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Setting up Presynaptic Structures at Specific Positions

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Summary

Precise formation of presynaptic structures at specific loci is critical for correctly wiring neuronal circuits. Recent findings have gradually revealed how essential cues from different sources inform the axon to define the presynaptic domain and to choose its postsynaptic target. Here, we review key molecular regulators which mediate instructive or repellent signals from multiple sources including the target cells, local guidepost cells, and distal guiding tissues.

Introduction

Neurons form highly complex neural circuits through precise synaptic connections. The accuracy of wiring relies on the ability of each single neuron to find its destined synaptic partners. Cell migration and axon guidance determine the position of neurons and target the axons to distinct regions of the nervous system [1,2]. Subsequently, the process of synaptic target selection and synapse formation takes place, during which the pre- and postsynaptic cells become juxtaposed against each other. The recruitment of active zone proteins and clusters of synaptic vesicles to the presynaptic sites transform the adjacent axonal membrane into a highly specialized presynaptic membrane. Growing evidence suggests that synapses not only form between specific pairs of cells but also at particular subcellular localizations. In this article, we will review the emerging findings that indicate various cues from the synaptic partner cell, local neighboring cells, the extracellular matrix, and distal guiding tissues define the location of presynaptic boutons. We will focus on recent discoveries of key molecular regulators for this process *in vivo*.

Target-derived signals induce presynaptic formation

The striking observation that neurons in dissociated cultures can form functional synapses argues strongly that postsynaptic cells are sufficient to induce the development of presynaptic terminals, and hence may contain information to specify the location of presynapses *in vivo*. The most intuitive and probably most accepted model for synaptic specificity is that specific recognition molecules between the synaptic partner cells bind and mutually induce the formation of pre- and postsynaptic specializations. Indeed, a number of postsynaptic adhesion molecules directly interact with presynaptic binding partner molecules to trigger presynaptic development. These cell adhesion molecules include Neurexin/Neurologin, SynCAM, leucine-rich repeat (LRR) domain proteins, Cadherins, Integrins and Immunoglobulin superfamily (IgSF) proteins [3-5].

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Neurexin/Neurologin and SynCAM are among the earliest identified synaptic cell adhesion molecules (CAMs). It was demonstrated that the postsynaptically localized membrane protein neurologin, when expressed in fibroblasts, was sufficient to induce morphological and functional presynaptic differentiation *in vitro* [6]. The homophilic adhesion molecule synCAM, which has been found at synapses, appears to have similar presynaptic inducing activity [7]. However, the exact roles for these proteins in synaptic specificity *in vivo* remain somewhat unclear [8,9].

LRRTM

Using a similar fibroblast expression assay in an unbiased screen, Craig and colleagues found that a family of transmembrane proteins containing leucine-rich repeat domains (LRRTM1-4) also possesses activity in inducing presynaptic differentiation, suggesting that multiple classes of membrane molecules can induce the formation of presynaptic terminals [10]. Furthermore, two recent studies identified neurexin 1 as the interaction partner of LRRTM2, a member of the LRRTM family [11,12]. They showed that the postsynaptic localized LRRTM2 bind to neurexin and induce presynapse formation through this interaction. Interestingly, *in vivo* analysis of specific synapse formation in the *Drosophila* neuromuscular junction system also led to the notion that at least four LRR proteins, *caps*, *trn*, *haf*, and *CG8651* contribute to the specificity of synaptogenesis [13].

EphrinB

The EphrinB subfamily of the Ephrin proteins can also be found at presynaptic terminals and promotes synapse formation and maturation in the *Xenopus* retinotectal system [14]. Using *in vitro* observations of HEK293T-cortical neuron co-cultures, Kayser and colleagues showed that expression of various isoforms of EphB2 was sufficient to trigger clustering of presynaptic molecules, potentially by activating ephrinB on the presynaptic terminals [15].

Neurofascin

Increasing anatomical and physiological evidence suggest that synaptic connections are precisely established at specific subcellular compartments. For example, Purkinje neurons are innervated by two types of GABAergic interneurons: stellate cells and basket cells [16]. While the stellate cells innervate spines and dendrites of Purkinje cells, basket cells specifically form synapses on the axon initial segment (AIS). At the AIS, a member of the L1 cell adhesion molecules, neurofascin, is concentrated and recruited by ankyrin-G [17]. Ango et al. found neurofascin186 (NF186) guides basket axon terminals towards the AIS. Furthermore, they showed that NF186 induces the branching and formation of presynaptic specializations of the basket axon, generating the characteristic brush-like “pinceau” synapses [18].

Sidekicks and Dscams

The precision of synaptic connectivity is evident in the inner plexiform layer (IPL) of the retina where different classes of bipolar, amacrine and retinal ganglion neurons (RGCs) innervate each other within this complex neuropil. Distinct populations of axons terminate at specific depths of the IPL, forming more than ten sublaminae [19,20]. The IgSF proteins *Sidekick-1*, *Sidekick-2* and DSCAMs are each expressed by distinct groups of RGCs and bipolar or amacrine neurons. The homophilic adhesion properties of these molecules form the foundation of the hypothesis that neurons expressing a particular Sidekick or DSCAM will connect to other neurons expressing the same adhesion molecule. Consistent with this idea, depletion of *sidekicks* disrupts the respective laminar-specific arborization of RGCs, while ectopic expression of Sidekick can redirect axons or dendrites to the respective sublaminae [21,22]. Similarly, Dscam and Dscam-like-1 (DscamL) are mainly involved in

homophilic interactions [20]. While the loss of Dscams disrupts the neuronal arborization in respective sublaminae of the IPL, overexpression leads neuronal processes to the sublaminae with enriched Dscam [20]. While it is not clear yet whether the Sidekicks or Dscam can directly stimulate synapse formation, they are good candidates for synapse inducing molecules that specify connection specificity.

Off-target-derived signals inhibit presynaptic formation

While the abovementioned target-derived molecules potentially specify the location of presynaptic terminals by stimulating synapse formation with a correct partner, it is conceivable that inhibitory factors derived from incorrect target cells prevent ectopic synapse formation with a wrong synaptic partner. Indeed, at least two sets of molecules fit the bill.

Semaphorins

Semaphorin proteins, initially characterized as axon guidance factors, can also influence local targeting and synaptic connectivity. Overexpression of SemaII in muscles prevents motor neuron innervation in flies [23]. Semaphorin receptors plexin A3, plexin A4, and neuropilin 2 are required for pruning transient axon collaterals and removing their ectopic synaptic contacts in the developing mouse CNS [24,25]. Recently, Sema3e and its receptor plexin D1 (PlxnD1) were shown to regulate two types of reflex circuitry in mice [26]. In wild-type animals, both cutaneous maximum (Cm) sensory neurons and triceps (Tri) sensory neurons express PlexD1. Cm motor neurons specifically express Sema3e which prevents synaptic contacts with Cm sensory neurons. In contrast, triceps (Tri) motor neurons do not express Sema3e and do form synapses with Tri sensory. Removal of Sema3e or PlexD1 results in an ectopic synaptic connection between Cm sensory neurons and Cm motor neurons. Ectopic expression of Sema3e in Tri motor neuron blocks its synapse formation with Tri sensory neurons. Therefore, through a repellent mechanism, Sema3e signaling determines whether Cm and Tri monosynaptic reflex circuitry can form.

Wnt4

Genetic analysis of the *Drosophila* NMJ system led to the discovery of another target-derived repulsive cue, Wnt4. Two similar larval ventral muscles, M12 and M13, are parallel and adjacent with comparable size and morphology. Still, they are innervated by different motor neurons. Inaki et al. found that Wnt4 is preferentially expressed in M13 [27]. *Wnt4* mutants displayed reduced motor neuron innervation on M12 and increased innervation on M13. Specific labeling revealed M12-specific motor neurons RP5 and V mistarget M13 in the *Wnt4* mutant. Conversely, ectopic expression of Wnt4 in M12 also caused reduced M12 innervation and augmented M13 innervation.

Taken together, this emerging literature suggests that semaphorins and Wnts secreted from local off-target cells can dramatically influence the locations of presynaptic terminals by inhibiting inappropriate synaptogenesis.

Guidepost-derived signals specify synaptic position

Development of synaptic circuits takes place concurrently with the development of non-neuronal cells and tissues such as glia and vasculature. It is becoming increasingly clear that neighboring non-neuronal cells can also play important roles in positioning presynaptic terminals at least in *C. elegans*.

SYG-1 and SYG-2

The *C. elegans* egg-laying motor neuron HSNL forms synapses onto the VC neurons and vulva muscles near the vulva. Surprisingly, genetically ablation of these target cells does not affect the location of presynaptic specification in HSNL. However, the removal of neighboring vulva epithelium cells does cause defects in synaptic specificity. Genetic analysis of this system led to the discovery of two IgSF proteins, SYG-1 and SYG-2. SYG-1 functions autonomously in HSNL and accumulates at the synaptic region during synaptogenesis. SYG-2 is expressed by vulva epithelium cells, and bind to SYG-1. Furthermore, ectopic expression of SYG-2 in secondary epithelial cells relocates SYG-1 and synaptic vesicles in HSNL, suggesting SYG-2 induces presynaptic development and instructs the location of synapses [28,29].

UNC-6/Netrin and UNC-40/DCC

Like the HSNL neuron, the interneuron AIY also utilizes an external cue provided by guidepost cells to form synapses with another interneuron RIA [30]. In a genetic screen, a mutant of netrin receptor *unc-40/DCC* was isolated based on the loss of AIY presynaptic structures apposed to RIA. Since the phenotype can be cell autonomously rescued by expressing *unc-40* in AIY, netrin signaling is unexpectedly required for presynaptic assembly. Interestingly, the netrin signal is derived from ventral cephalic sheath cells (VCSCs), which are astrocyte-like cells beneath the contact region between AIY and RIA [31,32]. Abnormal extension of VCSCs in *unc-34/enable* caused ectopic AIY presynapses and an elongated RIA process, suggesting the glia instructively match the connection between AIY and RIA.

Morphogenetic gradients specify subcellular localization of synapses

Besides local cell adhesion mechanisms, it has been known that many secretory morphogenic factors can also serve as prosynaptogenic cues, including Wnt family members, fibroblast growth factors (FGFs), and bone morphogenic protein (BMP) [33-35]. Recently, several findings have further unraveled how secretory factors pattern the presynaptic specification, identifying unexpected roles as anti-synaptogenic cues. Two studies utilizing the *C.elegans* tail motor neuron DA9 illustrate how environmental cues restrict presynaptic specification to a well-defined domain within the axon projection.

LIN-44/Wnt

The DA9 axon forms stereotyped *en passant* synapses in a discrete axonal segment, connecting with VD/DD neurons and dorsal body wall muscles. No presynaptic structures are present in the dorsal-posterior asynaptic domain or in the commissure region. It turns out that a Wnt gradient formed by secretion of LIN-44 by hypodermal cells in the tail prevents synapse formation in the “asynaptic” domain [36]. Ectopic synapse forms in this region in the *lin-44* mutant. Similar effects are found in the deficiency of *lin-17/frizzled*, which encodes a WNT receptor that functions in the DA9 axon. These results suggest that Wnts, signaling through LIN-17/Frizzled, locally suppress synapse formation in the proximal segment of the axon.

Netrin and UNC-5

In contrast to the WNT signal, which generates a gradient from the posterior to the anterior, the axon-guidance molecule, UNC-6/Netrin, is expressed in the ventral side and forms another gradient in the dorsal-ventral axis [32]. Poon et al. discovered abnormal ventral accumulation of presynaptic components at dendritic loci of DA9 in *unc-6* mutant animals [37]. Interestingly, the UNC-6/Netrin signal is transmitted by the UNC-5 receptor, but not the UNC-40 receptor as in AIY since *unc-5* mutants, but not *unc-40* mutants, show

phenotypes that are similar to *unc-6*. A genetic manipulation that only removes UNC-5 at a developmental stage after the axon is well guided can still cause mislocalized dendritic synaptic vesicles, suggesting the defect is not due to an axon guidance problem and Netrin signaling is constantly required for preventing ectopic synapses.

Taken together these findings in an invertebrate system suggest that even global extracellular cues can affect the positioning of presynaptic terminals.

Axon-axon interactions might specify the location of synapses

Apart from their axon guidance activities, ephrins and their receptor Ephs also regulate dendritic spine morphogenesis and synaptic maintenance (reviewed by Klein, 2009)[38]. In a very interesting study, Galimberti et al. studied how the dentate gyrus (DG) granule axons within the mossy fibers arrange their presynaptic terminals in a topographic manner. The mossy fiber axons form large boutons (Large Mossy Fiber Terminals, LMTs) onto CA3 pyramidal neurons in the hippocampus. They found that about half of these mossy fibers develop more than one intricate Terminal Arborizations (TAs), which contains a core LMT and multiple satellite LMTs connected to the core LMT through processes. Notably, mossy fibers form TAs in CA3 following a topographic rule based on their cell body location in the DG[39]. They further dissected different portions of the DG and CA3 tissues and identified a unique gradient of EphA4 in the DG. Treatment with EphA4 inhibitor abolishes the topographic distribution of TAs in slice cultures. Further studies will be required to understand the exact role of EphA4 *in vivo* and whether it autonomously regulates presynaptic structures or reversely signals postsynaptic components. Still, these data argue strongly that there are mechanisms to arrange presynaptic terminals within a neuropil. Axon-axon interaction might be one of the sources to provide such positional information. For example, in the fly visual system, the quantitative difference of the atypical cadherin Flamingo between photoreceptor growth cones is essential for target selection [40]. Similar arrangement of presynaptic terminals for *en passant* synapses is a robust phenomenon in invertebrate systems such as *C. elegans*[41]. It is highly likely that axon-axon interactions might also play important roles for the correct topographic placement of synapses.

Conclusion remarks

It appears that diverse mechanisms are utilized to specify the location of presynaptic terminals *in vivo*. Both inductive and inhibitory cues from target and non-target tissues play important roles to determine where and with whom synapses form.

The cellular and molecular studies on the mechanisms of presynapse formation have also revealed striking resemblance to axon guidance mechanisms. Direct cell-cell interaction, local cell matrix, guidepost cells, and long range morphogenic gradients define presynaptic domains. It is obvious that the consistency of target specificity is achieved combinatorially through multiple signaling pathways at different levels (Figure 1). Most signaling pathways discussed here also direct axonal growth in other systems. For example, the Netrin pathway plays important conserved roles in both axon guidance and synapse formation. Interestingly, through two sets of receptors, Netrin can be an attractive or repulsive signal to the growth cone while also promoting or inhibiting presynaptic specifications. How are these ligand and receptor systems coupled to diverse intracellular signaling pathways to mediate axon guidance and presynaptic assembly still remains to be determined.

Setting up presynaptic structures at specific positions requires diverse external cues and internal machineries. Much needs to be learned regarding the mechanisms that translate extracellular cues to internal regulation and coordination of assembly and disassembly presynaptic structures. Given the complexity of neuronal circuitry, genetic tools that can

label single neuron or subsets of neurons will be required to reveal detailed synaptic patterning and will enable studies to dissect the intricate cell-cell interactions and intracellular signaling pathways that achieve such precise connectivity during development and adult plasticity.

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Figure 1. The Selection of the Presynaptic Position

Inhibitory signals secreted from distal tissues prevent erroneous assembly of presynaptic structures. Instructive cues from apposed guidepost cells and correct targets induce the presynaptic bouton while repulsive signals from potential off-target cells preclude inaccurate connection.