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Synaptogenesis: unmasking molecular mechanisms using *Caenorhabditis elegans*

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Abstract

The nematode *Caenorhabditis elegans* is a research model organism particularly suited to the mechanistic understanding of synapse genesis in the nervous system. Armed with powerful genetics, knowledge of complete connectomics, and modern genomics, studies using *C. elegans* have unveiled multiple key regulators in the formation of a functional synapse. Importantly, many signaling networks display remarkable conservation throughout animals, underscoring the contributions of *C. elegans* research to advance the understanding of our brain. In this chapter, we will review up-to-date information of the contribution of *C. elegans* to the understanding of chemical synapses, from structure to molecules and to synaptic remodeling.

Keywords: synapse, *Caenorhabditis elegans*, review

Introduction

Chemical synapses are remarkable microstructures that not only support directional transfer of information between excitable cells but also individually process this information. Hence, synaptic dysfunction causes a wide range of neuropsychiatric disorders in humans. This chapter aims to provide accurate and up-to-date accounting of the *Caenorhabditis elegans* contribution to the understanding of chemical synapses, from structure to molecules and to remodeling. We will first emphasize unique and attractive features of *C. elegans* that account for its power to investigate synapse biology, especially using genetic strategies. We will then review key findings using the well-studied neuromuscular junctions as examples, further expand to mechanisms governing synapse-type specificity, and finally touch upon synapse maintenance and remodeling. Because of space limitation, we recommend highly informative reviews on related topics, such as axonal trafficking and synaptic vesicle dynamics (Richmond 2005; Blazie and Jin 2018; Gan and Watanabe 2018; Hendi *et al.* 2019), and biology of electrical synapses (Hall 2019; Jin *et al.* 2020).

Interrogating synapse biology: why *C. elegans*?

Synapses likely emerged early during evolution in the common ancestor of cnidarians and bilaterians more than one billion years ago (Emes and Grant 2012; Arendt 2020). Although evolution yields to striking diversification and increased complexity of animal nervous systems, the basic molecular composition and function mechanisms of synapses remain remarkably stable. Hence, it is possible to interrogate synapse structure and function in simple organisms, including *C. elegans*.

Among the criteria contributing to the visionary choice of Sydney Brenner in pushing *C. elegans* as a model organism were its anatomical simplicity, its invariant development, its amenability to electron microscopy (EM), and a life cycle compatible with high-throughput genetics. These features are especially salient when exploring synaptic biology. First, the pioneering serial EM reconstruction of adult *C. elegans* provided a comprehensive picture of its neuronal network (White *et al.* 1986). This heroic work revealed that individual neurons (302 in the adult hermaphrodite) were connected by relatively few synapses (less than 10,000 total, including neuromuscular junctions) and that connectivity was mostly reproducible among individuals of the same genotype. This opened a unique opportunity to identify specific synapses in a living animal and to quantitatively measure the impact of genetic perturbations on synapse formation and maintenance. The emergence of higher throughput techniques in EM image acquisition and computation enabled the connectome reconstruction of several individuals at distinct stages, marking a new era for the analysis of the *C. elegans* nervous system at single synapse resolution (Emmons *et al.* 2021; Witvliet *et al.* 2021). Second, because feeding and reproduction require little contribution of the *C. elegans* nervous system under laboratory conditions, mutants with severely impaired synaptic function can be propagated with ease, allowing thorough anatomical and physiological analysis. Recent advances in genetic toolkits, such as genome editing, provide means to fully inactivate synaptic transmission in specific

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neuron classes to analyze their intrinsic properties and their contribution to network functions. Third, elegant genetic strategies have been developed by the *C. elegans* research community to interrogate almost any biological question in the living organism. Because synaptic dysfunction translates into a wide range of behavioral defects, the very initial screens conducted by Sydney Brenner for "abnormal worms" already identified important genes involved in synaptic development and function (Brenner 1974). Further screens based on specific behaviors, sensitivity to drugs, or direct visualization of synaptic components uncovered many additional synaptic players. The recent development of super-efficient genome engineering strategies based on the CRISPR–Cas9 system now enables fast investigation of specific genes in synaptic function. Complementary to genetic tools, implementation of electrophysiology was critical for the analysis of synaptic function in wild type and mutants, initially at neuromuscular junctions (Richmond *et al.* 1999) and later at neuro-neuronal synapses (Liu *et al.* 2017). Parallel development of opto- and chemo-genetic tools to control neuronal activity provides efficient means to explore activity-dependent plasticity (Emmons *et al.* 2021). Altogether, *C. elegans* provides an extremely efficient system for sophisticated investigation of the biology of the synapse in vivo.

Anatomy of *C. elegans* synapses

Contribution of technological innovations in EM

Historically, EM was instrumental in solving synaptic organization, especially in *C. elegans*, because the size of the different synaptic compartments is smaller than the resolution limit of standard light microscopy. Initial work used chemical fixation of the worms. The quality of the ultrastructure allowed the description of synapses and the reconstruction of the nervous system connectivity (White *et al.* 1986). Amazingly, data acquired in the 70's were recently reanalyzed, in combination with image digitalization and reconstruction software, to generate landmark descriptions of the male *C. elegans* "connectome" almost 50 years after the initial acquisition of the micrographs! (Jarrell *et al.* 2012; Cook *et al.* 2019).

However, due to the impermeability of the worm's cuticle, classical chemical fixation EM techniques suffer some limitations because fixation is slow and the use of hyperosmotic buffers sometimes introduces neurite shrinking. As a more recent alternative, high-pressure freezing (HPF) enables the physical immobilization of an entire living adult in amorphic ice within milliseconds. Water is subsequently substituted by organic solvents at low temperature, followed by resin infiltration (McDonald 1999). Rapid physical immobilization minimizes the structural rearrangement that can occur at synapses during fixation (Rostaing *et al.* 2004; Weimer *et al.* 2006). HPF is now routinely used for synaptic analysis (Fig. 1a) (Mulcahy *et al.* 2018; Emmons *et al.* 2021; Witvliet *et al.* 2021) (Fig. 1a).

In addition, coupling optogenetics with HPF provided a means to capture very early synaptic events in intact animals, a few milliseconds after neuronal stimulation. This enabled the visualization of synaptic vesicle (SV) fusion and the discovery that SV fusion triggers ultrafast endocytosis at active zones, enabling SV recycling at the millisecond scale (Watanabe *et al.* 2013).

Analysis of the morpho-functional organization of synapses often necessitates its volumetric reconstruction. It usually relies on the imaging of serial ultrathin sections. While the x-y resolution reaches a few nanometers the z resolution is limited by the thickness of the sections (≈50 nm). Isometric visualization of *C. elegans* synapses with nanoscale resolution was provided by electron tomography (Weimer *et al.* 2006; Kittelmann, Hegermann, *et al.* 2013) (Fig. 1b and c). The more recent implementation of FIB-SEM (focused ion-beam coupled with scanning EM) technique to visualize the *C. elegans* nervous system now provides a means to generate large neuronal volumes with isotropic nanoscale resolution (Britz *et al.* 2021).

Ultrastructure of *C. elegans* synapses

In *C. elegans*, synapses are made "*en passant*" between adjacent processes, as is also frequently seen in the mammalian brain. Swelling of the presynaptic neurite forms the presynaptic bouton where synaptic vesicles (SVs) accumulate. Classical neurotransmitters are contained in clear SVs of about 40 nm in diameter, interconnected by a meshwork of filaments. Larger dense-core vesicles (DCVs) that contain neuropeptides and proprotein-processing enzymes are present at the periphery of the boutons. A track of microtubules and a narrow endoplasmic reticulum (ER) cisterna run along the entire length of the neurite and localize at the periphery of the bouton (Rolls *et al.* 2002; Weimer *et al.* 2006) (Fig. 1). Overall, the size of the presynaptic regions varies considerably among neurons and during development (Cook *et al.* 2019; Witvliet *et al.* 2021).

At classical synapses, neurotransmitter is released at the active zone (AZ), the specialized area of the presynaptic bouton where vesicles fuse with the plasma membrane. Electron-dense material, called the "dense projection" (DP), sits at the center of the AZ and is commonly used as a landmark to identify synapses. DPs are composed of core modules of about 50 nm wide by 30 nm high that assemble in branched structures between 100 and 400 nm in length oriented along the axis of the neurite (Kittelmann, Hegermann, *et al.* 2013). Filaments radiating from the core structure contact SVs docked at the plasma membrane and in the interior of the bouton (Weimer *et al.* 2006). The DP contains scaffolding molecules, such as SYD-2/liprin, UNC-10/Rim, CLA-1/Clarinet1 (see below), that are involved in the organization of the presynaptic bouton. DPs represent the functional equivalent of the electron-dense structures found at synapses in other species such as the presynaptic grid in mammals or the T-bar in Drosophila (Wichmann and Sigrist 2010; Siksou *et al.* 2011). At neuromuscular junctions, the AZ extends roughly over 200 nm on each side of the DP and is usually limited by adherens junctions at its periphery (Hammarlund *et al.* 2007). About 30 vesicles are in close contact with the plasma membrane at the AZ. These "docked" vesicles correspond to the physiologically-defined "primed" vesicles and constitute the readily releasable pool (Hammarlund *et al.* 2007). Immediately after SV fusion, membrane is recovered at the AZ within milliseconds by ultrafast endocytosis. Further away is a periactive zone where vesicle membrane is recovered by endocytosis with a time constant of 1.4 second (Kittelmann, Liewald, *et al.* 2013; Watanabe *et al.* 2013).

Besides synapses involved in fast neurotransmission, a fraction of "modulatory" synapses are thought to predominantly release neuropeptides. They are identified as periodic varicosities along the neurites of modulatory neurons, each filled with a cloud of DCVs (Lim *et al.* 2016; Witvliet *et al.* 2021). Some modulatory synapses are devoid of clear synaptic vesicles and most DCV-specific varicosities do not contain dense projections (DPs).

The postsynaptic domains are not readily identified by EM in *C. elegans*: unlike in vertebrates, there is little or no ultrastructural specialization of the postsynaptic membranes. In some cases, small postsynaptic densities are detected using specific visualization methods (Weimer *et al.* 2006). Hence, the identification of

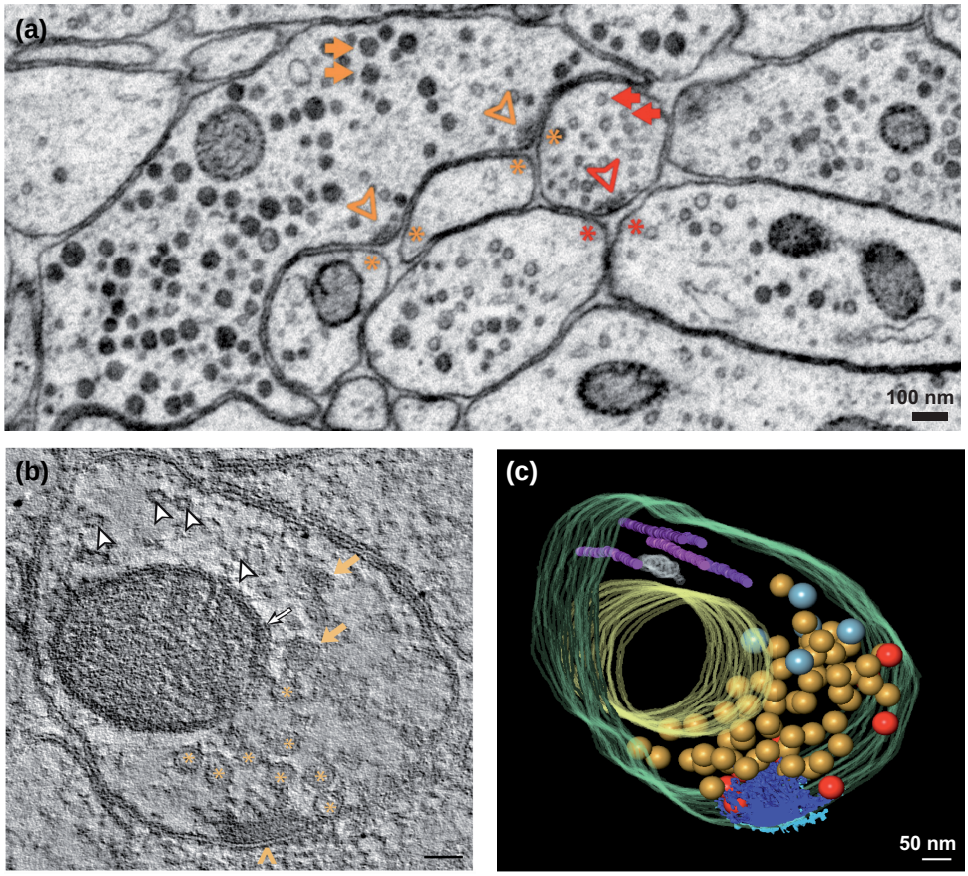

Fig. 1. Organization of *C. elegans* synapses. a) EM micrograph of the neuropil. Presynaptic termini of chemical synapses are characterized by a pool of clear synaptic vesicles (SVs) (red arrows) surrounding a dense projection (DP) (red arrowhead). Presynaptic termini of chemical synapses of modulatory neurons are characterized by mostly dense-core vesicles (orange arrows) distant from the AZ (orange arrowhead). Postsynaptic cells are marked by asterisks. (Adapted from Witvliet *et al.* (2021)). b) Virtual slice from an electron tomogram of a cholinergic neuromuscular junction (~1 nm thickness). SVs (asterisks), DP (open arrowhead), DCVs (orange arrows), microtubules (white arrowheads), and mitochondrion (white arrows). c) 3D model obtained by segmentation of the 250-nm-thick tomogram shown in (b). DCVs (bright blue) are situated at the rim of the SV pool (yellow). Docked SVs (red) touch the presynaptic membrane (green) and are mostly clustered around the DPs (dark blue), but some docked vesicles are located distantly to the DPs. Microtubules (violet) and smooth ER (white) are situated distant to the DPs beyond the mitochondrion (yellow), and both structures run along the longitudinal axis of the NMJ in parallel to DPs. Extracellular electron-dense material adjacent to DPs is annotated in turquoise. (Adapted from Stigloher *et al.* (2011).)

postsynaptic partners relies on the vicinity of the postsynaptic neuron membrane with the presynaptic DP (usually ≈100 nm). One presynaptic neuron may have more than one postsynaptic partner, forming polyadic synapses (two partners: dyadic synapses; three partners: triadic synapses; and so on). The polyadic nature of synapses was functionally validated at neuromuscular junctions where cholinergic neurons project onto several postsynaptic muscle processes and a downstream GABAergic inhibitory motoneuron (Liu, Chen, Hall, *et al.* 2007). In postsynaptic domains, neurotransmitter receptors and scaffolding molecules accumulate in front of presynaptic AZ (Gally and Bessereau 2003; Weimer *et al.* 2006) (see below). Recent studies have documented the existence of functional dendritic spines in the processes of GABAergic motor neurons (Philbrook *et al.* 2018; Cuentas-Condori *et al.* 2019), confirming the initial annotation of EM reconstructions (White *et al.* 1986). These spines concentrate dynamic actin-rich microdomains, and also contain smooth ER-like cisternae and ribosomes. Activation of presynaptic cholinergic neurons drives calcium transients, highly reminiscent of what is observed at dendritic spines in mammalian CNS. Spine-like protrusions are detected throughout the nervous system and increase during development to contribute 17% of synapses in the adult connectome. They may facilitate developmental changes, as developmentally dynamic connections were twice as likely to involve these protrusions than other connections (Witvliet *et al.* 2021).

Presynaptic molecules and their mechanisms

Presynaptic assembly requires interaction between presynaptic and postsynaptic cells followed by the assembly of the AZ, as well as the recruitment and clustering of synaptic vesicles (SVs) at the presynaptic site. Molecular dissection of mechanisms underlying presynaptic assembly was made possible by the Nobel-prize winning discovery of GFP as a versatile reporter in vivo (Chalfie *et al.* 1994). Synaptic vesicle clusters are readily visible using synaptic proteins fused to GFP, with synaptobrevin (SNB-1) and synaptogyrin (SNG-1) used in first generation reporters (Nonet 1999) and later RAB-3 and other synaptic factors, when driven under neuro-type-specific promoters. Two early pieces of evidence gave confidence on the authenticity of such labeling. First, the number and spacing of fluorescent clusters formed by SNB-1::GFP driven under the promoter of *unc-25*, encoding GAD, matched the estimated presynaptic terminals of GABAergic motor neurons (Jorgensen *et al.* 1995; Jin *et al.* 1999; Weimer *et al.* 2003). Second, in mutants of *unc-104* KIF1A, which

is dedicated for transporting synaptic vesicle precursors (Hall and Hedgecock 1991), SNB-1::GFP clusters are largely absent in presynaptic regions and instead are retained in the soma (Nonet 1999). Labeling of synapses in other cell types, primarily mechanosensory neurons, ASI chemosensory neurons, HSN motor neurons, DA9 cholinergic motor neurons, provided additional validation and also expanded the tool box for synaptic protein labeling. Multiple genetic screens, carried out with neuron-specific synaptic reporters, subsequently yielded a large number of mutants, based on visual inspection for number, location, and pattern of fluorescent synaptic clusters. Cloning of these mutants revealed an overlapping set of genes, most of which define a founding member of a protein family conserved in evolution. In this section, we summarize the core components of presynaptic assembly.

AZ scaffold proteins

SYD-1/mSYD1 and SYD-2/Liprin-α

Two AZ scaffold proteins, SYD-1 (SYnapse Defective-1) and SYD-2, were identified from the visual-based forward genetic screening for the disrupted SNB-1::GFP localization pattern in the D-type GABAergic motor neurons (Zhen and Jin 1999; Hallam *et al.* 2002). SYD-1 contains PDZ, C2, and RhoGAP domains, and defines a conserved protein family that includes Drosophila dSYD-1 and mammalian mSYD1A and mSYD1B. SYD-2 is a member of Liprin-α (for leukocyte common antigen related (LAR)-interacting protein) proteins, and has five coiled-coil domains, also known as Liprin-homology (LH) domains, and three SAM domains. In the *syd-1* and *syd-2* mutants, presynaptic structure is severely disrupted in multiple neuron types examined, suggesting that SYD-1 and SYD-2 are the essential scaffold proteins for presynaptic assembly in *C. elegans*. Consistently, SYD-2 positively regulates the size of electron-DPs, the hallmark of AZ (Kittelmann, Hegermann, *et al.* 2013). Both SYD-1 and SYD-2 are localized to the AZ, and form a protein complex with another AZ scaffold protein ELKS-1 (see below) to recruit other proteins to the presynaptic sites (Dai *et al.* 2006; Patel *et al.* 2006). Consistent with the critical functions of SYD-2 in AZ formation, SYD-2 serves as a structural scaffold of the AZ by recruiting a series of presynaptic proteins via its coiled-coil domain in its N-terminus and three SAM domains in the C-terminus (Taru and Jin 2011; Chia *et al.* 2013). The coiled-coil domain of SYD-2/Liprin-α binds to UNC-10/RIM (Schoch *et al.* 2002) in mammals and ELKS-1/ELKS (Dai *et al.* 2006). At the GABAergic synapses, SYD-2 recruits PTP-3A (LAR-type receptor tyrosine phosphatase) which then reinforces the localization of SYD-2 to the AZ through a positive feedback loop (Ackley *et al.* 2005). In the HSNs, RSY-1/PNISR (Pinin-interacting serine and arginine-rich protein) inhibits the formation of SYD-1-SYD-2-ELKS-1 complex, thereby preventing the presynaptic assembly (Patel *et al.* 2006).

ELKS-1/ELKS

ELKS-1 is an ortholog of mammalian PDZ-binding protein, ELKS/CAST/ERC, and known to physically interact with SYD-1, SYD-2, and UNC-10 (Deken *et al.* 2005; Dai *et al.* 2006; Patel and Shen 2009; McDonald *et al.* 2020). While *elks-1* single mutants do not exhibit structural and functional presynaptic defects, *elks-1* enhances synaptic vesicle clustering defects in *nab-1* mutants (Chia *et al.* 2012) (see *nab-1* section below), which is consistent with a modulating role for ELKS-1 in SV localization. In mammals, CAST and ELKS double knockout causes reduction in presynaptic voltage-gated Ca^{2+} channel number (Dong *et al.* 2018). Similar to mammals, it is reported that *elks-1* is involved in the presynaptic localization of UNC-2/CaV2 channel together with *unc-10/RIM* and *rmbp-1/RIM-binding protein* (Oh *et al.* 2021).

ELKS-1 and SYD-2 undergo liquid–liquid phase separation (LLPS) to form a condensate, which then forms a stable structure during synapse maturation (McDonald *et al.* 2020). In the *syd-2* and *elks-1* mutants that lack domains essential for their phase separation, the other AZ proteins such as UNC-10, CLA-1, UNC-13, and synaptic vesicle components such as RAB-3, failed to be recruited to the presynaptic sites in the hermaphrodite-specific neurons (HSNs). Interestingly, mutant SYD-2 and ELKS-1 proteins that lack domains for phase separation retain their ability to interact with other AZ components, such as SYD-1 and UNC-10 and are localized normally at the AZ. These data suggest that the ability of SYD-2 and ELKS-1 to phase separate is critical for their function as presynaptic organizers. Recent work showed that the oligomerization of human Liprin-α affects the phase separation of ELKS and RIM concentration in the condensate (Liang *et al.* 2021). The LLPS is, therefore, likely a conserved mechanism for AZ formation.

CLA-1

CLA-1 (Clarinet) is an AZ protein identified most recently (Xuan *et al.* 2017). Multiple CLA-1 isoforms share a common C-terminus, which contains C2 and PDZ domains that are conserved with mammalian AZ scaffold proteins, Piccolo/Bassoon and Drosophila Fife, although the rest of the CLA-1 proteins has little sequence similarity with known vertebrate presynaptic components. EM reconstruction of the neuromuscular junctions show reduced numbers of docked SVs associated with AZ and an increase in docked SVs around the AZ in *cla-1(null)* mutants (Xuan *et al.* 2017), suggesting CLA-1's function in SV localization. In addition, CLA-1 controls the AZ size. Synapses in the *cla-1 null* mutants contain smaller AZ judged by the size of DP. Consistently, SYD-2 localization at the AZ is partially dependent on *cla-1*, while CLA-1 localization at the AZ also depends on *syd-2* (Xuan *et al.* 2017). Therefore, CLA-1 appears to be an important structural component for the AZ formation and controls SV localization.

AZ proteins required for SV exocytosis

SVs are transported from soma to presynaptic sites via UNC-104/KIF1A motor proteins (Hall and Hedgecock 1991). At the presynaptic sites, there is a pool of undocked reserve SVs, some of which are recruited to the AZ membrane, and attached to the membrane (docking/priming) through the actions of AZ proteins and the SNAP receptor (SNARE) protein complex. Docked/primed SVs at the AZ undergo for Ca^{2+}-dependent exocytosis, and their release probability correlates with the distance from the DP.

UNC-10/RIM (Rab3a-interacting molecule) and RIMB-1 (rim-binding protein)

UNC-10 is localized at the AZ at least in part through the direct interaction with ELKS-1 (Deken *et al.* 2005). In both *C. elegans* and vertebrates, UNC-10/RIM at the AZ binds to the GTP-bound form of RAB-3, a SV-associated small GTPase, to recruit SVs to the AZ (Wang *et al.* 1997; Weimer *et al.* 2006; Gracheva *et al.* 2008). In *rab-3* and *unc-10* mutants, the number of docked SV near the DP is significantly reduced (Gracheva *et al.* 2008). UNC-10 also promotes SV docking/priming by activating UNC-13 (see below) through inhibiting the homodimerization of UNC-13 (Liu *et al.* 2019).

RIMB-1 is a homolog of RIM-binding proteins, which bind RIM and calcium channels (Kushibiki *et al.* 2019). In mammals, the PDZ domain of RIM physically interacts with N- and P/Q-type

Ca^{2+} channels containing CaV2.1 and CaV2.2 a1 subunits, respectively. In mice, knocking out RIM results in the reduced presynaptic localization of CaV2.1, and the impaired SV release (Kaeser *et al.* 2011). In *C. elegans*, UNC-10 and RIMB-1 control the presynaptic localization of UNC-2/Cav2 (Kushibiki *et al.* 2019; Oh *et al.* 2021). In the double mutants of *unc-10* and *rimb-1*, the presynaptic localization of UNC-2 is significantly reduced in the dorsal nerve cord. Thus, UNC-10 and RIMB-1 act together to control the Ca^{2+}-dependent SV exocytosis by promoting the presynaptic localization of Ca^{2+} channels.

UNC-13/MUNC13 and UNC-18/MUNC18

SVs undergo exocytosis for neurotransmitter release, which is controlled by the conserved SNARE protein complex and their regulatory AZ proteins, UNC-13/Munc13 and UNC-18/Munc18. The SNARE complex formation involves the assembly of the trans-SNARE containing Syntaxin and SNAP-25 on the presynaptic membrane, and the vesicle-SNARE containing Synaptobrevin on the SVs (Sutton *et al.* 1998). The complete SNARE assembly, or the formation of cis-SNARE complex leads to the membrane fusion and neurotransmitter release (Sauvola and Littleton 2021). The process of the partial SNARE complex assembly is the molecular manifestation of the SV docking/priming.

unc-13 and unc-18 loss-of-function (lof) mutants were originally isolated for their locomotion defects (Brenner 1974), and later shown to be critical in SV release (Ahmed *et al.* 1992; Hosono *et al.* 1992; Gengyo-Ando *et al.* 1993; Richmond *et al.* 1999; Kohn *et al.* 2000). Mammalian Munc13 forms an inhibitory homodimer through its C2A domain, or an active state of heterodimer with RIM to inhibit or promote SV docking/priming, respectively (Lu *et al.* 2006; Deng *et al.* 2011; Camacho *et al.* 2017). Similarly, the long isoform of UNC-13(L) in *C. elegans* can form an inactive homodimer or an active heterodimer with UNC-10/RIM (Liu *et al.* 2019). The active form of UNC-13 binds UNC-18, which recruits Syntaxin (UNC-64) and Synaptobrevin (SNB-1) through physical interaction, to promote SNARE complex assembly (Dulubova *et al.* 2007; Park *et al.* 2017; Sitarska *et al.* 2017). Interestingly, UNC-64 is accumulated in the neuronal cell body in *unc-18* mutants, suggesting that *unc-18* is also required for the anterograde trafficking of UNC-64 (McEwen and Kaplan 2008).

As transgenic overexpression of constitutively open form of UNC-64/Syntaxin partially bypasses the requirement of *unc-13* (Richmond *et al.* 2001), UNC-13 has been believed to serve as an "Syntaxin opener" to promote the SNARE complex assembly. Consistently, constitutively active UNC-13 enhances the release probability by opening UNC-64 (Li *et al.* 2019). However, open Syntaxin only partially suppressed the SV release defects of *unc-13/Munc13* in mammals and *C. elegans* (Richmond *et al.* 2001; Lai *et al.* 2017; Tien *et al.* 2020). Therefore, it is unlikely that UNC-13's function as a Syntaxin opener is the sole mechanism through which UNC-13 controls SV priming. The current model favors the idea that UNC-13 acts with UNC-18 to protect proper SNARE complex from the SNARE disassembly factors, NFS and αSNAP (He *et al.* 2017; Dittman 2019).

Consistent with the critical functions of UNC-13 and UNC-18 in SNARE complex assembly, the number of docked SVs near the DP are severely diminished in *unc-13* mutants, which results in the reduced release probability (Zhou *et al.* 2013). Similar release probability defects are observed in *unc-10* mutants that are incapable of mediating the UNC-13L-UNC-10 heterodimerization (Liu *et al.* 2019).

TOM-1/Tomosyn, another conserved AZ protein, contains an SNARE motif and acts antagonistically to UNC-13 and UNC-18 by competing for binding spots of UNC-64/Syntaxin (McEwen *et al.* 2006). The binding of TOM-1 to UNC-64 prevents UNC-18–UNC-64 interaction thereby inhibiting SV docking/priming in *C. elegans*, Drosophila, and mammals (Hatsuzawa *et al.* 2003; Gracheva *et al.* 2006; Sakisaka *et al.* 2008; Sauvola *et al.* 2021).

Regulatory mechanisms

NAB-1/Neurabin

NAB-1 is an ortholog of Neurabin-1 (neural tissue-specific F-actin binding protein), and originally found in a genome-wide RNAi screening for ardicarb resistance (Sieburth *et al.* 2005). NAB-1 is localized at the presynaptic sites of DA neurons, and *nab-1* mutants disrupted the localization pattern of AZ and SV markers (Sieburth *et al.* 2005). During HSN development, NAB-1 is transiently recruited to the ARP2/3-dependent branched F-actin network enriched at the presynaptic sites through its actin-binding domain (Chia *et al.* 2012). NAB-1 recruits SYD-1, which then indirectly recruits SYD-2 to the presynaptic sites. Consistently, loss of *nab-1* impairs presynaptic localization of SYD-1 and SYD-2, as well as the SV-associated protein, SNB-1 in the HSNs. Interestingly two AZ component genes, *sad-1(lof)* (see below) and *elks-1(lof)* greatly enhanced the SV localization defects of *nab-1(lof)* mutant (Chia *et al.* 2012). The synergistic effects of these AZ components suggest that parallel signaling cascades recruit SVs to the AZ.

SAD-1/BRSK (brain-specific serine/threonine kinase)1/2

SAD-1 (for Synapse of the Amphid Defective) is a presynaptically localized NAB-1 interacting serine–threonine kinase required for SV clustering (Crump *et al.* 2001; Hung *et al.* 2007). In the GABAergic motor neurons, SAD-1 localization depends on STRD-1, the *C. elegans* ortholog of STRADα, a STE-20-related pseudokinase (Kim *et al.* 2010). Presynaptic SAD-1 localization in the HSNs depends on *nab-1*, *syd-1*, and *syd-2*, suggesting that *sad-1* acts downstream of *syd-1* and *syd-2* (Patel *et al.* 2006; Chia *et al.* 2012). Consistently, loss of *sad-1* results in partially diffuse SV localization without affecting the SYD-2 localization (Crump *et al.* 2001; Patel *et al.* 2006). However, the synergistic effect of *sad-1* and *nab-1* on the SV localization in the HSNs (Chia *et al.* 2012) suggests that *sad-1* can also act in parallel to *nab-1*, which acts upstream of *syd-1* and *syd-2*. In addition to its function in presynaptic assembly, SAD-1 also controls the axon/dendrite polarity in several neuron types (Hung *et al.* 2007). The physical interaction between SAD-1 and NAB-1 is required for their functions in determining axon/dendrite polarity, but not for their functions in SV localization (Hung *et al.* 2007), highlighting the context-dependent functions of SAD-1 and NAB-1 in neuronal development.

RPM-1/Ubiquitination-protein degradation

RPM-1 (Regulator of Presynaptic Morphology-1) is a member of the conserved PHR (Pam-Highwire-Rpm-1) ubiquitin-protein E3 ligases, all of which are large proteins with multiple domains (Schaefer *et al.* 2000; Zhen *et al.* 2000; Grill *et al.* 2016). *rpm-1(lof)* mutants exhibit reduced synapse number and irregular synapse morphology along with other neuronal structural defects (Schaefer *et al.* 2000; Zhen *et al.* 2000; Nakata *et al.* 2005). Together with the F-box protein, FSN-1, RPM-1 negatively regulates the MAP kinase pathway consisting of DLK-1/MAPKKK, MKK-4/MAPKK, and PMK-3/MAPK by inducing the ubiquitin-dependent degradation of DLK-1 (Liao *et al.* 2004; Nakata *et al.* 2005). This interaction between PHR proteins and DLKs is one of the highly conserved mechanisms in the nervous system (Grill

et al. 2007, 2016). Additionally, multiple parallel acting partners of RPM-1 were identified through mass-spectrometry approaches. These include GLO-4/Guanine nucleotide exchange factor, PPM-2/protein phosphatase, ANC-1/Nesprin and others. GLO-4 and PPM-2 controls RPM-1-dependent synapse assembly via activating GLO-1/Rab GTPase or inhibiting DLK-1 independently of RPM-1's ubiquitin ligase function (Grill et al. 2007; Baker et al. 2014). ANC-1/Nesprin proteins anchor nuclear position, and the interaction of RPM-1 with ANC-1 modulates synapse formation through β-catenin BAR-1 (Tulgren et al. 2014).

UNC-43/CaMKII

Calcium/calmodulin-dependent protein kinase II (CaMKII) is one of the most abundant proteins in the brain, and plays pivotal roles in synaptic plasticity. In *C. elegans*, in addition to its well-known functions in the postsynaptic sites, *unc-43(lof)* mutants exhibit defects at the presynaptic sites. Presynaptic UNC-43 inhibits neurotransmitter release through phosphorylating SLO-1/BK channel (Liu, Chen, Ge, et al. 2007). EM studies showed up to 90% reduction in DCVs at the synapses of *unc-43* nonsense mutants (Hoover et al. 2014). The presynaptic sites labeled with SNB-1::GFP in GABAergic DD neurons are enlarged in both gain-of-function and loss-of-function mutants of *unc-43* (Caylor et al. 2013), while those labeled with GFP::RAB-3 in DA9 exhibit diffuse distribution along the axon with weaker clustering at the presynaptic sites (Chia et al. 2018).

Autophagy in synapse formation

In the AIY interneurons, the autophagy machinery promotes synaptogenesis (Stavoe et al. 2016). Loss-of-function mutants of many autophagy components such as *atg-9/ATG9* and *unc-51/ULK1* show defects in the presynaptic localization of AZ proteins and SVs (Stavoe et al. 2016). At the presynaptic sites, ATG-9 is found on a subpopulation of vesicles as ATG-9::GFP exhibit partial overlap with the SV markers, mCherry::RAB-3, and SNG-1::mCherry. The ATG-9 containing vesicles undergo activity-dependent exocytosis through SNARE complex, and clathrin-dependent endocytosis to control activity-dependent synaptic autophagy (Yang et al. 2022). Similar to the AIY neurons in which autophagy positively regulates synapse formation, the PLM neurons fail to form synaptic branch in the *unc-51* mutants (Crawley et al. 2019). However, transgenic expression of dominant negative *unc-51* suppresses the loss of synaptic branch phenotype of the *rpm-1(lof)* mutant (Crawley et al. 2019). It is therefore likely that autophagy is required for both promoting and inhibiting synapse formation. The inhibitory role for autophagy in synapse formation is consistent with the observation in mammals in which loss of autophagy machinery is also associated with synapse pruning defects (Tang et al. 2014).

Specification of postsynaptic compartments

Synaptic transmission requires the differentiation of specialized domains on postsynaptic cells, not only to localize receptors in the vicinity of neurotransmitter release sites, but also to assemble complex signal transduction platforms that shape the synaptic transfer function and support synaptic maintenance and plasticity. As compared to the molecular mechanisms involved in the differentiation and function of presynaptic compartments, the mechanisms involved in postsynaptic differentiation are less characterized in *C. elegans*. Best described synapses are the neuromuscular junctions (NMJs), where numerous genetic screens have identified diverse molecular players. Initial screens relied on the identification of mutants resistant to levamisole, a nematode-specific agonist of acetylcholine receptors, which causes muscle hypercontraction and kills the worms at high concentration (Lewis et al. 1980; Fleming et al. 1997). More recent screens relied on the visualization of fluorescently-tagged receptors, directly looking for mutants with abnormal receptor distribution (Pinan-Lucarre et al. 2014; Tu et al. 2015). We will first review the molecular organization of the neuromuscular system and then provide information on our current knowledge of postsynaptic differentiation at a few neuro-neuronal synapses.

Neuromuscular junctions

C. elegans NMJs best characterized at the postsynaptic level are formed between the motor neuron axons of the ventral and dorsal cords and body-wall muscle cells. Theses NMJs have features that are closer to neuro-neuronal synapses than to "standard" NMJs. In vertebrates or Drosophila, motoneurons establish a single NMJ with myofibers containing hundreds to thousands of nuclei, which differs from neuronal innervation, where a single neuron typically receives thousands of excitatory and inhibitory inputs, building a mosaic of specialized domains concentrating receptors to match presynaptic inputs.

The anatomical organization of the *C. elegans* neuromuscular system provides a means to interrogate a number of questions that may relate more closely to the innervation of vertebrate neurons. First, *C. elegans* body-wall muscle cells do not fuse and remain mononucleated. Second, they send dendrite-like extensions called "muscle arms" that contact and extend along the motoneurons and form "*en passant*" synapses. Third, each muscle cell receives both excitatory cholinergic and inhibitory GABAergic inputs from distinct classes of motoneurons (Fig. 2a). Hence, the *C. elegans* neuromuscular arrangement represents a very simple poly-neuronal innervation system. Specifically, it can be used to interrogate how specific compartments are built on the plasma membrane to concentrate different neurotransmitter receptors in front of the corresponding neurotransmitter release sites.

Interestingly, NMJ formation in *C. elegans* does not involve the same molecular pathways as for mammalian NMJs. For example, *agr-1*, which encodes the ortholog of agrin, a nerve-derived glycoprotein critical for NMJ differentiation in mice, does not play a role at *C. elegans* NMJs (Hrus et al. 2007). As presented below, most of the molecules involved in the organization of *C. elegans* NMJs have mammalian orthologs that are expressed in the CNS and potentially regulate neuronal synapses.

MADD-4/Punctin, an anterograde synaptic organizer for both cholinergic and GABAergic NMJs

The postsynaptic assembly of cholinergic and GABAergic NMJs in *C. elegans* relies on an anterograde synaptic organizer called MADD-4 (Muscle Arm Development Defective-4). MADD-4 belongs to a family of poorly characterized extracellular matrix proteins, the ADAMTS-like proteins, that contain multiple thrombospondin-repeat, immunoglobulin, and structurally unsolved domains (Apte 2009). There are two *madd-4* orthologs in vertebrates: *Punctin1/ADAMTSL1* and *Punctin2/ADAMTSL3*. The precise function of these genes is unknown. However, a variant of *Punctin1* was recently shown to cause a complex phenotype including congenital glaucoma, craniofacial, and other systemic features (Hendee et al. 2017). *Punctin2* is expressed in the brain and was identified as a susceptibility gene for schizophrenia (Dow et al. 2011). Whether these proteins are involved in synaptic organization has not been determined.

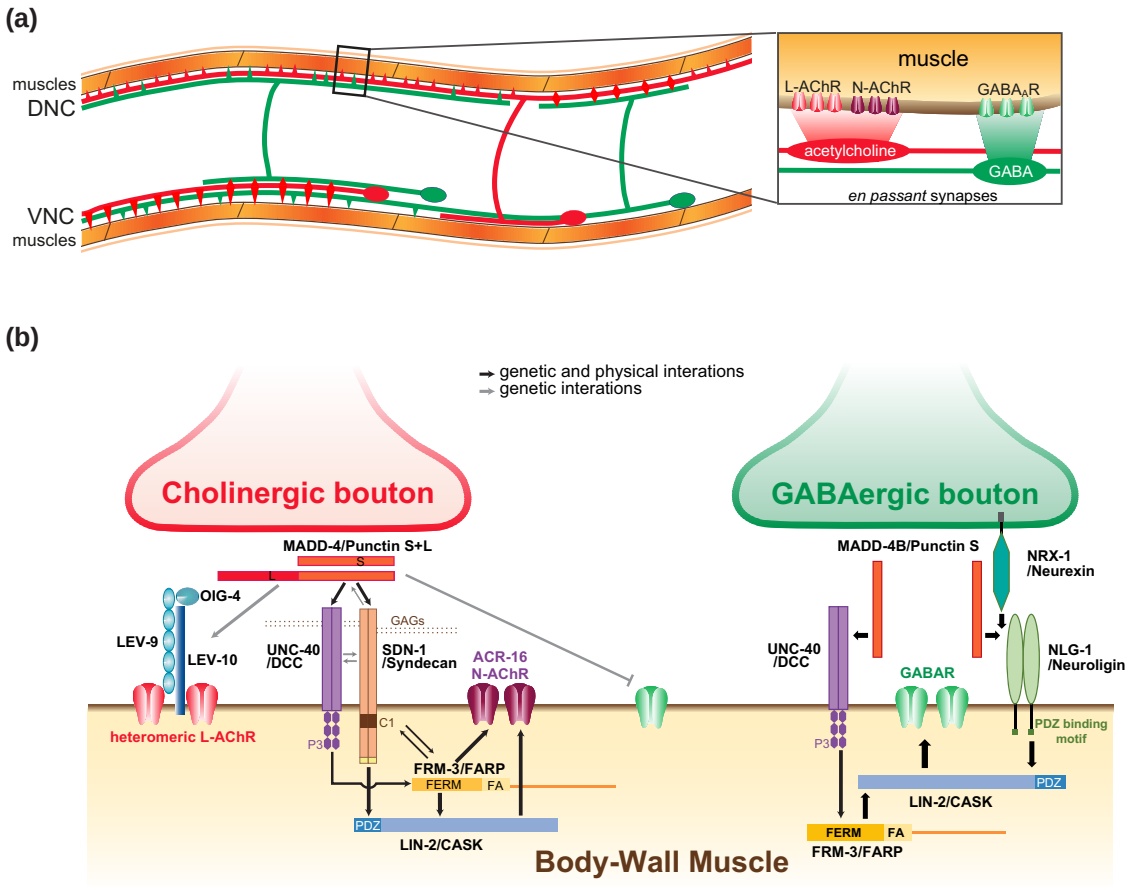

Fig. 2. Organization of neuromuscular junctions. a) Schematic organization of the *C. elegans* neuromuscular network. Mononucleated body-wall muscle cells are innervated by cholinergic (red) and GABAergic (green) motoneurons along the ventral and dorsal nerve cords (VNC and DNC). Cholinergic neurons form dyadic synapses activating muscle cells and GABAergic motoneurons that innervate opposite muscle cells. b) Molecular organization of postsynaptic domains. See main text for detailed description and references.

madd-4 generates long (MADD-4L) and short (MADD-4S) isoforms by use of alternative promoters. MADD-4S was initially found to attract muscle arm growth and be required for midline-oriented guidance in *C. elegans* (Seetharaman *et al.* 2011). The role of *madd-4* in synaptic organization was subsequently identified in a visual screen for mutants with abnormal positioning of fluorescently-tagged postsynaptic receptors at NMJs (Pinan-Lucarre *et al.* 2014). MADD-4L is only expressed in cholinergic motoneurons and secreted in the synaptic cleft where it triggers postsynaptic clustering of acetylcholine resceptors (AChRs). MADD-4S is expressed in both cholinergic and GABAergic neurons (Fig. 2). At cholinergic synapses, MADD-4S inhibits the attraction of GABA$_A$Rs by MADD-4L, possibly following heterodimerization of the L and S isoforms. At GABAergic synapses, MADD-4S promotes the clustering of GABA$_A$Rs in front of presynaptic GABA boutons. Genetic inactivation of MADD-4S does not alter presynaptic GABA boutons, but GABA$_A$Rs relocalize at cholinergic synapses. Conversely, forced expression of MADD-4L in GABAergic motoneurons in a *madd-4* null mutant triggers the colocalization of AChRs and GABA$_A$Rs opposed to GABAergic boutons (Pinan-Lucarre *et al.* 2014). These results demonstrated that the identity of pre- and postsynaptic domains can be genetically uncoupled in vivo.

Interestingly, the expression of MADD-4 is under direct regulation of the transcription factors that specify the terminal identity of motoneurons. The phylogenetically conserved transcription factor UNC-3 controls the expression of numerous genes required for cholinergic neurotransmission, but it also directly activates the transcription of *madd-4L* and S isoforms in cholinergic motoneurons (Kratsios *et al.* 2015). Similarly, the homeobox transcription factor UNC-30 controls the GABAergic identity of D-type motoneurons (Jin *et al.* 1994) and regulates the expression of *madd-4S* (Kratsios P and Hobert O, personal communication). Therefore, coordinated control of motoneuron identity and MADD-4 expression provides a means to ensure proper coupling between presynaptic identity and postsynaptic differentiation.

The GABAergic NMJ

The UNC-49 type-A GABA receptors are clustered in register with presynaptic GABAergic boutons and define the postsynaptic domains (Gally and Bessereau 2003). These receptors are generated from a single complex locus, which generates at least three different subunits (A, B, and C) by alternative splicing. A block of exons encodes most of the extracellular N-terminal domain, which is shared by all subunits, while exons coding for transmembrane regions are specific to each subunit. In *Xenopus* oocytes, functional GABA receptors can be reconstituted by expressing the B-subunit either alone or in combination with the C-subunit (Bamber *et al.* 1999). In vivo recordings and the pharmacological analyses of endogenous GABA$_A$Rs indicate that they are likely composed of UNC-49 B/C heteromers (Bamber *et al.* 2005). Synaptic clustering depends on presynaptic innervation and occurs concomitantly with presynaptic differentiation based on

the visualization of fluorescently-tagged synaptic proteins (Gally and Bessereau 2003). However, a detailed longitudinal analysis is still missing to ascertain precise temporal relationship between pre- and postsynaptic differentiation. Remarkably, in mutants that do not synthetize GABA, both pre- and postsynaptic structures are indistinguishable from wild type, demonstrating that "inhibitory" synapses differentiate in the absence of neurotransmission (Jin *et al.* 1999; Gally and Bessereau 2003). This situation is not unique since various synaptic types were also reported to differentiate in mammalian cell cultures and in mice in the absence of neurotransmitter release (Misgeld *et al.* 2002; Varoqueaux *et al.* 2002; Sigler *et al.* 2017).

MADD-4S localizes UNC-49 at the synapse through two parallel pathways. First, it localizes the synaptic adhesion molecule neuroligin NLG-1 at GABA synapses (Maro *et al.* 2015; Tu *et al.* 2015). Second, it localizes and activates the netrin receptor UNC-40/DCC (Seetharaman *et al.* 2011; Zhou *et al.* 2020), which, in turn, promotes the formation of an intracellular complex comprising the scaffolding proteins FRM-3/FARP and LIN-2/CASK (Tong *et al.* 2015; Zhou *et al.* 2020). This scaffold enables the synaptic recruitment of UNC-49 by NLG-1.

NLG-1/Neuroligin defines GABAergic postsynaptic domains

Neuroligins (NLs) are evolutionary ancient proteins that are readily detected in Bilaterians (Lenfant *et al.* 2014). The human genome encodes 5 NLs that support trans-synaptic adhesive functions at excitatory and inhibitory synapses and contribute to postsynaptic receptor clustering (for review, see Sudhof (2017)). The *C. elegans* genome contains only one NL-coding gene, *nlg-1*, which is expressed in multiple types of neurons and in muscle (Hunter *et al.* 2010). NLG-1 shares about 25% identity with human NLs and cannot be related to one specific paralog. However, the core protein organization is conserved between mammals and the nematode (Calahorro 2014). Three main NLG-1 isoforms are generated by alternative splicing of exons encoding cytoplasmic domains of the protein (Calahorro *et al.* 2015). This splicing seems developmentally regulated but the precise complement of NLG-1 isoforms expressed in neurons and muscle and its functional relevance remain to be analyzed.

In muscle, NLG-1 is only found at GABAergic NMJs and strictly colocalizes with the UNC-49 $GABA_A$Rs (Maro *et al.* 2015; Tong *et al.* 2015; Tu *et al.* 2015). *nlg-1(lof)* causes a redistribution of the $GABA_A$Rs out of the GABA receptor domains and a reduction of the frequency and amplitude of spontaneous miniature inhibitory postsynaptic currents (mIPSCs). The synaptic localization of NLG-1 depends on MADD-4S, which directly binds the NLG-1 ectodomain (Platsaki *et al.* 2020). The intracellular moiety of NLG-1 is dispensable for its synaptic localization but is required for its ability to cluster $GABA_A$Rs (Maro *et al.* 2015; Tu *et al.* 2015). In the absence of MADD-4S, NLG-1 and UNC-49 are recruited at cholinergic synapses by MADD-4L, while GABAergic presynaptic terminals remain the same as in the wild type, hence demonstrating that pre- and postsynaptic identities can be genetically uncoupled.

NRX-1/Neurexin is present at GABAergic NMJs

GABA motoneurons also express NRX-1, the sole ortholog of the mammalian neurexins that are presynaptic ligands of neuroligins (reviewed in Sudhof (2017)). NRX-1 is present at presynaptic sites of GABAergic NMJs, but is not required for the synaptic localization of NLG-1. A synergistic effect between *nrx-1(lof)* and *madd-4(lof)* initially suggested that NRX-1 might work in parallel with MADD-4 to promote the clustering of $GABA_A$Rs (Maro *et al.* 2015), although this phenotype was not observed by others (Tong *et al.* 2015) using a different *nrx-1(lof)* allele. Therefore, the positive interaction between NRX-1 and MADD-4 at GABA synapses remains to be further investigated in this system.

UNC-40/DCC regulates $GABA_A$Rs synaptic content

The synaptic content of $GABA_A$Rs depends on the UNC-6/netrin receptor UNC-40/DCC (Deleted in Colorectal Cancer) (Tu *et al.* 2015). This receptor has been implicated in a wide range of developmental events involving cellular migration and axonal navigation (Chan *et al.* 1996; Keino-Masu *et al.* 1996). It is a single transmembrane domain protein that does not contain any obvious catalytic domain. Upon netrin binding, UNC-40 is believed to dimerize, causing the intracellular domains to serve as a signaling platform to recruit or activate numerous downstream targets, including several signal transduction molecules that regulate cytoskeletal dynamics (for reviews, see Finci *et al.* (2015); Boyer and Gupton (2018)).

In *C. elegans*, UNC-40 plays a specific role in the neuromuscular system. First, it promotes the growth of muscle arms (Alexander *et al.* 2009). At an early larval stage, MADD-4S localizes UNC-40 at the tip of the muscle arms and, together with the guidance cue UNC-6/netrin, activates UNC-40. Thus, the number of muscle arms that project to the ventral and dorsal nerve cords is drastically reduced in *unc-40(lof)* mutants. However, the number of GABAergic boutons is unaffected and NLG-1 postsynaptic clusters remain readily detected. In addition, UNC-40 controls the amount of $GABA_A$Rs at synapses. In *unc-40* mutants, there is a 60% reduction of receptors at GABAergic NMJs. A constitutively activated version of UNC-40, which only contains the intracellular moiety of UNC-40 targeted to the plasma membrane, rescues the synaptic clustering of $GABA_A$Rs (Tu *et al.* 2015). Recent data indicate that UNC-40 promotes the recruitment of $GABA_A$Rs onto NLG-1 clusters by tethering an intracellular scaffold containing FRM-3 and LIN-2 that bridges $GABA_A$Rs and neuroligin (Tong *et al.* 2015; Zhou *et al.* 2020). Notably, UNC-40 is also present at cholinergic synapses where it regulates AChR content via a closely related mechanism (see below).

FRM-3/FARP and LIN-2/CASK stabilize $GABA_A$Rs at GABAergic NMJs

frm-3 and *lin-2* encode intracellular proteins that are required for UNC-49 localization at GABA synapses (Tong *et al.* 2015; Zhou *et al.* 2020). Their disruption causes 80% loss of synaptic receptors, while the overall number of receptors present at muscle cell surface remains the same as in the wild type, supporting a specific role for synaptic localization.

FRM-3 is the ortholog of the mammalian FARP1 and FARP2 proteins. FARPs are able to modulate F-actin assembly and regulate neuronal development and synaptogenesis by interacting with cell surface proteins such as SynCAM1 and class A Plexins (Cheadle and Biederer 2012, 2014). FRM-3 contains in its N-terminal part FERM (p4.1, Ezrin, Radixin, Moesin) and FA (FERM-Adjacent) domains that binds the C-terminal P3 domain of UNC-40. Interestingly, FERM-FA domains can oligomerize and bind protein and lipid partners, suggesting that FRM-3 might build up an oligomeric platform tethered to the plasma membrane by UNC-40.

In turn, FRM-3 physically interacts and recruits the adaptor protein LIN-2 in synaptic regions. LIN-2 is the ortholog of CASK, a member of the membrane-associated guanylate-kinase family. Most studies have focused on presynaptic roles for CASK, but sparse reports also suggest a role of CASK at the postsynaptic side (Chen and Featherstone 2011). LIN-2 is a multimodule protein

able to interact with neuroligin via its PDZ domain and with the TM3-TM4 cytoplasmic loop of UNC-49 via its SH3 and guanylate-kinase domains. Another study suggests that FRM-3 directly binds the UNC-49 TM3-4 loop, thereby recruiting LIN-2 to UNC-49 complexes (Tong *et al.* 2015). As a result, the recruitment of LIN-2 by FRM-3 forms an intracellular scaffold that bridges UNC-49 receptors to synaptic neuroligin clusters (Zhou *et al.* 2020). In addition, a NLG-1/neuroligin-dependent scaffold was initially proposed to stabilize $GABA_A$Rs at the synapse in parallel to FRM-3 and LIN-2 (Tong *et al.* 2015). Although this pathway does not seem to be predominant (Zhou *et al.* 2020) additional mechanisms such as extracellular interactions between NLG-1 and UNC-49 might contribute to the synaptic stabilization of $GABA_A$Rs.

The cholinergic NMJ

Cholinergic motoneurons establish dyadic synapses with body-wall muscle cells and GABAergic MN (Fig. 2). Acetylcholine release triggers the contraction of muscles on one side of the animal, and concomitantly activates downstream inhibitory MNs that project and relax opposite muscles, therefore causing local bending of the body. Two types of ionotropic AChRs are present at neuromuscular junctions (Richmond and Jorgensen 1999). First, heteromeric levamisole-sensitive AChRs (L-AChRs) can be activated by the nematode-specific cholinergic agonist levamisole, which causes muscle hypercontraction and death of wild-type worms at high concentrations (Lewis *et al.* 1980; Fleming *et al.* 1997). Genetic screens for complete resistance to levamisole have identified the structural subunits of these receptors, comprising three α subunits (LEV-8, UNC-38, and UNC-63) and two non- α subunits (LEV-1 and UNC-29) (Fleming *et al.* 1997; Culetto *et al.* 2004; Touroutine *et al.* 2005; Boulin *et al.* 2008). Second, homomeric nicotine-sensitive AChRs (N-AChRs) are insensitive to levamisole and formed by an ACR-16 homo-pentamer (Francis *et al.* 2005; Touroutine *et al.* 2005). N-AChR have striking similarity with mammalian α7 AChRs. While L-AChRs desensitize slowly and are poorly permeable to calcium, N-AChRs desensitize quickly and are more permeable to calcium (Boulin *et al.* 2008). The physiological role of N-AChR is not fully understood since *acr-16(0)* have no clear locomotory phenotype. Yet N-AChRs can support neuromuscular transmission since L-AChR mutant have partial reduction of mobility, while inactivation of both L- and N-AChRs causes complete paralysis. Remarkably, two distinct mechanisms are dedicated to the clustering of L- and N-AChRs. Note that *sdn-1* mutants (and likely *frm-3* and *lin-2*) decrease both ACR-16 and L-AChR clustering. So, there is some overlap in clustering mechanisms.

L-AChR synaptic clustering relies on an extracellular scaffold

Genetic screens for partial resistance to levamisole identified three proteins required for L-AChR clustering, all expressed by muscle cells. LEV–9 and OIG–4 are secreted proteins containing eight complement-control protein (CCP) domains and one Ig domain, respectively (Gendrel *et al.* 2009; Rapti *et al.* 2011). LEV-9 activation by C-terminal cleavage is required for proper L-AChR clustering but not for LEV-9 secretion (Briseno-Roa and Bessereau 2014). LEV–10 is a type I transmembrane protein whose extracellular domain containing five CUB domains supports its clustering activity (Gally *et al.* 2004). These three proteins form a physical complex with L-AChRs in the synaptic cleft. Genetic depletion of any of these proteins, including L-AChR subunits, disrupts their clustering and their synaptic localization, although cholinergic NMJs are still formed and N-AChRs remain properly localized. In the absence of MADD-4L, LEV-9 and LEV-10 still associate with L–AChRs but the clusters are fragmented and are distributed at the surface of the muscle cells outside of synaptic regions. MADD-4L likely localizes preformed L-AChR clusters at the synapse by direct interaction with the L-AChR-associated scaffolding molecules; however, biochemical evidence remains to be provided to demonstrate this model. Interestingly, the diffuse state of extrasynaptic receptors is likely not a default state simply explained by the lack of synaptic cues, but necessitates additional proteins to prevent spontaneous clustering as observed in *rsu-1* mutants where L-AChRs assemble into ectopic clusters and compete with synaptic receptor content (Pierron *et al.* 2016).

HPO-30, a member of the claudin superfamily of membrane proteins mostly characterized for their roles at tight junctions, was detected at cholinergic NMJs. HPO-30 physically interacts with L-AChRs and its disruption causes a strong decrease of L-AChRs at NMJs without extrasynaptic declustering (Sharma *et al.* 2018). Surprisingly, this phenotype is suppressed by inactivating *nlg-1*. Indeed, HPO-30 negatively regulates NLG-1 levels, which in turn appears to decrease L-AChR synaptic content. The molecular basis of the antagonistic interplay between NLG-1 and L-AChRs remains to be further explored. The NLG-1/NRX-1 complex was also reported to be present at cholinergic NMJs, but in a "flipped" orientation, with NLG-1 being presynaptic and NRX-1 postsynaptic (Hu *et al.* 2012) This system does not seem to regulate receptor content but shedding of NRX-1 ectodomain is proposed to serve as a negative retrograde signal of ACh release by decreasing the presynaptic abundance of UNC-36/α2δ, an auxiliary subunit of UNC-2/CaV2 calcium channels (Tong *et al.* 2017).

SDN-1/Syndecan, a core organizer of cholinergic NMJs required for N-AChR clustering:

Syndecans are a class of transmembrane heparan sulfate glycoproteins implicated in a wide range of biological processes. In *C. elegans*, SDN-1, the sole syndecan member, is widely expressed in epidermis, neurons and muscle and is especially important for axonal guidance. It is highly enriched at both cholinergic and GABAergic NMJs and carries most of heparan sulfate chains detected at these synapses (Attreed *et al.* 2012; Zhou *et al.* 2021). Genetic disruption of *sdn-1* causes a ≈60% decrease of L-AChRs and an almost complete disappearance of N-AChRs. The synaptic content of UNC-49 is also decreased by roughly 35% in these mutants. Genetic and biochemical evidence indicate that MADD-4 localizes SDN-1 and UNC-40 by direct interaction. Interestingly, MADD-4 and UNC-40 are reduced in *sdn-1(0)* mutants by 30% and 40%, respectively, suggesting the formation of an extracellular scaffold in the synaptic cleft (Zhou *et al.* 2021). It would also be interesting to test if SDN-1 might stabilize the interaction of MADD-4 and the L-AChR clustering machinery.

SDN-1 localizes N-AChRs at cholinergic NMJs by bridging extracellular matrix components with intracellular scaffolding proteins (Fig. 2), playing a very similar role as NLG-1 at GABAergic NMJs. Coincident clustering of SDN-1 and UNC-40 at cholinergic NMJs triggers the intracellular recruitment of the scaffolding molecules LIN-2/CASK and FRM-3/FARP by direct interaction with the PDZ domain binding site of SDN-1 and the C-terminal P3 domain of UNC-40, respectively. The FERM-FA domain of FRM-3/FARP also engages direct interaction with SDN-1, likely with its submembrane C1 domain. The resulting LIN-2/FRM-3 (CASK/FARP) complex then triggers synaptic clustering of N-AChRs. Remarkably, relocating the intracellular domain of SDN-1 exclusively at GABAergic NMJs positions N-AChRs in front of GABA

boutons, while L-AChR remains at cholinergic synapses (Zhou *et al.* 2021).

The central and specific role of SDN-1 for N-AChR clustering at cholinergic synapses raises questions about the mechanisms underlying this specificity. Not only is SDN-1 equally present at GABAergic NMJs, but its associated proteins UNC-40/DCC, FRM-3/FARP, and LIN-2/CASK are used at inhibitory NMJs to cluster $GABA_A$Rs. Additional components might need to be identified to solve this question, but it illustrates the functional versatility of individual components when assembled in higher order complexes.

Wnt signaling for N-AChR synaptic localization:

CAM-1, a ROR receptor tyrosine kinase, was shown to specifically promote the insertion of ACR-16 at postsynaptic sites, while it has no impact on L-AChRs and $GABA_A$ receptors (Francis *et al.* 2005; Babu *et al.* 2011; Jensen *et al.* 2012). It colocalizes and functions with LIN-17/Frizzled and DSH-1/Disheveled at postsynaptic sites, which eventually regulates HMP-2/β-catenin (Pandey *et al.* 2017).Together with LIN-17, CAM-1 serves as a coreceptor for the Wnt ligands CWN-2 and LIN-44 expressed in cholinergic motoneurons (Jensen *et al.* 2012; Pandey *et al.* 2017). Optogenetic stimulation of these neurons stimulates the secretion of CWN-2 and LIN-44 and enhances N-AChR-dependent currents. The basal secretion of Wnt ligands is negatively regulated in neurons by the tetraspan protein HIC-1 (Tikiyani *et al.* 2018), and the activity of CAM-1 is antagonized by RIG-3, an immunoglobulin superfamily protein expressed presynaptically that is proposed to interact trans-synaptically with the ectodomain of CAM-1 (Babu *et al.* 2011; Pandey *et al.* 2017).

Wnt-dependent regulation of ACR-16 provides a means to rapidly regulate synaptic strength in response to motoneuron activity. Its interplay with the assembly of the syndecan-dependent core clustering complex remains to be investigated.

Neuron–neuron synapses

Defining postsynaptic domains at cholinergic to GABAergic motoneuron synapses

Cholinergic motoneurons also synapse onto GABAergic neurons at dyadic synapses. The AChRs expressed by the GABAergic neurons are almost identical to the muscle L-AChRs, except that LEV-8 is replaced by the closely related ACR-12 subunit (Petrash *et al.* 2013; Philbrook *et al.* 2018). However, the molecules required for L-AChR clustering, such as MADD-4 and LEV-10, play a minor role for the clustering of ACR-12-containing receptors (ACR-12*). In contrast, NRX-1/neurexin, present in presynaptic cholinergic boutons, is necessary for the stabilization of spine-like protrusions on the dendrites of the GABAergic neurons and for the clustering of ACR-12* at the tip of these spines (Philbrook *et al.* 2018; Oliver *et al.* 2022). Interestingly, *nrx-1* disruption impairs the transmission between cholinergic and GABAergic neurons but has no functional impact on neuromuscular transmission (Hu *et al.* 2012; Philbrook *et al.* 2018). Since NLG-1 is dispensable in this process, NRX-1 and ACR-12* partner(s) remain to be identified. These results emphasize the remarkable diversity of the molecular pathways involved in trans-synaptic organization since the same presynaptic element uses two distinct molecular pathways to differentiate ACh-sensitive receptive domains on immediately adjacent cells.

Regulation of synaptic glutamate receptor content

The *C. elegans* genome encodes at least 10 subunits of cationic glutamate receptors homologous to AMPA, kainate, and NMDA receptors that are involved in several behaviors including locomotory control, mechanical and chemical sensation, and learning and memory (for review, see Brockie and Maricq (2006)). The mechanisms governing their synaptic localization have been mostly studied for the GLR-1 and GLR-2 subunits that form readily detectable puncta along the ventral cord of worms expressing GFP-tagged subunits in command interneurons. The use of GLR-1-GFP, in combination with genetic screens for mutants defective in glutamate-dependent behaviors, was extremely powerful to identify regulators of trafficking, membrane insertion, and dynamics of GLRs. However, a comprehensive understanding of the mechanisms controlling the postsynaptic differentiation of glutamatergic synapses is still in progress.

LIN-10/Mint is a PDZ domain-containing protein previously shown to be required for proper localization of GLR-1. LIN-10 localizes at glutamatergic synapses in the ventral cord independently from GLR-1, and in *lin-10* mutants GLR-1-GFP is diffusely distributed (Rongo *et al.* 1998). However, LIN-10 does not bind GLR-1 and might indirectly promote the insertion of the receptor in postsynaptic domains by promoting its recycling from endosomes (Glodowski *et al.* 2005; Zhang *et al.* 2012). LIN-10 is negatively regulated under hypoxia conditions, which leads to decreased GLR-1 synaptic content (Juo *et al.* 2007; Park *et al.* 2009, 2012; Park and Rongo 2016). MAGI-1, another PDZ-containing protein, localizes at some glutamatergic synapses. It physically binds GLR-2 but is not required for its synaptic localization, yet it counteracts the ubiquitin-dependent withdrawal of receptors observed in mechanically stimulated *magi-1* mutants (Emtage *et al.* 2009).

The trafficking of GLR-1 to postsynaptic sites requires UNC-43/CAMKII and the UNC-116/KIF5 kinesin motor-protein. In *unc-43* and *unc-116* mutants, GLR-1 is significantly decreased at postsynaptic sites and UNC-43 is required for activity-dependent insertion of GLRs at synapses (Rongo and Kaplan 1999; Hoerndli *et al.* 2013, 2015). CDK-5/Cyclin-dependent kinase and KLP-4/KIF13A, another kinesin family member, are also required for the trafficking of GLR-1 (Juo *et al.* 2007; Monteiro *et al.* 2012), but the exact interplay between *unc-43 and cdk-5* remains to be elucidated. The surface content of GLRs is dynamically regulated by ubiquitination together with clathrin-mediated endocytosis (Burbea *et al.* 2002; Juo and Kaplan 2004; Schaefer and Rongo 2006; Glodowski *et al.* 2007; Kowalski *et al.* 2011; Zhang *et al.* 2012; Dahlberg and Juo 2014; Garafalo *et al.* 2015). The muscle-released PVF-1 peptide was recently shown to increase surface content of GLRs through the VPS-35/retromer recycling complex by activating its cognate VEGF neuronal receptor homologs, VER-1 and VER-4 (Luth *et al.* 2021).

Mechanisms of synapse specificity

In the past two decades, genetic research in *C. elegans* revealed various secreted and contact-dependent signaling cues play crucial roles in spatial synaptic arrangement and target specificity during the development of nervous system. Here we will summarize and discuss these signaling cues in *C. elegans*.

Inhibitory cues that define the presynaptic position of DA9

The most posterior cholinergic motor neuron DA9 forms *en passant* synapses onto the dorsal body-wall muscles. While the DA9

axon extends to the middle of the worm body, it forms NMJs onto the most posterior dorsal body-wall muscles within a specific segment of its axon. The unique topographic organization and availability of the cell-specific markers made the DA9 neuron a platform to uncover various inhibitory cues that restrict synapses within a specific axonal domain.

Wnt signaling

C. elegans possesses five Wnt homologs (*mom-2, lin-44, egl-20, cwn-1,* and *cwn-2*) (Sawa and Korswagen 2013). Among them, LIN-44 is secreted from the hypodermal cells located at the tip of tail. LIN-44 recruits its receptor, LIN-17/Frizzled, to the posterior axonal region of DA9 to locally inhibit presynaptic assembly (Fig. 3) (Klassen and Shen 2007; Mizumoto and Shen 2013b). In the *lin-44(lof)* mutants, ectopic synapses are formed in the posterior dorsal axonal region of DA9. Loss-of-function mutants of *egl-20/wnt,* which is expressed from the cells in the preanal ganglion, enhances the *lin-44(lof)* mutant phenotype: ectopic synapse formation is also observed in the commissure and the ventral axonal region of DA9. *lin-17(lof)* mutants mimic the *lin-44; egl-20* double mutant phenotype (Klassen and Shen 2007). Therefore, two Wnts, LIN-44 and EGL-20, act through LIN-17 to locally inhibit synapse formation in the DA9 posterior axonal domain. Overexpression of LIN-44 from the *egl-20* promoter displaces DA9 synapses more anteriorly, and overexpression of LIN-44 from the dorsal muscle-specific promoter displaced DA9 presynapses to the dendrite in the ventral nerve cord (Klassen and Shen 2007). Therefore, LIN-44 appears to act as a gradient inhibitory cue for presynaptic assembly. Mutants of *dsh-1/Dishevelled*, which is the intracellular transducer of Wnt signaling, partially mimics the synapse patterning defects of the *lin-17* mutants (Klassen and Shen 2007). While the downstream factors of Wnt signaling in inhibiting synapse formation are not fully understood, recent work showed that another Frizzled receptor, MIG-1, which inhibits synapse formation in another DA neuron (DA8) (Mizumoto and Shen 2013b), inhibits synapse formation in DA9 by controlling the retrograde transport of synaptic vesicles (Balseiro-Gomez *et al.* 2022). MIG-1 inhibits the localization of atypical kinesin, VAB-8, which recruits microtubule minus end proteins such as PTRN-1/Petrnin and NOCA-1/Ninein, and increases pausing of retrograde transport for inducing presynaptic assembly (Balseiro-Gomez *et al.* 2022).

While Wnt signaling acts as inhibitory cue for presynaptic assembly through LIN-17, LIN-17 itself has an Wnt-independent function in promoting presynaptic assembly in DA9 together with NRX-1/Neurexin (Fig. 3) (Kurshan *et al.* 2018). *nrx-1(lof)* single mutants have reduced synapses in DA9 neuron. While *lin-17(lof)* single mutants do not exhibit severe reduction in synapse number, the *nrx-1; lin-17* double mutants show synergistic reduction in synapse number in DA9. This loss of synapse phenotype of *nrx-1; lin-17* double mutants can be rescued by expressing a mutant LIN-17(ΔCRD) that cannot bind Wnt proteins, suggesting that LIN-17 acts as a pro-synaptogenic factor when it does not interact with Wnt.

UNC-6/Netrin signaling

UNC-6/Netrin is the first axon guidance cue identified using *C. elegans* (Hedgecock *et al.* 1990; Ishii *et al.* 1992). UNC-6 is expressed in the ventral nerve cord neurons and body-wall muscles (Fig. 3) (Wadsworth *et al.* 1996). Ventrally expressed UNC-6 is crucial for axon guidance as well as the distal tip cells (DTCs) migration along the D-V axis (Su *et al.* 2000). UNC-6 acts as a repulsive guidance cue for neurons expressing UNC-5/UNC5 and UNC-40/DCC receptor complex, or as an attractive guidance cue for neurons expressing UNC-40/DCC homodimer (Leung-Hagesteijn *et al.* 1992; Chan *et al.* 1996; Wadsworth 2002).

In DA9, the ventrally secreted UNC-6 inhibits presynaptic assembly in the dendrite and ventral axon through UNC-5 (Fig. 3a and c) (Poon *et al.* 2008). Ectopic expression of *lin-44/Wnt* in the *unc-6*-expressing cells is sufficient to reduce ectopic synapses in the dendrite of *unc-6* mutants in an *unc-5*-independent manner. Similarly, expression of *unc-6* under the *lin-44* promoter is sufficient to rescue the DA9 synapse phenotype of *lin-44(lof)* mutants in a *lin-17*-independent manner. It is therefore possible that these cues use the same intracellular mechanisms to inhibit synaptogenesis. Two redundant Cyclin/CDK signaling pathways consisting of CYY-1/Cyclin Y and PCT-1/CDK, and CDKA-1/p35 and CDK-5 are the potential downstream signaling as the double mutants of these two pathway components such as *cyy-1; cdka-1* double mutants show ectopic presynaptic assembly phenotypes in the DA9 dendrite similar to *unc-6* mutants (Ou *et al.* 2010), though their genetic and molecular relationships are unknown.

Semaphorin-Plexin signaling in synaptic tiling

The EM reconstruction of the *C. elegans* nervous system revealed the complete but non-overlapping synaptic innervation within each motor neuron class thereby achieving tiled synaptic innervation along the verve cords (Fig. 3a) (White *et al.* 1976, 1986). In the loss-of-function mutants of transmembrane Semaphorin ligands (*smp-1* and *smp-2*) and their receptor *Plexin (plx-1)*, the tiled synaptic innervation of the two posterior DA neurons (DA8 and DA9) is compromised: the synapses of DA8 and DA9 are intermingled along their axons (Mizumoto and Shen 2013a). PLX-1 is localized at the anterior edge of the synaptic domain of DA9 in a *smp-1*- and DA8-DA9 axon contact-dependent manner, where it inactivates RAP-2/Rap2A small GTPase through its cytoplasmic RapGAP (GTPase-activating protein) domain to locally inhibit presynaptic assembly (Fig. 3b) (Mizumoto and Shen 2013a; Chen *et al.* 2018).

The hmolog of Rap2-interacting kinase TNIK (Traf2- and Nck-interacting kinase), *mig-15,* also acts in the same genetic pathway as *plx-1* and *rap-2* (Chen *et al.* 2018), while the molecular relationship between RAP-2 and MIG-15 remains elusive. *mig-15* mutants exhibit larger degree of overlap between the DA8 and DA9 synaptic domains compared with *plx-1(lof)* or *rap-2(lof)* mutants, which appears to be due to the increase in overall synapse number. This suggests that *mig-15* negatively controls the number of synapses formed in each neuron, in addition to its function in controlling synaptic tiling. Consistently, neuronal overexpression of *mig-15* reduced the number of presynaptic sites (Chen *et al.* 2018). Similar to the observation in DA8 and DA9, *mig-15* inhibits synapse formation in the PLM neuron (Crawley *et al.* 2017).

Glial and non-neuronal tissues as signaling guidepost cells to instruct synapse formation

UNC-6/Netrin from glia as a guidepost signal to instruct synapse formation

The AIY amphid interneuron forms synaptic connections with RIA interneuron within a specific axonal domain (Fig. 4a). This synapse location is determined by the UNC-6 from the glial cells (Colon-Ramos *et al.* 2007). Ventral cephalic sheath glial cells (CEPshVs) ensheath the AIY > RIA synaptic connection site (Wadsworth *et al.* 1996). In contrast to the DA9 dendrite, where UNC-6 inhibits presynaptic assembly through UNC-5, UNC-6 secreted from the glial cells promotes AIY presynaptic assembly by recruiting UNC-40/DCC in AIY (Colon-Ramos *et al.* 2007).

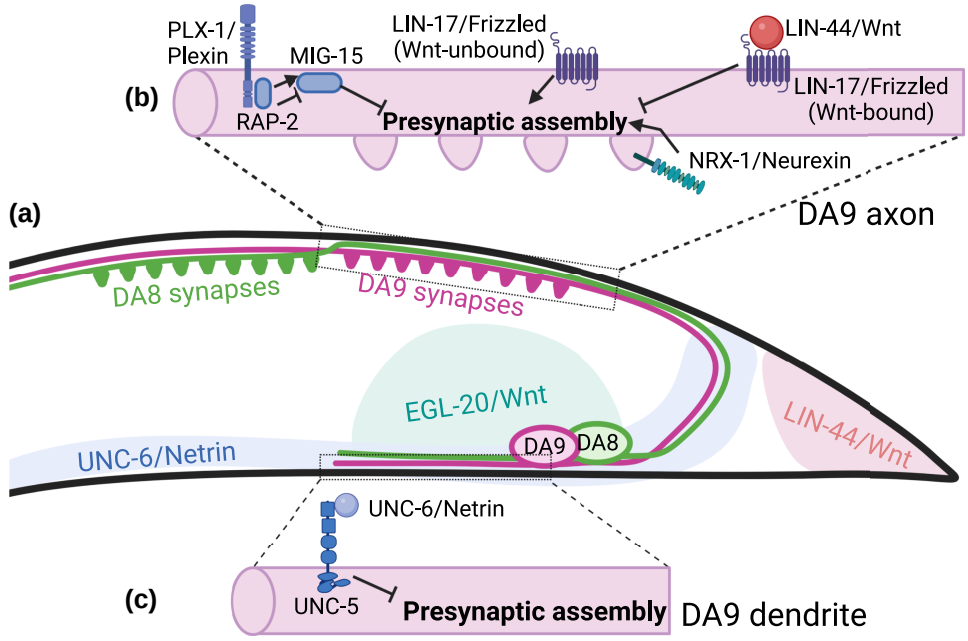

Fig. 3. Signaling molecules that specify spatial arrangement of DA9 neuromuscular junctions. a) UNC-6/Netrin, LIN-44/Wnt and EGL-20/Wnt form gradient distribution around DA9 to prevent the assembly of synapses. b) PLX-1/Plexin and their effectors locally inhibit presynaptic assembly in the distal axon. In the posterior axonal region, Wnt-bound LIN-17/Frizzled inhibits presynaptic assembly, while Wnt-unbound LIN-17 instructs synapse formation within the presynaptic domain. c) In the dendrite, UNC-5 inhibits presynaptic assembly.

In the AIY neuron, UNC-40 instructs presynaptic assembly through two downstream pathways, CED-5/DOCK180 and CED-10/Rac1, and SYD-2 and SYD-1 AZ proteins to recruit MIG-10/Lamellipodin to the presynaptic sites (Fig. 4a and b) (Stavoe and Colon-Ramos 2012; Stavoe *et al.* 2012). The localized MIG-10 recruits SVs via ABI-1/Abi1, a component of WAVE-regulatory complex (WRC) that organizes actin cytoskeleton. Recent work suggested that CDC-42 and PES-7/IQGAP may be required for the AIY synaptogenesis, while the relationship between CDC-42-PES-7 and UNC-40 remains elusive (Dong *et al.* 2020).

Consistent with the function of glial cells as a pro-synaptogenic guidepost cells, the increased glia-AIY contact sites in the *cima-1/SLC17* results in the ectopic presynaptic assembly in the AIY neuron (Shao *et al.* 2013).

SYG-1/Neph1 and SYG-2/Nephrin

Two transmembrane immunoglobulin domain superfamily (IgSF) proteins, SYG-1 and SYG-2, are essential for determining the position of presynaptic assembly in the HSNs (Shen and Bargmann 2003; Shen *et al.* 2004). HSNs form synapses onto vulval muscle

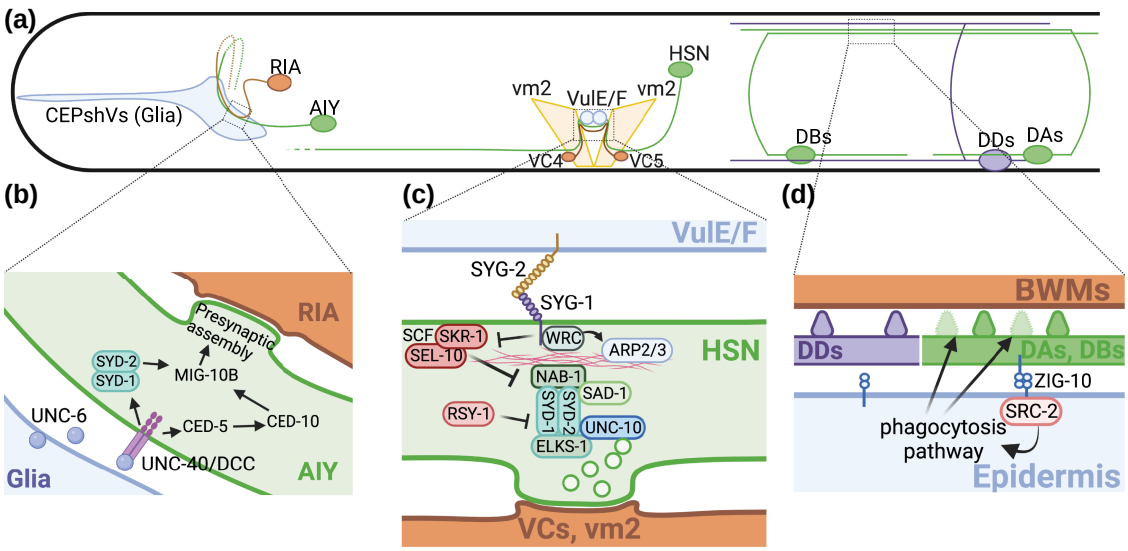

Fig. 4. Guidepost cells determine the position of synapses. a) Schematics of the neurons and their guidepost cells. b) AIY > RIA synaptic connection. Ventral cephalic sheath glial cells express UNC-6 to instruct synaptic connection between AIY > RIA. UNC-40/DCC induces synaptogenesis via two parallel pathways to recruit MIG-10/Lamellipodin. c) SYG-1 instructs synapse formation in the HSNs through interaction with SYG-2 in VulE/F. SYG-1 induces presynaptic assembly by promoting branched F-actin formation. d) Homophilic interaction of ZIG-10 expressed in epidermis and cholinergic motor neurons reduces the cholinergic synapse density through SRC-2-dependent phagocytosis pathway.

(vm2) cells and VC4/5 motor neurons for proper egg-laying behavior (Fig. 4a). SYG-2/Nephrin, which is exclusively expressed in the primary vulval epithelial cell lineage, recruits SYG-1/Neph1 in the HSNs, where it locally instructs presynaptic assembly. Ectopic expression of SYG-2 in the secondary vulval epithelial cells recruits SYG-1 at the HSN axonal region in contact with SYG-2-expressing epithelial cells to induce ectopic synapse formation. Therefore, the primary vulval epithelial cells serve as guidepost cells for HSN synaptogenesis. Protein structure studies show that the extracellular domains of SYG-1 and SYG-2 bind in an orthogonal geometry, and this conformation is essential for their function in instructing presynaptic assembly (Ozkan *et al.* 2014). In the HSNs, SYG-1 intracellular domain binds WVE-1, a core component of the WRC. Recruited WRC assembles Arp2/3-dependent branched F-actin at the presynaptic sites, which then recruits NAB-1 to instruct AZ assembly as described in the previous section (Fig. 4c) (Chia *et al.* 2012, 2014). This is in contrast to the AIY presynaptic assembly, in which AZ proteins instruct presynaptic F-actin formation, suggesting the presence of neuron-type-specific presynaptic assembly mechanisms. In addition to its instructive role in presynaptic assembly, SYG-1 also protects synapses from degradation. In the HSNs, the SCF (Skp1-cullin-F-box) E3 ubiquitin ligase complex composed of SKR-1/Skp1 and a F-box protein, SEL-10, induces synapse degradation. SYG-1 intracellular domain binds SKR-1 and inhibits SCF complex assembly, thereby creating a permissive environment for presynaptic assembly (Ding *et al.* 2007).

ZIG-10 two-immunoglobulin domain protein and phagocytosis modulate density of cholinergic NMJs

In *C. elegans*, the nerve cords are ensheathed by the epidermis, which also extends small protrusions near NMJs, analogous to glia cells in mammals. The ZIG-10 two-Ig domain transmembrane protein modulates the synaptic density of the cholinergic motor neurons. *zig-10* was identified from a genetic enhancer screening of the epileptic seizure-like convulsion phenotype of the gain-of-function mutant of *acr-2* (Cherra and Jin 2016). In the *zig-10(lof)* mutants, cholinergic motor neurons form excessive number of synapses. ZIG-10 is expressed in the epidermis and the cholinergic neurons, and the homophilic interaction between ZIG-10 in these tissues activates SRC-2 kinase in the epidermis, which then induces the CED-1/Draper-dependent phagocytosis of the cholinergic synapses (Fig. 4d).

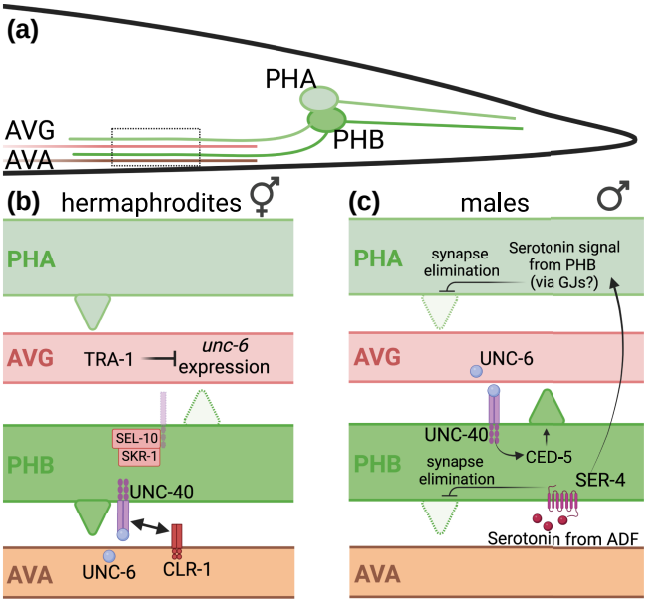

Fig. 5. Sexually dimorphic synaptic patterning. a) schematic of the PHA and PHB phasmid neurons and the AVA and AVG interneurons. b) Synaptic connection of PHA > AVG and PHB > AVA in hermaphrodites. c) Synaptic connection of PHB > AVG in males. The PHA > AVA synaptic connection is not shown in this diagram. GJs: gap junctions.

Sex-specific synapse specificity

In *C. elegans*, some neurons shared in hermaphrodites and males exhibit sexually dimorphic synaptic connection pattern. Recent works using GFP reconstitution across synaptic partners (GRASP), which visualizes the synaptic connections between two neurons (Feinberg *et al.* 2008), has enabled the studies on the sexually dimorphic synaptic connections among sex-shared neurons.

Sexually dimorphic UNC-6 expression specifies sex-specific synaptic connections

In adult hermaphrodites, the PHB sensory neurons synapse onto the AVA interneuron, and the PHA sensory neurons synapse onto the AVG interneuron (Fig. 5) (White *et al.* 1976, 1986). In adult males, PHBs synapse onto AVG, and PHAs synapse onto AVA. At the L1 stage, PHBs synapse onto both AVA and AVG interneurons in both sexes. By the L4 stage, the PHB > AVG synaptic connections are eliminated or pruned in hermaphrodites (Fig. 5b), while the PHB > AVA synaptic connections are pruned in males (Fig. 5c). This rearrangement coincides with the sex-specific behaviors, including vulva searching behavior mediated by the PHB > AVG connection in males (Oren-Suissa *et al.* 2016; Bayer *et al.* 2020).

The sexually dimorphic expression pattern of *unc-6* underlies the sex-specific synaptic pruning. In hermaphrodites, UNC-6 secreted from the AVA neuron specifies PHB > AVA synaptic connection through UNC-40 in the PHB axon (Fig. 4b) (Park, Knezevich, *et al.* 2011). Therefore, UNC-6 functions as a pro-synaptogenic factor through UNC-40 in PHB, similar to their functions in the AIY neurons. CLR-1, an LAR -type receptor tyrosine phosphatase, in AVA also controls UNC-6-depdendent PHB > AVA synaptic connectivity (Fig. 5b) (Varshney *et al.* 2018), while its molecular relationship with UNC-40 is not known. *unc-6* is expressed in AVG in both sexes at L1 stage. However, at the L4 stage, *unc-6* expression is lost in hermaphrodites, which is mediated by the expression of TRA-1/Transformer, a sex-determining transcription factor in hermaphrodites (Fig. 5b). TRA-1 antagonizes the LIN-11-dependent *unc-6* expression in AVG likely by directly binding to the TRA-1-binding sites located in the *unc-6* intron (Weinberg *et al.* 2018).

Mechanistically, UNC-6 protects UNC-40 from ubiquitin-mediated protein degradation (Salzberg *et al.* 2020). In hermaphrodites, loss of *unc-6* expression results in the UNC-40 degradation in PHBs through the physical interaction between UNC-40 intracellular domain and SEL-10, an F-box protein in the SCF ubiquitin ligase complex (Fig. 5b). In the *sel-10* mutants, UNC-40 degradation does not occur and the PHB > AVG connection is maintained in adult hermaphrodites. In adult males, UNC-6 expression from AVG protects UNC-40 from degradation, and UNC-40 maintains PHB > AVG synaptic connection partly through CED-5/DOCK180 (Weinberg *et al.* 2018).

The hermaphrodite-specific loss of PHB > AVG synaptic connection also depends on the expression of Doublesex/Mab-3-related transcription factor (DMRT), DMD-4 (Bayer *et al.* 2020). Similar to *sel-10(lof)* mutants, the PHB > AVG connection is maintained in the *dmd-4(lof)* mutant hermaphrodites. DMD-4 protein is detectable in hermaphrodites but not males at adult stage,

and it appears that ubiquitin binding stabilizes DMD-4 in hermaphrodites.

Sexually dimorphic serotonin signaling induces male-specific synaptic pruning

In males, PHA > AVG and PHB > AVA synaptic connections are pruned (Bayer and Hobert 2018). The male-specific synapse pruning is dependent on the serotonin signal from the sex-shared ADF neuron (Fig. 5c) (Bayer and Hobert 2018). The expression of the serotonin-synthesizing enzyme tryptophan hydroxylase (*tph-1*) in ADF is significantly higher in males than hermaphrodites. The serotonin is sensed by the 5HT1A serotonin receptor, SER-4, in the PHB neurons to induce pruning of the PHB > AVA connection (Fig. 5). In the loss-of-function mutants of *ser-4*, the PHA > AVG and PHB > AVA connections are maintained in adult males. While the PHA neurons do not express *ser-4*, they may indirectly respond to the serotonin signaling through male-specific gap junctions between PHA and PHB (Bayer and Hobert 2018). Interestingly, starvation-induced octopamine synthesis in the RIC interneurons inhibits the serotonin synthesis in ADF, which results in the persistence of hermaphrodite-specific PHA > AVG and PHB > AVA connections in males (Bayer and Hobert 2018).

Developmental synapse remodeling

Synapses can be modified in many ways throughout the lifetime of an animal, such as widespread formation of new synaptic connections during metamorphosis in insects and amphibians, or selective and precise refinement and consolidation of synapses during specific periods of development in mammalian visual cortex and neuromuscular junctions. In *C. elegans*, the remodeling of synaptic connectivity of GABAergic Dorsal D (DD) motor neurons during larval development, often called "DD remodeling" or "DD rewiring", represents a dramatic form of synapse plasticity.

Over four decades ago, realizing that the juvenile (L1) larvae have fewer ventral cord motor neurons than the adult locomotor circuit (Sulston and Horvitz 1977), John White and colleagues reconstructed the nerve cords of two young L1 larvae and discovered that the synapse positions for the embryonically born DD neurons differed from those in adult DDs (White *et al.* 1978). Six DD neurons have their soma evenly spaced along the ventral nerve cord and differentiate a side-down H shape morphology, with longitudinal neurites extending within ventral and dorsal nerve cords, connected by circumferential commissures (Fig. 6a, left). In young L1, the ventral neurites of DDs form presynaptic terminals to innervate ventral body muscle, and the dorsal neurites act as dendrites to receive synaptic inputs from the cholinergic DA and DB motor neurons (Fig. 6b, left), which are born in embryos and form dyad synapses onto dorsal body muscle and DDs. Around the time of post-embryonic cell division, which generates additional 53 ventral cord motor neurons (Sulston and Horvitz 1977), DDs undergo a complete connectivity switch such that they innervate dorsal body muscle and receive inputs from post-embryonic cholinergic VA and VB motor neurons, which also innervate ventral body muscle (White *et al.* 1986) (Fig. 6a and b, right panels). Very recently, a large-scale EM reconstruction of the ventral nerve cord from different timepoints of L1 to adult has been generated, which reveals exquisite ultrastructure dataset on DD remodeling and formation of mature locomotor circuitry (Witvliet *et al.* 2021; Mulcahy *et al.* 2022). This resource will have immense value for researchers to investigate synapse and circuit formation and refinement.

The entire process of DD remodeling, which entails formation of new synapses in dorsal neurites, elimination of pre-existing synapses in ventral neurites, and similar reversal of postsynaptic sites, happens without gross changes in neuronal morphology. Combining synapse visualization with genetics and genomics, studies over the past two decades have revealed that DD remodeling, along with formation of post-embryonic locomotor circuitry, is regulated by exceedingly complex genetic pathways that act in parallel and/or redundant manners (Kurup and Jin 2016; Cuentas-Condori and Miller 3rd 2020). Below, we review key molecular players and mechanisms emerged from these studies.

Factors that regulate the timing and initiation of DD remodeling

The use of transgenic GFP reporters for presynaptic terminals enabled in vivo visualization of DD remodeling (Hallam and Jin 1998). By correlating the appearance of SNB-1::GFP marker in the dorsal neurites of DDs with post-embryonic P neuroblast migration and division, it was determined that DDs initiate remodeling in mid-L1, approximately 10 hours post hatching, and that remodeling of individual DDs proceeds in an anterior to posterior manner. Elimination of pre-existing synapses in DD ventral neurites happens concurrently, although precise time course remains to be correlated with post-embryonic cell division. By late L2, remodeling of DD axonal domains is generally considered to be complete, as verified in the recent published developmental EM connectomics (Witvliet *et al.* 2021; Mulcahy *et al.* 2022). The timing of DD remodeling coincides with the birth of the VD neurons, which form synapses to the ventral body muscle, thereby replacing the juvenile DD synapses. However, the absence of VD or other ventral cholinergic motor neurons does not affect the initiation of DD remodeling. Both EM studies and live imaging of DD remodeling in *lin-6(lof)* mutants, where post-embryonic P cell division is defective (Horvitz *et al.* 1983), showed that DDs initiate and complete new synapse formation normally (Hallam and Jin 1998), with some synaptic inputs from the cholinergic DA and DB neurons remaining, a possible passive outcome in the absence of the VA and VB neurons (White *et al.* 1978).

Nuclear factor LIN-14 represses DD remodeling

The heterochronic gene *lin-14* was the first factor identified to regulate DD remodeling. LIN-14 is a novel nuclear protein and is well known as a master regulator of temporal patterns of cell division (Ambros and Horvitz 1987; Ruvkun and Giusto 1989). By immunofluorescence staining using anti-LIN-14 antibodies, it was observed that the expression levels of LIN-14 in DDs decrease at the onset of remodeling (Hallam and Jin 1998). Loss of *lin-14* results in precocious DD remodeling, the degree of which correlates with the reduced activity levels of *lin-14*. Constitutive expression of LIN-14 delays DD remodeling. *lin-14(lof)* does not appear to affect the duration of DD remodeling, judged by the intensity of the presynaptic SNB-1::GFP marker in DD dorsal neurites. These data identified the first role of *lin-14* in post-mitotic cells and also indicate that *lin-14* represses the initiation of DD remodeling. LIN-14 regulates expression of many genes at the transcriptional level (Hristova *et al.* 2005). One relevant target of LIN-14 in DD remodeling is *oig-1*, encoding a one-immunoglobin domain protein (Howell *et al.* 2015). OIG-1 is transiently expressed in DDs in embryo and early L1, i.e. pre-remodeling; and in *lin-14* null animals, this transient expression

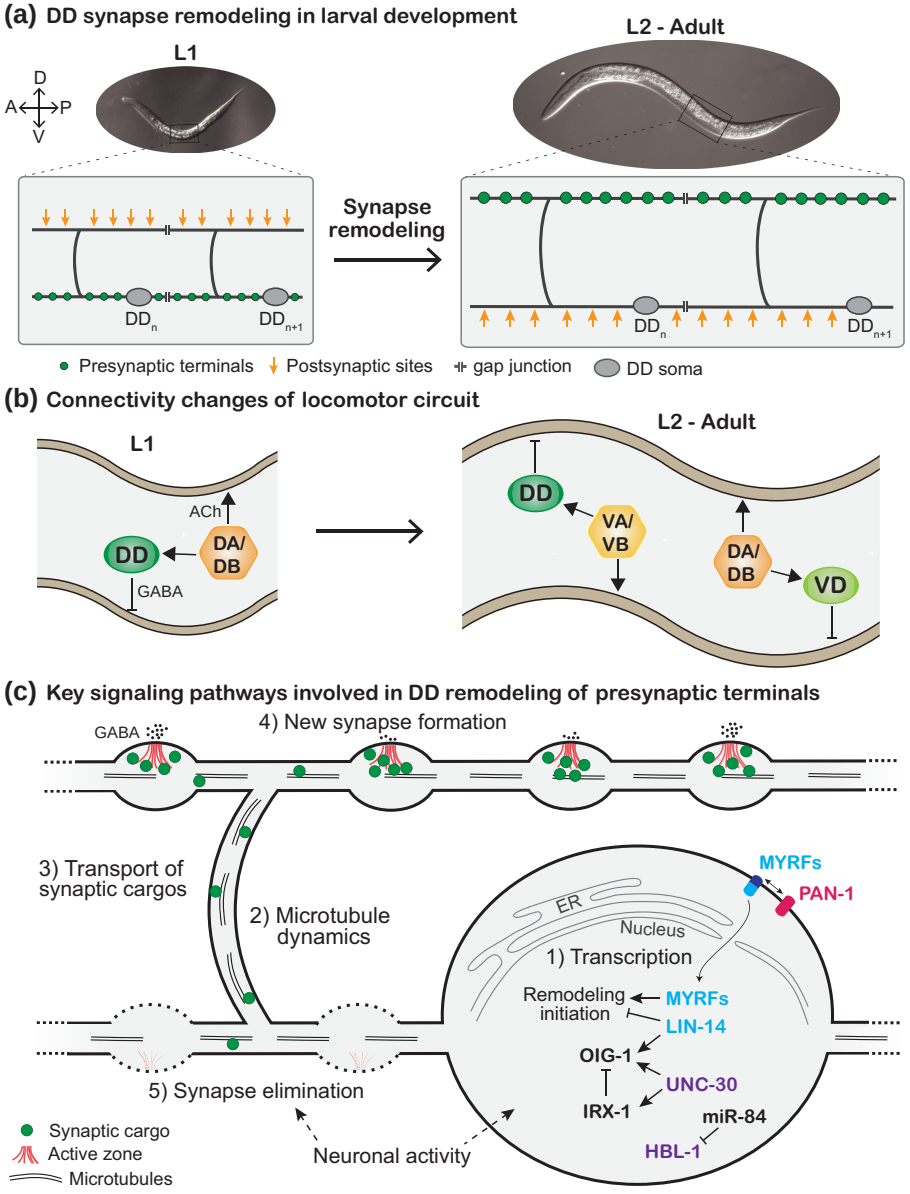

Fig. 6. Developmental synapse remodeling of DD motor neurons. a) Illustration of DD neuron morphology and connectivity in early and mid-L1 larvae (left) and late L2 to adults (right). b) Schematics of the ventral cord locomotor circuit in young L1s (left) and adults (right). In L1s DA/DB cholinergic neurons excite both dorsal body muscles (curved light brown shade) and DD neurons, which inhibit ventral body muscles. In adults VA/VB cholinergic neurons excite both ventral body muscles and DD neurons, which inhibit dorsal body muscles. c) Graphic summary of major regulators of DD synapse remodeling (see main text for detailed description and references). Nomarski images and art drawing are credited to Eugene Jennifer Jin.

is diminished. In young L1 of *oig-1* null mutants, presynaptic markers are detected in dorsal neurites of some DDs, along with clusters of the postsynaptic UNC-49 receptors in the dorsal body muscle and other defects in postsynaptic domain of DDs, suggesting precocious DD remodeling (He *et al.* 2015; Howell *et al.* 2015). Expression of wild type *oig-1* in juvenile DDs also depends on the UNC-30/Pitx transcription factor, which functions to specify GABAergic fate of both DD and VD neurons (Jin *et al.* 1994). EM studies of an *unc-30(lof)* mutant L1 reveal multiple differentiation defects, including DD neurite forming aberrant innervation to dorsal body muscle (Howell *et al.* 2015). These data led to the model that LIN-14 and UNC-30 co-regulate *oig-1* and other transcriptional targets to maintain synaptic connectivity of juvenile DDs (Howell *et al.* 2015) (Fig. 6c.1).

MYRF proteins and PAN-1 LRR-TM protein promote DD remodeling

The myelin regulatory factor (Myrf) family proteins are membrane-associated transcription factors, named based on the original reports that mouse Myrf regulates myelinogenesis genes (Bujalka *et al.* 2013; Li *et al.* 2013). They contain at the N-terminus a DNA-binding domain that is homologous to that of yeast Ndt80, followed by an Intramolecular Chaperone of Endosialidase (ICE) domain and a transmembrane domain predicted to be anchored to the ER. The ICE domain mediates the formation of a homotrimer, which triggers auto-cleavage and release of the DNA-binding domain, which can then enter the nucleus to mediate transcription. *C. elegans* has two MYRF homologs, MYRF-1 and MYRF-2. Both MYRFs are broadly expressed in many tissues

and neurons. *myrf-1* null mutants arrest in larvae, whereas *myrf-2* null mutants are healthy like wild type. Isolation of a missense mutation in *myrf-1* that blocks DD remodeling uncovered the roles of both *myrf* genes acting together to promote DD remodeling (Meng *et al.* 2017). The missense mutation of *myrf-1* alters the DNA-binding domain, and acts in a dominant-interfering manner, resulting in inhibition of both *myrf-1* and *myrf-2*. Indeed, DD remodeling occurs normally in single loss-of-function in *myrf-1* or *myrf-2*, whereas simultaneous loss of *myrf-1* and *myrf-2* blocks DD remodeling, recapitulating the missense mutation of *myrf-1*. Expression of both MYRFs in DDs is up-regulated in late L1, and both *myrf* genes act cell autonomously to promote DD remodeling. The ICE domain-dependent cleavage is critical for nuclear translocation of the DNA-binding domain of both MYRFs. The LRR-TM protein PAN-1 was identified as a binding partner of both MYRFs (Xia *et al.* 2021) (Fig. 6c.1). Loss of *pan-1* blocks DD remodeling, resembling loss of both *myrfs*. Unexpectedly, mechanistic dissection of MYRFs and PAN-1 interaction revealed that both MYRFs and PAN-1 are co-trafficked to the cell membrane of DD neurons. Moreover, the binding interaction between MYRFs and PAN-1 involves the C-terminal domain of MYRFs, which is localized extracellularly, although the protein binding may occur in the ER. PAN-1 is essential for nuclear localization and stabilization of N-terminus of MYRFs, although it remains to be addressed whether and how PAN-1 regulates cleavage of MYRFs. Future studies will reveal how MYRFs transcriptionally regulate genes that are involved in DD remodeling.

Multiple dynamic cellular processes facilitate completion of DD remodeling

Once DD remodeling is initiated, multiple events must happen to restructure axonal and dendritic domains so that pre- and postsynaptic cargos are delivered to the correct neurites and organized into subcellular structures. Work in the past decade has shown a remarkable theme that nearly every step towards formation and stabilization of new synapses and elimination of pre-existing synapses involves parallel pathways.

Formation of new synapses in DD dorsal neurites

Microtubules (MTs) are the primary cytoskeleton in transport of synaptic materials in neurons. In neurons with elaborate morphology, axons and dendrites generally have distinct polarities. *C. elegans* DD neurons have a pseudo-unipolar morphology, with branching to form circumferential commissures and bifurcation in the dorsal cord to form dorsal neurites. Using MT plus-end binding protein (EBP-2) marker, it was determined that MT polarity is plus-end out in ventral and dorsal neurites of both juvenile and adult DDs, indicating that DD remodeling of axon–dendrite identity is uncoupled from MT polarity (Kurup *et al.* 2015). A major regulatory mechanism underlying the delivery of axonal cargos to DD dorsal neurites during remodeling involves dynamic MTs (Fig. 6c.2-3). Upon initiation of DD remodeling the number of dynamic MTs is increased. Dynamic MTs facilitate synaptic vesicle transport along the DD neuron commissure by the kinesin motors UNC-104/Kinesin-3 and UNC-116/Kinesin-1. In mutant animals with low dynamic MTs during DD remodeling, new synapses in the dorsal neurite fail to be stabilized. The conserved MAP3Kinase DLK-1 is intimately linked to dynamic changes in MT cytoskeleton (Ghosh-Roy *et al.* 2012). During DD remodeling, DLK-1 mediates an increase in the number of dynamic MTs, partly through MT catastrophe factors like KLP-7/Kinesin-13 and SPAS-1/Spastin. A pulse of DLK-1 expression at the onset of DD remodeling facilitates new synapse formation. While loss of *dlk-1* alone results in a slight delay in the completion of DD remodeling, in conditions where MT structure is compromised due to a genetic alteration in TBA-1/α-tubulin, loss of *dlk-1* completely blocks DD remodeling (Kurup *et al.* 2015). Thus, temporal activation of DLK-1 synergizes with changes in MT cytoskeleton to promote DD remodeling.

Another parallel pathway that facilitates new synapse formation involves the kinase CDK-5 and the CYY-1/cyclin E (Park, Watanabe, *et al.* 2011). Single loss-of-function mutants for *cdk-5* or *cyy-1* exhibits incomplete remodeling, as presynaptic markers remain detectable in DD ventral neurites in older larvae and adults. Double mutants of *cyy-1; cdk-5* show a strong block of DD remodeling as presynaptic markers are mostly retained in ventral neurites, with few detectable in dorsal neurites. Pulsed expression of both genes even in older larvae of the double mutants can promote completion of DD remodeling, suggesting that they may maintain or stabilize new synapses. Intriguingly, while CDK-5 facilitates new synapse formation in DD dorsal neurites through enhancing UNC-104-mediated anterograde transport of presynaptic cargos (Fig. 6c.4), CYY-1 is found to be involved in elimination of pre-exiting synapses in juvenile DD ventral neurites. Additionally, patterning of new synapses in the re-structured dorsal neurites is mediated by coordinated transport involving both the kinesin UNC-104 and the dynein DHC-1 (Park, Watanabe, *et al.* 2011; Kurup *et al.* 2017). Collectively, these findings unveil the existence of multiple redundant pathways that regulate MT growth and motor-cargo interactions to initiate and complete DD remodeling (Fig. 6c.2-4).

Synapse elimination in DD ventral neurites

Concurrent with formation of new synapses in the dorsal neurite of DD neurons to body muscles, existing synapses are eliminated from the ventral neurite of DD neurons. Synapse elimination depends on intracellular calcium (Miller-Fleming *et al.* 2016), and also involves several pathways that appear to act in parallel. The classical CED apoptosis signaling pathway, including CED-9/BCL-2 and CED-3/Caspase, acts through GSNL-1, a gelsolin-related protein, to promote F-actin disassembly in DD synapse elimination (Meng *et al.* 2015) (Fig. 6c.5). The homeobox transcription factor IRX-1/Iroquois is expressed in L1 DDs and down-regulates expression of *oig-1* during L1/L2 transition (Petersen *et al.* 2011; He *et al.* 2015). *irx-1* null animals are L1 lethal; and down-regulation of *irx-1* by DD neuron RNAi causes delayed initiation of DD remodeling (Petersen *et al.* 2011). Interestingly, IRX-1 also regulates synapse elimination by controlling two parallel pathways (Miller-Fleming *et al.* 2021). In one pathway, IRX-1 promotes the expression of the UNC-8 DEG/ENaC cation channel subunit, which affects neuronal activity to disassemble synaptic vesicles and associated cytomatrix. In another pathway, IRX-1 selectively regulates disassembly of a subset of presynaptic cytomatrix involved in docking and priming synaptic vesicles. How CYY-1 functions in synapse elimination remains to be investigated.

Remodeling of postsynaptic sites of DD neurons

DD dendrites receive synaptic inputs from cholinergic motor neurons. Recent super-resolution microscopy and EM studies have revealed that DD dendrites have actin-rich spines that share key hallmarks of mammalian spines (Cuentas-Condori *et al.* 2019). These spines contain ER and ribosomes and also display calcium transients evoked by presynaptic activity as well as respond to activity-dependent signals that modulate spine density. The acetylcholine receptor subunit ACR-12 is localized to DD dorsal neurites in early L1 (Petrash *et al.* 2013; He *et al.* 2015). The trans-

synaptic adhesion protein Neurexin/NRX-1 is required in presynaptic cholinergic motor neurons to promote postsynaptic spine formation and maintenance in DD dendrites (Philbrook *et al.* 2018). As DD remodeling initiates, ACR-12::GFP in the dorsal neurites disappear, and nascent ACR-12::GFP puncta emerge on the ventral neurites. In *oig-1* mutants, the emergence of ACR-12::GFP puncta in the ventral neurites is accelerated (He *et al.* 2015; Howell *et al.* 2015). How spinogenesis is initiated during DD remodeling remains to be investigated.

Neuronal activity influences DD remodeling

Global circuit activity can alter the timing and duration of DD remodeling, such that delayed or accelerated DD remodeling is observed in genetic mutants that block or exaggerate synaptic transmission, such as loss-of-function mutants of *unc-13* and *tom-1*, respectively (Thompson-Peer *et al.* 2012). The circuit activity changes influence the expression of the Hunchback transcription factor HBL-1 and its microRNA regulator, miR-84 (Fig. 6c.1). In mutants lacking GABA transmission, DD remodeling can proceed to completion, with delayed elimination of ventral synapses (Jin *et al.* 1999; Miller-Fleming *et al.* 2016). Optogenetic activation of juvenile DD leads to precocious appearance of synapses in the dorsal neurites (Miller-Fleming *et al.* 2016). A target of neuronal activity is the DEG/ENaC cation channel subunit UNC-8. The levels of cAMP, revealed by in vivo FRET imaging, show a positive correlation with DD remodeling (Yu *et al.* 2017). Loss of *pde-4* causes some DD neurons to remodel precociously. In mutants that display precocious DD remodeling, cAMP levels are also elevated. CHIP-seq analyses, together with studies of genetic mutants, suggest that multiple transcriptional factors, such as LIN-14, IRX-1, and UNC-30 coordinate expression of cAMP regulators in DD neurons.

The COUP-TF UNC-55 represses remodeling of post-embryonic VD neurons

The post-embryonic VD motor neurons share all differentiated features as DDs, and their GABAergic fate is under the control of UNC-30 (Jin *et al.* 1994). From L2 to adults, VDs form synapses to ventral body muscles, replacing the function of L1 DD neurons. VDs do not undergo remodeling, which is due to selective expression of the COUP-TF nuclear hormone receptor UNC-55 in the VD neurons (Zhou and Walthall 1998; Shan *et al.* 2005). Loss of *unc-55* causes VD neurons to innervate dorsal body muscle. Moreover, ectopic expression of UNC-55 in L1 DD neurons can repress their remodeling, supporting the idea that lack of UNC-55 in DD neurons enables their ability to remodel. Studies of transcriptional targets of UNC-55 have led to the finding that UNC-55 represses the expression of the Iroquois-like homeodomain protein IRX-1 and the Hunchback-like transcription factor HBL-1, both of which are normally expressed in the DD neurons and promote remodeling (Petersen *et al.* 2011; Thompson-Peer *et al.* 2012). Thus, UNC-30 co-regulates transcriptional targets with LIN-14 in L1 DD neurons, such as *oig-1* and *irx-1*, to facilitate remodeling, and UNC-55 works with UNC-30 in VD neurons to inhibit synapse remodeling.

Concluding remarks and future perspective

C. elegans has proven to be an extremely fruitful system for identifying new factors and mechanisms involved in the biology of synapses. Over the last 20 years, countless studies have confirmed the striking conservation of neurochemical synapse organization through evolution, which enables the possibility to transfer new knowledge between distantly related animals, such as worms, flies, and mammals. Specifically, the ease of *C. elegans* handling enables in vivo analysis of synaptic components and regulators to detail levels that are way more complicated to achieve in the mouse.

However, in contrast to its anatomical simplicity, the genetic complexity of *C. elegans* is comparable to mammalian genomes, with ≈20,000 coding genes, including nematode-specific expansions of many neuron function-related gene families such as diverse ion channel families, sensory receptors and neurotransmitter receptors (Hobert 2013). At the moment, we must admit that the molecular mechanisms governing the formation and the composition of *C. elegans* synapses are totally unknown for probably more than three quarters of those! As in other animals, fundamental questions remain: what fraction of the synapses is genetically encoded and what depends on system activity? What are the molecular pathways that trigger the formation of a synapse between two specific neurons? What determines the molecular composition of a given synapse? What mechanisms control synaptic plasticity in response to the animal experience? Fortunately, these questions should greatly benefit from the recent explosion of new technologies that can be applied in *C. elegans*.

First, considerable efforts have been made to increase EM throughput to get access to the "connectome" of the nervous system of various animals. Recent reconstruction of eight isogenic *C. elegans* hermaphrodites at different post-embryonic ages showed that connections between neurons are dynamic during development (≈15% of connections) and that more than 40% of connections between two specific neurons are variable among isogenic animals (accounting for 16% of total synapses) (Witvliet *et al.* 2021). Hence, *C. elegans* synaptogenesis is clearly constrained by genetic information but is not 100% genetically hardwired. With the fast progress of automation and image annotation, it might be conceivable to get systematic connectome analysis of mutant strains in a near future. In parallel, strategies based on fluorescent markers have emerged to visualize synaptic contacts in living animals such as GRASP and iBLINC (Feinberg *et al.* 2008; Desbois *et al.* 2015). Although they are not as reliable as EM (see Emmons *et al.* 2021 for discussion) they might provide higher throughput information for connectomic analysis, maybe by combining these techniques with strains that facilitate individual neuron identification such as the NeuroPAL (Yemini *et al.* 2021). As emphasized, *C. elegans* synapses are relatively small as compared with mammals, and the implementation of super-resolution microscopies should help understanding the molecular organization of synapses. These techniques have been immensely fruitful to analyze the organization and the molecular dynamics of synapses in mammalian neurons (for review, see Choquet *et al.* (2021)). Implementing these techniques in *C. elegans* is still challenging and likely explains the limited number of studies published so far, but future developments should provide unique information on the molecular dynamics of synapses in intact nervous systems of wild-type and mutant animals.

Second, synapse study in *C. elegans* benefits from the revolution of new genetic tools. In *C. elegans*, CRISPR/Cas9-engineered strains can be obtained in one to few weeks (Dickinson and Goldstein 2016). This provides a means to generate accurate reporters for gene expression and subcellular protein localization by tagging endogenous gene products. Avoiding overexpression artifacts is of greatest importance to study compartmentalized structures such as synapses. In addition, any gene can be inactivated and multiple strategies have been developed to control spatial and temporal gene inactivation (Nance and Frokjaer-Jensen 2019).

Conditional degradation of proteins can now be achieved using auxin-induced degron (AID) (Zhang *et al.* 2015) or ZF1/ZIF-1 (Armenti *et al.* 2014) systems. The use of orthogonal strategies potentially allows the manipulation of any protein expression in any single neuron. This will be especially interesting to explore the function of essential genes whose global invalidation causes developmental arrest or lethality. So far, most of the genetic analyses have been limited to monogenic Mendelian genetics. Especially in forward genetic screens or in gene candidate analyses, behavioral, or cellular phenotypes are ultimately linked to mutations in one specific locus. However, in the field of synaptogenesis, few mutants have been demonstrated to disrupt the formation of synapses between given neurons, apart from mutations in transcription factors such as *unc-3*, *unc-4*, or *unc-30* (Miller *et al.* 1992; Howell *et al.* 2015; Kratsios *et al.* 2015). This suggests that parallel pathways contribute to synaptic formation and maintenance, and that disrupting a single gene is unlikely to disrupt the formation of a specific synapse. New genetic tools enable the parallel invalidation of multiple genes, which becomes of ultimate interest due to the implementation of another technical revolution, the single-cell transcriptomics.

Recently, the CeNGEN consortium used single-cell RNA sequencing (scRNA-seq) to generate gene expression profiles of all 302 neurons of the *C. elegans* nervous system (Taylor *et al.* 2021). In combination with the known topology of the nervous system, it could identify sets of adhesion molecules potentially involved in specific neurite interactions or in synaptogenesis, that can now be experimentally validated. By comparing the transcripts expressed in different neurons at different stages, or in wild-type and mutant backgrounds, it is possible to identify candidate genes controlling synaptogenesis (Palumbos *et al.* 2021). Proteomics approaches such as proximity labeling method using TurboID also allow for the discovery of novel synaptic components at the single neuron-type resolution (Artan *et al.* 2021).

Finally, a crucial question is to decipher the impact of neuronal activity on synapse formation and patterning. The activity-dependent expression of *hbl-1* was reported to influence the timing of synaptic remodeling of the GABAergic DD motor neurons (Thompson-Peer *et al.* 2012). Another study also revealed that synaptogenesis of the DVB neuron is activity dependent in males (Hart and Hobert 2018). On the other hand, many neurons in *C. elegans* including HSNs still form a normal number of synapses in the absence of neuronal activity (Patel *et al.* 2006), suggesting the presence of activity-dependent and independent mechanisms of synapse formation. With recent techniques such as expression of tetanus toxins or optogenetic inhibition of synaptic release with chromophore-assisted light inactivation (CALI) (Lin *et al.* 2013) to inhibit neurotransmitter release, histamine-gated chloride channels (Pokala *et al.* 2014) and channelrhodopsin (Bergs *et al.* 2018) to reversibly silence and activate neurons, respectively, we can further elucidate the mechanisms of activity-dependent synaptogenesis, synapse specificity and plasticity in *C. elegans*.

In conclusion, getting to a comprehensive understanding of the formation and patterning of the nervous system seems to be a realistic objective in *C. elegans* because of its simplicity and its experimental amenability. This endeavor might identify a few general principles that explain synaptic patterning at the molecular level. Alternatively, the global synaptic complement of *C. elegans* might involve a piece-meal process that uses a multiplicity of molecular strategies that emerged during evolution and were deployed in parallel at distinct synapses. Whatever the answers, these should tell us a lot about the formation of more complex nervous systems, including humans.

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Conflicts of interest

None declared.

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