner of both Nlg-1 (Banovic *et al*., 2010) and FasII (Thomas *et al*., 1997; Zito *et al*., 1997), suggesting multiple potential links between the cytoskeleton, PSD and transsynaptic adhesion.

Pre- and postsynaptic apposition

Loss of *nlg*-1, *ten-m,* or *ten-a* results in apposition defects in addition to the phenotypes in size, spacing and cytoskeletal organization described above. These animals exhibit numerous unapposed Brp puncta, suggesting a failure to accumulate GluRs at some synapses (Fig. 4C) (Banovic *et al*., 2010; Mosca *et al*., 2012). In fact, mutants in *nlg-1* often have entire boutons, termed “orphan boutons” completely lacking postsynaptic specializations (Banovic *et al*., 2010). Testing for epistasis with respect to specific defects, Mosca *et al*. (2012) conclude that these two adhesion pairs act in

partially overlapping pathways, with Teneurins primarily organizing the cytoskeleton and Nrx/Nlg primarily regulating apposition (Mosca *et al*., 2012).

Unapposed GluR clusters define a second category of apposition defect (Fig. 4C). DiAntonio and colleagues have characterized several mutations that exhibit this phenotype, highlighting distinct pathways that regulate presynaptic development. A mutation in *running-unapposed* (*rup*), which encodes *Drosophila rab3*, was identified in a screen for AZ mutants (Graf *et al*., 2012). *rab3*^{rup} mutants exhibit a dramatic loss of Brp-positive puncta and impaired neurotransmitter release. Most GluR clusters are unapposed, and existing Brp puncta are abnormally large. Ultrastructural analysis revealed that the number of electron-dense AZs in *rab3* mutant synapses is consistent with the number of GluR clusters, suggesting that unapposed GluRs do in fact have an apposing EM-defined AZ. However, T-bars are dramatically redistributed, with multiple T-bars located at single AZs. Thus, the number of putative release sites in *rab3* mutants is slightly reduced, but these sites are abnormally distributed to a smaller number of synapses. This study revealed a function for Rab3 in regulating the distribution of Brp - and therefore the release probability - of individual AZs, and supports the idea that AZs are individually modified.

Loss of the serine threonine kinase *unc-51* also results in many GluR clusters lacking Brp and impaired neurotransmitter release (Wairkar *et al*., 2009). Ultrastructurally, these mutants show an overall decrease in synaptic density and fewer T-bars per electron-dense AZ, indicating that *unc-51* mutants are defective in T-bar assembly, but not in formation of AZs per se. Further analysis revealed that Unc-51 promotes synaptic density and Brp accumulation by inhibiting the activity of the MAP kinase ERK.

Presynaptic inhibition of the serine-threonine phosphatase PP2A also results in many unapposed GluR clusters (Viquez *et al*., 2009). Ultrastructural analysis of these mutants revealed an overall decrease in synaptic density, but no change in the number of T-bars per AZ. Thus, in contrast to *rab3* and *unc-51* mutants, loss of PP2A impairs development of electron-dense AZs at some synapses. Loss of PP2A also impairs GluR clustering, as observed in live imaging experiments. PP2A acts antagonistically to the serine-threonine kinase GSK-3β, as inhibition of GSK-3β suppresses the PP2A AZ defects.

Contributions of the extracellular matrix to synaptic organization

The synaptic extracellular matrix (ECM), an array of glycoproteins and protepglycans filling the space between the pre- and postsynaptic membranes, plays an important role in regulating the synapse. ECM synaptomatrix proteins are organized into highly compartmentalized domains, mediate interactions at the pre- and postsynaptic membranes, and regulate transsynaptic signaling (reviewed by Dani and Broadie 2012).

Mind-the-gap (MTG) is a presynaptically secreted lectin found in the synaptic cleft at the NMJ (Rohrbough *et al*., 2007; Rushton *et al*., 2009). Lectins are carbohydrate-binding proteins with diverse cellular functions. Within the synaptic cleft, MTG organizes the ECM by regulating the distribution of lectin-binding glycans and integrins (Rushton *et al*., 2009), as well as the Jelly belly (Jeb)-Anaplastic lymphoma kinase (Alk) signaling complex (Rohrbough and Broadie, 2010). Mutants of *mtg* exhibit a severe impairment in synaptic transmission, ultrastructural defects both in the synaptic cleft and

in the postsynapse, and mislocalization of GluR puncta. Furthermore, Dlg, Pak, Dock and Pix are either reduced or severely mislocalized from *mtg* mutant synapses, suggesting that MTG plays a critical role upstream of these proteins to regulate GluR localization (Rohrbough *et al*., 2007). Interestingly, when *mtg* mutants are rescued with MTG lacking its carbohydrate-binding domain (CBD), postsynaptic GluR accumulation increases (Rushton *et al*., 2012). Furthermore, elimination of the CBD does not affect the ability of MTG to bind to glycans, supporting an unexpected function of this domain in limiting the PSD. How MTG acts from the ECM to instruct postsynaptic organization remains to be determined, but could involve intergrin signaling or some other cytoplasm-ECM signaling complex.

Other ECM proteins that regulate the synapse are the heparin sulfate proteoglycans (HSPGs) Dallylike (Dlp) and Syndecan (Sdc). Mutants in *dlp* show increased evoked release, along with an abnormally high density of small AZs (Johnson *et al*., 2006). Dlp binds and acts antagonistically to Lar, a receptor protein tyrosine phosphatase previously shown to regulate AZ size (Kaufmann *et al*., 2002; Johnson *et al*., 2006). Together, Dlp and Lar create a potential link between the ECM and the presynaptic membrane to regulate AZ function and morphology. The mechanism by which Dlp antagonizes Lar is unknown but could involve inhibition of the receptor function of Lar. Sdc also binds to LAR, and together they act to promote growth of the NMJ arbor (Johnson *et al*., 2006).

Recent RNAi screens have targeted glycan-related genes, including enzymes involved in the biosynthesis or modification of glycans. This approach has identified many factors that regulate the size of the NMJ arbor, the size of AZs, and/or neurotransmission (Dani *et al*., 2012, 2014). The α-*N*-acetylgalactosaminyltransferases *pgant3* and *pgant35A* are glycosylating enzymes identified in the screen. Loss of either *pgant* gene results in an increase in the number and size of Brp and GluR fields, along with increased evoked release. However, double *pgant* mutants have normal synapses, suggesting an antagonistic relationship between the *pgants*. Pgants appear to act by suppressing integrin signaling (Dani *et al*., 2014). Also identified by this method were *hs6st* and *sulf1*, which affect the sulfation state of heparin sulfate proteoglycan (HPSGs) (Dani *et al*., 2012). *hs6st* and *sulf1* differentially affect the levels of Wnt and BMP ligand in the extracellular space, thus modulating these transsynaptic signaling pathways. Many interesting questions remain about how the complexity of possible glycan modifications is interpreted at the synapse to regulate synaptic structure and function.

Investigating AZ organization at central synapses

Recent work has identified novel synaptic genes at *Drosophila* CNS synapses. Antennal lobe projection neurons (PNs) send axonal projections to the mushroom body calyx, where they form specialized synaptic clusters called microglumeruli. Within a microglomerulus, synapses are characterized by large presynaptic boutons surrounded by a claw-like dendritic specializations from multiple Kenyon cells (Yasuyama *et al*., 2002; Leiss *et al*., 2009). AZs, PSDs and components of the synaptic cleft can be clearly resolved at these mushroom body input synapses, providing a model to examine AZ organization at a central cholinergic synapse (Kremer *et al*., 2010; Nakayama *et al*., 2014). Microglomeruli also display activity-dependent structural plasticity, including changes in AZ density (Kremer *et al*., 2010).

Drep2 is related to the DNA fragmentation family of proteins that are targets of caspases during apoptosis. Surprisingly, Drep2 was found to be a synaptic protein highly expressed in the CNS, without any evident role in apoptosis (Andlauer *et al*., 2014). Drep2 is enriched at the PSD of mushroom body input synapses, colocalizing with the ACh receptor subunit Dα7 and directly apposing Brp. Loss of *drep-2* results in defects in olfactory short- and intermediate-term memory.

A novel matrix protein was also identified at cholinergic brain synapses. Hikaru genki (Hig) is a secreted immunoglobulin that localizes to the synaptic cleft of cholinergic synapses, including mushroom body input synapses (Nakayama *et al*., 2014). Loss of *hig* results in abnormal irregular distribution of Dlg and reduced levels of Ach subunits Dα7 and Dα6. This work reveals novel components of the extracellular matrix that can organize postsynaptic molecules.

Mushroom body input synapses may prove to be a second model synapse beyond the NMJ to examine synaptic organization and identify novel synaptogenic molecules. How similar AZ and PSD proteins are between glutamatergic, cholinergic and GABAergic synapses is an open question.

Molecular mechanisms regulating NMJ growth and plasticity

The NMJ arbor is both stereotypic, in that each innervated muscle has a predictable pattern of branches and boutons, and plastic, in that this pattern is regulated by activity. Structural plasticity of the NMJ is regulated by various pathways: regulation of excitability; anterograde, retrograde and autocrine signaling between pre- and postsynaptic partners; and signal modulation by the cytoskeleton, ECM and vesicle trafficking pathways.

Modes of growth, maintenance and plasticity

At the NMJ, the presynaptic terminal undergoes significant growth during the larval stages, expanding the size of the arbor and number of synapses in parallel with the dramatically increasing muscle size. The addition of boutons at type-I terminals during development was first observed using a GFP-tagged chimeric protein of the cytoplasmic domain of the Shaker K+ channel fused to the transmembrane and extracellular domains of CD8 (Zito *et al*., 1999). When expressed in muscle, this construct is targeted to postsynaptic membranes and highlights synaptic boutons, allowing live observation of bouton morphology through the larval cuticle. This study revealed two modes of bouton addition: either existing boutons stretch apart and form a new bouton between them, or new boutons are added onto the ends of existing strings of boutons (Fig. 5A).

Recent work has indicated an important role for spontaneous neurotransmission (minis) in regulating synaptic growth (Huntwork and Littleton, 2007; Choi *et al*., 2014). Newly formed boutons are small in size, and then undergo expansion, which is associated with maturation of T-bar architecture. Impeding all neurotransmission, by reducing levels of the glutamate transporter *vglut*, results in a decrease in bouton size. Interestingly, it is spontaneous - and not evoked - neurotransmission that is required for normal bouton expansion. To specifically impede miniature neurotransmission, Choi *et al*. (2014) compared *iGluR* mutants expressing either a functional ($iGluR^{WT}$) or nonfunctional ($iGluR^{MUT}$) rescue transgene. These animals have comparable evoked neurotransmission,

due to homeostatic compensation of quantal content in the *iGluR*^{MUT} animals (see Frank, 2014, and Homeostatic Plasticity section of this review). However, *iGluR*^{MUT} animals have severely reduced miniature neurotransmission that is accompanied by a strong reduction in bouton size. In contrast, specifically blocking evoked release with genetically encoded peptide toxins has no impact on bouton size. The effect of minis on bouton growth appears to be bidirectional, as bouton overgrowth in *cpx* mutants can be suppressed by inhibiting minis. Furthermore, mini-regulated growth is a local signaling event, which can be independently modulated at individual synaptic terminals. The molecular pathway of mini-regulated growth is not yet understood, but involves the GEF Trio and Rac1 (Choi *et al*., 2014). This work provides *in vivo* evidence for a specific function of minis in synaptic development. Rather than being simply a nonfunctional byproduct of neurotransmission, there is an emerging view of spontaneous release as an important component of neuronal signaling (for review, see Kavalali, 2015). How the synapse can distinguish spontaneous from evoked transmission is a fascinating open question for future studies. It is possible that populations of spontaneous-only AZs (Melom *et al*., 2013) could spatially distinguish postsynaptic signaling mechanisms, or that different kinetics of release of evoked and spontaneous transmission can be detected and integrated by the postsynaptic cell.

In addition to developmental growth of the synapse, a distinct mode of rapid presynaptic bouton budding has been observed in response to elevated neuronal activity (Ataman *et al*., 2008; Piccioli and Littleton, 2014). These structures, called ghost boutons, lack neurotransmission machinery and a postsynaptic scaffold, and are thus considered "immature" boutons (Fig. 5C). Ghost boutons can be elicited by experimental stimulation, such as incubating a dissected live preparation in elevated K+, but also occur spontaneously at wild type arbors, where ~1% of all boutons are classified as ghost boutons. While it is unclear to what extent ghost boutons contribute to normal synaptic growth, some do develop into mature boutons, acquiring pre- and postsynaptic neurotransmission machinery over time (Ataman *et al*., 2008). Furthermore, there is evidence that motor neurons normally form excess ghost boutons, the majority of which are eliminated in an engulfment pathway regulated by the receptor Draper (Fuentes-Medel *et al*., 2009). Many signaling pathways that govern developmental synaptic growth also regulate the ability of the NMJ to bud ghost boutons in response to activity (Ataman *et al*., 2008; Piccioli and Littleton, 2014).

Several molecular mechanisms have been identified that regulate synaptic maintenance at the NMJ. Since elaboration of the SSR requires the presence of a presynaptic nerve terminal, abnormal retraction events can be identified as Dlg-positive PSDs that are unapposed by any presynaptic markers. This assay has been used in numerous screens to identify mutations involved in synaptic maintenance, including the Dynactin protein complex (Eaton *et al*., 2002), the microtubule-binding protein Stathmin (Graf *et al*., 2011), the spectrin cytoskeleton (Pielage *et al*., 2005), Ankyrin-2 and its regulator Casein kinase 2 (Koch *et al*., 2008; Pielage *et al*., 2008; Bulat *et al*., 2014), the L1-type adhesion protein Neuroglian (Enneking *et al*., 2013) and the actin-capping protein Hu-li tai shao/Adducin (Hts/Adducin) (Pielage *et al*., 2011). Little is known about how the balance between synapse formation and elimination is regulated during development. Interestingly, Hts/Adducin contributes to both of these processes. In addition to frequent and severe retraction events, *hts/adducin* mutants exhibit a dramatic

overgrowth phenotype and the appearance of abundant small membrane protrusions (Pielage *et al*., 2011). Consistent with the actin-capping function of Hts/Adducin, these protrusions are rich in actin. They contain presynaptic proteins like Synapsin and Brp and are associated with small GluR clusters and low levels of Dlg, supporting the idea that these structures may be newly formed synapses. This study suggests a new model of how new synapses might be added at the NMJ, though it is unclear whether this mode of growth occurs in wild type animals.

Mechanisms regulating growth and plasticity

Neuronal activity

Altered neuronal activity leads to changes in synaptic growth at the NMJ. This effect can be observed by experimentally increasing activity, such as by inducing seizures (Guan *et al*., 2005) or by increasing larval locomotion (Sigrist *et al*., 2003). Both of these manipulations result in an increase in total bouton number. Furthermore, several mutant backgrounds that increase neuronal excitability also lead to pronounced bouton overgrowth. Double mutants for two voltage-gated K+ channels, *sh* and *ether a go-go* (*eag*), are hyperexcitable and exhibit increased bouton number (Budnik *et al*., 1990). Overgrowth is also observed in mutants for K+ channels *slowpoke* (*slo*), a Ca^{2+}-activated BK channel, and *seizure* (*sei*), a voltage-dependent ERG channel (Lee and Wu, 2010). Overgrowth in these mutants is in the form of "satellite" boutons, a distinct phenotype where many small boutons decorate normal-sized parent boutons along the arbor (Fig. 5C). Activity-dependent overgrowth is mediated by Ca^{2+}/cAMP signaling. Loss of *rutabaga* (*rut*), an adenylyl cyclase that produces cAMP, suppresses overgrowth in *eag Sh*, *slo* or *sei* mutant backgrounds (Zhong *et al*., 1992; Lee and Wu, 2010). Furthermore, Loss of *dunce* (*dnc*), a cAMP-specific phosphodiesterase, also suppresses overgrowth in *slo* and *sei* mutants, suggesting that optimal cAMP levels are required for activity-dependent overgrowth.

Synaptotagmin 4-dependent retrograde signaling

Structural plasticity in response to activity requires the transduction of postsynaptic Ca^{2+} signals into a retrograde signal. Synaptotagmin 4 (Syt4) was identified as a postsynaptic Ca^{2+} sensor required for several forms of plasticity at the NMJ, such as the synaptic overgrowth of hyperexcitability mutants, the induction of presynaptic miniature release following high-frequency stimulation, or the induction of ghost boutons (Yoshihara *et al*., 2005; Barber *et al*., 2009; Korkut *et al*., 2013; Piccioli and Littleton, 2014). The retrograde molecules released in a Syt4-dependent manner have yet to be identified. Interestingly, recent work established that Syt4 is transferred transsynaptically from the presynaptic terminal on exosomes (Korkut *et al*., 2013). Exosomes are signaling vesicles released from cells, and are increasingly recognized to have important functions in cell-cell communication events (Cocucci and Meldolesi, 2015). Importantly, exosomes allow transmembrane and membrane-associated proteins to act as paracrine signals. It is intriguing that Syt4, a regulator of retrograde signaling, can be delivered in an anterograde fashion to the postsynaptic cell. The release, transfer and uptake of exosomes at the NMJ are likely important points of signal regulation, and uncovering these

mechanisms will be important in future studies. As discussed below, transsynaptic Wnt signaling also occurs through release of exosomes.

Transsynaptic signaling

BMP

Proteins of the transforming growth factor-beta (TGF-β) superfamily, including the Bone morphogenetic proteins (BMPs), are highly conserved growth and differentiation factors. Signaling is initiated when a secreted BMP ligand binds to a pair of type-II BMP receptors at the membrane, which phosphorylate and recruit a pair of type-I BMP receptors. The type-I receptors then phosphorylate cytoplasmic R-Smads, which recruit co-Smads to form a complex. The Smad complex then translocates to the nucleus to regulate transcription of target genes. At the *Drosophila* NMJ, BMP signaling is a transsynaptic pathway that plays a role in synaptic growth and function. Forward genetic screens in *Drosophila* for mutations affecting synaptic growth first identified *wishful thinking* (*wit*), encoding a BMP type II receptor (Aberle *et al*., 2002), which functions in the presynaptic motor neuron. Subsequent work identified mutants for multiple members of this pathway, which exhibit synaptic undergrowth and reduced neurotransmitter release. These include the BMP ligand Glass bottom boat (Gbb), the type I receptors Saxophone (Sax) and Thickveins (Tkv), the R-Smad Mothers against decapentaplegic (Mad) and the co-Smad Medea (Med) (Aberle *et al*., 2002; Marqués *et al*., 2002; McCabe *et al*., 2003, 2004; Rawson *et al*., 2003). The Rho-type guanine nucleotide exchange factor (GEF) *trio* has been identified as a direct transcriptional target of this pathway in the nuclei of motorneurons (Ball *et al*., 2010). Characterization of BMP signaling during development has identified a critical period during the first larval instar, during which Mad signaling is both necessary and sufficient for NMJ growth and its modulation by activity (Berke *et al*., 2013).

While BMP signaling was initially characterized as retrograde, with muscle-derived Gbb ligand activating receptors in the neuron, recent work has described both anterograde and autocrine functions of this pathway. Tkv and phospho-Mad (p-Mad), which is typically used as hallmark of BMP signaling activation, are both found in the postsynaptic compartment (Dudu *et al*., 2006). Furthermore, synaptic bouton number is reduced when Mad is down-regulated in either neurons or muscles (Fuentes-Medel *et al*., 2012). This suggests that p-Mad signaling is required bi-directionally at the synapse.

Two functions of the BMP pathway - synaptic growth and neurotransmitter release – appear to depend on different sources of the Gbb ligand. In *gbb* mutants, NMJ undergrowth is rescued by muscle expression of Gbb, but neurotransmitter release is only fully rescued when Gbb is expressed in neurons (McCabe *et al*., 2003; Goold and Davis, 2007). How the neuron can discriminate between pre- and postsynaptic sources of ligand depends on the Gbb-binding protein Crimpy (Cmpy). Cmpy physically binds to a Gbb precursor protein and delivers Gbb to dense core vesicles at presynaptic terminals (James *et al*., 2014). Loss of *cmpy* results in overgrowth, which is rescued by knockdown of *gbb* in neurons (James and Broihier, 2011). Cmpy is also required for activity-dependent release of neuronal Gbb and for the ability of neuron Gbb to rescue neurotransmitter release in *gbb* mutants (James *et al*., 2014). These observations suggest that Cmpy regulates Gbb release in a way that allows the neuron to distinguish it from the muscle-

derived pool. The authors propose that activity-dependent release of Gbb from neurons may be distinguishable from constitutive Gbb release from muscle. Also, a C-terminal fragment of Cmpy is secreted along with Gbb in an activity-dependent manner (James *et al*., 2014). It will be interesting to determine whether this fragment is functional and regulates BMP signaling in the synaptic cleft.

BMP signaling at the NMJ is also required for synaptic stabilization and activity-dependent ghost bouton budding. Mutations in *wit* and *gbb* exhibit a significant increase in synaptic footprints (Eaton and Davis, 2005). Synaptic stabilization requires not only canonical Smad-dependent signaling, but also a parallel pathway involving LIM Kinase1 (DLIMK1), which binds the C-terminal tail of Wit. Both Smad/Trio signaling and Wit/Limk signaling regulate budding. Limk controls the activity of Cofilin, which promotes rapid bouton budding in response to activity (Piccioli and Littleton, 2014).

Interestingly, a glia-derived TGF-β signal also modulates Gbb-dependent retrograde signaling (Fuentes-Medel *et al*., 2012). The TGF-β ligand Maverick (Mav) is released from glia near the NMJ. Loss of Mav leads to downregulation of BMP signaling in both muscle and neuron. Mav activates Gbb transcription in the muscle, thus promoting secretion of the retrograde BMP ligand. Mav is thought to activate the activin-type receptor Punt (Put) on the muscle membrane, and whether it can also signal through a neuronal receptor remains an open question.

Wnt

Wnt signaling is another highly conserved pathway with diverse functions in the development and function of cells, including neurons. There are several divergent pathways that can be activated downstream of Wnt ligands, including a canonical pathway that results in nuclear import of β-catenin and transcriptional regulation. The role of these various pathways at neuromuscular junctions has been thoroughly reviewed elsewhere (Koles and Budnik, 2012b). At the fly NMJ, Wnt signaling is bidirectional and activates different downstream cascades in the pre- and post-synaptic cells. Loss of Wnt1/Wingless (Wg) results a decrease in bouton number, abnormally large and irregular boutons, a depletion of the SSR, increase in A-type clustering and an increase in the number of ghost boutons (Packard *et al*., 2002; Ataman *et al*., 2006; Korkut *et al*., 2009; Speese *et al*., 2012; Kerr *et al*., 2014). Loss of Wg also leads to abnormal microtubule bundling at the presynaptic cytoskeleton (Packard *et al*., 2002).

Wg is secreted by the neuron and activates its receptor Frizzled-2 (Fz2) in both pre- and postsynaptic cells. Wg binding Fz2 in the postsynaptic membrane leads to the endocytosis, cleavage and delivery of a C-terminal fragment of Fz2 to the nucleus (Mathew *et al*., 2005), in the so-called Fz nuclear import (FNI) pathway. Nuclear transport of Fz2-C requires the scaffolding protein Grip (Ataman *et al*., 2006) and nuclear import requires nucleoporin-associated proteins Importin-β11 (Imp-β11) and Importin-α2 (Imp-α2) (Mosca and Schwarz, 2010). In the nucleus, Fz2-C interacts with prominent ribonucleoprotein (RNP) particles containing transcripts that are important for synaptic development (Speese *et al*., 2012). The nuclear laminar component LamC and the Par protein aPKC play essential roles in this process. The RNP granules exit the nucleus through a nuclear envelope budding mechanism. This nuclear export process may be important for the delivery of synaptic transcripts to the NMJ. Indeed, knockdown of *lamC* impedes NMJ enrichment of *par6* transcripts.

Wg signaling also occurs in the presynaptic cell, where it activates several components of the canonical Wnt pathway, including Fz2, co-receptor Arrow (Arr), cytoplasmic phosphoprotein Dishevelled (Dsh) and glycogen synthase kinase 3b (GSK3b/Shaggy/Shg) (Miech *et al*., 2008). Unlike canonical Wnt signaling, the presynaptic pathway at the NMJ is independent of β-catenin, and instead appears to act locally at the synaptic terminal. Inhibiting this pathway results in defects in microtubule organization in the presynaptic cell as well as reduced bouton number and irregular bouton morphology. Presynaptic Wg signaling also activates the heterotrimeric G protein subunit Gαo, which interacts with Ankyrin 2 to promote microtubule organization and NMJ growth (Lüchtenborg *et al*., 2014). These findings suggest a presynaptic pathway that regulates microtubules and a bidirectional pathway for normal synaptic morphology. Interestingly, Arr appears to also signal in the postsynaptic cell, since expression of Arr in muscle rescues the synaptic growth defects in *arr* mutants (Miech *et al*., 2008). Thus, there may be additional parallel Wg signaling mechanisms at the NMJ that have not been fully elucidated.

Secretion of Wnt molecules presents an interesting problem because Wnt proteins typically contain lipid modifications that make them hydrophobic molecules. A variety of mechanisms have been described to facilitate diffusion of Wnts in the extracellular space (reviewed by Mikels and Nusse 2006; Koles and Budnik 2012a). The multipass transmembrane protein Evenness interrupted (Evi)/Wntless (Wls)/Sprinter (Spr) is required for Wg secretion in several tissues (Bänziger *et al*., 2006; Bartscherer *et al*., 2006; Goodman *et al*., 2006; Korkut *et al*., 2009). Recently, it was shown that Evi localizes to exosomes at the NMJ and a model was proposed that Wg is transported across the synaptic cleft on Evi-containing exosomes (Korkut *et al*., 2009; Koles and Budnik, 2012a).

Little is known about the migration or uptake of extracellular vesicles between cells. Evi-containing exosomes were observed in the cisternae of the SSR (Koles *et al*., 2012), where presumably the Wg ligand is accessible to Fz2 receptors. Interactions with the ECM likely contribute to Wg trafficking. Recent work showed that the distribution of Wg in the extracellular space is regulated by the secreted heparan sulfate proteoglycan (HSPG) Perlecan/Terribly reduced optic lobes (Trol). Loss of *trol* results in an upregulation of presynaptic and downregulation of postsynaptic Wg signaling (Kamimura *et al*., 2013). Trol localizes to the SSR and promotes the spread of extracellular Wg towards the postsynaptic cell, suggesting that *trol* regulates the balance of bidirectional signaling by impacting the pre-post synaptic gradient of Wg. There are several possible mechanisms of how Trol could regulate Wg distribution, including binding Wg, Evi, or another exosome component, or regulating the uptake of these compartments at the postsynaptic membrane. The regulation of extracellular trafficking of exosomes-like compartments is a fascinating topic for future studies.

Interestingly, glia were recently shown to be an additional source of Wg ligand at the NMJ (Kerr *et al*., 2014). Using RNAi to disrupt Wg secretion in specific cell types, the authors show that loss of glia-derived Wg results in increased A-type receptor clustering, but not NMJ size defects. In contrast, loss of neuron-derived Wg disrupts both of these processes. The GluR clustering defects likely results from downregulation of the FNI pathway (Speese *et al*., 2012). How the receiving cell distinguishes separate pools of Wg is an open question.

Wnt signaling also plays a role in ghost bouton budding. One prominent phenotype seen in *wg* pathway mutants is an abundance of ghost boutons (Fig. 5C), and this defect can be rescued by activating the FNI pathway (Mosca and Schwarz, 2010; Kamimura *et al*., 2013). In an assay for rapid activity-dependent budding of ghost boutons, in which formation of these structures is stimulated by high K+, evoked activity was shown to induce release of Wg (Ataman *et al*., 2008). Bidirectional Wg signaling is then required for rapid ghost bouton budding.

Neurotrophins

Neurotrophic signaling is essential in the vertebrate nervous system, regulating most aspects of development including neuronal survival, proliferation, differentiation, synaptic targeting and synaptic plasticity. Surprisingly, neurotrophins (NTs) and their receptors were only recently identified in *Drosophila*. A bioinformatics approach first identified DNT1 and DNT2 in the fly genome and both were shown to be required for neuronal survival (Zhu *et al*., 2008). The same study also demonstrated neurotrophic function of the protein Spätzle (Spz), which was previously identified as structurally similar to the vertebrate neurotrophin NGF (DeLotto and DeLotto, 1998; Mizuguchi *et al*., 1998; Weber *et al*., 2007). No orthologs of the vertebrate NT receptors have been found in *Drosophila*. Interesting, the Toll-like receptor (TLR) proteins Toll-6 and Toll-7 were shown to bind and genetically interact with DNT1 and DNT2, revealing an unexpected neurotrophic function for the Toll family in *Drosophila* (McIlroy *et al*., 2013).

All three DNTs also play a role in synaptic growth and morphology at the NMJ (Sutcliffe *et al*., 2013). The spz^2 allele is a point mutation in the pro-domain of Spz that results in loss of function, likely due to a defect in biosynthesis or secretion (Weber *et al*., 2007). Neuronal activation of Toll signaling rescues the semi-lethality of spz^2, suggesting a retrograde function. In spz^2 mutant larvae, bouton number and axonal terminal length are increased and the number of AZs per bouton is decreased, with the defects observed at muscle 4 but not muscles 6 and 7. Conversely, double deletion mutants for *DNT1, DNT2* exhibit increased bouton number, increased axon terminal length, and decreased AZ number at muscles 6 and 7 but not muscle 4. These results begin to suggest a neuron-type specific role for NT signaling in synaptic morphology.

Another TLR protein, Tollo, was recently shown to regulate synaptic growth (Ballard *et al*., 2014). Loss of Tollo results in a decrease in bouton number at the NMJ. However, synaptic function is not altered in these mutants. Consistent with this observation, the density of AZs per bouton is increased, resulting in a near normal number of AZs across the NMJ. Tollo is required in the presynaptic cell and interacts with the JNK signaling pathway to regulate NMJ organization. The authors identify the putative neurotrophin Spz3 as a potential ligand to activate Tollo activity.

Neuropeptides

Cholecystokinin (CCK)-like receptor (CCKLR), a putative neuropeptide receptor, was recently uncovered in a forward genetic screen for NMJ morphology mutants (Chen and Ganetzky, 2012). CCKLR is a G protein–coupled receptor (GPCR), which typically act by modulating second messenger systems in response to peptide ligand binding. CCKLR is required presynaptically at the NMJ. Loss of *CCKLR* results in dramatically

undergrown synapses, suggesting that CCKLR promotes NMJ growth. A predicted ligand, *drosulfakinin* (*dsk*), was characterized in the same study. Mutants for *dsk* exhibit a similar undergrowth phenotype, and genetic interaction experiments with *CCKLR* mutations support the fact that they act in a common pathway. Furthermore, DSK and CCKLR were shown to act through cAMP-PKA-CREB signaling to regulate synaptic growth. Surprisingly, this ligand-receptor interaction may be mediated at non-synaptic regions.

Transsynaptic adhesion

Synaptic cell adhesion molecules bridge the synaptic cleft and provide direct molecular connections between pre- and postsynaptic cells. Many adhesion molecules play crucial roles in axon guidance, targeting and synapse formation during development, notably Capricious, N-Cadherin, Dscam and FasII (reviewed by Sun and Xie, 2012; Schwabe *et al*., 2014). Adhesive interactions also contribute to the morphology and plasticity of the NMJ. The level of FasII at the synapse impacts NMJ size, and regulation of synaptic recruitment of FasII is important for structural plasticity (reviewed by Packard *et al*., 2003; Kristiansen and Hortsch, 2010).

As discussed above, the transsynaptic adhesion partners Nrx and Nlg are important regulators of AZ morphology and apposition. In addition, mutants for *nrx-1*, *nlg-1* and *nlg-2* each exhibit a dramatic decrease in the number of boutons at the NMJ (Li *et al*., 2007; Banovic *et al*., 2010; Sun *et al*., 2011; Chen *et al*., 2012). In contrast, loss of *nlg-3* results in bouton overgrowth (Xing *et al*., 2014). Loss of *nlg-1* or *nlg-2* has also been noted to produce irregularly shaped or large boutons (Chen *et al*., 2012). Loss of *ten-A* or *ten-M* similarly results in a decrease in bouton number and the presence of irregular large boutons (Mosca *et al*., 2012). All of these mutations also result in an increase in the number of ghost boutons per NMJ (Mosca *et al*., 2012). Thus, the Nrx-Nlg and Ten-A-Ten-M pairs regulate multiple aspects of synaptic morphology and function, highlighting the importance of appropriate adhesive connections across the synapse.

Neuroglian (Nrg), the *Drosophila* L1-type CAM, was recently identified in a genetic screen as a regulator of synapse stability (Enneking *et al*., 2013). The extracellular domains of L1 proteins mediate both hemophilic and heterophilic cell-cell interactions and their intracellular tails interact with a variety of partners inside the cell, including Ankyrins. In *Drosophila,* the binding of Nrg to its partner Ank2 is important both for synapse stability and for constraining NMJ growth.

Endocytosis regulators

Many proteins that regulate endocytosis are also negative regulators of synaptic growth. These include Rab11, a GTPase that regulates recycling endosomes; the early endosomal protein Spichthyin (Spict); Dap160/intersectin; Spinster, a component of late endosomes; endophilin, synaptojanin and AP180, regulators of clathrin-mediated endocytosis; Dynamin, which is required for membrane fission, and the F-BAR protein Nervous wreck (Nwk) (Sweeney and Davis, 2002; Coyle *et al*., 2004; Koh *et al*., 2004; Marie *et al*., 2004; Dickman *et al*., 2006; Khodosh *et al*., 2006; Wang *et al*., 2007). In particular, Dickman *et al* (2006) observed that many endocytosis mutants exhibit overgrowth in the form of satellite boutons (Fig. 5C).

Synaptic overgrowth in endocytosis mutants is linked to a failure to attenuate BMP signaling in the presynaptic cell. The binding of a ligand to its receptor is typically followed by endocytosis of the signaling complex, which remains active in early endosomal compartments. Downregulation of the signal is therefore achieved by progression through the endosomal system, with eventual dismantling and/or degradation of signaling complexes (reviewed by Wegner *et al*. 2011). At the NMJ, the BMP receptors Tkv and Wit accumulate in early endosomes in the presynaptic cell and signaling is upregulated when traffic is blocked from this compartment (Wang *et al*., 2007). Trafficking and signal attenuation of BMP receptors is regulated in part by Nwk. Nwk negatively regulates BMP signaling by interacting directly with Tkv (O'Connor-Giles *et al*., 2008). Nwk also interacts with endocytosis regulators Dap160 and Dynamin, and the actin regulatory complex WASp-Arp2/3 (O'Connor-Giles *et al*., 2008; Rodal *et al*., 2008). The mechanism by which Nwk impacts BMP receptor activity involves regulated traffic between endosomal compartments. Nwk localizes to a novel compartment that shares some features of recycling endosomes (Rodal *et al*., 2008, 2011). Nwk-containing compartments interact transiently with early endosomes that contain sorting nexin 16 (SNX16). The Nwk-SNX16 interaction is critical for attenuation of both the BMP and Wg cascades.

Protein degradation pathways

Ubiquitin is a covalent peptide modification that is reversibly added to proteins to regulate their stability and localization in cells (reviewed by Tian and Wu 2013). Addition of ubiquitin chains is catalyzed by ubiquitin ligases (called E1, E2, E3) and antagonized by deubiquitinating proteases. Mutations affecting the ubiquitin-proteasome system (UPS) also regulate synaptic morphology, by influencing the stability or activity of key synaptic proteins.

The Highwire-Wallenda-Jnk (Hiw-Wnd-Jnk) pathway regulates synaptic growth. Wnd is a dual leucine zipper kinase (DLK) that functions as a MAP Kinase Kinase Kinase (MAPKKK) and activates Jnk signaling. Work in *Drosophila* and *C. elegans* showed that in the absence of the E3 ubiquitin ligase Highwire (Hiw; RPM-1 in *C. elegans*), Wnd levels increase, overactivating the Jnk pathway (Nakata *et al*., 2005; Collins *et al*., 2006). Loss of function of *hiw* gives rise to a distinct synaptic morphology characterized by a greatly expanded arbor and abnormally small boutons (Fig. 5B) (Wan *et al*., 2000; Wu *et al*., 2005). Despite the increase in bouton number, these animals exhibit synaptic dysfunction with decreased quantal size and quantal content. Regulation of Wld levels by Hiw-dependent ubiquitination is counteracted by the deubiquitinating enzyme Fat facets (DiAntonio *et al*., 2001). E3 ubiquitin ligases can act alone or in multicomponent E3 complexes. Recent studies have identified the proteins DFsn, Rae1 and SkpA, which act in a complex with Hiw to regulate Wnd (Wu *et al*., 2007; Tian *et al*., 2011; Brace *et al*., 2014) and resemble the conserved SCF (Skp/Cullin/F-box) E3 complex.

The APC/C E3 ubiquitin ligase complex has also been implicated in synaptic growth. Components APC2, Cdc27 and Cdh1 localize to the NMJ. Mutants in *apc2* fail to ubiquitinate Liprin-α, which accumulates at the NMJ resulting in an increase in bouton number (van Roessel *et al*., 2004).

Translational regulation

Many forms of synaptic plasticity have been shown to depend on the local translation of synaptic transcripts. Several recent studies have described RNA-binding proteins that impact synaptic growth and plasticity at the NMJ through regulation of translation.

Fragile X mental retardation protein

FMRP is a translational repressor and its loss results in misregulation of target genes with consequences for synaptic structure and function (reviewed by Sidorov *et al*. 2013). Mutations in the human *FMR1* gene cause Fragile X syndrome, one of the most common forms of intellectual disability. The single *dfmr1* gene in *Drosophila* is a well-characterized model for studying FMRP function and targets (reviewed by Gatto and Broadie 2011). Loss of *dfmr1* results in overgrowth at synapses and increased synaptic function (Zhang *et al*., 2001). Multiple targets of dFMR1 have been characterized, including Futsch (Zhang *et al*., 2001), GluRs (Pan and Broadie, 2007) and the HSPGs Dlp and Sdc, leading to defects in Wg and BMP signaling (Friedman *et al*., 2013). There are likely many more targets of dFMR1 to be identified, and the fly NMJ remains an excellent system to reveal the function of FMRP targets in synaptic organization.

Other RNA binding proteins

Pumilio and Nanos are translational repressors that act in opposition to each other to affect bouton size, number and GluR subunit composition (Menon *et al*., 2004, 2009). Pum binds *nos* and *GluRIIA* mRNAs, and the authors propose regulatory interplay between Pum, Nos, GluRIIA and GluRIIB to control the GluR subunit ratio. Another RNA binding protein at the NMJ is Staufen, which has a well-characterized role in mRNA localization during *Drosophila* development. Staufen is required for the localization of Cora mRNA and protein, leading to reduced accumulation of A-type receptors (Gardiol and St Johnston, 2014). A broader role in regulating multiple synaptic transcripts was found for the heterogeneous nuclear ribonucleoprotein (hnRNP) Syncrip (Syp). Syp is present postsynaptically at the NMJ and its loss results in synaptic overgrowth and defective neurotransmission (Halstead *et al*., 2014; McDermott *et al*., 2014). Syp appears to interact with multiple transcripts encoding synaptic proteins, and it differentially affects the protein levels of several of these targets (McDermott *et al*., 2014). Its loss is also associated with increased levels of Gbb and p-Mad, indicating that one output of its activity could be an impact on BMP signaling (Halstead *et al*., 2014). These recent findings suggest a level of translational control at the synapse that is not fully appreciated to date. Mechanisms affecting the transport, localization, translation and degradation of transcripts are likely to add a new layer of complexity to our understanding of synaptic organization. Furthermore, how neural activity regulates synaptic transcripts is a fascinating question. One possible mechanism comes from Speese *et al* (2012), who describe a model whereby postsynaptic Wnt signaling leads to the nuclear import of the cleaved Fz2 receptor and subsequent regulated nuclear export of RNPs containing synaptic transcripts.

miRNAs

miRNAs are noncoding RNAs that regulate gene expression. miRNAs bind to target transcripts and recruit the RNA-induced silencing complex (RISC), which can repress translation or degrade the transcript (McNeill and Van Vactor, 2012). mRNAs are diverse and regulate numerous cellular processes, including neuronal development, but are only beginning to be characterized in *Drosophila*. *mir-8* was first characterized using a microRNA "sponge", antisense oligonucleotides directed against specific miRNAs to knock down their function (Loya *et al*., 2009). This study revealed a role for *mir-8* in NMJ morphogenesis. *mir-8* is postsynaptic and regulates levels of the effector protein Enabled (Ena), an actin regulator (Loya *et al*., 2009, 2014). *mir-8* was also identified in a screen for miRNAs that are differentially regulated by activity (Nesler *et al*., 2013). This same study demonstrated that *mir-8* regulates *wg* expression *in vitro*, supporting a role for *mir-8* in synaptic plasticity.

Homeostatic plasticity

Homeostatic mechanisms stabilize various cellular properties within a range of physiologically appropriate values. Thus, as synapses change in response to activity, homeostatic signaling processes are engaged to constrain the extent of these modifications. Homeostatic plasticity of synapses has been shown in a variety of animal models to include changes in excitability, regulation of neurotransmitter release, or regulation of postsynaptic neurotransmitter receptors. The molecular pathways that contribute to homeostatic plasticity in *Drosophila* NMJ and other systems has been recently reviewed (Davis, 2013; Davis and Müller, 2014; Frank, 2014). We will briefly discuss some key findings below.

Homeostatic plasticity at the NMJ can be observed when GluR function is impaired, leading to a compensatory increase in presynaptic release. For example, mutants for *GluRIIA* exhibit a significant decrease in miniature amplitude accompanied by an increase in quantal content, resulting in an overall normal amplitude of evoked release that restores muscle depolarization (Petersen *et al*., 1997; DiAntonio *et al*., 1999). A similar phenotype was observed with postsynaptic expression of constitutive active Protein Kinase A, and this effect was shown to be dependent on *GluRIIA* (Davis *et al*., 1998). Notably, this homeostatic compensation occurs with depletion of A-type receptors and not B-type receptors, reflecting an important difference in their electrophysiological properties. Consistent with this observation, mutations affecting A-type receptor clustering have been shown to exhibit homeostatic compensation, including *Pak*, *dorsal* and *cactus* (Albin and Davis, 2004; Heckscher *et al*., 2007).

Homeostatic compensation can also be induced acutely, on a timescale of minutes, through application of Philanthotoxin-433 (PhTx). PhTx impairs GluR function, resulting in a decrease in miniature amplitude and compensatory increase in quantal content, similar to the phenotype of *GluRIIA* animals (Frank *et al*., 2006). Whether acute and developmental homeostatic changes are regulated by the same mechanisms is still unclear. Current findings support overlapping but non-identical regulation of these two modes of plasticity (see Frank 2014). A recent forward-genetic screen used PhTx application to identify mutations that block acute homeostatic compensation (Dickman and Davis, 2009; Müller *et al*., 2011; Younger *et al*., 2013). This approach led to the characterization of the schizophrenia susceptibility gene *dysbindin* (*dysb*), which is required for both acute and developmental homeostatic plasticity (Dickman and Davis,

2009). Dysb associates with SVs and appears to promote homeostasis downstream or independently of Ca^{2+} influx at the synapse. In mammals and flies, Dysb associates with seven other proteins to form BLOC-1 (biogenesis of lysosome-related organelles), a complex implicated in several aspects of membrane trafficking and fusion (Mullin *et al*., 2011, 2015). Genetic interaction experiments support the idea that the BLOC-1 components Snapin and Blos-1 cooperate with Dysb to mediate homeostatic plasticity in *Drosophila* (Dickman *et al*., 2012; Mullin *et al*., 2015), and like Dysb, Snapin localizes to SVs (Dickman *et al*., 2012). The mechanism of how Dysb and its interactors function in homeostatic compensation is unclear, but might involve regulation of SNARE-mediated fusion events, as BLOC-1 components have been shown to interact with SNAREs and NSF (Ghiani *et al*., 2010; Gokhale *et al*., 2015), and overexpression of NSF rescues the ability to respond to PhTx in *dysb* mutants (Gokhale *et al*., 2015).

Key presynaptic changes that increase quantal content during synaptic homeostasis include Ca^{2+} influx through the voltage-gated Ca^{2+} channel Cac (Frank *et al*., 2006; Müller and Davis, 2012), and increased size of the readily releasable pool (RRP) (Weyhersmuller *et al*., 2011). These changes appear to be separable; for example, mutants for *rim* (*rab3-interacting molecule*) block homeostatic changes in RRP size, but exhibit normal Ca^{2+} influx (Müller *et al*., 2012). RIM localizes to AZs and regulates Cac localization (Graf *et al*., 2012), but how RIM contributes to RRP regulation during homeostatic plasticity is unclear. As Rab3-GAP (Rab3 GTPase-activating protein) is also required presynaptically for synaptic homeostasis (Müller *et al*., 2011), there is mounting evidence for a Rab 3 signaling module regulating this process. The regulation of Cac function and Ca^{2+} influx during homeostatic modulation is still poorly understood. One regulatory pathway involves the Rho-type guanine exchange factor Ephexin (Exn) (Frank *et al*., 2009). Synaptic homeostasis is blocked in *exn* mutants and mutants for the Eph receptor (*Eph*). Genetic interaction experiments support modulation of Cac function by Exn, Eph and the effector Cdc42 during synaptic homeostasis.

Other regulators of homeostatic plasticity include the transcription factor Gooseberry (Marie *et al*., 2010), the K+ channel proteins Shal and Shaker (Bergquist *et al*., 2010) and TOR signaling (Penney *et al*., 2012). BMP and Wnt signaling pathways have also been implicated (Goold and Davis, 2007; Marie *et al*., 2010). Multiplexin, the *Drosophila* homolog of the matrix protein Collagen XV/XVIII, was recently demonstrated to be required for synaptic homeostasis by regulating presynaptic Ca^{2+} influx (Wang *et al*., 2014). Multiplexin is cleaved to produce Endostatin, which is thought to function as a transsynaptic signal. In addition, presynaptic insertion of an ENaC channel into synaptic membranes appears to contribute to homeostatic potentiation, resulting in increased depolarization of the membrane and enhanced presynaptic Ca^{2+} influx (Davis, 2013; Younger *et al*., 2013). These studies suggest a complex regulatory network on both sides of the synapse.

In addition to potentiation of release due to inhibition of GluR function, an opposite response can be observed when the glutamate concentration in SVs is increased. Overexpressing the vesicular glutamate transporter (vGlut) increases the amount of glutamate stored in SVs, resulting in larger spontaneous mini amplitude events (Daniels *et al*., 2004). The enhanced current from single SV fusion events is compensated by a homeostatic decrease in vesicle release probability, maintaining the normal level of membrane depolarization to an action potential. Animals overexpressing vGlut exhibit

reduced presynaptic levels of Cac, and no change in the size of the RRP, suggesting that the primary mechanism of homeostatic depression is through regulation of Ca^{2+} channel abundance at the synapse (Gaviño *et al*., 2015). Several mutations that block homeostatic potentiation have no effect on homeostatic depression in vGlut-overexpressing animals, consistent with the idea that these processes are regulated by distinct mechanisms (Gaviño *et al*., 2015). The *Drosophila* NMJ remains an excellent genetic system to reveal and characterize factors contributing to homeostatic potentiation and depression.

Conclusion

The *Drosophila* synapse as a model for human neurological diseases

At the turn of the 21st century, cross-genomic analysis of the fly and human genomes revealed that ~75% of human disease genes have conserved sequences in *Drosophila* (Bier, 2005). In the 15 years since, *Drosophila* has become an increasingly popular tool for studying human disease. Indeed, many of the molecular pathways discussed in this review have some human disease connection, and work in the fly model often provides important insight into the basic biology that might underlie disease pathology. Excellent examples include Nrx and Nlg, which are associated with autism spectrum disorders (ASDs) (Südhof, 2008), or Wnt signaling, which is associated with ASD, Schizophrenia and Alzheimer's disease (De Ferrari and Moon, 2006). Given the emerging view of autism, schizophrenia, Fragile X syndrome, and other neurodevelopmental diseases as "synaptopathies" (Mitchell, 2011; Grant, 2012), the *Drosophila* NMJ is highly relevant as a model system to investigate the function of synaptic genes and uncover novel interacting pathways.

Other efforts seek to model neurodegenerative proteinopathies, for which the temporal and tissue-specific overexpression tools available in *Drosophila* can be used to recapitulate expression of a mutant protein. Polyglutamine (PolyQ) expansion disorders were some of the first diseases to be studied using this approach, by overexpressing PolyQ proteins associated with Spinocerebellar ataxia type 3 (Warrick *et al*., 1998) or Huntington's disease (Jackson *et al*., 1998), and *Drosophila* remains a useful model for examining PolyQ protein dynamics and neurodegeneration (Krench and Littleton, 2013). Other notable neurodegenerative diseases modeled in flies include Parkinson's disease, Alzheimer's disease and prion diseases (Bier, 2005; Rincon-Limas *et al*., 2012).

Outlook

Over forty years of research on synaptic biology in *Drosophila* has led to a detailed picture of synaptic assembly, function and plasticity. The genes described in this review encode proteins of the synaptic membrane, cytoskeleton, vesicle trafficking system, nucleus and extracellular matrix, reflecting the complex layers of regulation required to maintain and modulate neural function at the cellular and molecular level. In particular, the past decade has brought significant developments in imaging technologies, allowing dynamic imaging of molecules at high resolution, with the potential to provide new and surprising information about protein mobility and interactions.

Evolutionary conservation of genes allows findings in *Drosophila* to provide key insights into the function of mammalian excitatory synapses, the study of disease, and

cellular processes in general. We anticipate that the *Drosophila* NMJ will continue to be a fruitful model synapse, providing a toolbox for genetic manipulation and *in vivo* accessibility not possible in other model organisms. Future work will undoubtedly continue to dissect the function of synaptic proteins and uncover novel functionalities through forward genetic screens.

Figure Legends

Figure 1. A. Anatomy of the *Drosophila* NMJ. A muscle 4 NMJ is shown with immunolabeling for the AZ protein Brp (magenta) and a postsynaptic GluR subunit (green). EM images of a bouton and a T-bar AZ are shown on the right. B. The anatomy of the muscle 6/7 NMJ from the late embryonic stage to the mature 3rd instar stage is shown. Immunocytochemistry highlights the nerve (anti-HRP, green) and AZs (anti-Brp, magenta). Scale bar is 10 mm in both images.

Figure 2. A. Molecular structure of the fusion machine containing synaptotagmin and the assembled SNARE complex (syntaxin - red; SNAP-25 - green; synaptobrevin - blue). The SV membrane and plasma membrane are also displayed. B. Representative excitatory junctional currents (EJCs) evoked by nerve stimulation from late stage embyros of synaptotagmin null mutants (syt1 -/-), null mutants rescued with a wildtype synaptotagmin construct (Syt1 WT), or null mutants rescued with constructs that disrupt Ca^{2+} binding to C2A (C2A*) or C2B (C2B*) (modified from Yoshihara *et al*., 2010). Two traces are shown for each genotype, with the representative structures of Syt1 shown above (X indicates loss of Ca^{2+} binding). Stimulation artifacts correspond to the onset of nerve stimulation. Null mutations lacking Syt1 show a loss of synchronous release and enhanced asynchronous release (arrows). Syt1 lacking C2A Ca^{2+} binding shows normal synchronous release, but cannot suppress asynchronous fusion. Syt1 lacking C2B Ca^{2+} binding shows a dramatic reduction in synchronous release, but is capable of preventing asynchronous fusion. C. Model of complexin regulation of SNARE assembly (modified from Bykhovskaia *et al*., 2013). Cpx (magenta) stabilizes a partially zippered SNARE bundle, leading to separation of the vesicle and plasma membranes (right). In the absence of Cpx, synaptobrevin fully zippers onto the t-SNARE complex, bringing the two membranes together to drive lipid bilayer fusion. D. Postsynaptic current recordings of spontaneous mini release events from 3rd instar NMJs of control (black), $cpx^{-/-}$ (red), $syt1^{-/-}$ (aqua) and $syt1^{-/-}$; $cpx^{-/-}$ double null (grey) synapses (modified from Jorquera *et al*., 2012). The loss of Cpx leads to a dramatic increase in spontaneous release that is abolished in the absence of Syt1.

Figure 3. Diagram of AZs within synaptic boutons. The bottom panel shows mapping of individual release site probabilities for evoked and spontaneous release using myr-GCaMP expressed in the postsynaptic compartment to visualize single SV fusion events (modified from Melom *et al*., 2013). AZs can show low or high release probabilities for both spontaneous and evoked release, with no correlation observed between the two modes of fusion. In addition, ~25% of AZs appear to be spontaneous only sites that lack evoked fusion events.

Figure 4. A. A single bouton at the NMJ (blue) embedded in the folds of the subsynaptic reticulum of the muscle (olive green). AZs within the bouton are apposed by GluRs. B. Schematic of the AZ and PSD. The AZ consists of Brp molecules decorated by SVs. Cac localizes to the center of the AZ, beneath Brp. DRBP forms a ring around the base of the Brp scaffold. Syd-1 and Liprin-α also localize within the AZ cytomatrix. On the

postsynaptic side, A-type and B-type GluR subunits cluster opposite the AZ. Postsynaptic proteins important for organizing the synapse include components of the postsynaptic spectrin cytoskeleton, the scaffolding protein Dlg, and transsynaptic adhesion complexes that span the synaptic cleft. C. Schematic illustration of synaptic organization defects. Mature wild type synapses exhibit regular size and spacing of synapses and precise apposition of pre- and postsynaptic specializations. Defects of synaptic organization can include changes in subunit composition, aberrant T-bar ultrastructure, enlarged GluR clusters and multiple T-bars. Defects of apposition include AZs lacking a GluR cluster or GluR clusters lacking AZs. Unapposed GluRs can occur through redistribution of Brp, lack of T-bars or complete lack of an electron dense AZ.

Figure 5. A. During development, boutons are added to the growing NMJ arbor. Either existing boutons stretch apart and form a new bouton between them, or new boutons are added onto the ends of existing strings of boutons. B. Examples of synaptic growth defects. The neuronal membrane is highlighted in magenta and the PSD protein Dlg is shown in green. A wildtype NMJ at muscle 4 is compared to a *highwire* mutant, which exhibits a greatly expanded arbor and abnormally small boutons. Other aberrant growth patterns are also shown. Satellite boutons, small boutons decorating a normal-sized parent bouton, are characteristic of loss-of-function mutations in endocytosis regulators. Ghost boutons, which lack a postsynaptic scaffold, are characteristic of mutants in postsynaptic Wnt signaling. Scale bars = 10 μm.

References

Aberle, H., Haghighi, A.P., Fetter, R.D., McCabe, B.D., Magalhães, T.R., and Goodman, C.S. (2002) wishful thinking encodes a BMP type II receptor that regulates synaptic growth in *Drosophila*. *Neuron* **33**: 545–58.

Adolfsen, B. and Littleton, J.T. (2001) Genetic and molecular analysis of the synaptotagmin family. *Cell. Mol. Life Sci.* **58**: 393–402.

Adolfsen, B., Saraswati, S., Yoshihara, M., and Littleton, J.T. (2004) Synaptotagmins are trafficked to distinct subcellular domains including the postsynaptic compartment. *J. Cell Biol.* **166**: 249–60.

Albin, S.D. and Davis, G.W. (2004) Coordinating structural and functional synapse development: postsynaptic p21-activated kinase independently specifies glutamate receptor abundance and postsynaptic morphology. *J. Neurosci.* **24**: 6871–9.

Andlauer, T.F.M., Scholz-Kornehl, S., Tian, R., Kirchner, M., Babikir, H.A., Depner, H., Loll, B., Quentin, C., Gupta, V.K., Holt, M.G., Dipt, S., Cressy, M., Wahl, M.C., Fiala, A., Selbach, M., Schwärzel, M., and Sigrist, S.J. (2014) Drep-2 is a novel synaptic protein important for learning and memory. *Elife* **3**: e03895.

Aravamudan, B., Fergestad, T., Davis, W.S., Rodesch, C.K., and Broadie, K. (1999) *Drosophila* UNC-13 is essential for synaptic transmission. *Nat. Neurosci.* **2**: 965–71.

Ataman, B., Ashley, J., Gorczyca, D., Gorczyca, M., Mathew, D., Wichmann, C., Sigrist, S.J., and Budnik, V. (2006) Nuclear trafficking of *Drosophila* Frizzled-2 during synapse development requires the PDZ protein dGRIP. *Proc. Natl. Acad. Sci. U. S. A.* **103**: 7841–6.

Ataman, B., Ashley, J., Gorczyca, M., Ramachandran, P., Fouquet, W., Sigrist, S.J., and Budnik, V. (2008) Rapid activity-dependent modifications in synaptic structure and function require bidirectional Wnt signaling. *Neuron* **57**: 705–18.

Atwood, H.L., Govind, C.K., and Wu, C.F. (1993) Differential ultrastructure of synaptic terminals on ventral longitudinal abdominal muscles in *Drosophila* larvae. *J. Neurobiol.* **24**: 1008–24.

Babcock, M., Macleod, G.T., Leither, J., and Pallanck, L. (2004) Genetic analysis of soluble N-ethylmaleimide-sensitive factor attachment protein function in *Drosophila* reveals positive and negative secretory roles. *J. Neurosci.* **24**: 3964–73.

Bachmann, A., Kobler, O., Kittel, R.J., Wichmann, C., Sierralta, J., Sigrist, S.J., Gundelfinger, E.D., Knust, E., and Thomas, U. (2010) A perisynaptic ménage à trois between Dlg, DLin-7, and Metro controls proper organization of *Drosophila* synaptic junctions. *J. Neurosci.* **30**: 5811–24.

Ball, R.W., Warren-Paquin, M., Tsurudome, K., Liao, E.H., Elazzouzi, F., Cavanagh, C., An, B.-S., Wang, T.-T., White, J.H., and Haghighi, A.P. (2010) Retrograde BMP signaling controls synaptic growth at the NMJ by regulating trio expression in motor neurons. *Neuron* **66**: 536–49.

Ballard, S.L., Miller, D.L., and Ganetzky, B. (2014) Retrograde neurotrophin signaling through Tollo regulates synaptic growth in *Drosophila*. *J. Cell Biol.* **204**: 1157–72.

Bang, M.L. and Owczarek, S. (2013) A matter of balance: role of neurexin and neuroligin at the synapse. *Neurochem. Res.* **38**: 1174–89.

Banovic, D., Khorramshahi, O., Owald, D., Wichmann, C., Riedt, T., Fouquet, W., Tian, R., Sigrist, S.J., and Aberle, H. (2010) *Drosophila* neuroligin 1 promotes growth and postsynaptic differentiation at glutamatergic neuromuscular junctions. *Neuron* **66**: 724–38.

Bänziger, C., Soldini, D., Schütt, C., Zipperlen, P., Hausmann, G., and Basler, K. (2006) Wntless, a conserved membrane protein dedicated to the secretion of Wnt proteins from signaling cells. *Cell* **125**: 509–22.

Barber, C.F., Jorquera, R.A., Melom, J.E., and Littleton, J.T. (2009) Postsynaptic regulation of synaptic plasticity by synaptotagmin 4 requires both C2 domains. *J. Cell Biol.* **187**: 295–310.

Bartscherer, K., Pelte, N., Ingelfinger, D., and Boutros, M. (2006) Secretion of Wnt ligands requires Evi, a conserved transmembrane protein. *Cell* **125**: 523–33.

Benzer, S. (1967) Behavioral mutants of *Drosophila* isolated by countercurrent distribution. *Proc. Natl. Acad. Sci. U. S. A.* **58**: 1112–9.

Bergquist, S., Dickman, D.K., and Davis, G.W. (2010) A hierarchy of cell intrinsic and target-derived homeostatic signaling. *Neuron* **66**: 220–34.

Berke, B., Wittnam, J., McNeill, E., Van Vactor, D.L., and Keshishian, H. (2013) Retrograde BMP signaling at the synapse: a permissive signal for synapse maturation and activity-dependent plasticity. *J. Neurosci.* **33**: 17937–50.

Biederer, T. and Sudhof, T.C. (2001) CASK and protein 4.1 support F-actin nucleation on neurexins. *J. Biol. Chem.* **276**: 47869–76.

Bier, E. (2005) *Drosophila*, the golden bug, emerges as a tool for human genetics. *Nat. Rev. Genet.* **6**: 9–23.

Van der Bliek, A.M. and Meyerowitz, E.M. (1991) Dynamin-like protein encoded by the *Drosophila* shibire gene associated with vesicular traffic. *Nature* **351**: 411–4.

Blunk, A.D., Akbergenova, Y., Cho, R.W., Lee, J., Walldorf, U., Xu, K., Zhong, G., Zhuang, X., and Littleton, J.T. (2014) Postsynaptic actin regulates active zone spacing and glutamate receptor apposition at the *Drosophila* neuromuscular junction. *Mol. Cell. Neurosci.* **61**: 241–54.

Bogdanik, L., Framery, B., Frölich, A., Franco, B., Mornet, D., Bockaert, J., Sigrist, S.J., Grau, Y., and Parmentier, M.-L. (2008) Muscle dystroglycan organizes the postsynapse and regulates presynaptic neurotransmitter release at the *Drosophila* neuromuscular junction. *PLoS One* **3**: e2084.

Brace, E.J., Wu, C., Valakh, V., and DiAntonio, A. (2014) SkpA restrains synaptic terminal growth during development and promotes axonal degeneration following injury. *J. Neurosci.* **34**: 8398–410.

Broadie, K. and Bate, M. (1993a) Activity-dependent development of the neuromuscular synapse during *Drosophila* embryogenesis. *Neuron* **11**: 607–19.

Broadie, K. and Bate, M. (1993b) Development of the embryonic neuromuscular synapse of *Drosophila melanogaster*. *J. Neurosci.* **13**: 144–66.

Broadie, K., Bellen, H.J., DiAntonio, A., Littleton, J.T., and Schwarz, T.L. (1994) Absence of synaptotagmin disrupts excitation-secretion coupling during synaptic transmission. *Proc. Natl. Acad. Sci. U. S. A.* **91**: 10727–31.

Broadie, K., Prokop, A., Bellen, H.J., O'Kane, C.J., Schulze, K.L., and Sweeney, S.T. (1995) Syntaxin and synaptobrevin function downstream of vesicle docking in *Drosophila*. *Neuron* **15**: 663–73.

Bruckner, J.J., Gratz, S.J., Slind, J.K., Geske, R.R., Cummings, A.M., Galindo, S.E., Donohue, L.K., and O'Connor-Giles, K.M. (2012) Fife, a *Drosophila* Piccolo-RIM homolog, promotes active zone organization and neurotransmitter release. *J. Neurosci.* **32**: 17048–58.

Budnik, V., Zhong, Y., and Wu, C.F. (1990) Morphological plasticity of motor axons in *Drosophila* mutants with altered excitability. *J. Neurosci.* **10**: 3754–68.

Buhl, L.K., Jorquera, R.A., Akbergenova, Y., Huntwork-Rodriguez, S., Volfson, D., and Littleton, J.T. (2013) Differential regulation of evoked and spontaneous neurotransmitter release by C-terminal modifications of complexin. *Mol. Cell. Neurosci.* **52**: 161–72.

Bulat, V., Rast, M., and Pielage, J. (2014) Presynaptic CK2 promotes synapse organization and stability by targeting Ankyrin2. *J. Cell Biol.* **204**: 77–94.

Bulgari, D., Zhou, C., Hewes, R.S., Deitcher, D.L., and Levitan, E.S. (2014) Vesicle capture, not delivery, scales up neuropeptide storage in neuroendocrine terminals. *Proc. Natl. Acad. Sci. U. S. A.* **111**: 3597–601.

Bykhovskaia, M., Jagota, A., Gonzalez, A., Vasin, A., and Littleton, J.T. (2013) Interaction of the complexin accessory helix with the C-terminus of the SNARE complex: molecular-dynamics model of the fusion clamp. *Biophys. J.* **105**: 679–90.

Cantera, R., Kozlova, T., Barillas-Mury, C., and Kafatos, F.C. (1999) Muscle structure and innervation are affected by loss of Dorsal in the fruit fly, *Drosophila melanogaster*. *Mol. Cell. Neurosci.* **13**: 131–41.

Chao, H.-W., Hong, C.-J., Huang, T.-N., Lin, Y.-L., and Hsueh, Y.-P. (2008) SUMOylation of the MAGUK protein CASK regulates dendritic spinogenesis. *J. Cell Biol.* **182**: 141–55.

Chen, K. and Featherstone, D.E. (2005) Discs-large (DLG) is clustered by presynaptic innervation and regulates postsynaptic glutamate receptor subunit composition in *Drosophila*. *BMC Biol.* **3**: 1.

Chen, K. and Featherstone, D.E. (2011) Pre and postsynaptic roles for *Drosophila* CASK. *Mol. Cell. Neurosci.* **48**: 171–82.

Chen, K., Gracheva, E.O., Yu, S.-C., Sheng, Q., Richmond, J., and Featherstone, D.E. (2010) Neurexin in embryonic *Drosophila* neuromuscular junctions. *PLoS One* **5**: e11115.

Chen, K., Merino, C., Sigrist, S.J., and Featherstone, D.E. (2005) The 4.1 protein coracle mediates subunit-selective anchoring of *Drosophila* glutamate receptors to the postsynaptic actin cytoskeleton. *J. Neurosci.* **25**: 6667–75.

Chen, X. and Ganetzky, B. (2012) A neuropeptide signaling pathway regulates synaptic growth in *Drosophila*. *J. Cell Biol.* **196**: jcb.201109044–.

Chen, Y.-C., Lin, Y.Q., Banerjee, S., Venken, K., Li, J., Ismat, A., Chen, K., Duraine, L., Bellen, H.J., and Bhat, M.A. (2012) *Drosophila* Neuroligin 2 is required presynaptically and postsynaptically for proper synaptic differentiation and synaptic transmission. *J. Neurosci.* **32**: 16018–16030.

Chen, Y.A., Scales, S.J., Patel, S.M., Doung, Y.C., and Scheller, R.H. (1999) SNARE complex formation is triggered by Ca2+ and drives membrane fusion. *Cell* **97**: 165–74.

Cho, R.W., Kümmel, D., Li, F., Baguley, S.W., Coleman, J., Rothman, J.E., and Littleton, J.T. (2014) Genetic analysis of the Complexin trans-clamping model for cross-linking SNARE complexes *in vivo*. *Proc. Natl. Acad. Sci. U. S. A.* **111**: 10317–22.

Cho, R.W., Song, Y., and Littleton, J.T. (2010) Comparative analysis of *Drosophila* and mammalian complexins as fusion clamps and facilitators of neurotransmitter release. *Mol. Cell. Neurosci.* **45**: 389–97.

Choi, B.J., Imlach, W.L., Jiao, W., Wolfram, V., Wu, Y., Grbic, M., Cela, C., Baines, R.A., Nitabach, M.N., and McCabe, B.D. (2014) Miniature neurotransmission regulates *Drosophila* synaptic structural maturation. *Neuron* **82**: 618–34.

Chouhan, A.K., Zhang, J., Zinsmaier, K.E., and Macleod, G.T. (2010) Presynaptic mitochondria in functionally different motor neurons exhibit similar affinities for Ca2+ but exert little influence as Ca2+ buffers at nerve firing rates in situ. *J. Neurosci.* **30**: 1869–81.

Cocucci, E. and Meldolesi, J. (2015) Ectosomes and exosomes: shedding the confusion between extracellular vesicles. *Trends Cell Biol.*

Cohen, A.R., Woods, D.F., Marfatia, S.M., Walther, Z., Chishti, A.H., Anderson, J.M., and Wood, D.F. (1998) Human CASK/LIN-2 binds syndecan-2 and protein 4.1 and localizes to the basolateral membrane of epithelial cells. *J. Cell Biol.* **142**: 129–38.

Collins, C.A., Wairkar, Y.P., Johnson, S.L., and DiAntonio, A. (2006) Highwire restrains synaptic growth by attenuating a MAP kinase signal. *Neuron* **51**: 57–69.

Coyle, I.P., Koh, Y.-H., Lee, W.-C.M., Slind, J., Fergestad, T., Littleton, J.T., and Ganetzky, B. (2004) Nervous wreck, an SH3 adaptor protein that interacts with Wsp, regulates synaptic growth in *Drosophila*. *Neuron* **41**: 521–34.

Currie, D.A., Truman, J.W., and Burden, S.J. (1995) *Drosophila* glutamate receptor RNA expression in embryonic and larval muscle fibers. *Dev. Dyn.* **203**: 311–6.

Dani, N. and Broadie, K. (2012) Glycosylated synaptomatrix regulation of trans-synaptic signaling. *Dev. Neurobiol.* **72**: 2–21.

Dani, N., Nahm, M., Lee, S., and Broadie, K. (2012) A targeted glycan-related gene screen reveals heparan sulfate proteoglycan sulfation regulates WNT and BMP trans-synaptic signaling. *PLoS Genet.* **8**: e1003031.

Dani, N., Zhu, H., and Broadie, K. (2014) Two protein N-acetylgalactosaminyl transferases regulate synaptic plasticity by activity-dependent regulation of integrin signaling. *J. Neurosci.* **34**: 13047–65.

Daniels, R.W., Collins, C.A., Chen, K., Gelfand, M. V, Featherstone, D.E., and DiAntonio, A. (2006) A single vesicular glutamate transporter is sufficient to fill a synaptic vesicle. *Neuron* **49**: 11–6.

Darnell, J.C., Van Driesche, S.J., Zhang, C., Hung, K.Y.S., Mele, A., Fraser, C.E., Stone, E.F., Chen, C., Fak, J.J., Chi, S.W., Licatalosi, D.D., Richter, J.D., and Darnell, R.B. (2011) FMRP stalls ribosomal translocation on mRNAs linked to synaptic function and autism. *Cell* **146**: 247–61.

Davis, G.W. (2013) Homeostatic signaling and the stabilization of neural function. *Neuron* **80**: 718–28.

Davis, G.W., DiAntonio, A., Petersen, S.A., and Goodman, C.S. (1998) Postsynaptic PKA controls quantal size and reveals a retrograde signal that regulates presynaptic transmitter release in *Drosophila*. *Neuron* **20**: 305–15.

Davis, G.W. and Müller, M. (2014) Homeostatic Control of Presynaptic Neurotransmitter Release. *Annu. Rev. Physiol.*

DeLotto, Y. and DeLotto, R. (1998) Proteolytic processing of the *Drosophila* Spätzle protein by easter generates a dimeric NGF-like molecule with ventralising activity. *Mech. Dev.* **72**: 141–8.

DeMill, C.M., Qiu, X., Kisiel, M., Bolotta, A., and Stewart, B.A. (2014) Investigation of the juxtamembrane region of neuronal-Synaptobrevin in synaptic transmission at the *Drosophila* neuromuscular junction. *J. Neurophysiol.* **112**: 1356–66.

Desai, S.A. and Lnenicka, G.A. (2011) Characterization of postsynaptic Ca2+ signals at the *Drosophila* larval NMJ. *J. Neurophysiol.* **106**: 710–21.

DiAntonio, A., Haghighi, A.P., Portman, S.L., Lee, J.D., Amaranto, A.M., and Goodman, C.S. (2001) Ubiquitination-dependent mechanisms regulate synaptic growth and function. *Nature* **412**: 449–52.

DiAntonio, A., Petersen, S.A., Heckmann, M., and Goodman, C.S. (1999) Glutamate receptor expression regulates quantal size and quantal content at the *Drosophila* neuromuscular junction. *J. Neurosci.* **19**: 3023–32.

DiAntonio, A. and Schwarz, T.L. (1994) The effect on synaptic physiology of synaptotagmin mutations in *Drosophila*. *Neuron* **12**: 909–20.

Dickman, D.K. and Davis, G.W. (2009) The schizophrenia susceptibility gene dysbindin controls synaptic homeostasis. *Science* **326**: 1127–30.

Dickman, D.K., Horne, J.A., Meinertzhagen, I.A., and Schwarz, T.L. (2005) A slowed classical pathway rather than kiss-and-run mediates endocytosis at synapses lacking synaptojanin and endophilin. *Cell* **123**: 521–33.

Dickman, D.K., Lu, Z., Meinertzhagen, I.A., and Schwarz, T.L. (2006) Altered synaptic development and active zone spacing in endocytosis mutants. *Curr. Biol.* **16**: 591–8.

Dickman, D.K., Tong, A., and Davis, G.W. (2012) Snapin is critical for presynaptic homeostatic plasticity. *J. Neurosci.* **32**: 8716–24.

Dudu, V., Bittig, T., Entchev, E., Kicheva, A., Jülicher, F., and González-Gaitán, M. (2006) Postsynaptic mad signaling at the *Drosophila* neuromuscular junction. *Curr. Biol.* **16**: 625–35.

Eaton, B.A. and Davis, G.W. (2005) LIM Kinase1 controls synaptic stability downstream of the type II BMP receptor. *Neuron* **47**: 695–708.

Eaton, B.A., Fetter, R.D., and Davis, G.W. (2002) Dynactin is necessary for synapse stabilization. *Neuron* **34**: 729–41.

Ehmann, N., van de Linde, S., Alon, A., Ljaschenko, D., Keung, X.Z., Holm, T., Rings, A., DiAntonio, A., Hallermann, S., Ashery, U., Heckmann, M., Sauer, M., and Kittel, R.J. (2014) Quantitative super-resolution imaging of Bruchpilot distinguishes active zone states. *Nat. Commun.* **5.**:

Elias, G.M. and Nicoll, R.A. (2007) Synaptic trafficking of glutamate receptors by MAGUK scaffolding proteins. *Trends Cell Biol.* **17**: 343–52.

Enneking, E.-M., Kudumala, S.R., Moreno, E., Stephan, R., Boerner, J., Godenschwege, T.A., and Pielage, J. (2013) Transsynaptic coordination of synaptic growth, function, and stability by the L1-Type CAM Neuroglian. *PLoS Biol.* **11**: e1001537.

Fatt, P. and Katz, B. (1952) Spontaneous subthreshold activity at motor nerve endings. *J. Physiol.* **117**: 109–28.

Featherstone, D.E., Rushton, E., and Broadie, K. (2002) Developmental regulation of glutamate receptor field size by nonvesicular glutamate release. *Nat. Neurosci.* **5**: 141–6.

Featherstone, D.E., Rushton, E., Rohrbough, J., Liebl, F., Karr, J., Sheng, Q., Rodesch, C.K., and Broadie, K. (2005) An essential *Drosophila* glutamate receptor subunit that functions in both central neuropil and neuromuscular junction. *J. Neurosci.* **25**: 3199–208.

Feeney, C.J., Karunanithi, S., Pearce, J., Govind, C.K., and Atwood, H.L. (1998) Motor nerve terminals on abdominal muscles in larval flesh flies, *Sarcophaga bullata*: comparisons with *Drosophila*. *J. Comp. Neurol.* **402**: 197–209.

Fergestad, T., Davis, W.S., and Broadie, K. (1999) The stoned proteins regulate synaptic vesicle recycling in the presynaptic terminal. *J. Neurosci.* **19**: 5847–60.

Fernandes, A.C., Uytterhoeven, V., Kuenen, S., Wang, Y.-C., Slabbaert, J.R., Swerts, J., Kasprowicz, J., Aerts, S., and Verstreken, P. (2014) Reduced synaptic vesicle protein degradation at lysosomes curbs TBC1D24/sky-induced neurodegeneration. *J. Cell Biol.* **207**: 453–62.

De Ferrari, G. V and Moon, R.T. (2006) The ups and downs of Wnt signaling in prevalent neurological disorders. *Oncogene* **25**: 7545–53.

Fouquet, W., Owald, D., Wichmann, C., Mertel, S., Depner, H., Dyba, M., Hallermann, S., Kittel, R.J., Eimer, S., and Sigrist, S.J. (2009) Maturation of active zone assembly by *Drosophila* Bruchpilot. *J. Cell Biol.* **186**: 129–45.

Frank, C.A. (2014) Homeostatic plasticity at the *Drosophila* neuromuscular junction. *Neuropharmacology* **78**: 63–74.

Frank, C.A., Kennedy, M.J., Goold, C.P., Marek, K.W., and Davis, G.W. (2006) Mechanisms underlying the rapid induction and sustained expression of synaptic homeostasis. *Neuron* **52**: 663–77.

Frank, C.A., Pielage, J., and Davis, G.W. (2009) A presynaptic homeostatic signaling system composed of the Eph receptor, ephexin, Cdc42, and CaV2.1 calcium channels. *Neuron* **61**: 556–69.

Friedman, S.H., Dani, N., Rushton, E., and Broadie, K. (2013) Fragile X mental retardation protein regulates trans-synaptic signaling in *Drosophila*. *Dis. Model. Mech.* **6**: 1400–13.

Fuentes-Medel, Y., Ashley, J., Barria, R., Maloney, R., Freeman, M., and Budnik, V. (2012) Integration of a retrograde signal during synapse formation by glia-secreted TGF-β ligand. *Curr. Biol.* **22**: 1831–8.

Fuentes-Medel, Y., Logan, M.A., Ashley, J., Ataman, B., Budnik, V., and Freeman, M.R. (2009) Glia and muscle sculpt neuromuscular arbors by engulfing destabilized synaptic boutons and shed presynaptic debris. *PLoS Biol.* **7**: e1000184.

Gardiol, A. and St Johnston, D. (2014) Staufen targets coracle mRNA to *Drosophila* neuromuscular junctions and regulates GluRIIA synaptic accumulation and bouton number. *Dev. Biol.* **392**: 153–67.

Gatto, C.L. and Broadie, K. (2011) *Drosophila* modeling of heritable neurodevelopmental disorders. *Curr. Opin. Neurobiol.* **21**: 834–41.

Gaviño, M.A., Ford, K.J., Archila, S., and Davis, G.W. (2015) Homeostatic synaptic depression is achieved through a regulated decrease in presynaptic calcium channel abundance. *Elife* **4.**:

Ghiani, C.A., Starcevic, M., Rodriguez-Fernandez, I.A., Nazarian, R., Cheli, V.T., Chan, L.N., Malvar, J.S., de Vellis, J., Sabatti, C., and Dell'Angelica, E.C. (2010) The dysbindin-containing complex (BLOC-1) in brain: developmental regulation, interaction with SNARE proteins and role in neurite outgrowth. *Mol. Psychiatry* **15**: 115, 204–15.

Ghosh, R., Vegesna, S., Safi, R., Bao, H., Zhang, B., Marenda, D.R., and Liebl, F.L.W. (2014) Kismet positively regulates glutamate receptor localization and synaptic transmission at the *Drosophila* neuromuscular junction. *PLoS One* **9**: e113494.

Giraudo, C.G., Eng, W.S., Melia, T.J., and Rothman, J.E. (2006) A clamping mechanism involved in SNARE-dependent exocytosis. *Science* **313**: 676–80.

Gokhale, A., Mullin, A.P., Zlatic, S.A., Easley, C.A., Merritt, M.E., Raj, N., Larimore, J., Gordon, D.E., Peden, A.A., Sanyal, S., and Faundez, V. (2015) The N-ethylmaleimide-sensitive factor and dysbindin interact to modulate synaptic plasticity. *J. Neurosci.* **35**: 7643–53.

González-Gaitán, M. and Jäckle, H. (1997) Role of *Drosophila* alpha-adaptin in presynaptic vesicle recycling. *Cell* **88**: 767–76.

Goodman, R.M., Thombre, S., Firtina, Z., Gray, D., Betts, D., Roebuck, J., Spana, E.P., and Selva, E.M. (2006) Sprinter: a novel transmembrane protein required for Wg secretion and signaling. *Development* **133**: 4901–11.

Goold, C.P. and Davis, G.W. (2007) The BMP ligand Gbb gates the expression of synaptic homeostasis independent of synaptic growth control. *Neuron* **56**: 109–23.

Gorczyca, D., Ashley, J., Speese, S., Gherbesi, N., Thomas, U., Gundelfinger, E., Gramates, L.S., and Budnik, V. (2007) Postsynaptic membrane addition depends on the Discs-Large-interacting t-SNARE Gtaxin. *J. Neurosci.* **27**: 1033–44.

Graf, E.R., Daniels, R.W., Burgess, R.W., Schwarz, T.L., and DiAntonio, A. (2009) Rab3 dynamically controls protein composition at active zones. *Neuron* **64**: 663–77.

Graf, E.R., Heerssen, H.M., Wright, C.M., Davis, G.W., and Diantonio, A. (2011) Stathmin is Required for Stability of the *Drosophila* Neuromuscular Junction. *J. Neurosci.* **31**: 15026–34.

Graf, E.R., Valakh, V., Wright, C.M., Wu, C., Liu, Z., Zhang, Y.Q., and DiAntonio, A. (2012) RIM promotes calcium channel accumulation at active zones of the *Drosophila* neuromuscular junction. *J. Neurosci.* **32**: 16586–96.

Grant, S.G.N. (2012) Synaptopathies: diseases of the synaptome. *Curr. Opin. Neurobiol.* **22**: 522–9.

Guan, Z., Saraswati, S., Adolfsen, B., and Littleton, J.T. (2005) Genome-wide transcriptional changes associated with enhanced activity in the *Drosophila* nervous system. *Neuron* **48**: 91–107.

Guichet, A., Wucherpfennig, T., Dudu, V., Etter, S., Wilsch-Bräuniger, M., Hellwig, A., González-Gaitán, M., Huttner, W.B., and Schmidt, A.A. (2002) Essential role of endophilin A in synaptic vesicle budding at the *Drosophila* neuromuscular junction. *EMBO J.* **21**: 1661–72.

Guo, X., Macleod, G.T., Wellington, A., Hu, F., Panchumarthi, S., Schoenfield, M., Marin, L., Charlton, M.P., Atwood, H.L., and Zinsmaier, K.E. (2005) The GTPase dMiro is required for axonal transport of mitochondria to *Drosophila* synapses. *Neuron* **47**: 379–93.

Hallermann, S., Kittel, R.J., Wichmann, C., Weyhersmüller, A., Fouquet, W., Mertel, S., Owald, D., Eimer, S., Depner, H., Schwärzel, M., Sigrist, S.J., and Heckmann, M. (2010) Naked dense bodies provoke depression. *J. Neurosci.* **30**: 14340–5.

Halstead, J.M., Lin, Y.Q., Durraine, L., Hamilton, R.S., Ball, G., Neely, G.G., Bellen, H.J., and Davis, I. (2014) Syncrip/hnRNP Q influences synaptic transmission and regulates BMP signaling at the *Drosophila* neuromuscular synapse. *Biol. Open* **3**: 839–49.

Hamanaka, Y., Park, D., Yin, P., Annangudi, S.P., Edwards, T.N., Sweedler, J., Meinertzhagen, I.A., and Taghert, P.H. (2010) Transcriptional orchestration of the regulated secretory pathway in neurons by the bHLH protein DIMM. *Curr. Biol.* **20**: 9–18.

Harrison, S.D., Broadie, K., van de Goor, J., and Rubin, G.M. (1994) Mutations in the *Drosophila* Rop gene suggest a function in general secretion and synaptic transmission. *Neuron* **13**: 555–66.

Haucke, V., Neher, E., and Sigrist, S.J. (2011) Protein scaffolds in the coupling of synaptic exocytosis and endocytosis. *Nat. Rev. Neurosci.* **12**: 127–38.

Heckscher, E.S., Fetter, R.D., Marek, K.W., Albin, S.D., and Davis, G.W. (2007) NF-kappaB, IkappaB, and IRAK control glutamate receptor density at the *Drosophila* NMJ. *Neuron* **55**: 859–73.

Heerssen, H., Fetter, R.D., and Davis, G.W. (2008) Clathrin dependence of synaptic-vesicle formation at the *Drosophila* neuromuscular junction. *Curr. Biol.* **18**: 401–9.

Hewes, R.S., Gu, T., Brewster, J.A., Qu, C., and Zhao, T. (2006) Regulation of secretory protein expression in mature cells by DIMM, a basic helix-loop-helix neuroendocrine differentiation factor. *J. Neurosci.* **26**: 7860–9.

Hida, Y. and Ohtsuka, T. (2010) CAST and ELKS proteins: structural and functional determinants of the presynaptic active zone. *J. Biochem.* **148**: 131–7.

Hoang, B. and Chiba, A. (2001) Single-cell analysis of *Drosophila* larval neuromuscular synapses. *Dev. Biol.* **229**: 55–70.

Huntwork, S. and Littleton, J.T. (2007) A complexin fusion clamp regulates spontaneous neurotransmitter release and synaptic growth. *Nat. Neurosci.* **10**: 1235–7.

Ikeda, K., Ozawa, S., and Hagiwara, S. (1976) Synaptic transmission reversibly conditioned by single-gene mutation in *Drosophila melanogaster*. *Nature* **259**: 489–91.

Iyer, J., Wahlmark, C.J., Kuser-Ahnert, G.A., and Kawasaki, F. (2013) Molecular mechanisms of COMPLEXIN fusion clamp function in synaptic exocytosis revealed in a new *Drosophila* mutant. *Mol. Cell. Neurosci.* **56**: 244–54.

Jackson, G.R., Salecker, I., Dong, X., Yao, X., Arnheim, N., Faber, P.W., MacDonald, M.E., and Zipursky, S.L. (1998) Polyglutamine-expanded human huntingtin transgenes induce degeneration of *Drosophila* photoreceptor neurons. *Neuron* **21**: 633–42.

James, R.E. and Broihier, H.T. (2011) Crimpy inhibits the BMP homolog Gbb in motoneurons to enable proper growth control at the *Drosophila* neuromuscular junction. *Development* **138**: 3273–3286.

James, R.E., Hoover, K.M., Bulgari, D., McLaughlin, C.N., Wilson, C.G., Wharton, K.A., Levitan, E.S., and Broihier, H.T. (2014) Crimpy enables discrimination of presynaptic and postsynaptic pools of a BMP at the *Drosophila* neuromuscular nunction. *Dev. Cell* **31**: 586–98.

Jan, L.Y. and Jan, Y.N. (1976) Properties of the larval neuromuscular junction in *Drosophila melanogaster*. *J. Physiol.* **262**: 189–214.

Jepson, J.E.C., Shahidullah, M., Liu, D., le Marchand, S.J., Liu, S., Wu, M.N., Levitan, I.B., Dalva, M.B., and Koh, K. (2014) Regulation of synaptic development and function by the *Drosophila* PDZ protein Dyschronic. *Development* **141**: 4548–57.

Jiao, W., Masich, S., Franzén, O., and Shupliakov, O. (2010) Two pools of vesicles associated with the presynaptic cytosolic projection in *Drosophila* neuromuscular junctions. *J. Struct. Biol.* **172**: 389–94.

Johansen, J., Halpern, M.E., Johansen, K.M., and Keshishian, H. (1989) Stereotypic morphology of glutamatergic synapses on identified muscle cells of *Drosophila* larvae. *J. Neurosci.* **9**: 710–725.

Johnson, K.G., Tenney, A.P., Ghose, A., Duckworth, A.M., Higashi, M.E., Parfitt, K., Marcu, O., Heslip, T.R., Marsh, J.L., Schwarz, T.L., Flanagan, J.G., and Van Vactor, D. (2006) The HSPGs Syndecan and Dallylike bind the receptor phosphatase LAR and exert distinct effects on synaptic development. *Neuron* **49**: 517–31.

Jorquera, R.A., Huntwork-Rodriguez, S., Akbergenova, Y., Cho, R.W., and Littleton, J.T. (2012) Complexin controls spontaneous and evoked neurotransmitter release by regulating the timing and properties of synaptotagmin activity. *J. Neurosci.* **32**: 18234–45.

Kaeser, P.S. and Regehr, W.G. (2014) Molecular mechanisms for synchronous, asynchronous, and spontaneous neurotransmitter release. *Annu. Rev. Physiol.* **76**: 333–63.

Kamimura, K., Ueno, K., Nakagawa, J., Hamada, R., Saitoe, M., and Maeda, N. (2013) Perlecan regulates bidirectional Wnt signaling at the *Drosophila* neuromuscular junction. *J. Cell Biol.* **200**: 219–33.

Kaplan, W.D. and Trout, W.E. (1969) The behavior of four neurological mutants of *Drosophila*. *Genetics* **61**: 399–409.

Karunanithi, S., Marin, L., Wong, K., and Atwood, H.L. (2002) Quantal size and variation determined by vesicle size in normal and mutant *Drosophila* glutamatergic synapses. *J. Neurosci.* **22**: 10267–76.

Kasprowicz, J., Kuenen, S., Swerts, J., Miskiewicz, K., and Verstreken, P. (2014) Dynamin photoinactivation blocks Clathrin and α-adaptin recruitment and induces bulk membrane retrieval. *J. Cell Biol.* **204**: 1141–56.

Katz, B. and Miledi, R. (1969) Spontaneous and evoked activity of motor nerve endings in calcium Ringer. *J. Physiol.* **203**: 689–706.

Katz, B. and Miledi, R. (1967) The timing of calcium action during neuromuscular transmission. *J. Physiol.* **189**: 535–44.

Kaufmann, N., DeProto, J., Ranjan, R., Wan, H., and Van Vactor, D. (2002) *Drosophila* liprin-alpha and the receptor phosphatase Dlar control synapse morphogenesis. *Neuron* **34**: 27–38.

Kavalali, E.T. (2015) The mechanisms and functions of spontaneous neurotransmitter release. *Nat. Rev. Neurosci.* **16**: 5–16.

Kawasaki, F., Felling, R., and Ordway, R.W. (2000) A temperature-sensitive paralytic mutant defines a primary synaptic calcium channel in *Drosophila*. *J. Neurosci.* **20**: 4885–9.

Kawasaki, F. and Ordway, R.W. (2009) Molecular mechanisms determining conserved properties of short-term synaptic depression revealed in NSF and SNAP-25 conditional mutants. *Proc. Natl. Acad. Sci. U. S. A.* **106**: 14658–63.

Kawasaki, F. and Ordway, R.W. (1999) The *Drosophila* NSF protein, dNSF1, plays a similar role at neuromuscular and some central synapses. *J. Neurophysiol.* **82**: 123–30.

Kawasaki, F., Zou, B., Xu, X., and Ordway, R.W. (2004) Active zone localization of presynaptic calcium channels encoded by the cacophony locus of *Drosophila*. *J. Neurosci.* **24**: 282–5.

Kerr, K.S., Fuentes-Medel, Y., Brewer, C., Barria, R., Ashley, J., Abruzzi, K.C., Sheehan, A., Tasdemir-Yilmaz, O.E., Freeman, M.R., and Budnik, V. (2014) Glial wingless/Wnt regulates glutamate receptor clustering and synaptic physiology at the *Drosophila* neuromuscular junction. *J. Neurosci.* **34**: 2910–20.

Khodosh, R., Augsburger, A., Schwarz, T.L., and Garrity, P.A. (2006) Bchs, a BEACH domain protein, antagonizes Rab11 in synapse morphogenesis and other developmental events. *Development* **133**: 4655–65.

Kidokoro, Y. (2006) Vesicle trafficking and recycling at the neuromuscular junction: two pathways for endocytosis. *Int. Rev. Neurobiol.* **75**: 145–164.

Kim, Y.-J., Bao, H., Bonanno, L., Zhang, B., and Serpe, M. (2012) *Drosophila* Neto is essential for clustering glutamate receptors at the neuromuscular junction. *Genes Dev.* **26**: 974–87.

Kim, Y.-J. and Serpe, M. (2013) Building a synapse: A complex matter. *Fly (Austin).* **7**: 146–52.

Kittel, R.J., Wichmann, C., Rasse, T.M., Fouquet, W., Schmidt, M., Schmid, A., Wagh, D.A., Pawlu, C., Kellner, R.R., Willig, K.I., Hell, S.W., Buchner, E., Heckmann, M., and Sigrist, S.J. (2006) Bruchpilot promotes active zone assembly, Ca2+ channel clustering, and vesicle release. *Science* **312**: 1051–4.

Koch, I., Schwarz, H., Beuchle, D., Goellner, B., Langegger, M., and Aberle, H. (2008) *Drosophila* ankyrin 2 is required for synaptic stability. *Neuron* **58**: 210–22.

Koenig, J.H. and Ikeda, K. (1989) Disappearance and reformation of synaptic vesicle membrane upon transmitter release observed under reversible blockage of membrane retrieval. *J. Neurosci.* **9**: 3844–60.

Koh, T.-W., Korolchuk, V.I., Wairkar, Y.P., Jiao, W., Evergren, E., Pan, H., Zhou, Y., Venken, K.J.T., Shupliakov, O., Robinson, I.M., O'Kane, C.J., and Bellen, H.J. (2007) Eps15 and Dap160 control synaptic vesicle membrane retrieval and synapse development. *J. Cell Biol.* **178**: 309–22.

Koh, T.-W., Verstreken, P., and Bellen, H.J. (2004) Dap160/intersectin acts as a stabilizing scaffold required for synaptic development and vesicle endocytosis. *Neuron* **43**: 193–205.

Kohsaka, H., Takasu, E., and Nose, A. (2007) *In vivo* induction of postsynaptic molecular assembly by the cell adhesion molecule Fasciclin2. *J. Cell Biol.* **179**: 1289–300.

Koles, K. and Budnik, V. (2012a) Exosomes go with the Wnt. *Cell. Logist.* **2**: 169–173.

Koles, K. and Budnik, V. (2012b) Wnt signaling in neuromuscular junction development. *Cold Spring Harb. Perspect. Biol.* **4.**:

Koles, K., Nunnari, J., Korkut, C., Barria, R., Brewer, C., Li, Y., Leszyk, J., Zhang, B., and Budnik, V. (2012) Mechanism of Evi-exosome release at synaptic boutons. *J. Biol. Chem.*

Korkut, C., Ataman, B., Ramachandran, P., Ashley, J., Barria, R., Gherbesi, N., and Budnik, V. (2009) Trans-synaptic transmission of vesicular Wnt signals through Evi/Wntless. *Cell* **139**: 393–404.

Korkut, C., Li, Y., Koles, K., Brewer, C., Ashley, J., Yoshihara, M., and Budnik, V. (2013) Regulation of postsynaptic retrograde signaling by presynaptic exosome release. *Neuron* **77**: 1039–46.

Kremer, M.C., Christiansen, F., Leiss, F., Paehler, M., Knapek, S., Andlauer, T.F.M., Förstner, F., Kloppenburg, P., Sigrist, S.J., and Tavosanis, G. (2010) Structural long-term changes at mushroom body input synapses. *Curr. Biol.* **20**: 1938–44.

Krench, M. and Littleton, J.T. Modeling Huntington disease in *Drosophila*: Insights into axonal transport defects and modifiers of toxicity. *Fly (Austin).* **7**: 229–36.

Kristiansen, L. V and Hortsch, M. (2010) Fasciclin II: the NCAM ortholog in *Drosophila melanogaster*. *Adv. Exp. Med. Biol.* **663**: 387–401.

Kurdyak, P., Atwood, H.L., Stewart, B.A., and Wu, C.F. (1994) Differential physiology and morphology of motor axons to ventral longitudinal muscles in larval *Drosophila*. *J. Comp. Neurol.* **350**: 463–72.

Laprise, P. and Tepass, U. (2011) Novel insights into epithelial polarity proteins in *Drosophila*. *Trends Cell Biol.* **21**: 401–8.

Lee, H.-G., Zhao, N., Campion, B.K., Nguyen, M.M., and Selleck, S.B. (2013) Akt regulates glutamate receptor trafficking and postsynaptic membrane elaboration at the *Drosophila* neuromuscular junction. *Dev. Neurobiol.* **73**: 723–43.

Lee, J., Guan, Z., Akbergenova, Y., and Littleton, J.T. (2013) Genetic analysis of synaptotagmin C2 domain specificity in regulating spontaneous and evoked neurotransmitter release. *J. Neurosci.* **33**: 187–200.

Lee, J. and Littleton, J.T. (2015) Transmembrane tethering of Synaptotagmin to synaptic vesicles controls multiple modes of neurotransmitter release. *Proc. Natl. Acad. Sci. U. S. A.* **In Press**: 3793–8.

Lee, J. and Wu, C.-F. (2010) Orchestration of stepwise synaptic growth by k+ and Ca2+ channels in *Drosophila*. *J. Neurosci.* **30**: 15821–33.

Leiss, F., Groh, C., Butcher, N.J., Meinertzhagen, I.A., and Tavosanis, G. (2009) Synaptic organization in the adult *Drosophila* mushroom body calyx. *J. Comp. Neurol.* **517**: 808–24.

Li, J., Ashley, J., Budnik, V., and Bhat, M.A. (2007) Crucial role of *Drosophila* neurexin in proper active zone apposition to postsynaptic densities, synaptic growth, and synaptic transmission. *Neuron* **55**: 741–55.

Littleton, J.T. (2000) A genomic analysis of membrane trafficking and neurotransmitter release in *Drosophila*. *J. Cell Biol.* **150**: F77–82.

Littleton, J.T., Bai, J., Vyas, B., Desai, R., Baltus, A.E., Garment, M.B., Carlson, S.D., Ganetzky, B., and Chapman, E.R. (2001) synaptotagmin mutants reveal essential functions for the C2B domain in Ca2+-triggered fusion and recycling of synaptic vesicles *in vivo*. *J. Neurosci.* **21**: 1421–1433.

Littleton, J.T., Barnard, R.J., Titus, S.A., Slind, J., Chapman, E.R., and Ganetzky, B. (2001) SNARE-complex disassembly by NSF follows synaptic-vesicle fusion. *Proc. Natl. Acad. Sci. U. S. A.* **98**: 12233–8.

Littleton, J.T., Chapman, E.R., Kreber, R., Garment, M.B., Carlson, S.D., and Ganetzky, B. (1998) Temperature-sensitive paralytic mutations demonstrate that synaptic exocytosis requires SNARE complex assembly and disassembly. *Neuron* **21**: 401–13.

Littleton, J.T. and Ganetzky, B. (2000) Ion channels and synaptic organization: analysis of the *Drosophila* genome. *Neuron* **26**: 35–43.

Littleton, J.T., Stern, M., Perin, M., and Bellen, H.J. (1994) Calcium dependence of neurotransmitter release and rate of spontaneous vesicle fusions are altered in *Drosophila* synaptotagmin mutants. *Proc. Natl. Acad. Sci. U. S. A.* **91**: 10888–92.

Littleton, J.T., Stern, M., Schulze, K., Perin, M., and Bellen, H.J. (1993) Mutational analysis of *Drosophila* synaptotagmin demonstrates its essential role in Ca(2+)-activated neurotransmitter release. *Cell* **74**: 1125–34.

Liu, K.S.Y., Siebert, M., Mertel, S., Knoche, E., Wegener, S., Wichmann, C., Matkovic, T., Muhammad, K., Depner, H., Mettke, C., Bückers, J., Hell, S.W., Müller, M., Davis, G.W., Schmitz, D., and Sigrist, S.J. (2011) RIM-binding protein, a central part of the active zone, is essential for neurotransmitter release. *Science* **334**: 1565–9.

Loya, C.M., Lu, C.S., Van Vactor, D., and Fulga, T.A. (2009) Transgenic microRNA inhibition with spatiotemporal specificity in intact organisms. *Nat. Methods* **6**: 897–903.

Loya, C.M., McNeill, E.M., Bao, H., Zhang, B., and Van Vactor, D. (2014) miR-8 controls synapse structure by repression of the actin regulator enabled. *Development* **141**: 1864–74.

Lüchtenborg, A.-M., Solis, G.P., Egger-Adam, D., Koval, A., Lin, C., Blanchard, M.G., Kellenberger, S., and Katanaev, V.L. (2014) Heterotrimeric Go protein links Wnt-Frizzled signaling with ankyrins to regulate the neuronal microtubule cytoskeleton. *Development* **141**: 3399–409.

Mackler, J.M., Drummond, J.A., Loewen, C.A., Robinson, I.M., and Reist, N.E. (2002) The C(2)B Ca(2+)-binding motif of synaptotagmin is required for synaptic transmission *in vivo*. *Nature* **418**: 340–4.

Marie, B., Pym, E., Bergquist, S., and Davis, G.W. (2010) Synaptic homeostasis is consolidated by the cell fate gene gooseberry, a *Drosophila* pax3/7 homolog. *J. Neurosci.* **30**: 8071–82.

Marie, B., Sweeney, S.T., Poskanzer, K.E., Roos, J., Kelly, R.B., and Davis, G.W. (2004) Dap160/intersectin scaffolds the periactive zone to achieve high-fidelity endocytosis and normal synaptic growth. *Neuron* **43**: 207–19.

Marqués, G., Bao, H., Haerry, T.E., Shimell, M.J., Duchek, P., Zhang, B., and O'Connor, M.B. (2002) The *Drosophila* BMP type II receptor Wishful Thinking regulates neuromuscular synapse morphology and function. *Neuron* **33**: 529–43.

Marrus, S.B., Portman, S.L., Allen, M.J., Moffat, K.G., and DiAntonio, A. (2004) Differential localization of glutamate receptor subunits at the *Drosophila* neuromuscular junction. *J. Neurosci.* **24**: 1406–15.

Martina, J.A., Bonangelino, C.J., Aguilar, R.C., and Bonifacino, J.S. (2001) Stonin 2: an adaptor-like protein that interacts with components of the endocytic machinery. *J. Cell Biol.* **153**: 1111–20.

Mathew, D., Ataman, B., Chen, J., Zhang, Y., Cumberledge, S., and Budnik, V. (2005) Wingless signaling at synapses is through cleavage and nuclear import of receptor DFrizzled2. *Science* **310**: 1344–7.

Matkovic, T., Siebert, M., Knoche, E., Depner, H., Mertel, S., Owald, D., Schmidt, M., Thomas, U., Sickmann, A., Kamin, D., Hell, S.W., Bürger, J., Hollmann, C., Mielke, T., Wichmann, C., and Sigrist, S.J. (2013) The Bruchpilot cytomatrix determines the size of the readily releasable pool of synaptic vesicles. *J. Cell Biol.* **202**: 667–83.

McCabe, B.D., Hom, S., Aberle, H., Fetter, R.D., Marques, G., Haerry, T.E., Wan, H., O'Connor, M.B., Goodman, C.S., and Haghighi, A.P. (2004) Highwire regulates presynaptic BMP signaling essential for synaptic growth. *Neuron* **41**: 891–905.

McCabe, B.D., Marqués, G., Haghighi, A.P., Fetter, R.D., Crotty, M.L., Haerry, T.E., Goodman, C.S., and O'Connor, M.B. (2003) The BMP homolog Gbb provides a retrograde signal that regulates synaptic growth at the *Drosophila* neuromuscular junction. *Neuron* **39**: 241–54.

McDermott, S.M., Yang, L., Halstead, J.M., Hamilton, R.S., Meignin, C., and Davis, I. (2014) *Drosophila* Syncrip modulates the expression of mRNAs encoding key synaptic proteins required for morphology at the neuromuscular junction. *RNA* **20**: 1593–606.

McIlroy, G., Foldi, I., Aurikko, J., Wentzell, J.S., Lim, M.A., Fenton, J.C., Gay, N.J., and Hidalgo, A. (2013) Toll-6 and Toll-7 function as neurotrophin receptors in the *Drosophila melanogaster* CNS. *Nat. Neurosci.* **16**: 1248–56.

McMahon, H.T., Missler, M., Li, C., and Südhof, T.C. (1995) Complexins: cytosolic proteins that regulate SNAP receptor function. *Cell* **83**: 111–9.

McNeill, E. and Van Vactor, D. (2012) MicroRNAs shape the neuronal landscape. *Neuron* **75**: 363–79.

Megighian, A., Zordan, M., Pantano, S., Scorzeto, M., Rigoni, M., Zanini, D., Rossetto, O., and Montecucco, C. (2013) Evidence for a radial SNARE super-complex mediating neurotransmitter release at the *Drosophila* neuromuscular junction. *J. Cell Sci.*

Melom, J.E., Akbergenova, Y., Gavornik, J.P., and Littleton, J.T. (2013) Spontaneous and evoked release are independently regulated at individual active zones. *J. Neurosci.* **33**: 17253–63.

Mendoza, C., Olguin, P., Lafferte, G., Thomas, U., Ebitsch, S., Gundelfinger, E.D., Kukuljan, M., and Sierralta, J. (2003) Novel isoforms of Dlg are fundamental for neuronal development in *Drosophila*. *J. Neurosci.* **23**: 2093–2101.

Mendoza-Topaz, C., Urra, F., Barría, R., Albornoz, V., Ugalde, D., Thomas, U., Gundelfinger, E.D., Delgado, R., Kukuljan, M., Sanxaridis, P.D., Tsunoda, S., Ceriani, M.F., Budnik, V., and Sierralta, J. (2008) DLGS97/SAP97 is developmentally upregulated and is required for complex adult behaviors and synapse morphology and function. *J. Neurosci.* **28**: 304–14.

Menon, K.P., Andrews, S., Murthy, M., Gavis, E.R., and Zinn, K. (2009) The translational repressors Nanos and Pumilio have divergent effects on presynaptic terminal growth and postsynaptic glutamate receptor subunit composition. *J. Neurosci.* **29**: 5558–72.

Menon, K.P., Sanyal, S., Habara, Y., Sanchez, R., Wharton, R.P., Ramaswami, M., and Zinn, K. (2004) The translational repressor Pumilio regulates presynaptic morphology and controls postsynaptic accumulation of translation factor eIF-4E. *Neuron* **44**: 663–76.

Miech, C., Pauer, H.-U., He, X., and Schwarz, T.L. (2008) Presynaptic local signaling by a canonical wingless pathway regulates development of the *Drosophila* neuromuscular junction. *J. Neurosci.* **28**: 10875–84.

Mikels, A.J. and Nusse, R. (2006) Wnts as ligands: processing, secretion and reception. *Oncogene* **25**: 7461–8.

Mitchell, K.J. (2011) The genetics of neurodevelopmental disease. *Curr. Opin. Neurobiol.* **21**: 197–203.

Mizuguchi, K., Parker, J.S., Blundell, T.L., and Gay, N.J. (1998) Getting knotted: a model for the structure and activation of Spätzle. *Trends Biochem. Sci.* **23**: 239–42.

Morel, V., Lepicard, S., Rey, A.N., Parmentier, M.-L., and Schaeffer, L. (2014) *Drosophila* Nesprin-1 controls glutamate receptor density at neuromuscular junctions. *Cell. Mol. Life Sci.* **71**: 3363–79.

Mosca, T.J., Hong, W., Dani, V.S., Favaloro, V., and Luo, L. (2012) Trans-synaptic Teneurin signalling in neuromuscular synapse organization and target choice. *Nature* **484**: 237–41.

Mosca, T.J. and Schwarz, T.L. (2010) The nuclear import of Frizzled2-C by Importins-beta11 and alpha2 promotes postsynaptic development. *Nat. Neurosci.* **13**: 935–43.

Müller, M. and Davis, G.W. (2012) Transsynaptic control of presynaptic Ca2+ influx achieves homeostatic potentiation of neurotransmitter release. *Curr. Biol.*

Müller, M., Liu, K.S.Y., Sigrist, S.J., and Davis, G.W. (2012) RIM controls homeostatic plasticity through modulation of the readily-releasable vesicle pool. *J. Neurosci.* **32**: 16574–85.

Müller, M., Pym, E.C.G., Tong, A., and Davis, G.W. (2011) Rab3-GAP controls the progression of synaptic homeostasis at a late stage of vesicle release. *Neuron* **69**: 749–62.

Mullin, A.P., Gokhale, A., Larimore, J., and Faundez, V. (2011) Cell biology of the BLOC-1 complex subunit dysbindin, a schizophrenia susceptibility gene. *Mol. Neurobiol.* **44**: 53–64.

Mullin, A.P., Sadanandappa, M.K., Ma, W., Dickman, D.K., VijayRaghavan, K., Ramaswami, M., Sanyal, S., and Faundez, V. (2015) Gene dosage in the dysbindin schizophrenia susceptibility network differentially affect synaptic function and plasticity. *J. Neurosci.* **35**: 325–38.

Nakata, K., Abrams, B., Grill, B., Goncharov, A., Huang, X., Chisholm, A.D., and Jin, Y. (2005) Regulation of a DLK-1 and p38 MAP kinase pathway by the ubiquitin ligase RPM-1 is required for presynaptic development. *Cell* **120**: 407–20.

Nakayama, M., Matsushita, F., and Hama, C. (2014) The matrix protein Hikaru genki localizes to cholinergic synaptic clefts and regulates postsynaptic organization in the *Drosophila* brain. *J. Neurosci.* **34**: 13872–13877.

Nesler, K.R., Sand, R.I., Symmes, B.A., Pradhan, S.J., Boin, N.G., Laun, A.E., and Barbee, S.A. (2013) The miRNA pathway controls rapid changes in activity-dependent synaptic structure at the *Drosophila melanogaster* neuromuscular junction. *PLoS One* **8**: e68385.

Nishikawa, K. and Kidokoro, Y. (1995) Junctional and extrajunctional glutamate receptor channels in *Drosophila* embryos and larvae. *J. Neurosci.* **15**: 7905–15.

O'Connor-Giles, K.M., Ho, L.L., and Ganetzky, B. (2008) Nervous wreck interacts with thickveins and the endocytic machinery to attenuate retrograde BMP signaling during synaptic growth. *Neuron* **58**: 507–18.

Owald, D., Fouquet, W., Schmidt, M., Wichmann, C., Mertel, S., Depner, H., Christiansen, F., Zube, C., Quentin, C., Körner, J., Urlaub, H., Mechtler, K., and Sigrist, S.J. (2010) A Syd-1 homologue regulates pre- and postsynaptic maturation in *Drosophila*. *J. Cell Biol.* **188**: 565–79.

Owald, D., Khorramshahi, O., Gupta, V.K., Banovic, D., Depner, H., Fouquet, W., Wichmann, C., Mertel, S., Eimer, S., Reynolds, E., Holt, M., Aberle, H., and Sigrist, S.J. (2012) Cooperation of Syd-1 with Neurexin synchronizes pre- with postsynaptic assembly. *Nat. Neurosci.* **advance on**: 1219–26.

Packard, M., Koo, E.S., Gorczyca, M., Sharpe, J., Cumberledge, S., and Budnik, V. (2002) The *Drosophila* Wnt, wingless, provides an essential signal for pre- and postsynaptic differentiation. *Cell* **111**: 319–30.

Packard, M., Mathew, D., and Budnik, V. (2003) FASt remodeling of synapses in *Drosophila*. *Curr. Opin. Neurobiol.* **13**: 527–34.

Pallanck, L., Ordway, R.W., and Ganetzky, B. (1995) A *Drosophila* NSF mutant. *Nature* **376**: 25.

Pan, L. and Broadie, K.S. (2007) *Drosophila* fragile X mental retardation protein and metabotropic glutamate receptor A convergently regulate the synaptic ratio of ionotropic glutamate receptor subclasses. *J. Neurosci.* **27**: 12378–89.

Park, D., Hadžić, T., Yin, P., Rusch, J., Abruzzi, K., Rosbash, M., Skeath, J.B., Panda, S., Sweedler, J. V, and Taghert, P.H. (2011) Molecular organization of *Drosophila* neuroendocrine cells by Dimmed. *Curr. Biol.* **21**: 1515–24.

Park, D., Li, P., Dani, A., and Taghert, P.H. (2014) Peptidergic cell-specific synaptotagmins in *Drosophila*: localization to dense-core granules and regulation by the bHLH protein DIMMED. *J. Neurosci.* **34**: 13195–207.

Peled, E.S. and Isacoff, E.Y. (2011) Optical quantal analysis of synaptic transmission in wild-type and rab3-mutant *Drosophila* motor axons. *Nat. Neurosci.* **14**: 519–26.

Peled, E.S., Newman, Z.L., and Isacoff, E.Y. (2014) Evoked and spontaneous transmission favored by distinct sets of synapses. *Curr. Biol.* **24**: 484–493.

Penney, J., Tsurudome, K., Liao, E.H., Elazzouzi, F., Livingstone, M., Gonzalez, M., Sonenberg, N., and Haghighi, A.P. (2012) TOR Is required for the retrograde regulation of synaptic homeostasis at the *Drosophila* neuromuscular junction. *Neuron* **74**: 166–178.

Petersen, S.A., Fetter, R.D., Noordermeer, J.N., Goodman, C.S., and DiAntonio, A. (1997) Genetic analysis of glutamate receptors in *Drosophila* reveals a retrograde signal regulating presynaptic transmitter release. *Neuron* **19**: 1237–1248.

Piccioli, Z.D. and Littleton, J.T. (2014) Retrograde BMP signaling modulates rapid activity-dependent synaptic growth via presynaptic LIM kinase regulation of cofilin. *J. Neurosci.* **34**: 4371–81.

Pielage, J., Bulat, V., Zuchero, J.B., Fetter, R.D., and Davis, G.W. (2011) Hts/Adducin controls synaptic elaboration and elimination. *Neuron* **69**: 1114–31.

Pielage, J., Cheng, L., Fetter, R.D., Carlton, P.M., Sedat, J.W., and Davis, G.W. (2008) A presynaptic giant ankyrin stabilizes the NMJ through regulation of presynaptic microtubules and transsynaptic cell adhesion. *Neuron* **58**: 195–209.

Pielage, J., Fetter, R.D., and Davis, G.W. (2006) A postsynaptic spectrin scaffold defines active zone size, spacing, and efficacy at the *Drosophila* neuromuscular junction. *J. Cell Biol.* **175**: 491–503.

Pielage, J., Fetter, R.D., and Davis, G.W. (2005) Presynaptic spectrin is essential for synapse stabilization. *Curr. Biol.* **15**: 918–28.

Qin, G., Schwarz, T., Kittel, R.J., Schmid, A., Rasse, T.M., Kappei, D., Ponimaskin, E., Heckmann, M., and Sigrist, S.J. (2005) Four different subunits are essential for expressing the synaptic glutamate receptor at neuromuscular junctions of *Drosophila*. *J. Neurosci.* **25**: 3209–18.

Ramachandran, P., Barria, R., Ashley, J., and Budnik, V. (2009) A critical step for postsynaptic F-actin organization: regulation of Baz/Par-3 localization by aPKC and PTEN. *Dev. Neurobiol.* **69**: 583–602.

Ramaswami, M., Krishnan, K.S., and Kelly, R.B. (1994) Intermediates in synaptic vesicle recycling revealed by optical imaging of *Drosophila* neuromuscular junctions. *Neuron* **13**: 363–75.

Rao, S., Lang, C., Levitan, E.S., and Deitcher, D.L. (2001) Visualization of neuropeptide expression, transport, and exocytosis in *Drosophila melanogaster*. *J. Neurobiol.* **49**: 159–72.

Rao, S., Stewart, B.A., Rivlin, P.K., Vilinsky, I., Watson, B.O., Lang, C., Boulianne, G., Salpeter, M.M., and Deitcher, D.L. (2001) Two distinct effects on neurotransmission in a temperature-sensitive SNAP-25 mutant. *EMBO J.* **20**: 6761–71.

Rasse, T.M., Fouquet, W., Schmid, A., Kittel, R.J., Mertel, S., Sigrist, C.B., Schmidt, M., Guzman, A., Merino, C., Qin, G., Quentin, C., Madeo, F.F., Heckmann, M., and Sigrist, S.J. (2005) Glutamate receptor dynamics organizing synapse formation *in vivo*. *Nat. Neurosci.* **8**: 898–905.

Rawson, J.M., Lee, M., Kennedy, E.L., and Selleck, S.B. (2003) *Drosophila* neuromuscular synapse assembly and function require the TGF-beta type I receptor saxophone and the transcription factor Mad. *J. Neurobiol.* **55**: 134–50.

Reist, N.E., Buchanan, J., Li, J., DiAntonio, A., Buxton, E.M., and Schwarz, T.L. (1998) Morphologically docked synaptic vesicles are reduced in synaptotagmin mutants of *Drosophila*. *J. Neurosci.* **18**: 7662–73.

Renden, R., Berwin, B., Davis, W., Ann, K., Chin, C.T., Kreber, R., Ganetzky, B., Martin, T.F., and Broadie, K. (2001) *Drosophila* CAPS is an essential gene that regulates dense-core vesicle release and synaptic vesicle fusion. *Neuron* **31**: 421–37.

Richmond, J.E., Weimer, R.M., and Jorgensen, E.M. (2001) An open form of syntaxin bypasses the requirement for UNC-13 in vesicle priming. *Nature* **412**: 338–41.

Rieckhof, G.E., Yoshihara, M., Guan, Z., and Littleton, J.T. (2003) Presynaptic N-type calcium channels regulate synaptic growth. *J. Biol. Chem.* **278**: 41099–108.

Rikhy, R., Kumar, V., Mittal, R., and Krishnan, K.S. (2002) Endophilin is critically required for synapse formation and function in *Drosophila melanogaster*. *J. Neurosci.* **22**: 7478–84.

Rincon-Limas, D.E., Jensen, K., and Fernandez-Funez, P. (2012) *Drosophila* models of proteinopathies: the little fly that could. *Curr. Pharm. Des.* **18**: 1108–22.

Rodal, A.A., Blunk, A.D., Akbergenova, Y., Jorquera, R.A., Buhl, L.K., and Littleton, J.T. (2011) A presynaptic endosomal trafficking pathway controls synaptic growth signaling. *J. Cell Biol.* **193**: 201–17.

Rodal, A.A. and Littleton, J.T. (2008) Synaptic endocytosis: illuminating the role of clathrin assembly. *Curr. Biol.* **18**: R259–61.

Rodal, A.A., Motola-Barnes, R.N., and Littleton, J.T. (2008) Nervous wreck and Cdc42 cooperate to regulate endocytic actin assembly during synaptic growth. *J. Neurosci.* **28**: 8316–25.

Van Roessel, P., Elliott, D.A., Robinson, I.M., Prokop, A., and Brand, A.H. (2004) Independent regulation of synaptic size and activity by the anaphase-promoting complex. *Cell* **119**: 707–18.

Rohrbough, J. and Broadie, K. (2010) Anterograde Jelly belly ligand to Alk receptor signaling at developing synapses is regulated by Mind the gap. *Development* **137**: 3523–33.

Rohrbough, J., Rushton, E., Woodruff, E., Fergestad, T., Vigneswaran, K., and Broadie, K. (2007) Presynaptic establishment of the synaptic cleft extracellular matrix is required for post-synaptic differentiation. *Genes Dev.* **21**: 2607–28.

Roos, J. and Kelly, R.B. (1998) Dap160, a neural-specific Eps15 homology and multiple SH3 domain-containing protein that interacts with *Drosophila* dynamin. *J. Biol. Chem.* **273**: 19108–19.

Ruiz-Canada, C., Ashley, J., Moeckel-Cole, S., Drier, E., Yin, J., and Budnik, V. (2004) New synaptic bouton formation is disrupted by misregulation of microtubule stability in aPKC mutants. *Neuron* **42**: 567–80.

Ruiz-Canada, C., Budnik, V., Ruiz-Cañada, C., and Budnik, V. (2006) Introduction on the use of the *Drosophila* embryonic/larval neuromuscular junction as a model system to study synapse development and function, and a brief summary of pathfinding and target recognition. *Int. Rev. Neurobiol.* **75**: 217–236.

Rushton, E., Rohrbough, J., and Broadie, K. (2009) Presynaptic secretion of mind-the-gap organizes the synaptic extracellular matrix-integrin interface and postsynaptic environments. *Dev. Dyn.* **238**: 554–71.

Rushton, E., Rohrbough, J., Deutsch, K., and Broadie, K. (2012) Structure-function analysis of endogenous lectin mind-the-gap in synaptogenesis. *Dev. Neurobiol.* **72**: 1161–79.

Saitoe, M., Tanaka, S., Takata, K., and Kidokoro, Y. (1997) Neural activity affects distribution of glutamate receptors during neuromuscular junction formation in *Drosophila* embryos. *Dev. Biol.* **184**: 48–60.

Sanyal, S., Tolar, L.A., Pallanck, L., and Krishnan, K.S. (2001) Genetic interaction between shibire and comatose mutations in *Drosophila* suggest a role for snap-receptor complex assembly and disassembly for maintenance of synaptic vesicle cycling. *Neurosci. Lett.* **311**: 21–4.

Saraswati, S., Adolfsen, B., and Littleton, J.T. (2007) Characterization of the role of the Synaptotagmin family as calcium sensors in facilitation and asynchronous neurotransmitter release. *Proc. Natl. Acad. Sci. U. S. A.* **104**: 14122–7.

Schaub, J.R., Lu, X., Doneske, B., Shin, Y.-K., and McNew, J.A. (2006) Hemifusion arrest by complexin is relieved by Ca2+-synaptotagmin I. *Nat. Struct. Mol. Biol.* **13**: 748–50.

Schmid, A., Hallermann, S., Kittel, R.J., Khorramshahi, O., Frölich, A.M.J., Quentin, C., Rasse, T.M., Mertel, S., Heckmann, M., and Sigrist, S.J. (2008) Activity-dependent site-specific changes of glutamate receptor composition *in vivo*. *Nat. Neurosci.* **11**: 659–66.

Schmid, A., Qin, G., Wichmann, C., Kittel, R.J., Mertel, S., Fouquet, W., Schmidt, M., Heckmann, M., and Sigrist, S.J. (2006) Non-NMDA-type glutamate receptors are essential for maturation but not for initial assembly of synapses at *Drosophila* neuromuscular junctions. *J. Neurosci.* **26**: 11267–77.

Schulze, K.L. and Bellen, H.J. (1996) *Drosophila* syntaxin is required for cell viability and may function in membrane formation and stabilization. *Genetics* **144**: 1713–24.

Schulze, K.L., Broadie, K., Perin, M.S., and Bellen, H.J. (1995) Genetic and electrophysiological studies of *Drosophila* syntaxin-1A demonstrate its role in nonneuronal secretion and neurotransmission. *Cell* **80**: 311–20.

Schulze, K.L., Littleton, J.T., Salzberg, A., Halachmi, N., Stern, M., Lev, Z., and Bellen, H.J. (1994) rop, a *Drosophila* homolog of yeast Sec1 and vertebrate n-Sec1/Munc-18 proteins, is a negative regulator of neurotransmitter release *in vivo*. *Neuron* **13**: 1099–108.

Schuster, C.M., Davis, G.W., Fetter, R.D., and Goodman, C.S. (1996) Genetic dissection of structural and functional components of synaptic plasticity. I. Fasciclin II controls synaptic stabilization and growth. *Neuron* **17**: 641–54.

Schuster, C.M., Ultsch, A., Schloss, P., Cox, J.A., Schmitt, B., and Betz, H. (1991) Molecular cloning of an invertebrate glutamate receptor subunit expressed in *Drosophila* muscle. *Science* **254**: 112–4.

Schwabe, T., Gontang, A.C., and Clandinin, T.R. (2014) More than just glue. *Cell Adh. Migr.* **3**: 36–42.

Shakiryanova, D., Klose, M.K., Zhou, Y., Gu, T., Deitcher, D.L., Atwood, H.L., Hewes, R.S., and Levitan, E.S. (2007) Presynaptic ryanodine receptor-activated calmodulin kinase II increases vesicle mobility and potentiates neuropeptide release. *J. Neurosci.* **27**: 7799–806.

Shakiryanova, D., Tully, A., Hewes, R.S., Deitcher, D.L., and Levitan, E.S. (2005) Activity-dependent liberation of synaptic neuropeptide vesicles. *Nat. Neurosci.* **8**: 173–8.

Shakiryanova, D., Zettel, G.M., Gu, T., Hewes, R.S., and Levitan, E.S. (2011) Synaptic neuropeptide release induced by octopamine without Ca2+ entry into the nerve terminal. *Proc. Natl. Acad. Sci. U. S. A.* **108**: 4477–81.

Sidorov, M.S., Auerbach, B.D., and Bear, M.F. (2013) Fragile X mental retardation protein and synaptic plasticity. *Mol. Brain* **6**: 15.

Sigrist, S.J., Reiff, D.F., Thiel, P.R., Steinert, J.R., and Schuster, C.M. (2003) Experience-dependent strengthening of *Drosophila* neuromuscular junctions. *J. Neurosci.* **23**: 6546–56.

Smith, L.A., Wang, X., Peixoto, A.A., Neumann, E.K., Hall, L.M., and Hall, J.C. (1996) A *Drosophila* calcium channel alpha1 subunit gene maps to a genetic locus associated with behavioral and visual defects. *J. Neurosci.* **16**: 7868–79.

Söllner, T., Whiteheart, S.W., Brunner, M., Erdjument-Bromage, H., Geromanos, S., Tempst, P., and Rothman, J.E. (1993) SNAP receptors implicated in vesicle targeting and fusion. *Nature* **362**: 318–24.

Speese, S.D., Ashley, J., Jokhi, V., Nunnari, J., Barria, R., Li, Y., Ataman, B., Koon, A., Chang, Y.-T., Li, Q., Moore, M.J., and Budnik, V. (2012) Nuclear envelope budding enables large ribonucleoprotein particle export during synaptic Wnt signaling. *Cell* **149**: 832–846.

Staples, J. and Broadie, K. (2013) The cell polarity scaffold Lethal Giant Larvae regulates synapse morphology and function. *J. Cell Sci.* **126**: 1992–2003.

Stevens, R.J., Akbergenova, Y., Jorquera, R.A., and Littleton, J.T. (2012) Abnormal synaptic vesicle biogenesis in *Drosophila* synaptogyrin mutants. *J. Neurosci.* **32**: 18054–67, 18067a.

Stewart, B.A., Mohtashami, M., Trimble, W.S., and Boulianne, G.L. (2000) SNARE proteins contribute to calcium cooperativity of synaptic transmission. *Proc. Natl. Acad. Sci. U. S. A.* **97**: 13955–60.

Stewart, B.A., Schuster, C.M., Goodman, C.S., and Atwood, H.L. (1996) Homeostasis of synaptic transmission in *Drosophila* with genetically altered nerve terminal morphology. *J. Neurosci.* **16**: 3877–86.

Stimson, D.T., Estes, P.S., Rao, S., Krishnan, K.S., Kelly, L.E., and Ramaswami, M. (2001) *Drosophila* stoned proteins regulate the rate and fidelity of synaptic vesicle internalization. *J. Neurosci.* **21**: 3034–44.

Stimson, D.T., Estes, P.S., Smith, M., Kelly, L.E., and Ramaswami, M. (1998) A product of the *Drosophila* stoned locus regulates neurotransmitter release. *J. Neurosci.* **18**: 9638–49.

Stowers, R.S., Megeath, L.J., Górska-Andrzejak, J., Meinertzhagen, I.A., and Schwarz, T.L. (2002) Axonal transport of mitochondria to synapses depends on milton, a novel *Drosophila* protein. *Neuron* **36**: 1063–77.

Striegel, A.R., Biela, L.M., Evans, C.S., Wang, Z., Delehoy, J.B., Sutton, R.B., Chapman, E.R., and Reist, N.E. (2012) Calcium binding by Synaptotagmin's C2A domain is an essential element of the electrostatic switch that triggers synchronous synaptic transmission. *J. Neurosci.* **32**: 1253–1260.

Sudhof, T.C. (2004) The synaptic vesicle cycle. *Annu. Rev. Neurosci.* **27**: 509–47.

Südhof, T.C. (2008) Neuroligins and neurexins link synaptic function to cognitive disease. *Nature* **455**: 903–11.

Südhof, T.C. and Rothman, J.E. (2009) Membrane fusion: grappling with SNARE and SM proteins. *Science* **323**: 474–7.

Sun, M. and Xie, W. (2012) Cell adhesion molecules in *Drosophila* synapse development and function. *Sci. China. Life Sci.* **55**: 20–6.

Sun, M., Xing, G., Yuan, L., Gan, G., Knight, D., With, S.I., He, C., Han, J., Zeng, X., Fang, M., Boulianne, G.L., and Xie, W. (2011) Neuroligin 2 is required for synapse development and function at the *Drosophila* neuromuscular junction. *J. Neurosci.* **31**: 687–99.

Sutcliffe, B., Forero, M.G., Zhu, B., Robinson, I.M., and Hidalgo, A. (2013) Neuron-type specific functions of DNT1, DNT2 and Spz at the *Drosophila* neuromuscular junction. *PLoS One* **8**: e75902.

Sutton, R.B., Fasshauer, D., Jahn, R., and Brunger, A.T. (1998) Crystal structure of a SNARE complex involved in synaptic exocytosis at 2.4 A resolution. *Nature* **395**: 347–53.

Sweeney, S.T., Broadie, K., Keane, J., Niemann, H., and O'Kane, C.J. (1995) Targeted expression of tetanus toxin light chain in *Drosophila* specifically eliminates synaptic transmission and causes behavioral defects. *Neuron* **14**: 341–351.

Sweeney, S.T. and Davis, G.W. (2002) Unrestricted synaptic growth in spinster-a late endosomal protein implicated in TGF-beta-mediated synaptic growth regulation. *Neuron* **36**: 403–16.

Takamori, S., Holt, M., Stenius, K., Lemke, E.A., Grønborg, M., Riedel, D., Urlaub, H., Schenck, S., Brügger, B., Ringler, P., Müller, S.A., Rammner, B., Gräter, F., Hub, J.S., De Groot, B.L., Mieskes, G., Moriyama, Y., … Jahn, R. (2006) Molecular anatomy of a trafficking organelle. *Cell* **127**: 831–46.

Tessier, C.R. and Broadie, K. (2012) Molecular and genetic analysis of the *Drosophila* model of fragile X syndrome. *Results Probl. Cell Differ.* **54**: 119–56.

Thomas, U., Kim, E., Kuhlendahl, S., Koh, Y.H., Gundelfinger, E.D., Sheng, M., Garner, C.C., and Budnik, V. (1997) Synaptic clustering of the cell adhesion molecule Fasciclin II by Discs-Large and its role in the regulation of presynaptic structure. *Neuron* **19**: 787–799.

Tian, X., Li, J., Valakh, V., DiAntonio, A., and Wu, C. (2011) *Drosophila* Rae1 controls the abundance of the ubiquitin ligase Highwire in post-mitotic neurons. *Nat. Neurosci.* **14**: 1267–75.

Tian, X. and Wu, C. (2013) The role of ubiquitin-mediated pathways in regulating synaptic development, axonal degeneration and regeneration: insights from fly and worm. *J. Physiol.* **591**: 3133–43.

Tolar, L.A. and Pallanck, L. (1998) NSF function in neurotransmitter release involves rearrangement of the SNARE complex downstream of synaptic vesicle docking. *J. Neurosci.* **18**: 10250–6.

Uytterhoeven, V., Kuenen, S., Kasprowicz, J., Miskiewicz, K., and Verstreken, P. (2011) Loss of skywalker reveals synaptic endosomes as sorting stations for synaptic vesicle proteins. *Cell* **145**: 117–32.

Valakh, V., Naylor, S.A., Berns, D.S., and Diantonio, A. (2012) A large-scale RNAi screen identifies functional classes of genes shaping synaptic development and maintenance. *Dev. Biol.* **366**: 163–71.

Vanlandingham, P.A., Barmchi, M.P., Royer, S., Green, R., Bao, H., Reist, N., and Zhang, B. (2014) AP180 couples protein retrieval to clathrin-mediated endocytosis of synaptic vesicles. *Traffic* **15**: 433–50.

Verstreken, P., Kjaerulff, O., Lloyd, T.E., Atkinson, R., Zhou, Y., Meinertzhagen, I.A., and Bellen, H.J. (2002) Endophilin mutations block clathrin-mediated endocytosis but not neurotransmitter release. *Cell* **109**: 101–12.

Verstreken, P., Koh, T.-W., Schulze, K.L., Zhai, R.G., Hiesinger, P.R., Zhou, Y., Mehta, S.Q., Cao, Y., Roos, J., and Bellen, H.J. (2003) Synaptojanin is recruited by endophilin to promote synaptic vesicle uncoating. *Neuron* **40**: 733–48.

Verstreken, P., Ly, C. V, Venken, K.J.T., Koh, T.-W., Zhou, Y., and Bellen, H.J. (2005) Synaptic mitochondria are critical for mobilization of reserve pool vesicles at *Drosophila* neuromuscular junctions. *Neuron* **47**: 365–78.

Verstreken, P., Ohyama, T., Haueter, C., Habets, R.L.P., Lin, Y.Q., Swan, L.E., Ly, C. V, Venken, K.J.T., De Camilli, P., and Bellen, H.J. (2009) Tweek, an evolutionarily conserved protein, is required for synaptic vesicle recycling. *Neuron* **63**: 203–15.

Vijayakrishnan, N., Phillips, S.E., and Broadie, K. (2010) *Drosophila* rolling blackout displays lipase domain-dependent and -independent endocytic functions downstream of dynamin. *Traffic* **11**: 1567–78.

Vilinsky, I., Stewart, B.A., Drummond, J., Robinson, I., and Deitcher, D.L. (2002) A *Drosophila* SNAP-25 null mutant reveals context-dependent redundancy with SNAP-24 in neurotransmission. *Genetics* **162**: 259–71.

Viquez, N.M., Füger, P., Valakh, V., Daniels, R.W., Rasse, T.M., and DiAntonio, A. (2009) PP2A and GSK-3beta act antagonistically to regulate active zone development. *J. Neurosci.* **29**: 11484–94.

Wagh, D.A., Rasse, T.M., Asan, E., Hofbauer, A., Schwenkert, I., Dürrbeck, H., Buchner, S., Dabauvalle, M.-C., Schmidt, M., Qin, G., Wichmann, C., Kittel, R., Sigrist, S.J., and Buchner, E. (2006) Bruchpilot, a protein with homology to ELKS/CAST, is required for structural integrity and function of synaptic active zones in *Drosophila*. *Neuron* **49**: 833–44.

Wairkar, Y.P., Fradkin, L.G., Noordermeer, J.N., and DiAntonio, A. (2008) Synaptic defects in a *Drosophila* model of congenital muscular dystrophy. *J. Neurosci.* **28**: 3781–9.

Wairkar, Y.P., Toda, H., Mochizuki, H., Furukubo-Tokunaga, K., Tomoda, T., and Diantonio, A. (2009) Unc-51 controls active zone density and protein composition by downregulating ERK signaling. *J. Neurosci.* **29**: 517–28.

Wan, H.I., DiAntonio, A., Fetter, R.D., Bergstrom, K., Strauss, R., and Goodman, C.S. (2000) Highwire regulates synaptic growth in *Drosophila*. *Neuron* **26**: 313–29.

Wang, P., Saraswati, S., Guan, Z., Watkins, C.J., Wurtman, R.J., and Littleton, J.T. (2004) A *Drosophila* temperature-sensitive seizure mutant in phosphoglycerate kinase disrupts ATP generation and alters synaptic function. *J. Neurosci.* **24**: 4518–29.

Wang, T., Hauswirth, A.G., Tong, A., Dickman, D.K., and Davis, G.W. (2014) Endostatin is a trans-synaptic signal for homeostatic synaptic plasticity. *Neuron* **83**: 616–29.

Wang, X., Shaw, W.R., Tsang, H.T.H., Reid, E., and O'Kane, C.J. (2007) *Drosophila* spichthyin inhibits BMP signaling and regulates synaptic growth and axonal microtubules. *Nat. Neurosci.* **10**: 177–85.

Warrick, J.M., Paulson, H.L., Gray-Board, G.L., Bui, Q.T., Fischbeck, K.H., Pittman, R.N., and Bonini, N.M. (1998) Expanded polyglutamine protein forms nuclear inclusions and causes neural degeneration in *Drosophila*. *Cell* **93**: 939–49.

Weber, A.N.R., Gangloff, M., Moncrieffe, M.C., Hyvert, Y., Imler, J.-L., and Gay, N.J. (2007) Role of the Spatzle Pro-domain in the generation of an active toll receptor ligand. *J. Biol. Chem.* **282**: 13522–31.

Weber, T., Zemelman, B. V, McNew, J.A., Westermann, B., Gmachl, M., Parlati, F., Söllner, T.H., and Rothman, J.E. (1998) SNAREpins: minimal machinery for membrane fusion. *Cell* **92**: 759–72.

Wegner, C.S., Rodahl, L.M.W., and Stenmark, H. (2011) ESCRT proteins and cell signalling. *Traffic* **12**: 1291–7.

Weyhersmuller, A., Hallermann, S., Wagner, N., and Eilers, J. (2011) Rapid active zone remodeling during synaptic plasticity. *J. Neurosci.* **31**: 6041–6052.

Wong, M.Y., Cavolo, S.L., and Levitan, E.S. (2015) Synaptic neuropeptide release by Dynamin-dependent partial release from circulating vesicles. *Mol. Biol. Cell*.

Wong, M.Y., Zhou, C., Shakiryanova, D., Lloyd, T.E., Deitcher, D.L., and Levitan, E.S. (2012) Neuropeptide delivery to synapses by long-range vesicle circulation and sporadic capture. *Cell* **148**: 1029–38.

Wu, C., Daniels, R.W., and DiAntonio, A. (2007) DFsn collaborates with Highwire to down-regulate the Wallenda/DLK kinase and restrain synaptic terminal growth. *Neural Dev.* **2**: 16.

Wu, C., Wairkar, Y.P., Collins, C.A., and DiAntonio, A. (2005) Highwire function at the *Drosophila* neuromuscular junction: spatial, structural, and temporal requirements. *J. Neurosci.* **25**: 9557–66.

Wu, M.N., Fergestad, T., Lloyd, T.E., He, Y., Broadie, K., and Bellen, H.J. (1999) Syntaxin 1A interacts with multiple exocytic proteins to regulate neurotransmitter release *in vivo*. *Neuron* **23**: 593–605.

Wu, M.N., Littleton, J.T., Bhat, M.A., Prokop, A., and Bellen, H.J. (1998) ROP, the *Drosophila* Sec1 homolog, interacts with syntaxin and regulates neurotransmitter release in a dosage-dependent manner. *EMBO J.* **17**: 127–39.

Xing, G., Gan, G., Chen, D., Sun, M., Yi, J., Lv, H., Han, J., and Xie, W. (2014) *Drosophila* Neuroligin3 regulates neuromuscular junction development and synaptic differentiation. *J. Biol. Chem.* **289**: M114.574897–.

Xue, L., Zhang, Z., McNeil, B.D., Luo, F., Wu, X.-S., Sheng, J., Shin, W., and Wu, L.-G. (2012) Voltage-dependent calcium channels at the plasma membrane, but not vesicular channels, couple exocytosis to endocytosis. *Cell Rep.* **1**: 632–8.

Xue, M., Lin, Y.Q., Pan, H., Reim, K., Deng, H., Bellen, H.J., and Rosenmund, C. (2009) Tilting the balance between facilitatory and inhibitory functions of mammalian and *Drosophila* Complexins orchestrates synaptic vesicle exocytosis. *Neuron* **64**: 367–80.

Yao, C.-K., Lin, Y.Q., Ly, C. V, Ohyama, T., Haueter, C.M., Moiseenkova-Bell, V.Y., Wensel, T.G., and Bellen, H.J. (2009) A synaptic vesicle-associated Ca2+ channel promotes endocytosis and couples exocytosis to endocytosis. *Cell* **138**: 947–60.

Yasuyama, K., Meinertzhagen, I.A., and Schürmann, F.-W. (2002) Synaptic organization of the mushroom body calyx in *Drosophila melanogaster*. *J. Comp. Neurol.* **445**: 211–26.

Yoshihara, M., Adolfsen, B., Galle, K.T., and Littleton, J.T. (2005) Retrograde signaling by Syt 4 induces presynaptic release and synapse-specific growth. *Science* **310**: 858–63.

Yoshihara, M., Guan, Z., and Littleton, J.T. (2010) Differential regulation of synchronous versus asynchronous neurotransmitter release by the C2 domains of synaptotagmin 1. *Proc. Natl. Acad. Sci. U. S. A.* **107**: 14869–74.

Yoshihara, M. and Littleton, J.T. (2002) Synaptotagmin I functions as a calcium sensor to synchronize neurotransmitter release. *Neuron* **36**: 897–908.

Yoshihara, M., Rheuben, M.B., and Kidokoro, Y. (1997) Transition from growth cone to functional motor nerve terminal in *Drosophila* embryos. *J. Neurosci.* **17**: 8408–26.

Yoshihara, M., Ueda, A., Zhang, D., Deitcher, D.L., Schwarz, T.L., and Kidokoro, Y. (1999) Selective effects of neuronal-synaptobrevin mutations on transmitter release evoked by sustained versus transient Ca2+ increases and by cAMP. *J. Neurosci.* **19**: 2432–41.

Younger, M.A., Müller, M., Tong, A., Pym, E.C., and Davis, G.W. (2013) A presynaptic ENaC channel drives homeostatic plasticity. *Neuron* **79**: 1183–96.

Zarnescu, D.C., Jin, P., Betschinger, J., Nakamoto, M., Wang, Y., Dockendorff, T.C., Feng, Y., Jongens, T.A., Sisson, J.C., Knoblich, J.A., Warren, S.T., and Moses, K. (2005) Fragile X protein functions with lgl and the par complex in flies and mice. *Dev. Cell* **8**: 43–52.

Zhang, B. (2003) Genetic and molecular analysis of synaptic vesicle recycling in *Drosophila*. *J. Neurocytol.* **32**: 567–89.

Zhang, B., Koh, Y.H., Beckstead, R.B., Budnik, V., Ganetzky, B., and Bellen, H.J. (1998) Synaptic vesicle size and number are regulated by a clathrin adaptor protein required for endocytosis. *Neuron* **21**: 1465–75.

Zhang, Y.Q., Bailey, A.M., Matthies, H.J., Renden, R.B., Smith, M.A., Speese, S.D., Rubin, G.M., and Broadie, K. (2001) *Drosophila* fragile X-related gene regulates the MAP1B homolog Futsch to control synaptic structure and function. *Cell* **107**: 591–603.

Zheng, C.-Y., Seabold, G.K., Horak, M., and Petralia, R.S. (2011) MAGUKs, synaptic development, and synaptic plasticity. *Neuroscientist* **17**: 493–512.

Zhong, Y., Budnik, V., and Wu, C.F. (1992) Synaptic plasticity in *Drosophila* memory and hyperexcitable mutants: role of cAMP cascade. *J. Neurosci.* **12**: 644–51.

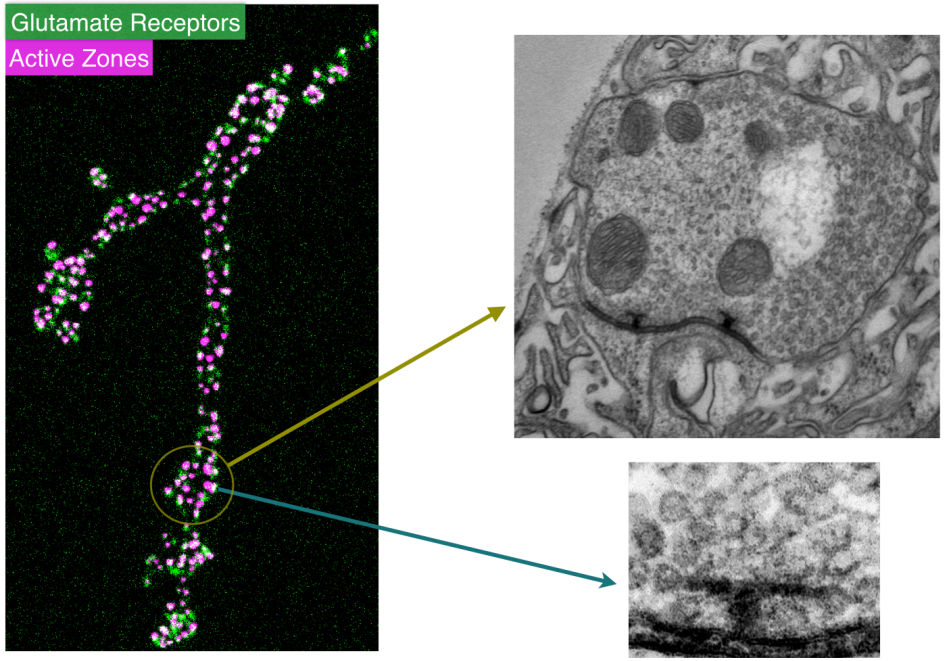
Zhu, B., Pennack, J.A., McQuilton, P., Forero, M.G., Mizuguchi, K., Sutcliffe, B., Gu, C.-J., Fenton, J.C., and Hidalgo, A. (2008) *Drosophila* neurotrophins reveal a common mechanism for nervous system formation. *PLoS Biol.* **6**: e284.

Zito, K., Fetter, R.D., Goodman, C.S., and Isacoff, E.Y. (1997) Synaptic clustering of Fasciclin II and Shaker: essential targeting sequences and role of Dlg. *Neuron* **19**: 1007–1016.

Zito, K., Parnas, D., Fetter, R.D., Isacoff, E.Y., and Goodman, C.S. (1999) Watching a synapse grow: noninvasive confocal imaging of synaptic growth in *Drosophila*. *Neuron* **22**: 719–29.

Figure 1

A Anatomy of the *Drosophila* NMJ



B Dramatic expansion of the NMJ during development

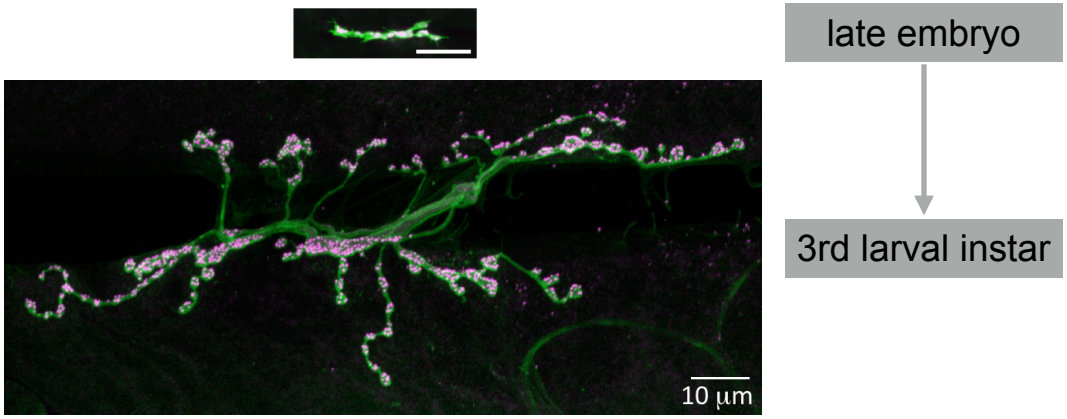
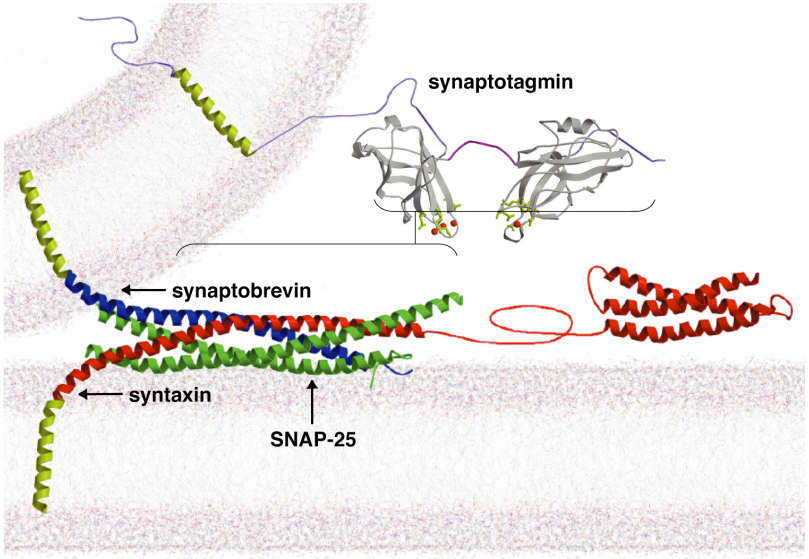
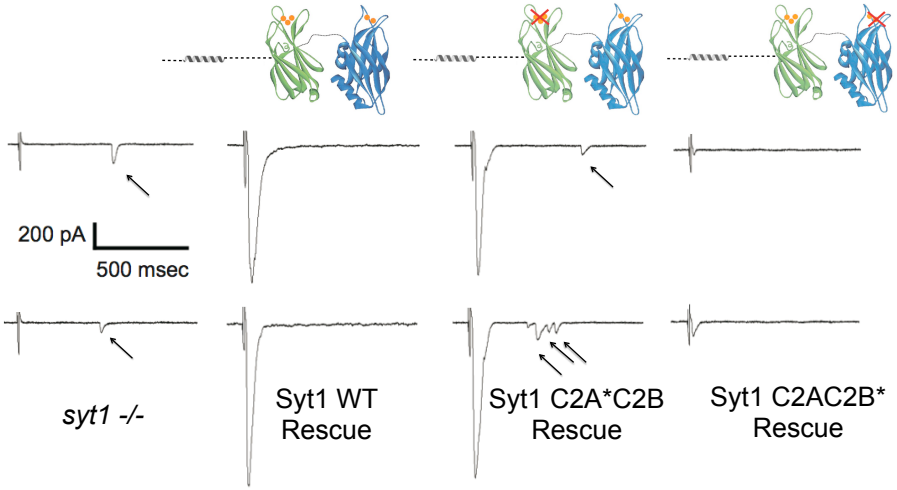


Figure 2

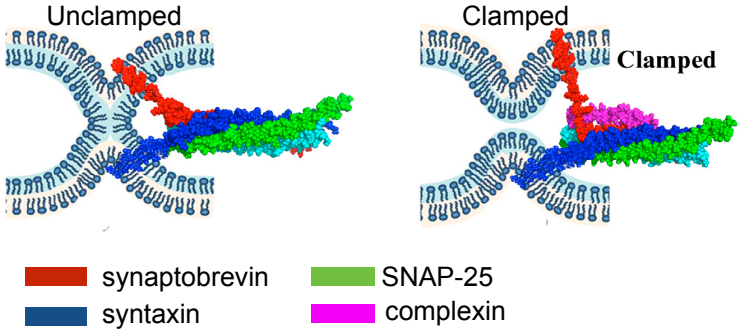
A Molecular structure of the fusion machinery



B The tandem C2 domains of Syt1 differentially regulate synchronous and asynchronous release



C Model of Complexin regulation of SNARE assembly



D Increased mini frequency in *cpx* is suppressed by *syt1*

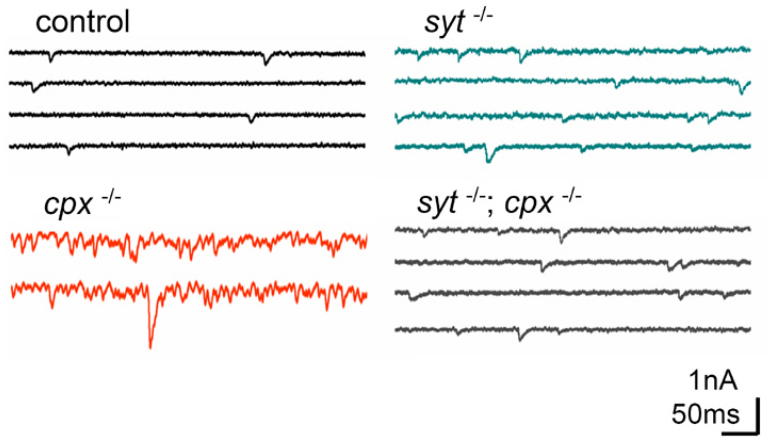
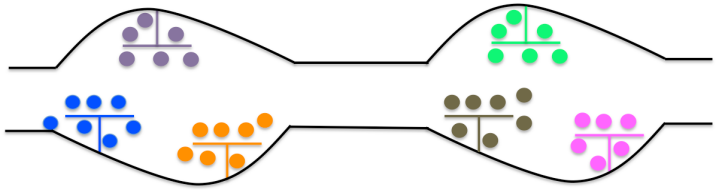
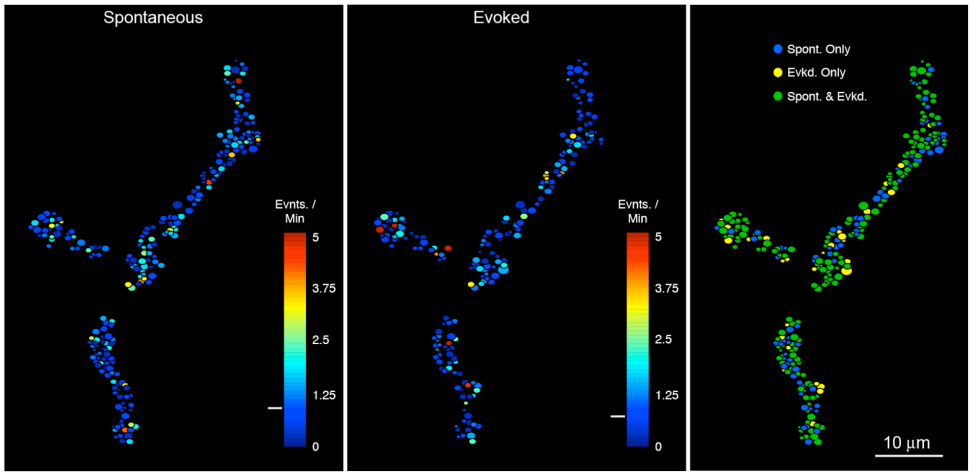


Figure 3

A Diagram of AZs within synaptic boutons



B Mapping of individual release site probabilities



C Classifications of AZs by release probabilities

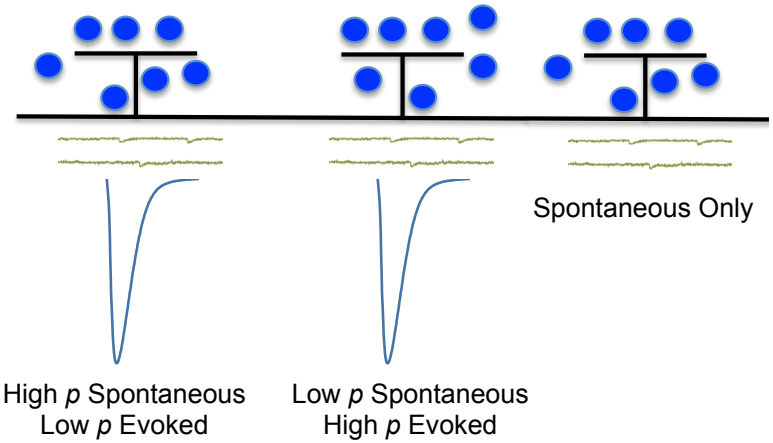
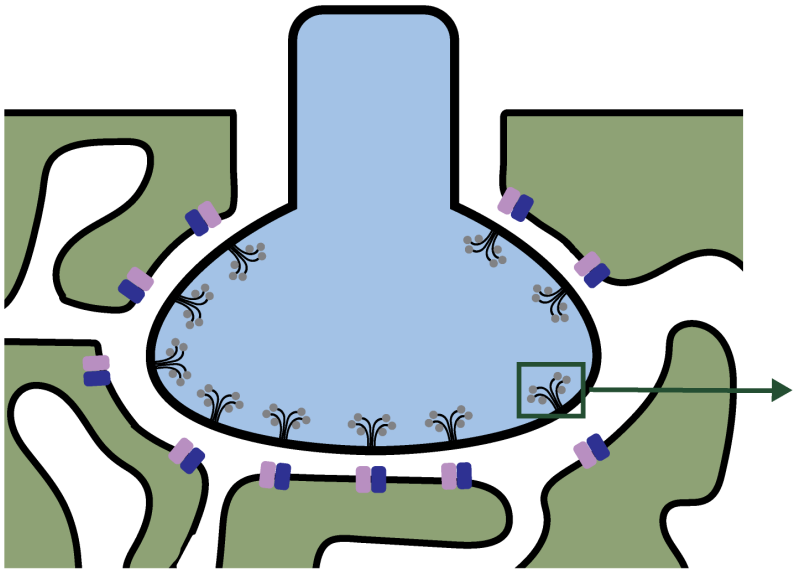
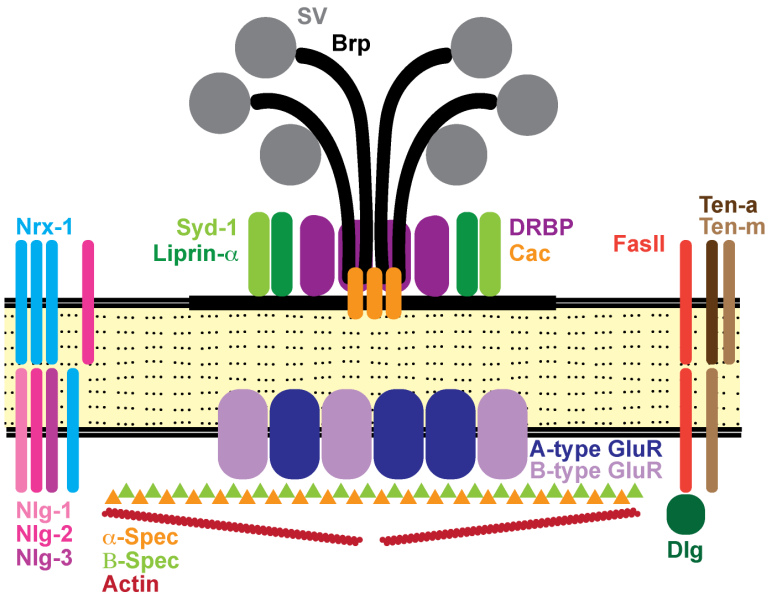


Figure 4

A Synaptic bouton and subsynaptic reticulum



B The active zone and postsynaptic density



C Synaptic organization defects

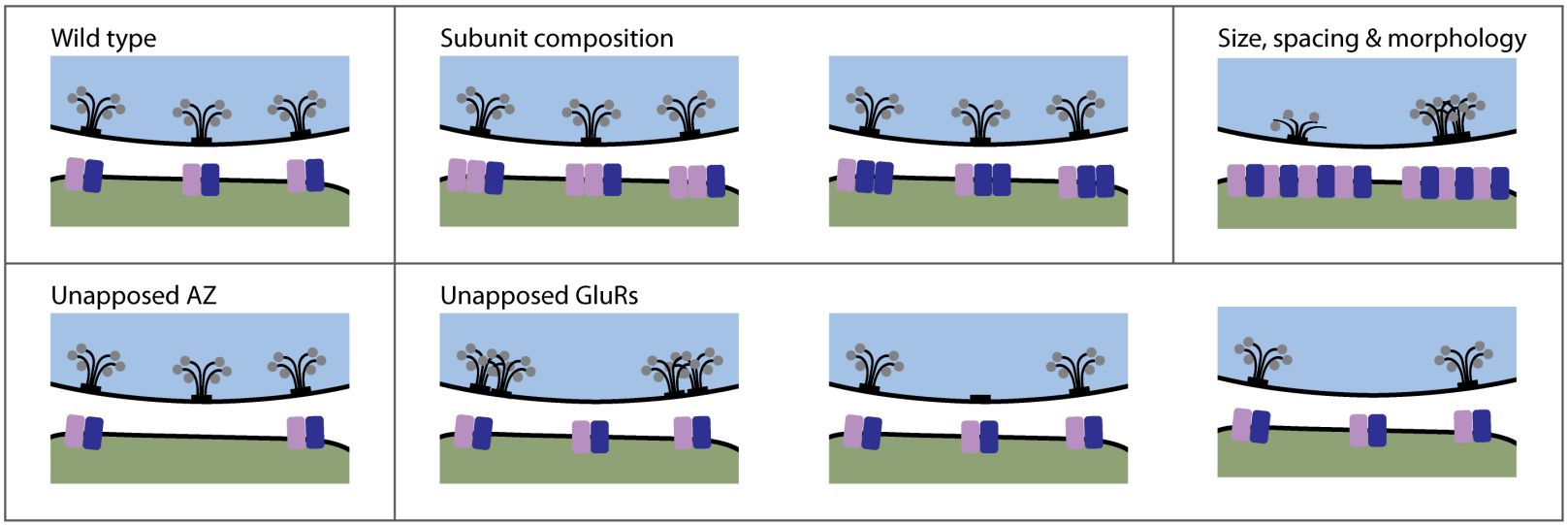
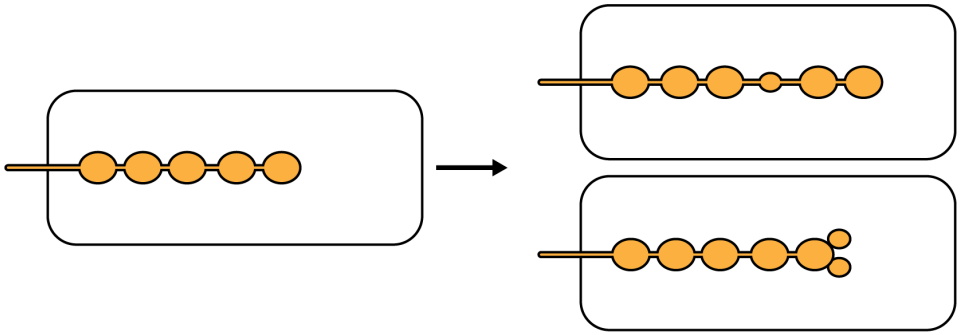


Figure 5

A Addition of new boutons at the NMJ



B Synaptic growth defects