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Expanding views of presynaptic terminals: new findings from *Caenorhabditis elegans*

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Abstract

The unique ability of chemical synapses to transmit information relies on the structural organization of presynaptic terminals. Empowered by forward genetics, research using *Caenorhabditis elegans* has continued to make pivotal contributions to discover conserved regulators and pathways for presynaptic development. Recent advances in microscopy have begun to pave the path for linking molecular dynamics with subsynaptic structures. Studies using diverse reporters for synapses further broaden the landscape of regulatory mechanisms underlying presynaptic differentiation. The identification of novel regulators at transcriptional and post-transcriptional levels raises new questions for understanding synapse formation at the genomic scale.

Introduction

A presynaptic terminal is defined as a specialized region of axons, where synaptic vesicles (SVs) cluster and surround presynaptic dense projections to form the active zone (AZ). Presynaptic differentiation occurs frequently as a result of the intrinsic properties of active zone proteins and vesicular intermediates at defined locations in axons [1]. Hundreds of proteins are estimated to be present in presynaptic compartments [2,3]. The longstanding questions remain to be how different components assemble into orderly organized entities at presynaptic terminals. What holds SVs and AZ proteins in microspace? How SV and AZ proteins interact with each other during transport and assembly? What mechanisms control the amount of SV and AZ proteins? Genetics-driven functional studies in *Caenorhabditis elegans* have identified rate-limiting factors and provided critical insights into important pathways. In this mini-review, we highlight new findings that address unique aspects of presynaptic development. For additional information on related topics, we recommend several excellent reviews [4–6].

Towards an ultraview of synapses: insights from new microscopy

Synapses are rich in molecules and organelles, which are organized into orderly but finite structures that are, unfortunately, below the resolution limit for conventional fluorescent microscopy. In the past few years, breakthroughs in super-resolution microscopy have begun

to overcome the optic limitations [7]. These include STED (stimulated emission depletion) microscopy that utilizes a second red-shifted laser beam for de-excitation to achieve sub-100 nm resolution, and single-molecule imaging technology such as PALM (photoactivation localization microscopy) and STORM (stochastic optical reconstruction microscopy) that rely on photoactivatable fluorescent proteins or fluorophores. Application of super-resolution microscopy to synapses is beginning to provide us thrilling views of synaptic molecules in their native settings. An example is the analyses of the *Drosophila* Bruchpilot, a divergent member of the conserved active zone ELKS proteins, using STED microscopy. The high-resolution images reveal that Bruchpilot takes on an extended conformation with its N-terminus close to voltage-gated calcium channels and its C-terminus extending away from AZ, towards SV clusters [4]. Concomitant with these advances is the question whether the super-resolution imaging methods can be used to analyze the dynamics of presynaptic components. A study using FIONA (fluorescence imaging with one-nanometer accuracy) reports the detection of moving puncta of fluorescently tagged ELKS-1 proteins with sub-10 nm in 5 ms resolution in live *C. elegans* [8•]. The versatility of transgenic labeling and transparency of *C. elegans* make this organism a favorite concept-proof test ground for new methodologies.

Nevertheless, understanding synaptic cellular structures remains to be the task of ultrastructural analyses using electron microscopy (EM). Instantaneous fixation by high pressure freezing (HPF) followed by freeze substitution allows better preservation of molecules, organelles and cytoskeletons [9]. Combined with EM tomography technology, Stigloher *et al.* [10•] recently reported a three dimensional super-view of presynaptic architecture. SVs are connected with one another by 25-nm long filamentous structures called connectors. Docked SVs are positioned to AZ through additional fine filaments that emanate radially from a compact core structure of dense projections. Null mutants for AZ proteins UNC-10/RIM or SYD-2/Liprin- α show reduced evoked vesicle release and a reduction of the number of SVs contacting dense projection filaments, supporting a role of these two proteins in tethering of docked SVs to AZ. Similar filamentous network of connectors has also been described in mammalian CNS synapses [11]. Future research will uncover the molecular basis of connectors and the formation of presynaptic filamentous network.

Fluorescent labeling has revolutionized the way by which we understand the relationship between molecules and structures. However, a technical hurdle is to preserve fluorescence from labeled molecules under ultrastructural fixation conditions. A breakthrough came from a study by Watanabe *et al.* [12••]. They painstakingly optimized fixation conditions and embedding plastics, and developed a compromised condition that allowed them to successfully combine super-resolution light microscopy with scanning EM. By aligning images of fluorescent particles with synapse ultrastructures, they mapped the SYD-2/Liprin- α signals from PALM and STED microscopy to the dense projection (Figure 1(a)–(a'')). Another promising reagent that has the property to be observed directly for light microscopy and EM is a genetically encoded singlet oxygen generator called miniSOG [13••]. MiniSOG is a flavin-binding fluorescent protein of 106 amino acids, generated through a mutagenesis screen of the LOV (light, oxygen, and voltage) domain of Arabidopsis phototropin. Upon blue light illumination, miniSOG produces green fluorescence and also quantum yield of singlet oxygen that can convert diaminobenzidine into electron dense precipitates detectable by EM. MiniSOG-tagging is feasible in intact *C. elegans*, and has helped to resolve the precise localization of SynCAM1 and SynCAM2 at synaptic junctions in tissues of mouse brain. Transgenic *C. elegans* expressing tagged molecules at endogenous levels can now be readily generated using MosSCI insertion method [14]. It is optimistic that an ultraview of the subcellular localization of major synaptic proteins can be achieved in the near future.

Novel mechanisms for localization of AZ proteins

AZ formation is essential for ensuring the tight organization of SVs in close proximity to voltage-gated calcium channels (VGCCs) and plasma membrane. Fluorescent labeling of SV proteins in *C. elegans* has proven to be tremendously successful in uncovering the mechanisms underlying synapse formation, and has led to the identification of key regulators of AZ assembly [15,16]. AZ proteins exhibit remarkable amino acid sequence and function conservation. A general consensus is that biogenesis and localization of AZ proteins are largely distinct from those of SV proteins. However, little is known about how they are targeted and localized to presynaptic terminals. Two genetic screens in *C. elegans* directly probed into the mechanisms underlying clustering of VGCC and AZ proteins at presynaptic sites.

The presynaptic pore-forming $\alpha 1$ subunit of VGCC is encoded by the *unc-2* gene [17]. Saheki and Bargmann [18••] generated a functional GFP-UNC-2 transgene and isolated mutants that show decreased synaptic GFP-UNC-2, and increased somatic expression. Importantly, in these mutants other AZ proteins and SV markers have normal synaptic expression. These mutants affect UNC-36, the $\alpha 2/\delta$ subunit of VGCC, and a novel gene named CALF-1 (calcium channel localization factor-1). CALF-1 is a type I transmembrane protein and localized at somatic endoplasmic reticulum (ER); and UNC-36 also shows ER localization, in addition to axonal expression. In *calf-1* mutants the somatically retained GFP-UNC-2 colocalizes with ER markers. In a pulse-chase experiment using a photoconvertible DENDRA2-UNC-2 reporter the authors show that transient expression of CALF-1 in *calf-1* mutants can induce pre-existing UNC-2 to translocate from soma to synapses. These observations demonstrate that ER exit plays a direct role in synaptic delivery of UNC-2. CALF-1 contains multiple Arginine-based ER-sorting motifs. Similar motifs are present in a large number of ion channels and known to regulate ER exit of channels [19,20]. However, the trans-configuration of these motifs in CALF-1 implies a mechanism analogous to ER exit of major histocompatibility complex (MHC). Studies of AZ protein assembly in vertebrate synapses have identified an obligated Golgi assembly for their delivery to synaptic terminals [21]. The ER regulation of UNC-2 via CALF-1 and UNC-36 adds a new angle to our understanding of AZ protein biogenesis and synaptic delivery (Figure 2).

The notion that synaptic localization of AZ proteins involves distinct regulatory mechanisms is also echoed in studies using GFP-SYD-2. Loss of function of *syd-2/Liprin- α* causes a diffuse localization of SVs, while gain of function of *syd-2* promotes presynaptic assembly [22–24]. In a large visual screen, the Zhen group isolated mutants that specifically alter GFP-SYD-2 and other active zone proteins, without apparent effects on SVs [25]. One gene encodes the UNC-7 innexin [26••]. Innexins are multi-transmembrane proteins homologous to mammalian pannexin and form gap junctions in invertebrates. At the ultrastructural level, synapses in *unc-7* mutants have an overall increased volume of AZ per synapse, comparing to wild type. *unc-7* is required in presynaptic neurons during early phase of synaptogenesis. In mature synapses UNC-7 and its cognate partner UNC-9 co-localize near chemical synaptic regions where no gap junctions are present. It is possible that transient gap junctions could be present at earlier stages, as studies in other organisms have shown electrical coupling via gap junctions precedes formation of chemical synapses [27]. Alternatively, the role of UNC-7 in AZ formation may be independent of the establishment of gap junctions, as some gap junction proteins have been shown to act as adhesion proteins in neuronal migration [28,29]. Pannexins can form hemi-channels to control flux of ions and second messengers. Interestingly, the AZ defects in *unc-7* are suppressed by loss of function in *nca-1*, a voltage-gated cation leak channel that regulates neuronal excitability [30]. Thus,

these two genetic screens have revealed surprising roles of previously unknown pathways in the multi-layered regulation for AZ components (Figure 2).

New findings on biogenesis and transport of presynaptic vesicles

Vesicular cargos with distinct biochemical and ultrastructural properties are reported to carry SV or AZ proteins [21,31,32]. SV proteins are mostly associated with clear core vesicles, often called synaptic vesicle precursors (SVPs), which are primarily transported by the KIF1A/UNC-104 Kinesin-3 family [33,34]. AZ proteins are associated and trafficked in vesicles of dense core appearances. Recent findings in *C. elegans* have uncovered an unexpected regulatory role of AZ proteins in SVP transport. Klassen *et al.* [35••] identified an Arf-like small G protein, ARL-8, loss of function in which causes a systematic mislocalization of SV and AZ proteins to asynaptic segments of the axons in DA9 motor neurons. Through correlative EM analyses, they find that the ectopic SV protein puncta contain large clusters of clear core vesicles and AZ-like structures without apparent postsynaptic structures. By live imaging, they further reveal that although in *arl-8* mutants the velocity of antero- and retrograde transport is unaltered, the incidents of moving particles are significantly reduced, accompanied with an increase in the size of stable particles. These data indicate that ARL-8 normally suppresses aggregation of transporting vesicles. Interestingly, the SVP aggregation defects are suppressed by loss of function in several AZ proteins including SYD-2/liprin- α , implying a close crosstalk between SV biogenesis and trafficking with those of AZ proteins (Figure 2). SYD-2 has been shown to bind UNC-104/KIF1A through coiled-coil and SAM domains [36]. In *syd-2* mutants UNC-104-GFP particles show an excessive retrograde movement, due to a reduced net anterograde movement and velocity. Binding of SYD-2 to UNC-104 may enhance the clustering of UNC-104 on the SVP surface to promote anterograde transport of SV cargos (Figure 2).

In addition to the core components, presynaptic terminals also contain another morphologically distinct population of vesicles, in general called dense-core vesicles (DCVs). DCVs are 60–80 nm in diameter, and present in a distributed manner throughout presynaptic terminals, notably at a distance away from the dense projection of the AZ. DCVs carry neuropeptide transmitters, but are highly heterogeneous in content. A common way to visualize DCVs is by tagging proneuropeptides [36,37]. Recent studies have revealed that the Rab2 small GTPase encoded by the *unc-108* gene plays a key role in DCV maturation [38,39]. UNC-108/RAB-2 localizes at the Golgi apparatus in cell bodies. In wild type animals DCV markers are mostly overlapped with the early endosomal markers in soma, while in *unc-108/rab-2* mutants, they largely overlapped with markers for late endosomes and lysosomes. Moreover, in axons loss of function of *unc-108/rab-2* does not change the number of DCVs, but appears to reduce the amount of neuropeptides packaged in DCVs. UNC-108/RAB-2 recruits RIC-19, the *C. elegans* orthologue of the human diabetes auto-antigen ICA69 [39] (Figure 2). Although it is not yet clear whether DCVs directly contribute to the formation of presynaptic terminals, these findings begin to provide a glimpse of the distinct mechanisms underlying DCV biogenesis.

Diverse regulatory roles of microtubules at presynaptic terminals

Remodeling of cytoskeleton precedes the formation of presynaptic terminals, and is required for synapse maintenance. Much has learned about the actin cytoskeleton in SV dynamics [40], while microtubules (MTs) have received lesser attention, partly because classical EM fixation preserves few MTs in synaptic boutons. The improved fixation by high pressure freezing has made MT abundantly visible in synapses [9]. Do MTs contribute to synapse formation or simply act as highways for trafficking? A series of recent studies have begun to

reveal surprisingly diverse effects of MT cytoskeleton in presynaptic development and signaling.

Tubulins have a conserved globular structure with 12 helices (H1–H12) [41,42]. A gain-of-function mutation in an α -tubulin, TBA-1, alters an invariant glycine to arginine in the C-terminal H11-12 loop, and causes a reduction of synapse number and irregular morphology and size of synapses in motor neurons [43]. Interestingly, a mutation in the same loop of human α -tubulin (TUBA1A) has been reported to cause defects in neuronal migration and axon tract formation [44]. The H11–12 loop is in general thought to provide a binding surface for MT-associated proteins and motors [45]. It remains to be tested whether the mutant tubulins affect trafficking specificity, such as cargo and motor interactions.

Tubulins are highly abundant and in general perceived as less likely to be rate-limiting in synapse development. By examining mechanosensory neuron (TRN) development and function, Bounoutas *et al.* uncovered an intriguing interaction between tubulins and protein ubiquitination. An F-Box protein MEC-15 is required for both proper localization of a SV marker and mechanosensation [46•]. The organization of MT cytoskeleton and axonal transport is grossly normal in *mec-15* mutants. Remarkably, the synaptic defects in *mec-15* mutants are completely suppressed by eliminating one copy of a β -tubulin gene, but dominantly enhanced by an α -tubulin mutation that reduces MT acetylation. It is not yet known whether MEC-15 directly regulates the abundance of tubulins. The conserved RPM-1/Ring-finger and FSN-1/F-box E3 ligase also regulates TRN synapse development [47]. However, *mec-15* genetically synergizes with *rpm-1*, indicating that these two E3 ligases act in parallel [46•].

Extensive post-translational modifications of MTs have been implicated in the versatile regulation of their dynamic states. An interesting study reports that colchicine treatment to *C. elegans* causes destabilization of acetylated MTs in TRN, and leads to a systematic reduction of levels of proteins that function in mechanosensory transduction [48]. Using a cleverly designed protein stability reporter, the authors further find that this colchicine-induced effect depends on the DLK-1 MAP kinase pathway. DLK-1 is the substrate for RPM-1 E3 ligase, and elevated activity of DLK-1 causes abnormal synapse development [49]. These new observations strengthen the important roles of protein ubiquitination in synapses, and also raise our attention to how regulation of MT cytoskeleton dynamics is coupled with synapse organization pathways.

New regulators of gene expression in presynaptic development

Synapse formation occurs rapidly and involves a large number of proteins. Transcriptional and translational controls ensure appropriate amount of synaptic proteins for building synapses [50,51]. New studies from *C. elegans* have uncovered previously unknown regulators, and expanded our knowledge of gene regulatory themes in synaptogenesis.

The conserved single-stranded DNA-binding proteins (SSDPs) are binding partners of LIM-homeodomain proteins, and previously known to regulate axis formation in *Xenopus* [52,53], wing development in *Drosophila* [52,53], and neural patterning and axonal projections in zebrafish [54]. A study by Zheng *et al.* [55•] recently revealed an important role of *C. elegans* ortholog of SSDP, SAM-10, in TRN development. In *sam-10* mutants synaptic varicosity of TRN is overextended, but with underdeveloped synaptic morphology. Additionally, the position of synaptic branches is misplaced. Interestingly, SAM-10 proteins are initially localized in the cytoplasm in early embryo, and are translocated into the nucleus before formation of synaptic branches. Loss of function of *C. elegans* Lim-domain-binding-protein, LDB-1, phenocopies *sam-10* mutants, and prevents SAM-10 from translocating into the nucleus (Figure 2). A transcriptional target of SAM-10/LDB-1 is PRK-2, an ortholog of

mammalian Pim kinases, and appears to function in synaptic branch development. The roles of SAM-10/LDB-1 in presynaptic terminal differentiation may involve additional novel target proteins.

Nuclear pre-mRNA 3'-end processing is a major event coupled with transcription. It determines the generation of the 3' untranslated region (UTR) and is vital the production of mature mRNA [56]. A genetic enhancer screen in *rpm-1* mutants uncovered a novel nuclear protein, SYDN-1 (Synaptic defective enhancer-1), studies of which led to the discovery of an unexpected regulatory role of nuclear pre-mRNA processing [57•]. *sydn-1* mutants display mild abnormality in axon and synapse morphology, but exhibit striking genetic synergy with numerous synapse development pathways. To understand the function of SYDN-1, the authors carried out a genetic suppressor screen of *sydn-1* and isolated a mutation in the gene encoding polyadenylation factor subunit-2 (PFS-2), a conserved member of pre-mRNA 3'-end processing machinery. Inactivation of other conserved 3'-end processing factors results in similar suppression effects, supporting a conclusion that SYDN-1 is a general repressor for nuclear pre-mRNA 3'-end processing (Figure 2). One function of SYDN-1 appears to regulate subnuclear pattern of pre-mRNA processing factors. The finding of SYDN-1 adds to an increasing list of RNA-binding proteins in presynaptic development and function, including the CELF/BRUNOL protein UNC-75 and the novel RNA-binding protein SYD-9 [58,59]. Interestingly, each of these RNA binding proteins shows specific synergistic genetic interactions with pathways that affect different aspects of presynaptic development or transmission. These findings show that complex parallel signaling pathways operate at the level of pre-mRNA regulation. A challenge will be to define the unique pools of RNA targets using modern genomic approaches.

Conclusion

The expanding technology in microscopy has provided us unprecedented views of presynaptic terminals. Discovery of new structures and molecular pathways continues to raise questions for a deeper understanding of the regulation of synapse formation. With the new imaging tools and the expansion of genomic information, challenges remain to elucidate how common pathways underlie the diversity of synapses and how regulation of synapses can respond to physiological stimuli in temporal and spatial precision. The rich resources of *C. elegans* genetics with the added advantage of *in vivo* imaging of synapse structure and activity in intact animals offer ample opportunities to uncover the missing links in our knowledge.

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Figure 1.

Towards an ultraview of presynaptic structures in *C. elegans*.

(a)–(a''): Localization of the active zone protein, SYD-2/Liprin- α by super-resolution fluorescent imaging on electron micrographs. STED image (a), (a') and (a'') of a thin section (70 nm) from a worm expressing SYD-2/Liprin- α -Citrine was overlaid with the EM image (a), (a') and (a'') from the same section (a), (a') and (a''). Scale bar 500 nm. Images are adapted with permission from Watanabe *et al.* [12••].

Figure 2.

Schematic summary of multiple steps coordinating the formation of presynaptic terminals. SAM-10 and SYDN-1 localize in the nucleus and control gene expression at the transcription and pre-mRNA 3' end processing steps. A novel protein CALF-1 and the VGCC $\alpha 2/8$ subunit UNC-36 act as cofactors in ER to gate the sorting or exit of the pore-forming calcium channel UNC-2 to presynaptic terminals. AZ proteins are probably preassembled in the Golgi apparatus. Biogenesis and maturation of DCVs require UNC-108/RAB-2 and RIC-19, respectively. Transport of SVPs is regulated by the ARF like protein ARL-8, and SYD-2 acts as an adaptor to link SVPs to the motor protein UNC-104. Two innexins, UNC-7 and UNC-9, co-localize near chemical synapses and regulate AZ formation.