

# Transmission, Development, and Plasticity of Synapses

Kathryn P. Harris and J. Troy Littleton<sup>1</sup>

Department of Biology, Department of Brain and Cognitive Sciences, The Picower Institute for Learning and Memory, Massachusetts Institute of Technology, Cambridge, Massachusetts 02139

**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, 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 (NMJ), 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.

**KEYWORDS** FlyBook; *Drosophila*; synapse; neurotransmitter release; synaptic plasticity; active zone; synaptic vesicle

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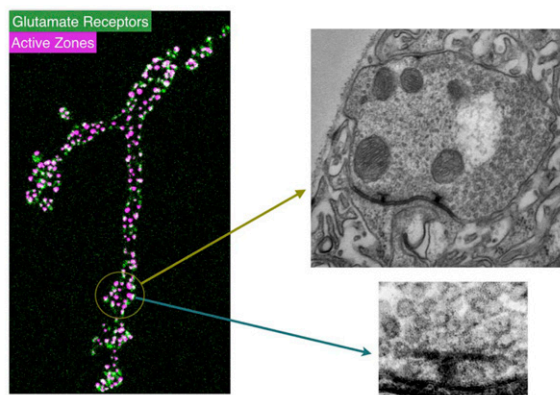
## Analyzing Synaptic Function at the *Drosophila* Neuromuscular Junction

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.

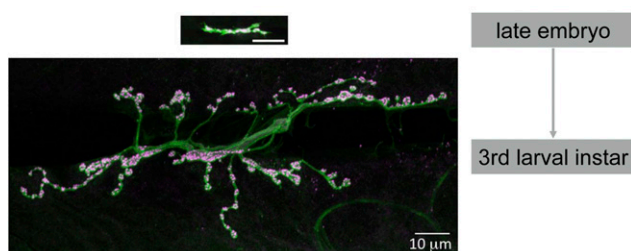
The developmental events underlying formation of the NMJ have been well characterized (Broadie and Bate 1993b). During

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 (Figure 1A). AZs are readily identifiable by electron microscopy (EM) as electron-dense T-bar structures (Feeney *et al.* 1998) (Figure 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.

## A Anatomy of the *Drosophila* NMJ



## B Dramatic expansion of the NMJ during development



**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 third instar stage is shown. Immunocytochemistry highlights the nerve (anti-HRP, green) and AZs (anti-Brp, magenta). Bar, 10  $\mu$ m in both images.

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 post-synaptic surface area, the innervating axon adjusts its synaptic arbor by increasing varicosity number 5- to 10-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 first instar synapse and the more mature third instar synapse (Figure 1B). Of the 30 muscles present in each abdominal hemisegment, 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.

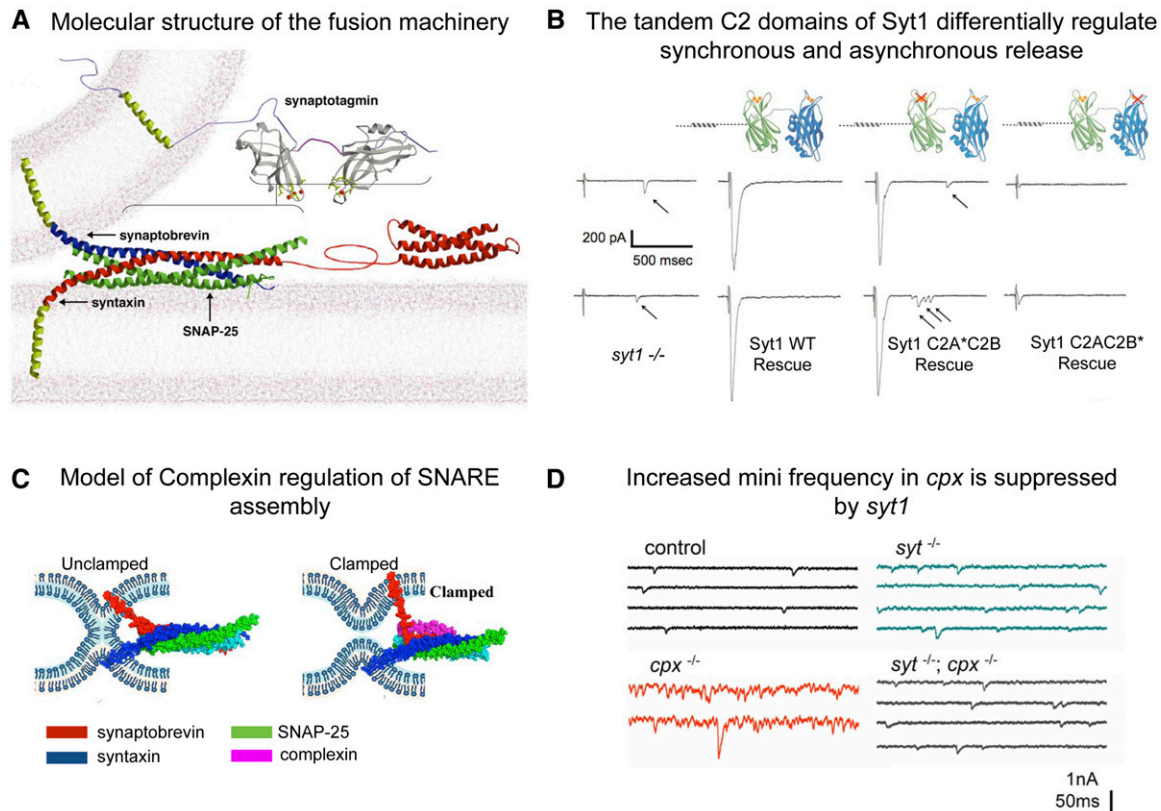
For physiological analysis, embryos or larvae are dissected along the dorsal midline, exposing the ventral musculature. Motor neuron axons can then be cut from where they exit the

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 third 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 third instar counterpart (Yoshihara *et al.* 2005; Barber *et al.* 2009). The embryonic synapse is also less susceptible to compensation mechanisms than the third instar synapse. Despite these advantages, the difficulty in recording from embryonic terminals has made the third 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 third 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 (Figure 2A) (Sutton *et al.* 1998; Weber *et al.* 1998). The rapid influx of  $\text{Ca}^{2+}$  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  $\text{Ca}^{2+}$ -triggered fusion of the SV and plasma membrane bilayers specifically at the AZ (for review, see Südhof and Rothman 2009). Current models



**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 embryos of synaptotagmin null mutants (*syt1*<sup>-/-</sup>), null mutants rescued with a wild-type synaptotagmin construct (Syt1 WT), or null mutants rescued with constructs that disrupt Ca<sup>2+</sup> 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<sup>2+</sup> 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<sup>2+</sup> binding shows normal synchronous release, but cannot suppress asynchronous fusion. Syt1 lacking C2B Ca<sup>2+</sup> 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 third instar NMJs of control (black), *cpx*<sup>-/-</sup> (red), *syt1*<sup>-/-</sup> (aqua), and *syt1*<sup>-/-</sup>; *cpx*<sup>-/-</sup> double null (gray) 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.

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<sup>2+</sup>. 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<sup>2+</sup>. Second, upon Ca<sup>2+</sup> influx, sensors in the vicinity of the AZ detect the rise in Ca<sup>2+</sup> 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-Synaptobrevin (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<sup>2+</sup> 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 third 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 *et al.* 2001b; 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  $\text{Ca}^{2+}$  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 hexameric 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 postexocytosis role at the synapse to disassemble *cis*-SNARE complexes (those residing in the same membrane) after fusion (Littleton *et al.* 2001b; 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  $\alpha$ -SNAP protein, which is required to tether NSF to the SNARE complex, are embryonic lethal (Babcock *et al.* 2004). Recordings done in hypomorphic  $\alpha$ -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  $\text{Ca}^{2+}$ -dependent SV fusion. Several proteins bind directly to assembled SNARE complexes, including Synaptotagmin 1 (Syt1) and Complexin (Cpx). Syts are a family of vesicular  $\text{Ca}^{2+}$  sensors with two cytoplasmic  $\text{Ca}^{2+}$  binding C2 domains, termed C2A and C2B (Adolfson and Littleton 2001; Sudhof 2004). Syt1 is one of the most well-studied vesicle trafficking proteins and functions to sense  $\text{Ca}^{2+}$  influx and regulate fusion of SVs (Littleton *et al.* 1993, 1994, 2001a; Broadie *et al.* 1994; Diantonio and Schwarz 1994; Reist *et al.* 1998; Yoshihara and Littleton 2002; Mackler *et al.* 2002; Saraswati *et al.* 2007; Yoshihara *et al.* 2010; Jorquera *et al.* 2012; Striegel *et al.* 2012; Lee *et al.* 2013b; 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 (Kaesler and Regehr 2014). *Drosophila syt1* null mutants lack the fast synchronous component of evoked fusion and show enhanced asynchronous and spontaneous release as well (Figure 2B) (Littleton *et al.* 1993; Yoshihara and Littleton 2002; Yoshihara *et al.* 2010; Jorquera *et al.* 2012; Lee *et al.* 2013b; Lee and Littleton 2015). These data suggest that two kinetically distinct  $\text{Ca}^{2+}$  sensors exist, with Syt1 mediating the rapid, synchronous component of transmitter release (Yoshihara and Littleton 2002), and a second unknown  $\text{Ca}^{2+}$  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  $\text{Ca}^{2+}$  binding sites in C2A or C2B in the background of *Drosophila syt1* null mutants (Figure 2B). Animals expressing mutations that disrupt  $\text{Ca}^{2+}$  binding to C2A have normal synchronous release, but do not suppress asynchronous release (Yoshihara *et al.* 2010). In contrast, rescue with  $\text{Ca}^{2+}$  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  $\text{Ca}^{2+}$  binding to C2A suppressing asynchronous release and  $\text{Ca}^{2+}$  binding to C2B driving synchronous fusion. Based on these 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  $\text{Ca}^{2+}$  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  $\text{Ca}^{2+}$ -independent properties of the protein (Lee

*et al.* 2013b). Beyond lipid penetration by the C2 domains following  $\text{Ca}^{2+}$  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 (Figure 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* (Figure 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  $\text{Ca}^{2+}$ -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.*

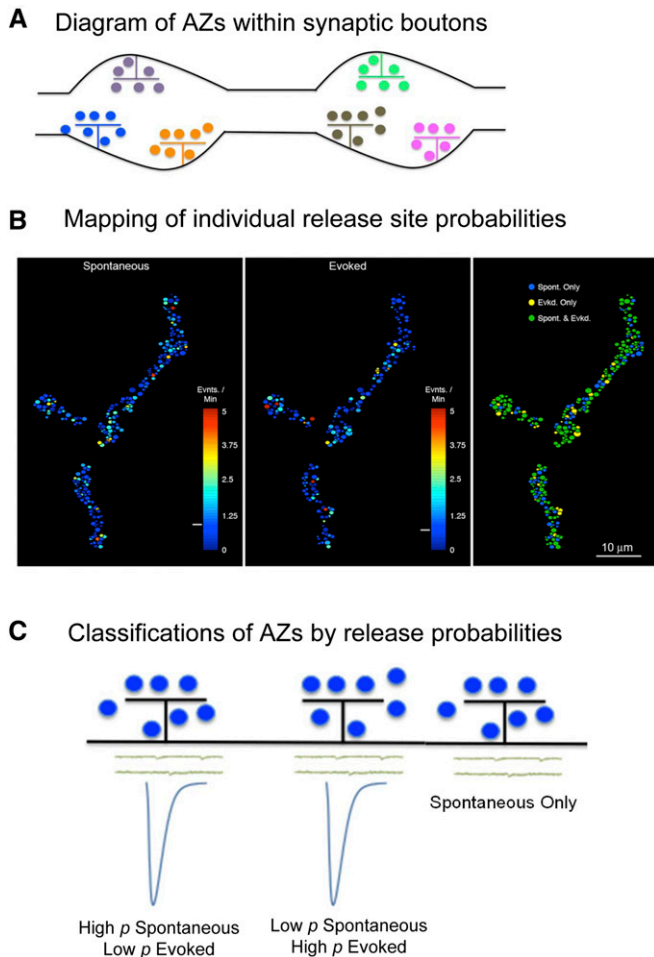
(2012) have shown that Cpx can regulate the  $\text{Ca}^{2+}$  cooperativity and speed of SV release. These properties suggest Cpx can help couple Syt1 to the SNARE fusion process to enhance its  $\text{Ca}^{2+}$  sensitivity and the speed of vesicle release.

### 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  $\text{Ca}^{2+}$  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



**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.

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), Tweak (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  $\text{Ca}^{2+}$  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; Kasprovicz *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  $\text{Ca}^{2+}$  channels open, allowing  $\text{Ca}^{2+}$  influx and the activation of the fusion machinery for SV release. *Drosophila* contains a single member of the  $\text{Ca}_v2$  family of N/P/Q-type presynaptic  $\text{Ca}^{2+}$  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  $\text{Ca}^{2+}$  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  $\text{Ca}^{2+}$  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 (Figure 3), a technique that has not been achieved at synaptic connections in other model systems.

Two genetically encoded  $\text{Ca}^{2+}$  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  $\text{K}^+$  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  $\text{Ca}^{2+}$  influx through GluRs that occurs following SV fusion. Regions of interest (ROIs) defined by these spatially segregated  $\text{Ca}^{2+}$  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  $\text{Ca}^{2+}$  signals

that peak in ~50 msec and decay to baseline by ~300 msec, with a change in baseline fluorescence ( $\Delta F$ ) of 40–100%. Subsequent fusion events at the same site produce similar  $\text{Ca}^{2+}$  profiles, allowing vesicle fusion to be measured at defined ROIs until the sensor begins to bleach (~10 min 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 (Figure 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  $\text{Ca}^{2+}$  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  $\text{Ca}^{2+}$  channels. Other features of spontaneous release that have been characterized through this optical imaging approach include the identification of nonuniform release probability rates across individual AZs, with an average rate of ~1 mini event per 80 sec (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  $\text{Ca}^{2+}$ -independent at *Drosophila* NMJs (Jorquera *et al.* 2012; Lee *et al.* 2013b), evoked fusion is triggered by the influx of  $\text{Ca}^{2+}$  at AZs. Using GCaMP to image release, synchronous fusion of SVs across many AZs gives rise to the evoked response. At normal extracellular  $\text{Ca}^{2+}$  concentrations, multivesicular release at individual AZs was not commonly observed, as  $\Delta F$  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 nonuniform nature of release probability per AZ for spontaneous fusion, evoked release probabilities were highly variable across the population as well (Figure 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 sites, 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  $\text{Ca}^{2+}$  channels at a single AZ, thereby controlling the level of presynaptic  $\text{Ca}^{2+}$  entry. A higher density of  $\text{Ca}^{2+}$  channels would lead to increased  $\text{Ca}^{2+}$  levels at an AZ, and thereby increase the likelihood that the presynaptic release machinery would be activated. An alternative model is that presynaptic  $\text{Ca}^{2+}$  channel density and  $\text{Ca}^{2+}$  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 to 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  $\text{Ca}^{2+}$  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  $\text{Ca}^{2+}$  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 Syt $\alpha$  and Syt $\beta$  (Adolfson *et al.* 2004; Park *et al.* 2014). In addition, one well-characterized DCV-specific regulator of release has been identified—the  $\text{Ca}^{2+}$ -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  $\text{Ca}^{2+}$ -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 extrasynaptic 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 *et al.* 2001a). 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  $\text{Ca}^{2+}$  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

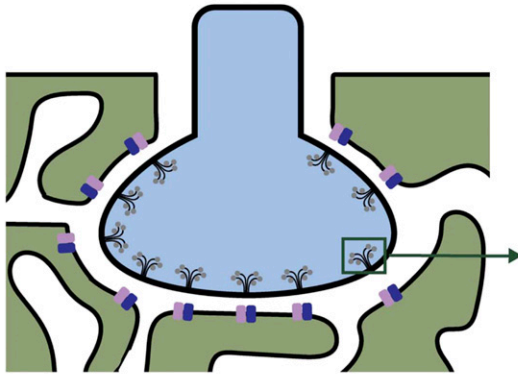
The AZ is a region of the presynaptic membrane that is highly specialized to facilitate neurotransmitter release. The AZ contains docked SVs and clusters of voltage-gated  $\text{Ca}^{2+}$  channels to create local high concentrations of  $\text{Ca}^{2+}$  for synaptic release. The AZ also contains a proteinaceous 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 (e.g., Bassoon, Piccolo, RIM, ELKS/CAST; see Hida and Ohtsuka 2010), and homologs of many of these are found at *Drosophila* synapses.

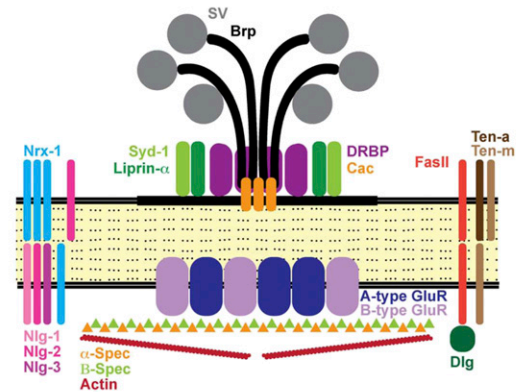
The CAZ of *Drosophila* synapses is readily identifiable by EM as a T-bar structure (Figure 1A) (Feeney *et al.* 1998). EM tomography provides a detailed picture of CAZ ultrastructure. A T-bar consists of a central core that is ~200 nm wide and 50 nm high and is attached to the plasma membrane by a grid 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, members of 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  $\text{Ca}^{2+}$  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-positive 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  $\text{Ca}^{2+}$  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<sup>nude</sup>*) 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 (DRBP) forms a ring at the base of the Brp core, where it surrounds a cluster of  $\text{Ca}^{2+}$  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  $\text{Ca}^{2+}$  channel levels, and impairment of

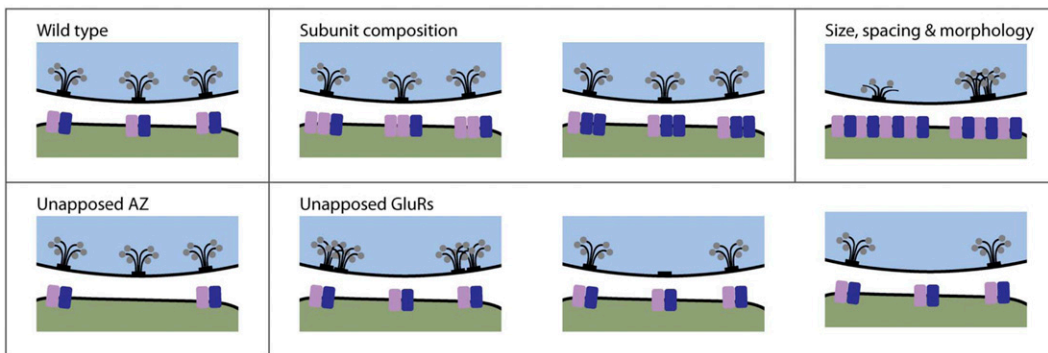
## A Synaptic bouton and subsynaptic reticulum



## B The active zone and postsynaptic density



## C Synaptic organization defects



**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- $\alpha$  also localize within the AZ cytoplasm. 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.

evoked release (Liu *et al.* 2011). Syd-1 and Liprin- $\alpha$  localize to the periphery of the CAZ (Fouquet *et al.* 2009; Oswald *et al.* 2010). Mutants of *syd-1* and *liprin- $\alpha$*  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; Oswald *et al.* 2010). Syd-1 clusters Liprin- $\alpha$  at the CAZ along with the transsynaptic adhesion protein Neurexin (Nrx), which is important for transsynaptic signaling for PSD organization and apposition (Oswald *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<sup>+</sup> channel Slowpoke (SLO) and together they regulate the presynaptic cytoskeleton, AZ size, and neurotransmission.

In summary, AZ components serve to cluster SVs, organize Ca<sup>2+</sup> channels at release sites, and bind transsynaptic partners to facilitate clustering of GluRs (Figure 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 (Figure 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) (Figure 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<sup>+</sup> 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 photobleaching (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 toward increased GluRIIA incorporation.

**Modulation of GluR subunit composition:** Several signaling pathways regulate GluR subunit composition and it appears that distinct modules support A-type vs. 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/Neurologin (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- $\kappa$ B signaling pathway. The transcription factor NF- $\kappa$ B/Dorsal is enriched at the PSD and appears to function locally at the synapse without any nuclear role. Dorsal acts along with inhibitory molecule IkB/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 (Lee *et al.* 2013a). It is unclear how Dorsal, Cactus, and Pelle affect GluRs. Observations from Heckscher *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; Waikar *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 (Figure 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; Oswald *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; Oswald *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 (Oswald *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 Ten-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 adapter 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 (Figure 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 (Figure 4C). DiAntonio and colleagues (Graf *et al.* 2012) 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. *rab3<sup>rup</sup>* 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-3 $\beta$ , as inhibition of GSK-3 $\beta$  suppresses the PP2A AZ defects.

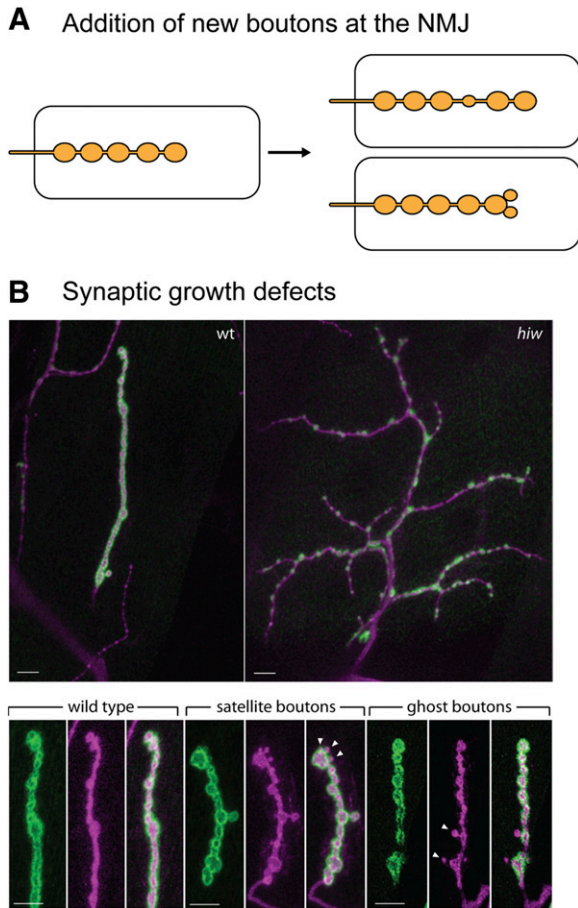
**Contributions of the extracellular matrix to synaptic organization:** The synaptic extracellular matrix (ECM), an array of glycoproteins and proteoglycans filling the space

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 integrin signaling or some other cytoplasm-ECM signaling complex.

Other ECM proteins that regulate the synapse are the heparan 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  $\alpha$ -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



**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 wild-type 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. Bars, 10  $\mu$ m.

*et al.* 2014). Also identified by this method were *hs6st* and *sulf1*, which affect the sulfation state of HSPGs (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 microglomeruli. Within a microglomerulus, synapses are characterized by

large presynaptic boutons surrounded by 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 Da7 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 Da7 and Da6. 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<sup>+</sup> 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 (Figure 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<sup>WT</sup>*) or nonfunctional (*iGluR<sup>MUT</sup>*) rescue transgene. These animals have comparable evoked neurotransmission, due to homeostatic compensation of quantal content in the *iGluR<sup>MUT</sup>* animals (see Frank 2014 and *Homeostatic Plasticity* section of this review). However, *iGluR<sup>MUT</sup>* 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 (Figure 5C). Ghost boutons can be elicited by experimental stimulation, such as incubating a dissected live preparation in elevated K<sup>+</sup>, but also occur spontaneously at wild-type arbors, where ~1% of all bou-

tons 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<sup>+</sup> 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<sup>+</sup> channels *slowpoke* (*slo*), a Ca<sup>2+</sup>-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 (Figure 5C). Activity-dependent overgrowth is mediated by  $\text{Ca}^{2+}$ /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  $\text{Ca}^{2+}$  signals into a retrograde signal. Synaptotagmin 4 (Syt4) was identified as a postsynaptic  $\text{Ca}^{2+}$  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- $\beta$ ) 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 a 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 bidirectionally 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 neuronal 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- $\beta$  signal also modulates Gbb-dependent retrograde signaling (Fuentes-Medel *et al.* 2012). The TGF- $\beta$  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  $\beta$ -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 postsynaptic cells. Loss of Wnt1/Wingless (Wg) results in a decrease in bouton number, abnormally large and irregular boutons, a depletion of the SSR, an 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- $\beta$ 11 (Imp- $\beta$ 11) and Importin- $\alpha$ 2 (Imp- $\alpha$ 2) (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, knock-down 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, coreceptor 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  $\beta$ -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 Gao, 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 up-regulation of presynaptic and down-regulation of postsynaptic Wg signaling (Kamimura *et al.* 2013). Trol localizes to the SSR and promotes the spread of extracellular Wg toward the postsynaptic cell, suggesting that *trol* regulates the balance of bidirectional signaling by impacting the pre- and postsynaptic 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 exosome-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 (Figure 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<sup>+</sup>, 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*<sup>2</sup> allele is a point mutation in the prodomain 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 semilethality of *spz*<sup>2</sup>, suggesting a retrograde function. In *spz*<sup>2</sup> 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 orga-

nization. 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 acts 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 nonsynaptic 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 Nr<sub>x</sub> and Nl<sub>g</sub> 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 Nr<sub>x</sub>–Nl<sub>g</sub> 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 Spichthyn (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 (Figure 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. Down-regulation 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 Tkvl and Wit accumulate in early endosomes in the presynaptic cell and signaling is up-regulated 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 Tkvl (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 *Caenorhabditis 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 (Figure 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 Wnd 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- $\alpha$ , 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 Staufien, which has a well-characterized role in mRNA localization during *Drosophila* development. Staufien 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) Syncrin (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 in-

crease 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<sup>2+</sup> 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<sup>2+</sup> influx through the voltage-gated Ca<sup>2+</sup> 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  $\text{Ca}^{2+}$  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 Rab3 signaling module regulating this process. The regulation of Cac function and  $\text{Ca}^{2+}$  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  $\text{K}^+$  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  $\text{Ca}^{2+}$  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  $\text{Ca}^{2+}$  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.* 2006). 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  $\text{Ca}^{2+}$  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 Nr1 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 proteopathies, 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 40 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, and nucleus and extracellular matrix, reflecting the complex layers of regulation required to maintain and modulate neural function at the cellular and molecular levels. 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.

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Communicating editor: J. Carlson