Presynaptic Morphogenesis, Active Zone Organization and Structural Plasticity in Drosophila

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Abstract

Effective adaptation of neural circuit function to a changing environment requires many forms of plasticity. Among these, structural plasticity is one of the most durable, and is also an intrinsic part of the developmental logic for the formation and refinement of synaptic connectivity. Structural plasticity of presynaptic sites can involve the addition, remodeling, or removal of pre- and post-synaptic elements. However, this requires coordination of morphogenesis and assembly of the subcellular machinery for neurotransmitter release within the presynaptic neurons, as well as coordination of these events with the postsynaptic cell. While much progress has been made in revealing the cell biological mechanisms of postsynaptic structural plasticity, our understanding of presynaptic mechanisms is less complete.

Introduction

The chemical synapse is the fundamental building block of nervous system connectivity. Each site of neurotransmitter release (active zone) requires coordinated assembly and maintenance of multiple protein complexes on both sides of this highly specialized intercellular junction. In addition, cellular morphogenesis is required to build the nerve terminal that houses active zones in precise register with corresponding postsynaptic structures. However, as the organism encounters new or changing information, neural networks must adapt to modulate their output. One key adaptive response of the synapse is structural plasticity, allowing synaptic sites to be added or remodeled in order to change their functional properties. Yet the complexity of synapse architecture demands that many cell biological processes are orchestrated in concert by neurons, target cells and neighboring glia, in order to accomplish such change. Although much has been learned about mechanisms that achieve postsynaptic morphogenesis and assembly in response to patterns of neural activity using vertebrate models, we know considerably less about the corresponding process of presynaptic structural plasticity. In this brief review, we will highlight recent advances made in a model excitatory glutamatergic synapse that has offered a useful in vivo platform to discover the machinery that regulates and executes presynaptic development and plasticity: the neuromuscular junction (NMJ) of *Drosophila melanogaster*. The fabric of discoveries from many laboratories reveals that structural plasticity at this synapse is controlled by a complex sequence of factors and communication between the motor neuron and its cellular partners.

The Organization of the *Drosophila* Neuromuscular Junction

The *Drosophila* larval neuromuscular junction (NMJ) is accessible to a powerful combination of sophisticated genetic tools, *in vivo* electrophysiology and imaging [1-4]. In this system, motor axons extend to make synaptic connections in the late embryo; however, due to the rapid growth of the larval stages ("instars"), the NMJ must continue to expand as part of its normal developmental program. During this developmental process, NMJ growth adapts to patterns of neural activity [5, 6]. In the so-called third instar stage, after a period of morphogenetic growth, mature motor terminals branch across their target muscles and form many presynaptic boutons that each house many active zones closely apposed to neurotransmitter receptors (Figure 1A & B). The largest class of boutons (type 1b &1s) are surrounded by a complex in-folded subsynaptic reticulum (SSR) of muscle membranes that extend endfeet rich in ionotropic glutamate receptors (iGluR) juxtaposed to active zones (AZs; Figure 1B). AZs cluster and dock synaptic vesicles (SVs) for evoked or spontaneous release to trigger postsynaptic currents (Figure 2). Interestingly, recent studies suggest that a subpopulation of AZs produce most of the spontaneous or "miniature" excitatory junctional potentials (mEJPs) [7]**, and that mEJPs are the principal drivers of developmental NMJ growth [8]**, however, such release sites may simply reflect different states of AZ maturation or differences in release machinery stoichiometry that could change over time [9].

Structural growth of the larval NMJ involves budding of new boutons, either within or at the termini of the axonal arbor (Figure 1C) [10]. Nascent 1b boutons bud at the SSR perimeter and fill with SVs within minutes of increased stimulation using pulses of high potassium [11-13]**; however, bouton maturation requires subsequent assembly of presynaptic AZs and postsynaptic specializations before boutons can become functional, stable and reach full size (Figure 1D). Postsynaptic multi-subunit iGluR clusters (GluRIIA/B plus GluRIIC-E) are initially populated by GluRIIA containing complexes that desensitize slowly and promote structural synaptic plasticity; over time, GluRIIB subunits compete for occupancy with GluRIIA, and shift the response properties of each synaptic site through regulatory mechanisms local to each site [14]. Bouton retraction and engulfment by muscle or adjacent glia can also prune boutons that fail to stabilize [15].

When an action potential (AP) depolarizes the presynaptic plasma membrane, Ca^{2+}-channels open and Ca^{2+} flows into the nerve terminal to evoke the exocytosis of synaptic

vesicles (SVs). Prior reactions, including docking, priming and fusion of SVs (see below), need to take place for the AZ to attain release competence. To establish tight temporal and spatial control, the release of SVs occurs exclusively at a restricted and highly specialized area of the presynaptic plasma, the above mentioned AZ [16],[17] that is closely and precisely aligned with the post-synaptic density. The AZ membrane confines and clusters Ca^{2+}-channels, function supported by a proteinaceous scaffold (or cytomatrix) decorating this specialized membrane [34, 35] (Figure 2).

Activity Induced Budding of Presynaptic Terminals

The NMJ system has been ideal for systematic identification and analysis of mutations altering synaptic growth and architecture. In fact, mutations in many different synaptic signaling pathways and effector genes have been identified by genetic screens, revealing that synapse morphogenesis requires a complex conversation between motor neurons and muscle that requires a combination of secreted factors (particularly important BMP and Wnt-family members [18, 19]), synaptic adhesion molecule (e.g. FasiclinII, Neurexin and Neuroligan, Teneurins [20-22]), and extracellular matrix [23]. Although activity-dependent control of NMJ morphogenesis was discovered decades ago [5], the advent of a simple acute stimulation paradigm [11]** created an opportunity to study mechanisms required to initiate bouton formation. Moreover, fluorescent imaging of living dissections or intact larvae now provide an increasingly dynamic view of NMJ morphogenesis and the role and nature of cell biological regulations involved, opening a new chapter in investigation of plasticity mechanisms using this model synapse.

Spaced stimulation of the NMJ with pulses of high potassium induces the formation of presynaptic filopodia-like extensions (“synaptopods”) and immature boutons lacking AZs (“ghost boutons” shown during budding in Figure 1D). This growth response requires activity-dependent neuronal exosome release of the Wnt family member wingless (wg) [11**, 24]. Wg triggers distinct canonical and non-canonical signaling responses on both sides of the synapse to promote synapse growth and maturation [11**, 25], with a major effector output of changes in the microtubule cytoskeleton [e.g. [26]]. Interestingly, glial release of Wg into the NMJ is also important, but appears selective to synapse maturation [27]. However, the precise intracellular events that couple synaptic transmission to structural growth are just coming into focus.

Presynaptic Calcium and Bouton Initiation

Calcium and cyclic nucleotide-gated kinases were identified in pioneering studies of the events that trigger synapse plasticity [28]. Consistent with a conserved molecular logic [1,28], protein kinase A (PKA) was recently shown to mediate acute bouton budding in *Drosophila* through the SV-associated substrate protein Synapsin [13]. Synapsin clusters rapidly accumulate at sites of new bouton initiation and are thought to play a key role in trafficking of SVs into nascent boutons before the assembly of AZs. In parallel, PKA phosphorylation of the synaptic vesicle fusion clamp Complexin controls the rate of spontaneous glutamate release, providing an important link between synapse activity and structural expansion of the NMJ [29]. Importantly, the spatial resolution of PKA activation at the NMJ is highly localized even to individual boutons, likely the result of the patterned distribution of phosphodiesterase (PDE) proteins [30]. PKA is also important in the early pruning of exuberant synaptic contacts [31], highlighting the multifunctional impact of synaptic activity.

In addition to PKA, the Camodulin-dependent kinase II (CaMKII), known for some time to regulate postsynaptic NMJ development [32], is also necessary for efficient bouton formation induced by acute stimulation [33]. Although it is not yet clear if this presynaptic function for CaMKII depends on its shared substrate Synapsin, local accumulation of CaMKII in motor terminals after stimulation does require rapid protein synthesis, as observed in mammalian systems [34]. Indeed, CaMKII local translation was shown some time ago to be necessary for memory formation in the *Drosophila* CNS [35], implicating the RNA Interference Silencing Complex (RISC) that mediates microRNA function. More recently, microRNA miR-289 was shown to block CaMKII-dependent bouton budding [33], and is altered in expression in the larval CNS subjected to acute stimulation [36]. In addition to non-coding RNAs, trans-synaptic signaling pathways required for activity-dependent NMJ plasticity are also under translational control of presynaptic RNA binding proteins such as Cup and eIF4E [37]. Cup is thought to restrict presynaptic BMP signaling by repressing translation of BMP modulators such as Endophillin. The BMP pathway can have multiple effects on synaptic development given the many roles of this trans-synaptic pathway (see below). Thus, multiple factors could converge on key mRNAs to tune gene expression in response to changes in neural activity.

Retrograde BMP Signaling Coordinates Presynaptic Structure and Function

Although retrograde synaptic BMP signaling was initially discovered based on static analysis of NMJ and AZ morphology defects [18], recent analysis using acute stimulation revealed that this pathway is essential for initial bouton budding acting largely through the actin cytoskeleton [12]. Bouton initiation also relies on non-canonical LIM-kinase (LIMK) that also controls the ongoing stability of mature boutons [12, 38]. LIMK appears to induce actin remodeling via its substrate Cofilin in concert with the guanine-nucleotide exchange factor (GEF) Trio known to be a downstream output of the canonical BMP signaling pathway [12, 39]. Although during an early (embryonic and first instar) developmental window canonical BMP signaling is required for neurotransmission and later competence for plasticity [40], acute postsynaptic responses govern subsequent release of the BMP-family ligand Glass-bottom boat (Gbb) from muscles, thus linking plasticity to synapse output in space and time. While Gbb is also released via dense core vesicles by nerve terminals, the Gbb co-factor Crimpy (Cmpy) has been shown to keep presynaptic information distinct from the postsynaptic signal [41]**.

To be fully functional, nascent boutons must rapidly mature via assembly of AZs and tightly apposed GluR clusters. Interestingly, a novel form of non-canonical and Gbb-independent BMP signaling appears to drive NMJ maturation by linking local synaptic accumulation and function of the SMAD mothers against Dpp (Mad) directly to the GluRIIA subunit, presumably via direct binding to the type II receptor Wishful thinking (Wit) [42]*. Although the mechanism by which phospho-Mad at the AZ stabilizes expression and clustering of GluRIIA subunits is an open question, the process appears to integrate glial input from the transforming growth factor beta/BMP-family factor Maverick [42**, 43], highlighting the complexity of intercellular interaction to control synapse formation and maturation. As in the case of presynaptic BMP signal transduction, retrograde BMP signaling is also regulated by postsynaptic RNA-binding proteins (RNABP), such as Syncrip/hnRNP Q [44], that offer a means to coordinately tune levels of multiple synaptic effector genes. Interestingly, a notorious translational inhibitory RNABP, the Fragile X Mental Retardation Protein (FMRP), was recently shown act on Wit/BMPR2 receptor expression to regulate signaling via LIMK1, thus suggesting a role of local translation in a conserved link between activity-dependent structural plasticity and disease [45].

New intracellular and extracellular modulators of BMP signaling have been recently defined, raising the question of whether additional components will be essential for acute

activity-dependent NMJ plasticity. For example, immunoglobulin superfamily proteins in the Defective proboscis extension response (Dpr) and Dpr-interacting protein (DIP) families have recently been shown to interact antagonistically with synaptic BMP signaling at the NMJ [46]**. This raises the possibility that NMJ synaptogenesis and plasticity are also sculpted by factors that control target specificity. Moreover, novel intracellular modulators of BMP signaling, such as the Thickvein (Tkv) type I BMP receptor regulators Ube3a (Angelmen Syndrome ubiquitin ligase) and S6 Kinase Like (S6KL) [47, 48], as well as the inner nuclear membrane protein MAN1 [49], may help set presynaptic sensitivity to plasticity signals.

Additional Retrograde Signaling via Syt4 Modulates Synaptic Adhesion

To tightly couple presynaptic remodeling with synaptic transmission, retrograde signals need to be regulated by activity-dependent events such as calcium flux. Much as presynaptic calcium sensors regulate release of neurotransmitters, it turns out that Synaptotagmin 4 (Syt4) functions as a postsynaptic calcium sensor to trigger release of a signal essential for activity-dependent NMJ growth [50]. Although Syt4 is neuronally expressed, the protein is exported to muscle via exosomes [51]. Recent work reveals that Syt4 collaborates with Syntaxin 4 (Syx4) supplied by the postsynaptic cell in order to achieve calcium-dependent retrograde release of the synaptic adhesion molecule Neuroligin 1 (Nlg1) that binds to presynaptic Neurexin 1 [52]. Like Syt4, Nlg1 is required for acute potassium-induced bouton budding, however, Ngl1 released by the Syt4 retrograde pathway triggers only addition of boutons [50], despite the fact that Nlg1 is also known to control AZ assembly [53]. This finding suggests that bouton initiation and subsequent formation of stable sites of AZ and GluR apposition require distinct phases of signaling. However, the precise relationship of Syt4-triggered retrograde vesicular release and Gbb delivery is not yet known.

Although NMJ arbor and bouton architecture is clearly important, these structures as such cannot contribute to synaptic output without AZs and associated GluR clusters. Along with Teneurins and receptor phosphatases [21, 54], Neurexins are key regulators of AZ form and function [55]*. These different classes of cell surface receptors all appear to connect to scaffolding and structural proteins linked to the presynaptic cytomatrix that forms part of the AZ or surrounding structure. Ultimately, progress in understanding structural plasticity executed via AZs is tightly linked to how well we understand the functional organization of AZs per se.

General molecular organization of AZ scaffolds

In fact, the cytoplasm of the presynaptic bouton is populated with several hundred protein species in copy numbers ranging over several orders of magnitude [56]**. Despite this complexity, invertebrate genetic analysis and biochemical analysis predominantly from rodent brains has now converged into the identification of a rather small conserved set of large scaffold proteins that form the core of the AZ scaffold. These canonical protein families are: ELKS/CAST family, RIM-superfamily including the mammalian Piccolo, Bassoon, RIM-BP, (M)UNC-13, Liprin-α and SYD-1 [9, 57-59]. A multitude of techniques, particularly conditional genetics, electron and super-resolution light microscopy have been combined with electrophysiological/biophysical analysis to elucidate the organizational principle of these structures. Analysis is complicated by multiple potentially parallel interactions, a situation which when analysed with loss-of-function genetics results in at least partial functional 'redundancy' between AZ scaffold components thus complicating stringent functional analysis and necessitating the simultaneous manipulations of several genetic loci. Nonetheless, analyses and results from invertebrate [60, 61] and rodent preparations [62]* have increasingly converged into a coherent picture concerning the roles of scaffold proteins. It appears that RIM, RBP and ELKS structurally and functionally interact at AZs, with double mutants between these factors leading to a large absence of active zone organization, in both structural and functional terms [63-65]**. Thus, these large scaffold proteins collectively organize active zones. A major challenge now is to in detail decipher how different domains of these large scaffold proteins intersect and organize the "functionalities" of the SV cycle, including docking, priming and SV fusion.

AZ scaffolds in the control of SV release that couple structural and functional properties

Notably, a novel assay [66]** in which a transgenic Ca^{2+} sensor (GCamp5.0) is positioned just opposite presynaptic AZs now allows the measurement of the likelihood of evoked and spontaneous release at individual AZs of neuromuscular synapses of Drosophila larvae [7, 9, 67, 68]. Using this approach, it was found that the probability of evoked release (in response to a single action potential) directly scales with the levels of the AZ scaffold protein Bruchpilot

(BRP, a member of the ELKS family), and thus also with the ultrastructural size at a single individual AZ level. Thereby, increasing AZ component BRP favored evoked over spontaneous transmission [68]. Similarly, in rat hippocampal neurons, evoked release per AZ scales with the ultrastructural AZ area and the local amounts Ca^{2+} channels as well as the scaffold proteins RIM1/2 and Bassoon [69, 70]. Very recent analysis has further elucidated the underpinnings of why AZ scaffold size controls evoked release. Docking, the targeted SV localization to the AZ plasma membrane, and priming, the maturation of SVs into a readily releasable pool (RRP), were shown to require the neuronal SNAREs Syntaxin, SNAP25 and VAMP2 [71-73]. A conformational change from closed to open Syntaxin required to engage all 3 neuronal SNAREs, is thought to be catalyzed by the essential priming factor Munc/Unc13, thus establishing a RRP and enhancing SV replenishment following exocytosis [72, 74-77]*. To couple SV release to electrical stimulation by APs, Ca^{2+} ions entering the cell through voltage gated Ca^{2+} channels activate the Ca^{2+} sensor Synaptotagmin on the SV to trigger fusion [71]. The efficacy of synaptic transmission largely depends on the distance between SVs and voltage gated Ca^{2+} channels. Close proximity is required for fast and immediate responses, which may require active localization of RRP SVs, a process referred to as positional priming [78-80]. Previous work showed that ELKS/BRP-family proteins, RIMs and RBPs are required to ensure proper Ca^{2+} channel-SV topology, RIM determines Ca^{2+} channel density, and the levels of these proteins predict release at single AZs [60-62, 69, 81, 82]. Recent findings also reveal that a presynaptic pool of the PSD-95 ortholog Discs Large (Dlg) contribute to Ca^{2+} channel localization and the spatial gradient of AZ size [83]. In addition, Liprin-α was shown to organize AZ composition [84-86]. Intravital imaging of the AZ assembly-trajectory of the *Drosophila* neuromuscular junction (NMJ) showed that an early Syd-1/Liprin-α scaffold protein complex initiates AZ-assembly. This protein complex precedes a second one containing BRP and RBP by hours in the assembly process [84, 87].

Recent analysis [65] showed that two distinct scaffold protein complexes define the spatio-temporal organization of two Unc13 isoforms during AZ maturation. Unc13B appears together with the “early” Liprin-α/Syd-1 scaffold and its AZ accumulation is specifically dependent on this scaffold. At mature AZs, Unc13B remains clustered at larger distances from Ca^{2+} channels (>100 nm). Later during the assembly process, Unc13A is positioned and stabilized in discrete clusters via the ELKS/BRP/RBP scaffold in a close proximity to the presynaptic Ca^{2+} channels (<100 nm). In line with a function in molecular priming and vesicle

positioning, we find that Unc13A dominates release not only by enhancing the number of docked and primed vesicles, but also by regulating release probability, latency and sensitivity to Ca^{2+} buffers. These results [65] are in line with two coexisting functional exocytosis pathways with identical Ca^{2+} sensing and fusion mechanism at mature AZs, differentially controlled by two Unc13 isoforms whose precise spatio-temporal positioning within AZ scaffold slots [88] determines AZ maturation and function. Notably, subsequent analysis from rodent synapses also showed that ELKS/BRP-family proteins, RIMs and RBPs collectively organize the localization of the critical munc13 isoform Munc13-1 [63, 64, 89], again emphasizing fundamental conservation of AZ scaffold mechanisms coupling release function with molecular organization. What might the mechanistic rationale in behind the close relation in between scaffold size and function? On the one hand, the AZ scaffold might provide 'SV fusion slots' where close proximity between Ca^{2+} channels and the SVs fusion machinery is established [88, 90, 91]. Additionally, as AZ scaffold components BRP, RIM-BP and RIMs promote Ca^{2+} channels clustering at AZs, the correlation between an AZ scaffold size and evoked release probability might also result from increased Ca^{2+} channels density and thus increased Ca2+ influx at larger AZs.

The AZ scaffold most likely takes active roles in recruiting SVs to the release process, as well as to couple exo- with endocytic activity and thereby to close the SV cycle [92]. Notably, the presynaptic terminal is filled with filamentous material that would appear to limit vesicular diffusion. A recent systematic electron-tomographic analysis [93] started to illustrate how the cooperative attachment and release of three distinct filament types facilitate the movement of SVs to the AZs to become docked in preparation for release. Consistent with direct contacts in between AZ components, a hypomorphic allele of BRP lacking merely the last 1% of the C-terminal amino acids (17 of 1740) still allowed for the formation of AZ scaffolds that were largely bare of SVs [94]. While basal glutamate release was unchanged, paired-pulse and sustained stimulation provoked depression, indicating that the tethering of SVs at the AZ scaffold is important also in functional terms. Interestingly, SVs contacting AZ scaffolds (in this case large ribbon-type AZ scaffolds) could be recently imaged directly [95]. Indeed, using EM tomography, the Piccolo-related scaffolding molecule Fife was recently shown to collaborate with RIM to regulate AZ structure, SV docking and Ca2+ coupling efficiency [96]*.

Evidence of AZ structural plasticity

In the face of these results, the obvious question is whether, and if so, how fast AZs might be able to undergo plasticity processes to change their functional status, and thus to support learning and memory processes. While deeper studies to address the interplay in between AZ architecture and AZ function are needed, several studies have reported dynamic changes of AZs scaffolds operating on the minute timescale in different synaptic preparations, which occur either spontaneously or due to different experimental conditions including reduction of postsynaptic glutamate receptor (GluR) activity or modulation of activity dependent. In cultures of hippocampal neurons, every fifth axospinous synapse spontaneously altered its size by more than 50% during a 30 min time interval, and these changes correlated closely with the equally dynamic alterations of SV release probability For the NMJ synapses of *Drosophila*, pharmacological blockade of postsynaptic glutamate receptors is known to trigger a homeostatic compensation. Already 10 minutes after drug application a significant increase in BRP intensity as a measure of AZ scaffold size was measured, showing that presynaptic scaffolds can change their sizes rather rapidly to mediate presynaptic strengthening [97].

BRP molecule number has been quantified with semi-quantitative super-resolution light microcopy and estimated to be approximately 140 BRP protein monomers per AZ while forming discrete sub-clusters [84, 98]. At the *Drosophila* neuromuscular junction, a spatial gradient of AZ size with larger and BRP enriched presynapses at the distal compared to the proximal end of the terminal correlating with larger and faster distal postsynaptic responses has been described [99]. Finally, a recent study analyzing AZ dynamics at *Drosophila* photoreceptor synapses reported activity dependent changes in scaffold size and composition. Here, prolonged light exposure triggered a disassembly process, which seemingly operates in reverse to the AZ assembly process as previously elucidated by intravital imaging in *Drosophila* larvae [53, 55, 87]. The canonical Wnt pathway and microtubule dynamics in conjunction with the Kinesin-3 motor was implicated in the control of rapid AZ reassembly at photoreceptor synapses [100].

In summary, several recent studies collectively suggest that modulations of the AZ scaffold extent and composition are mechanisms by which the SV release function could be adapted to activity-related or homeostatic demands. These programs seem to span a range of time scales, likely ranging from minutes to days. Future analysis will have to characterize the routes and kinetics of proteins to and from the AZ scaffolds and define the different pools of AZ proteins contributing to scaffold dynamics.

This said, the small GTPase Rab3 was recently implicated in steering the “local dynamics” of AZ scaffolds [101]. Loss of Rab3 provokes a striking misdistribution of scaffold AZ component BRP over the AZs of *Drosophila* neuromuscular junctions. With an elegant experimental setup allowing for temporally restricted re-expression of Rab3 in a *rab3* mutant background, BRP misdistribution could be reverted within 6-9 hours, suggesting a dynamic reshuffling of scaffold material between pre-existing AZs. To execute this function, Rab3 requires GTP-binding and membrane attachment, but surprisingly not the GTP hydrolytic activity, potentially suggesting a Rab3-dependent vesicle docking mechanism in this context [102]. A more recent study also showed that the Rab3 GDP-GTP exchange factor (Rab3-GEF) acts in conjunction with Rab3 to control AZ protein composition [103]. A transgenically expressed GTP-locked variant of Rab3 accumulates at the NMJ at wild-type levels and fully rescues the rab3 mutant but is unable to rescue the rab3-GEF mutant. These results suggest that although Rab3-GEF acts upstream of Rab3 to control Rab3 localization and likely GTP-binding, it also might act downstream to regulate scaffold dynamics, potentially as a Rab3 effector at the synapse. Recent analysis of Unc-104 indicates that this KIF1A MT motor ortholog is required for the delivery of Rab3 to specific synaptic sites [104], thus defining events upstream of this key regulator.

Conclusions and Future Directions

Decades of work have revealed substantial complexity in the intracellular and intercellular mechanisms that orchestrate the adaptive structural plasticity of the synapse. We now appreciate that synapse growth and remodeling involve a sequence of events controlled by multiple highly-conserved signaling pathways linked to neural activity. These pathways are controlled by different cellular participants (presynaptic neurons, target cells and glia), but must ultimately converge to coordinately regulate neuronal morphogenesis and the assembly of active zones. The accessibility of *Drosophila* to a variety of *in vivo* approaches, from physiology to genetics, has made the NMJ a useful platform to identify the machinery necessary for synapse formation, maturation or elimination. Although we are now poised to dissect the underlying mechanisms, very few of the genes know for essential roles in building the synapse have actually been tested for key roles in acute activity-dependent plasticity. This contrast between a rich palette of pathways and components, and a relatively open canvas, predicts a very exciting future of discovery for this model system. As NMJ terminals are unique in their accessibility to imaging, we anticipate that combining intravital and super-

resolution imaging will allow to draw an increasing sophisticated and dynamic image of the molecular trafficking and diffusion controlling synapse assembly and plasticity. In fact, a most recent study [105] allowed to directly measure and accurately quantify the lateral diffusion and trapping of syntaxin1A in nanoclusters which dynamically regulate neurotransmitter release. Thus, it looks that we just begun to exploit the NMJ system to decipher fundamental and profound insights into the dynamic underpinning of synapses directly from the in vivo situation.

Figure Legends

Figure 1. Overall Architecture of the Drosophila Neuromuscular Junction

(A) A view of NMJs on the ventral longitudinal muscles (M7, M6, M13 and M12) shows an overview of branched motor neuron arbor (green) studded with boutons (arrows); boxed regions correspond to magnifired views in panels B and C. **(B)** Each large type 1b bouton is embedded within a complex folded subsynaptic reticulum (SSR, purple) of muscle membrane. SSR endfeet appose presynaptic active zones (AZ, green) that cluster synaptic vesicles (SVs); mitochondria (mt) also fill the nerve terminal along with other membranous organieeles and protein complexes (not shown). **(C)** During NMJ growth, nascent boutons are added interstitially or at terminal boutons through a process of budding. Underlying microtubule bundles are highlighted in bright green. **(D)** The process of terminal bouton budding and maturation occurs in stages and can be triggered by acute bursts of synaptic activity or developmental signaling. Phenotypic endpoint analysis can distinguish effects on the bouton maturation process, leaving persistent immature boutons that lack AZ/iGluR sites (“ghost boutons”; upper right) or boutons that fail to grow to normal size (“satellite boutons”; not shown), or effects on the pruning process that leave temporary remnants of the postsynaptic cytomatrix (“footprints”; lower left).

Figure 2. Cartoon Schematic of Mature Active Zone Organization at the NMJ

(A) A transmission electron micrograph of a type 1b bouton (green) shows a site of synaptic neurotransmitter release, or active zone (AZ, red box inset B corresponds to cartoon in panel B); the t-bar structure is marked with an asterisk. Scale bar is 50nm. **(B)** Each AZ is formed through assembly of multiple synaptic scaffolding proteins required for recruitment of glutamate-filled synaptic vesicles (SVs). AZ formation is a sequential process. Initially, Liprin-

α and Syd-1 [blue and pink wedges] associate with Unc13B [tangerine sickle] and voltage-sensitive calcium channels [yellow] containing the Cac subunit, to form a weak release site where GluRIIA-containing receptor complexes begin to accumulate. As AZ formation continues, BRP [pink sickle] and Unc13A complexes are inserted to form large clusters that define the ultrastructural T-bar that is observed in the mature AZ. As postsynaptic receptor clusters consolidate, GluRIIB subunits accumulate, and the efficiency of the synapse increases.

Figure 3. Unc13A and B localize in distinct distances from the presynaptic Ca2+ channels

(A,B) Two-color super-resolution ("STED") images are shown of individual planar AZs from 3rd instar larvae of the genotypes stated stained with the indicated antibodies. (A,B) Unc13A is localized in close proximity to motoneuronal overexpressed Cac^{GFP}, surrounding it (A) while Unc13B localized in a larger distance (B). **(C)** Mean intensity profile of Cac (gold), motoneuronal overexpressed $Unc13A^{GFP}$ (red) or $Unc13B^{GFP}$ (blue) immunoreactivity plotted from the center of the AZ (the BRP signal was used as a reference, with the center of the BRP ring set to zero). The intensity maximum of the average fluorescence profile was found 30 nm from the AZ center for Cac, 60 nm for $Unc13A^{GFP}$ and at 120 nm for $Unc13B^{GFP}$.

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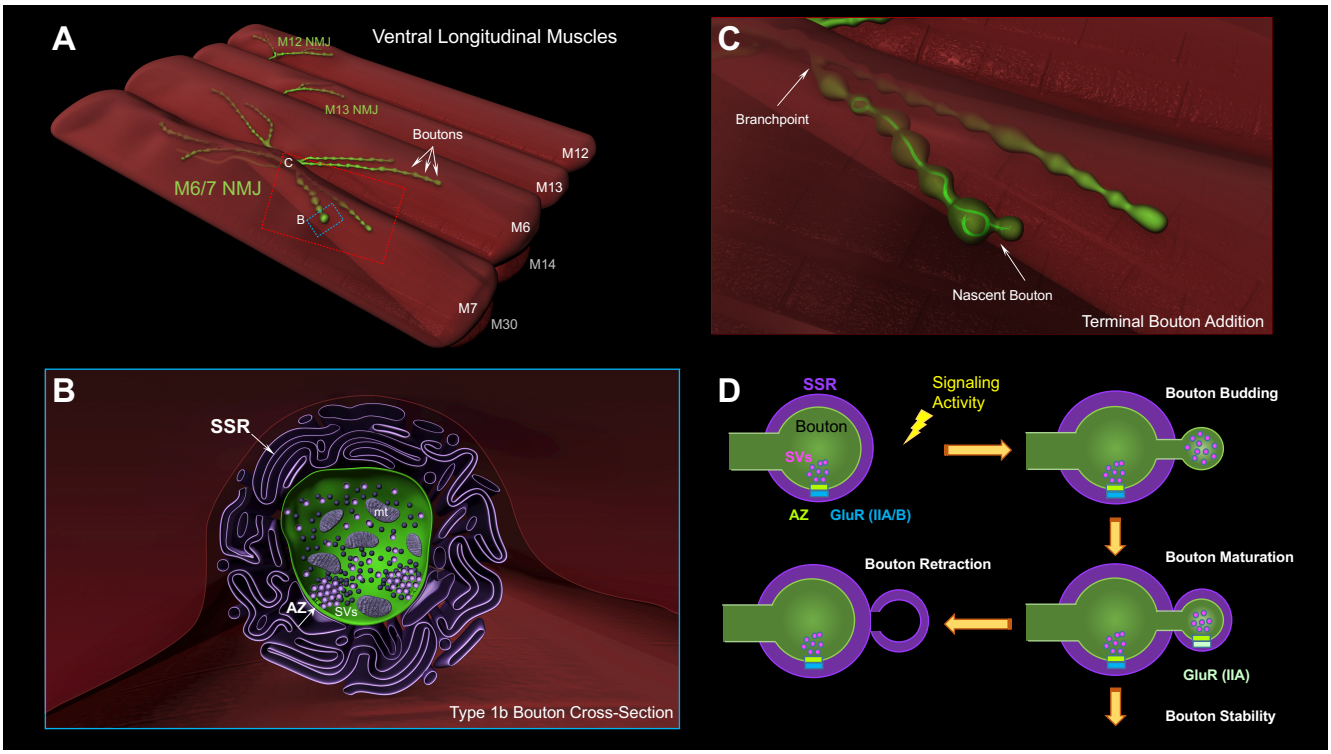
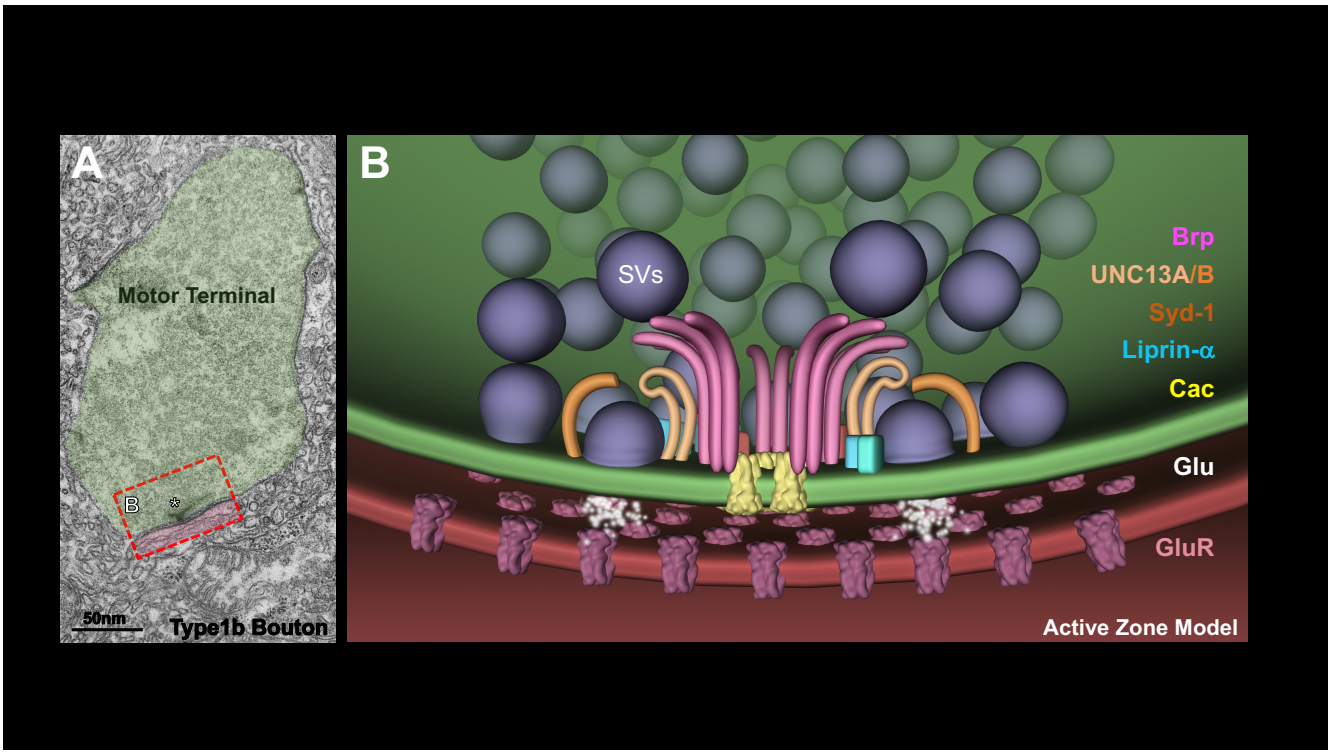

A
Ventral Longitudinal Muscles
M12 NMJ
M13 NMJ
Boutons
M12
M13
M6/7 NMJ
C
B
M6
M14
M7
M30
C
Branchpoint
Nascent Bouton
Terminal Bouton Addition
B
SSR
mt
AZ
SVs
Type 1b Bouton Cross-Section
D
SSR
Bouton
SVs
AZ
GluR (IIA/B)
Signaling Activity
Bouton Budding
Bouton Maturation
GluR (IIA)
Bouton Stability
Bouton Retraction

Figure 1


A
Motor Terminal
B
50nm
Type1b Bouton
B
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Syd-1
Liprin-α
Cac
Glu
GluR
Active Zone Model

Figure 2

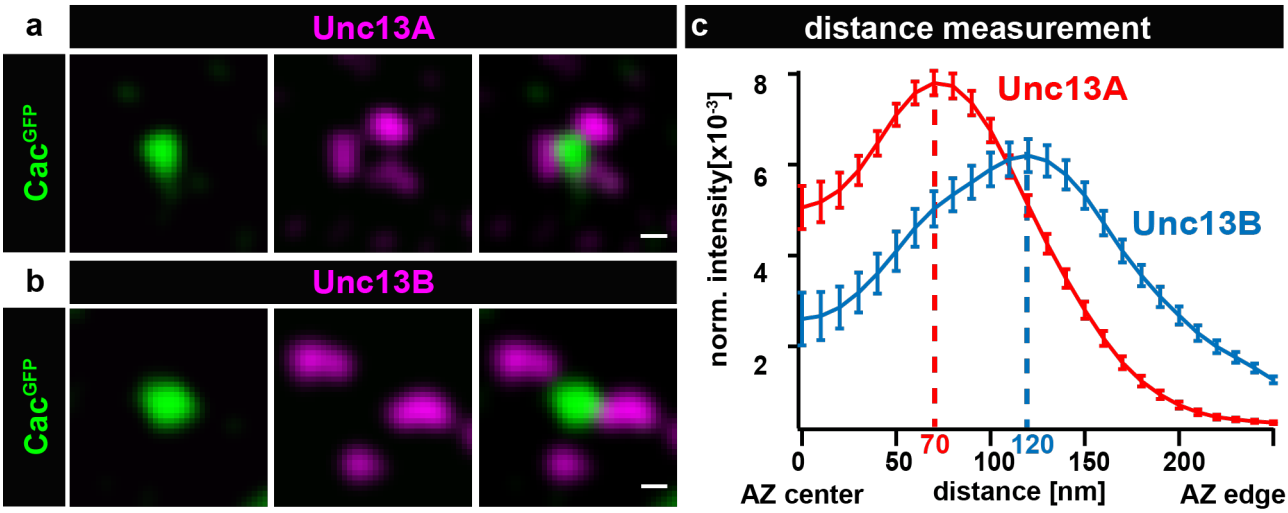
a
Unc13A
Cac^GFP
b
Unc13B
Cac^GFP
c
distance measurement
norm. intensity[x10^-3]
8
6
4
2
Unc13A
Unc13B
0
50
70
100
120
150
200
AZ center
distance [nm]
AZ edge

Figure 3