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Genetics and Cell Biology of Building Specific Synaptic Connectivity

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Abstract

The assembly of specific synaptic connections during development of the nervous system represents a remarkable example of cellular recognition and differentiation. Neurons employ several different cellular signaling strategies to solve this puzzle, which successively limit unwanted interactions and reduce the number of direct recognition events that are required to result in a specific connectivity pattern. Specificity mechanisms include the action of contact-mediated and long-range signals that support or inhibit synapse formation, which can take place directly between synaptic partners or with transient partners and transient cell populations. The molecular signals that drive the synaptic differentiation process at individual synapses in the central nervous system are similarly diverse and act through multiple, parallel differentiation pathways. This molecular complexity balances the need for central circuits to be assembled with high accuracy during development while retaining plasticity for local and dynamic regulation.

Keywords

recognition; trans-synaptic signaling; guidepost cell; synapse elimination

INTRODUCTION

The central nervous system is a gigantic network of neurons connected by an astronomical number of synapses. The connection specificity is critically important for the function of neuronal circuits. The structural organization of neuronal circuits emerges from a complex set of developmental events that include cell fate determination, cell migration, axon guidance, axonal and dendritic branch layer formation, synapse formation, and the activity-dependent maturation of synaptic circuits. Each of these successive steps contributes to wiring specificity by gradually restricting the availability of potential synaptic partners.

In this review, we focus on the cellular and molecular mechanisms that generate synapse specificity during the process of synapse formation. We define synapse specificity as the cellular process that enables pre- and postsynaptic cells to select each other as synaptic partners among the surrounding cells and that allows synapses to form at particular subcellular locations. Neuronal activity plays profound roles in modifying synaptic circuits

after their initial establishment. This topic has been reviewed extensively in several previous articles (Cohen & Greenberg 2008, Katz & Shatz 1996); therefore, we do not delve deeply into this issue. Instead, we focus on the recent advances in our understanding of the molecular mechanisms that specify connectivity.

Many anatomical studies suggest that neurons have the exquisite ability to choose their appropriate synaptic partners from a dense array of potential targets. Quantitative analyses of a neuron's ability to select synaptic targets come from serial electron microscopy reconstruction studies. A reconstruction of an arbor from a retinal ganglion axon in the lateral geniculate nucleus revealed that it only selects 4 cells as synaptic partners from a total of 43 contacting cells (Hamos et al. 1987). A systematic survey of the *Caenorhabditis elegans* nervous system showed that one out of six contacting neurons form synapses onto each other (White 1986). Both of these studies highlight the fact that neurons actively choose synaptic partners from surrounding cells.

Neurons are highly differentiated cells with complex morphologies and distinct functional compartments. Emerging anatomical and physiological evidence suggests that synapses are often formed onto specific subcellular compartments between neurons. For example, inhibitory synapses formed onto the perisomatic domain of a postsynaptic neuron have profound impact on the action potential that fires in the postsynaptic cell, whereas inhibitory synapses that are formed onto distal dendrites primarily affect dendritic calcium spikes (Miles et al. 1996, Pouille & Scanziani 2004). Developmentally, this subcellular specificity phenomenon begs the question of how such a precise innervation pattern is generated during development.

Finally, the stoichiometry of synaptic connectivity is precisely regulated at two levels: first, with respect to the number of different presynaptic partners that innervate a single postsynaptic cell; and second, with respect to the number of synapses that are formed between a single afferent and its target cell. Some afferents sparsely innervate select targets but achieve reliable activation of the postsynaptic partners through efficacious synapses, whereas other inputs require activation in concert with other inputs to be effective. The observed reproducibility of synaptic connection stoichiometry implies that this parameter is tightly controlled in developing circuits. In this article, we first describe different cell recognition events to shed light on how specificity is achieved during development. We detail examples of direct pre- and postsynaptic matching decisions and the functions of glia and guidepost cells in circuit formation. Then, we discuss molecular mechanisms that drive assembly and remodeling of synaptic connectivity and provide examples of the regulation of wiring molecules at the transcriptional level. Throughout the article, we combine information obtained in vertebrate and invertebrate model systems, using genetic and cell biological approaches, to identify general principles underlying the generation of synaptic specificity in the nervous system.

HOW IS SPECIFICITY GENERATED?

Conceptually, developing axons and dendrites can use a variety of cellular and molecular mechanisms to choose their synaptic targets and appropriate subcellular compartments. First, positive selection cues on the membranes of pre- and postsynaptic neurons can locally induce the assembly of pre- and postsynaptic differentiation. Under this mode of selection, synaptic partners express distinct sets of adhesion molecules or secrete anterograde and retrograde signals that drive the synaptic differentiation process (Figure 1a). Indeed, several synaptic signaling molecules are sufficient to induce the assembly of the pre- and postsynaptic apparatus. In the case of the vertebrate neuromuscular junction, interactions

between axon, muscle, and components of the basal lamina can achieve a similar outcome of patterned synaptic connections.

Second, inhibitory cues presented on neurites or released from the local environment can prevent synapses from forming between particular neurons or at certain subcellular locations. Similar to the concept of repellents in axon guidance, diffusible and membrane tethered cues may exert negative constraints against synapse formation (Figure 1*b*). This is a relatively new area with less experimental evidence. However, a few studies show that negative regulators of synapse formation might also be used to encode connection specificity (discussed below). Interestingly, negative cues can be presented by a potential interaction partner or act in the form of a morphogenetic gradient that is generated by adjacent cells in the target area (Figure 1*c*).

Third, synapse elimination is a powerful way of achieving synaptic specificity (Figure 1*e*). This has been well documented in classical studies of neuromuscular junction maturation as well as for many connections in the central nervous system. This type of regulation allows an initial, promiscuous phase of synapse formation, which leads to excessive synapse formation onto many targets, followed by a second phase of synapse elimination during which specific groups of synapses are eliminated. In many cases, this elimination process is driven by neuronal activity and serves as a way for neural activity to carve connectivity during the maturation of the synaptic circuit.

Last, besides the spatial regulation mentioned above, the temporal regulation of competence can serve as a way to limit synaptic partner choices (Figure 1). For example, axons establish many cellular contacts during axonal migration. However, neurons might not be competent to assemble synapses during this early phase of development, therefore avoiding ectopic connections. Once axons reach their target field, molecular programs for synapse formation are then upregulated. Indeed, several glia- and target-derived priming factors increase the number of synapses and induce the maturation of synapses and changes in gene expression in afferents (Christopherson et al. 2005, Diaz et al. 2002, Kalinovsky & Scheiffele 2004, Umemori et al. 2004). Importantly, the above-mentioned mechanisms are not mutually exclusive. Multiple strategies are likely used by the same neuron at different stages of development to establish, maintain, or remodel its synaptic connectivity.

Direct Matching of Synaptic Partners

The complex cellular environment during synapse development *in vivo* makes it difficult to discern whether synaptogenesis is initiated by contact between pre- and postsynaptic neurons and to exclude the involvement of surrounding cells. However, the fact that dissociated pure neuronal cultures can form functional synapses argues strongly that synapse formation is a cellular process that can be executed by synaptic partner cells alone (Bartlett & Banker 1984). Hence, one intuitive model for synapse specificity is that the recognition between synaptic partners induces synapse formation. This model suggests that designated synaptic partners possess molecular tags that direct synaptic connectivity. In this scenario, the recognition tags might either initiate synapse formation themselves or they promote selective cell-cell contacts and thereby selectively engage a common synaptogenic core machinery. The possibility that nonpartner cells in the target area might inhibit synapse formation, therefore avoiding the formation of inappropriate synapses, is less appreciated. Examples for each of these mechanisms, recognition and inhibitory signals, are discussed below.

Mutual attraction of synaptic partners by synaptic adhesion molecules—A simple model for the selective attraction between synaptic partner cells is the adhesion molecule-mediated match making accomplished by the coordinated expression of

homophilic adhesion molecules in pre- and postsynaptic partners (Fannon & Colman 1996, Shapiro et al. 2007). An array of homophilic adhesion molecules has been identified, which includes cadherins and immunoglobulin-superfamily (IgSF) proteins. The substantial molecular diversification of isoforms within such gene families provides a molecular basis for an adhesive code with the potential for encoding a multitude of selective cell-cell interactions. Therefore, appropriate axons and target dendrites might be sorted into a common synaptic domain in the same way that cells with different cadherin expression can be sorted into different clusters of cells during early embryonic development (reviewed in Steinberg 2007).

This homophilic adhesion model for wiring has been tested extensively in the retina in which synapses between amacrine and bipolar cell interneurons that are formed with retinal ganglion cell dendrites are organized in a highly ordered laminar arrangement in the inner plexiform layer (IPL). Subpopulations of interneurons and retinal ganglion cells express different variants of a closely related group of IgSF-proteins: Sidekick-1, Sidekick-2, Dscam, and DscamL (Yamagata & Sanes 2008, Yamagata et al. 2002). Each of the IgSF proteins engages in strictly homophilic interactions. Interneuron processes and ganglion cell dendrites that express the same IgSF isoform elaborate arbors and synapses in the same IPL sublaminae. Importantly, the IgSF protein repertoire of synaptic partners is instructive for their connectivity. When the IgSF content of cells is perturbed by either RNA interference or the misexpression of other isoforms, the processes misproject into inappropriate sublaminae (Yamagata & Sanes 2008). Therefore, this homophilic adhesion system directs the lamina-specific targeting of neuronal processes. Whether Sidekick and Dscam proteins trigger the initiation of synapse assembly in these laminae remains to be explored. Notably, Sidekick and Dscam proteins are not restricted to the retina but are broadly expressed in neuronal populations throughout the CNS, in which they may have analogous roles in the formation of lamina-specific synapses.

Mutual attraction through synaptic adhesion molecules has also been implicated in sculpting the subcellular specificity of neuronal connections. The vast majority of excitatory synapses are formed on dendritic spines, whereas specific inhibitory presynaptic inputs are restricted to perisomatic domains, dendritic domains, or the axon initial segment. In principal neurons of the primary visual cortex, this innervation specificity persists even in the absence of functional sensory and thalamic inputs, which suggests that subcellular specificity is genetically encoded (Di Cristo et al. 2004). Subcellular domain-specific inputs are also observed in Purkinje cells in the cerebellum where two classes of GABAergic inputs from stellate and basket cells are segregated into the dendritic domain and the axon initial segment, respectively. The restriction of basket cell inputs to the initial segment depends on the IgSF protein NrCAM and the cytoplasmic ankyrin scaffold because the position of basket cell terminals becomes more diffuse in knockout mice that lack the protein (Ango et al. 2004). Notably, only the initial segment restriction, but not the formation of basket cell-Purkinje cell synapses, is abolished in the absence of NrCAM, which suggests that subcellular specificity and synapse formation are controlled by different signaling systems. Notably, the dendritic domain innervation of Purkinje cells by stellate cells does not appear to be mediated by a similar mutual attraction between synaptic partners but by glial-derived signals (discussed below).

Repulsion from nonsynaptic partners in the innervation field—Although mutual attractive mechanisms between synaptic partners can be used to specify connectivity as discussed above, repulsive signals derived from inappropriate target cells also prevent abnormal innervation. The best understood examples of such mechanisms come from studies of the development of neuromuscular connectivity of the fruit fly. Each *Drosophila* abdominal hemisegment contains 30 muscles that are innervated by approximately 40 axons.

Each of these axons establishes muscle-specific neuromuscular junctions. Many membrane-tethered and secreted molecules contribute to the specificity of these synaptic connections. Some cues, such as FasII and SemaII, are expressed in all muscles and act as general pro- and antisynaptogenic forces, respectively. Interestingly, NetrinB is only expressed by a subset of muscles, in which it attracts certain axons while repelling others (Winberg et al. 1998). The interplay of multiple positive and negative cues led the authors to propose that synaptic specificity in this system does not depend on unique, synapse-specific signals that act similar to a key-lock mechanism. Instead, growth cones assess the relative balance of attractive and repulsive forces and establish synapses with the best available partner.

In a more recent study using the same system, Nose and colleagues focused on two similar adjacent muscles, M12 and M13, that only differ in their innervation patterns by different motor neurons. Through single-cell microarray analysis, they found that several genes are differentially expressed between these two cells. For example, Wnt4 is enriched in M13 but not in M12. In the absence of Wnt4 or its receptor Drizzled 2, neurons that normally only innervate M12 also synapse onto M13. The ectopic expression of Wnt4 in M12 inhibits synapse formation by MN12. These data strongly suggest that Wnt4 determines synaptic specificity by inhibiting synapse formation with inappropriate targets (Inaki et al. 2007).

Guideposts in Specificity

Although direct interactions between synaptic partners can transform into synapses, pre- and postsynaptic neurons are likely to contact many other cells during the time of synapse formation. Is it possible that these other cell-cell interactions between nonpartner cells also impact synapse formation? Guidepost cells, or intermediate targets for axon guidance events, were discovered previously. These cells are located at critical positions along the axonal trajectory where turns are often made (Bate 1976). From studies on synapse formation, there is now accumulating evidence that similar transient target interactions with cells other than pre- and postsynaptic partners might also play critical roles in synaptic partner choices, the regulation of synapse numbers, and synapse elimination.

Transient neuronal populations as synaptic placeholders—During the development of several systems, presynaptic neurons extend their axonal processes to the appropriate target field before postsynaptic partners are fully differentiated. In such cases, transient neuronal populations can act as placeholders to provide cues for target field selection by presynaptic axons.

Examples of placeholder cells are Cajal-Retzius cells and GABAergic interneurons in the hippocampus (reviewed in Sanes & Yamagata 1999). In mature hippocampus, afferent axons from the entorhinal cortex form synapses on the distal dendrites of pyramidal neurons in the stratum lacunosum-moleculare (SLM) layer, while commissural/associational fibers form synapses on the more proximal part of these same pyramidal dendrites in the stratum radiatum (SR). Developmentally, entorhinal axons enter the SLM layer, before the majority of pyramidal dendrites have arrived in this area. Two classes of early-born neurons, calretinin-positive Cajal-Retzius (CR) cells and calbindin-positive GABAergic interneurons, are present in the SLM and SR, respectively, when entorhinal and commissural axons enter the target field (Soriano et al. 1994, Super et al. 1998). Cajal-Retzius and GABAergic interneurons form transient synaptic contacts with entorhinal axons and commissural axons, respectively, and both cell populations undergo cell death later during development (Super et al. 1998). The ablation of CR cells prevents layer-specific innervation by entorhinal axons, which suggests that CR cells are required for the layer-specific innervation of these axons (Del Rio et al. 1997). Therefore, CR cells serve as transient placeholders that allow for layer-specific innervation in the hippocampus. Interestingly, Cajal-Retzius cells also

form transient synaptic contacts with pyramidal cells in the cortex (Frotscher 1998), which suggests that CR cells may also act as transient placeholders for cortical maturation.

Another example of such placeholder cells is the subplate cell in the developing visual cortex. In the mature mammalian visual system, axons that originated from the lateral geniculate nucleus (LGN) innervate layer 4 neurons in the visual cortex. However, during development, LGN axons arrive in the cortical target field long before layer 4 neurons have migrated into the region. While LGN axons wait for layer 4 neurons to migrate and mature, they form transient synaptic connections with subplate cells. Ablation studies have demonstrated that subplate neurons are necessary for the proper axon guidance of LGN neurons into layer 4 cortex (Ghosh et al. 1990), as well as for the formation of ocular dominance columns (Ghosh & Shatz 1992, Kanold et al. 2003). Thus, the transient synaptic interaction between subplate cells and thalamic axons is critical for the establishment of mature visual cortical circuits.

Glia and glia-like cells in local synaptic connectivity—In the mammalian nervous system, glial cells outnumber neurons by tenfold. It is not surprising that glia play important roles in the function and development of the nervous system (Barres 2008). Glial cells can secrete critical axon guidance molecules and serve as intermediate targets to specify axon trajectory (Learte & Hidalgo 2007). The role of glia or glia-like cells in regulating local connectivity and synapse formation is starting to be elucidated by recent studies.

How might glia cells coordinate the recognition between pre- and postsynaptic partners? One elegant example was reported by Josh Huang and colleagues during their studies in the development of cerebellar circuits. They showed that axons from stellate interneurons are organized and guided towards Purkinje cell dendrites by an intermediate scaffold of Bergmann glial (BG) cells. In the absence of an L1 type molecule that is required for the association between the stellate axon and the BG, synapse formation between mistargeted stellate axons and Purkinje dendrites is reduced and synapses cannot be maintained, which leads to a progressive atrophy of axon terminals. Hence, the BG provides a substrate for the meeting between the stellate axon and the postsynaptic dendrite (Ango et al. 2008).

A remarkable parallel to these mechanisms can be found in the nematode *C. elegans*, despite its much simpler nervous system. A recent study found that two glia-like sheath cells coordinate the innervation between the interneurons AIY and RIA in the thermotaxis circuit of *C. elegans*. The processes of sheath cells converge at a location where AIY forms synapses onto RIA. Sheath cells secrete the axon guidance molecule, UNC-6/Netrin, which elicits distinct UNC-40/DCC-dependent responses in the RIA and AIY neurons, regulating axon guidance of RIA and synapse formation of AIY, respectively (Colon-Ramos et al. 2007).

A third example of glia-like cells that act as scaffolds for synapse formation comes from studies of egg-laying HSN motor neurons in *C. elegans*. A group of guidepost epithelial cells determines the subcellular localization of HSN synapses through the heterologous interaction between two transmembrane immunoglobulin superfamily proteins, SYG-1 and SYG-2, which bind to each other and function as receptor and ligand to mediate the recognition between guidepost cells and the HSNL axon. SYG-2 is expressed transiently by guidepost cells during the early stages of HSNL synaptogenesis. SYG-1 functions in the presynaptic HSNL neuron and localizes to synapses early during synapse formation. In loss-of-function *syg-1* and *syg-2* mutants, the HSNL axon fails to form synaptic connections with its normal targets and instead forms synapses to adjacent cells that do not normally receive synaptic input from the HSNL. Therefore, guidepost cells function as a placeholder of the

presynaptic specialization of HSN through the action of a pair of Ig superfamily proteins (Shen & Bargmann 2003, Shen et al. 2004).

Besides serving as guidepost cells to specify location and partner selection, glial cells can also determine the number and functionality of synapses. For example, astrocyte-conditioned medium greatly enhances the number of synapses and the synaptic transmission of purified retinal ganglion cells (Ullian et al. 2001). Astrocytes secrete multiple factors to control various aspects of synapse formation. Glia produce cholesterol that facilitates the maturation of dendrites and the presynaptic terminal (Mauch et al. 2001). Astrocytes also secrete thrombospondin (TSP), which is sufficient to induce morphologically defined synapses in vitro (Christopherson et al. 2005). TSP1/2 double-knockout animals showed significantly reduced synapse numbers, which suggests that these astrocyte-derived molecules are required for synapse formation in vivo. Recent studies indicate that two synaptic proteins, neuroligin-1 (Xu et al. 2009) and the accessory calcium channel subunit $\alpha_2\delta-1$, serve as TSP receptors (Eroglu et al. 2009). The glial cell line-derived neurotrophic factor (GDNF) and its GPI-anchored receptor GFR α 1 induce the differentiation of presynaptic specializations (Ledda et al. 2007). Collectively, these data argue that glial cells play important roles in determining the partnership, subcellular localization, and density of synapses during development.

Morphogenetic gradients regulate local connectivity through the inhibition of synapse formation—Organization centers and graded diffusible signals play critical roles in cell fate determination (Lee & Jessell 1999) and axon guidance (Tessier-Lavigne & Goodman 1996). Recent studies in *C. elegans* suggest that similar gradients may shape synaptic connections directly. Worm neurons form en passant synapses on specific segments of their axons. For example, a particular motor neuron DA9 synapses onto the postsynaptic target muscle in a restricted domain, which only spans a small area of the entire axon. Although the posterior axonal segment contacts target cells, it is devoid of presynaptic terminals. A putative Wnt gradient formed by two Wnts, LIN-44 and EGL-20, is responsible for inhibiting synapse formation in this local area. Because both Wnts are only expressed in a small group of cells in the tail and wild-type DA9, synapses are not localized at the most posterior segment of the axon where the Wnt concentration is high. Furthermore, the loss of Wnts leads to a shift of synaptic distribution into the posterior region. When LIN-44 is ectopically expressed, it inhibits synapse formation in adjacent axon segments. Therefore, a local Wnt gradient shapes the DA9 synaptic domain through its antisynaptogenic activity (Klassen & Shen 2007).

Interestingly, another morphogenetic gradient, formed by the classic axon guidance molecule UNC-6/netrin, appears to exclude presynapse from the ventral axon of DA9. UNC-6 is expressed by ventral tissues and forms a ventral high-dorsal low gradient (Wadsworth et al. 1996). In the absence of *unc-6* or its repulsive receptor *unc-5*, a significant amount of presynaptic vesicle precursors and active zone proteins are localized to the ventral dendrite and axon (Poon et al. 2008). Hence, the Wnts and netrin, with different expression patterns, inhibit presynapse formation along different parts of the DA9 axon.

Morphogenetic gradients are well known for their functions in determining cell fate and guiding axons. How could their activity in shaping synaptic connections be distinguished from their early roles in the developing nervous system? A divergence of their activities might be at the level of receptor or at the level of downstream players. At least in the case of *C. elegans* Wnts, the diverse activities of Wnts appear to be mediated by the same receptor LIN-17/Frizzled. LIN-44/EGL-20 gradients exhibit distinct developmental roles in several classes of neurons. EGL-20 controls the migration of HSN and the Q neuroblast along the anterior-posterior (A-P) axis (Maloof et al. 1999), whereas several mechanosensory neurons

utilize Wnt signaling to orient their anteroposterior polarity (Hilliard & Bargmann 2006, Pan et al. 2006, Prasad & Clark 2006). In many cases, the same receptor LIN-17 mediates these diverse cellular behaviors (Hilliard & Bargmann 2006, Klassen & Shen 2007, Pan et al. 2006, Prasad & Clark 2006). These results argue that diverse downstream signaling pathways dictate the various cellular responses of Wnts.

MOLECULAR MECHANISMS OF SPECIFIC SYNAPSE ASSEMBLY

After the initial recognition events, pre- and postsynaptic cells undergo a series of changes, which transform the incipient membrane contacts into highly specialized pre- and postsynaptic domains. In the process of this transformation, pre- and postsynaptic-derived factors drive structural and functional changes in developing synapses (Figure 2). Many of these factors are sufficient to drive synaptic differentiation to a remarkable extent and, accordingly, are considered synaptogenic molecules. Below, we review our knowledge of such synaptogenic molecules in a few established experimental systems.

BIDIRECTIONAL ORGANIZATION OF SYNAPTIC STRUCTURES BY ADHESION COMPLEXES

The close apposition of synaptic membranes and the mechanical stability of synaptic structures have long highlighted the substantial trans-synaptic adhesive interactions that are partly mediated through trans-synaptic adhesion molecules. Cell biological studies have demonstrated that adhesion molecules not only glue synaptic partners together but that they organize functional aspects of the synaptic structure (Biederer et al. 2002, Linhoff et al. 2009, Scheiffele et al. 2000, Woo et al. 2009). This ability of synaptic adhesion molecules to drive synaptic differentiation was tested in fibroblast-neuron coculture assays in which individual neuronal adhesion molecules expressed in nonneuronal cells were tested for their ability to recruit pre- or postsynaptic components in cultured neurons (Biederer & Scheiffele 2007, Scheiffele et al. 2000). Although most adhesion molecules only promote the formation of contacts between transfected fibroblasts and neuronal cells, a specific subset of proteins exhibits synaptogenic activities in such assays. The neuroligin-neurexin complex provides one such bidirectional synapse differentiation signal. Postsynaptic neuroligins expressed in fibroblasts are sufficient to trigger the accumulation of active zone components and synaptic vesicles in axons downstream of the presynaptic neuroligin receptor. These presynaptic specializations are functional, contain a pool of recycling synaptic vesicles, and release neurotransmitters upon depolarization (Dean et al. 2003, Fu et al. 2003, Scheiffele et al. 2000). Transgenic mice that overexpress NL1, a neuroligin isoform primarily associated with glutamatergic synapses, exhibit a twofold increase in the size of glutamatergic presynaptic specializations and an increase in the number of synaptic vesicles per terminal (Dahlhaus et al. 2009, Hines et al. 2008). Importantly, this synaptogenic activity of the neuroligin-neurexin complex is not solely a retrograde signal that organizes the presynaptic compartment but acts bidirectionally. The expression of NL1 in hippocampal neurons in vitro and in vivo promotes the formation of dendritic spines, drives the recruitment of postsynaptic scaffolding molecules and NMDA-receptors to synaptic sites, and thereby, promotes the assembly of silent synapses (Chih et al. 2005, Dahlhaus et al. 2009, Gerrow et al. 2006, Sara et al. 2005). In vitro, this postsynaptic differentiation process can be elicited solely by the contact of dendrites with the presynaptic NL-receptor neuroligin expressed in fibroblasts (Chih et al. 2006, Graf et al. 2004, Nam & Chen 2005).

Several other synaptogenic adhesion complexes have been identified using assay systems that are similar to those used for the characterization of the neuroligin-neurexin complex. An unbiased expression library screen using the fibroblast coculture assay isolated LRRTM1, a member of the leucine-rich repeat family of synaptic adhesion proteins with synaptogenic

activity (Linhoff et al. 2009). Further candidate gene approaches characterized synapse-organizing activities for SynCAMs (IgSF proteins from the Nectin protein family) (Biederer et al. 2002) and for additional leucine-rich repeat proteins, which include netrin-G-ligands (NGL1, -2, and -3) and LRRTM2 (Kim et al. 2006, Linhoff et al. 2009, Woo et al. 2009). Interestingly, LRRTM2 binds with nanomolar affinity to neurexin-1, and knockdown of neurexin-1 in cultured hippocampal neurons abrogates LRRTM2-induced presynaptic differentiation (de Wit et al. 2009, Ko et al. 2009). These recent findings highlight an unexpected cross-talk between the neuroligin-neurexin complex and leucine-rich repeat proteins.

How can a single trans-synaptic adhesion complex drive a near-complete program for pre- and postsynaptic differentiation? The synaptic signaling mechanisms downstream of any synaptogenic adhesion molecule discussed above are poorly understood. One model for the assembly of pre- and postsynaptic specializations is the nucleation of cytoplasmic subsynaptic scaffolds, which facilitates the recruitment and retention of ion channels at incipient synaptic sites. Similar to NGLs and SynCAM, the neuroligin and neurexin proteins carry PDZ-binding motifs on their C-termini that couple the adhesion molecules to scaffolding proteins (Ichtchenko et al. 1995, Ushkaryov et al. 1992). In the case of pre-synaptic neurexins, interactions with adapter proteins Lin2/CASK and Lin10/Mint may provide a link to voltage-gated calcium channels at glutamatergic synapses (Maximov & Bezprozvanny 2002). In the postsynaptic compartment, NL1 may bind through cytoplasmic PDZ-domain interactions to PSD95 and thereby recruit NMDA-Rs to glutamatergic synapses (Irie et al. 1997). Although the relevance of PSD95 or other scaffolding molecules for the NL1-mediated synaptic recruitment of NMDA-Rs remains to be tested, a conceptually similar model has been examined for NL2, a member of the neuroligin protein family that is concentrated at glycinergic and GABAergic synapses (Varoqueaux et al. 2004). Via its cytoplasmic tail, NL2 binds and activates collybistin, which in turn tethers gephyrin at the postsynaptic membrane and recruits glycine and GABA-A-receptors (Poulopoulos et al. 2009). In the hippocampus of neuroligin-2 knockout mice, the perisomatic accumulation of gephyrin and GABA-A receptors is reduced, which highlights an essential role for NL2 in nucleating a cytoplasmic protein platform of GABAergic and glycinergic synapses.

Although loss-of-function studies in mice and invertebrate systems have uncovered severe structural phenotypes at neuromuscular synapses (Sanes & Lichtman 1999; see below), alterations in central synapse numbers for most of the bi-directional synaptic organizers described so far have been modest. The loss of the synaptogenic leucine-rich repeat protein LRRTM1 does not appear to result in a significant change in synapse density in the hippocampus (Linhoff et al. 2009). Similarly, the ablation of netrinG1 and netrinG2, respective ligands of NGL1 and -2 receptors, perturbs NGL localization but leaves synaptic connectivity intact (Nishimura-Akiyoshi et al. 2007). Neuroligin-1, -2, -3 triple-knockout animals exhibit only a 15% decrease in synapse density in the brain stem, a much smaller alteration than observed with acute perturbation experiments in vitro (Chih et al. 2005, Levinson et al. 2005, Nam & Chen 2005, Varoqueaux et al. 2006). In addition, KO mice exhibit more severe reductions in synaptic transmission owing to defects in the recruitment of postsynaptic neurotransmitter receptors (Chubykin et al. 2007, Varoqueaux et al. 2006). Alpha-neurexin knockout mice exhibit synaptic transmission defects owing to the reduced function of presynaptic voltage-gated calcium channels (Missler et al. 2003). On the structural level, these mice still form central synapses, although symmetric synapse density in the brain is reduced by 50%, and the elaboration of dendrites and the number of dendritic spines in glutamatergic neurons appear to be significantly reduced (Dudanova et al. 2007, Missler et al. 2003). This means that although synaptogenic adhesion complexes can drive a broad program of pre- and postsynaptic differentiation, only certain aspects of these

functions are essential for synapse formation in vivo. Notably, neurexin loss-of-function phenotypes in *Drosophila* are more dramatic. Glutamatergic neuromuscular junctions in *Drosophila* mutants that lack neurexin have severely reduced numbers of synaptic buttons and detaching pre- and postsynaptic membranes (Li et al. 2007). The comparably milder phenotypes with respect to the structural assembly of individual synapses in vertebrates highlight the increased molecular complexity of the vertebrate system characterized by the presence of multiple, partially redundant, parallel trans-synaptic pathways. Most likely, these pathways endow the synapse assembly process in the mammalian brain with the necessary robustness to ensure functional connectivity while still allowing for the necessary plasticity observed in CNS networks. Several such parallel pathways that act as anterograde or retrograde trans-synaptic signals are discussed below.

ANTEROGRADE ORGANIZERS

Anterograde Organizers at Mammalian Neuromuscular Junctions

We refer to presynaptic-derived factors that drive postsynaptic maturation as anterograde organizers (Figure 2b). Arguably, the best understood factor of this group is agrin, an extracellular matrix protein found at the synaptic cleft of neuromuscular junctions (NMJs) in vertebrate animals (Nitkin et al. 1987). The motor axon derived agrin is indispensable for the clustering of postsynaptic acetyl choline receptor (AChR) at the postsynaptic membrane. Experimental evidence that supports agrin's role as an anterograde inducer has been reviewed extensively by Kummer and colleagues (Kummer et al. 2006). Although the agrin-MuSK-rapsyn pathway has been well established on the basis of cell biology and genetic experiments, MuSK may not constitute the receptor for agrin because it does not bind to agrin. Recently, two groups reported the discovery that a member of the LDL receptor family, Lrp4, associates with MuSK and serves as a receptor for agrin in the process of NMJ formation (Kim et al. 2008, Zhang et al. 2008). Agrin binds directly to the extracellular domain of LRP4 and promotes the formation of an LRP4-MuSK complex. Agrin binding to LRP4 also triggers the phosphorylation of LRP4 and MuSK intracellular domains, which suggests that it might initiate the signaling of these receptor complexes. The activation of MuSK stabilizes AChR clusters at the postsynaptic membrane through a rapsyn-dependent mechanism; however, the exact molecular pathway downstream of MuSK has not been elucidated (Gautam et al. 1995).

Interestingly, motor neurons also release ACh, which acts as a negative signal to disperse noninnervated clusters of receptors and refine synaptic receptor clusters. In choline acetyltransferase (ChAT) mutant mice in which ACh is absent, AChR clusters are abnormally large. In ChAT/agrin double mutants, many AChR clusters are maintained, whereas they are largely absent in agrin single mutants (Lin et al. 2005, Misgeld et al. 2005). These genetic data strongly suggest that ACh and agrin antagonize each other and fine tune the size and location of AChR clusters. Downstream of ACh, the cyclin-dependent kinase, Cdk5, may be an effector in dispersing AChRs. The activity of Cdk5 is regulated by p35 and its proteolytic product p25, which is an even stronger activator of Cdk5 (Lin et al. 2005). One recent study showed that the ACh-induced activation of muscle cells stimulated the activity of a protease, Calpain, which cleaves p35 and produces p25. This calpain-dependent activity links the release of ACh and the activation of Cdk5 in the process of dispersing AChR clusters (Chen et al. 2007). Why would motor neurons release two antagonistic factors for postsynaptic development? The answer may be that the ranges of action for these two factors are different. Whereas ACh depolarizes the entire post-synaptic membrane and disperses AChR clusters across the entire postsynaptic muscle, the activity of agrin may be restricted to synapses. Therefore, through a global dispersal activity and a local clustering activity, the motor axons mold the postsynaptic AChRs juxtaposed to the presynaptic terminal.

Anterograde organizers and direct interactions with neurotransmitter receptors

—The identification of trans-synaptic signaling pathways that orchestrate the synaptic recruitment of AChR at neuromuscular synapses prompted the search for proteins with comparable activities in the CNS. Several anterograde inducers released from the presynaptic partner have been identified that organize postsynaptic components. Similar to the agrin-MuSK-rapsyn cascade, researchers anticipated that such factors would act through the recruitment of cytoplasmic scaffolds, which in turn tether neurotransmitter receptors at postsynaptic sites. However, more recently, for glutamatergic synapses, a different mechanism of action emerged for several of these anterograde inducers, namely a direct extracellular interaction of the differentiation signals with postsynaptic glutamate receptors.

Ionotropic glutamate receptors are tetramers, generally composed of dimers of two different subunits. In addition to ligand-binding and pore-forming domains, glutamate receptor subunits contain a large extracellular N-terminal domain (NTD) that shares sequence homology with the bacterial periplasmic amino acid-binding protein (also called the LIVBP-domain). Remarkably, the direct attachment of trans-synaptic differentiation signals to these NTDs provides an anterograde signal for the synaptic recruitment of postsynaptic glutamate receptors. The first such factors to be identified are neuronal pentraxins, which consist of two secreted proteins, the neuronal activity regulated pentraxin (NARP) and neuronal pentraxin 1 (NP1), and the trans-membrane neuronal pentraxin receptor (NPR). NARP and NP1 associate directly with AMPA-receptor (GluA) subunits (O'Brien et al. 1999). NP1 released from glutamatergic axons is sufficient to drive the synaptic aggregation of GluA4-containing receptors through interaction with the NTD (Sia et al. 2007). In vivo, this NARP/NP1 synaptic differentiation machinery localizes and acts selectively at glutamatergic shaft synapses formed on GABAergic interneurons. Specifically, the number of GluA4-containing synaptic structures is reduced (although not abolished) within the hippocampal dentate gyrus in pentraxin triple knockout mice (Sia et al. 2007). Similar mechanisms of NTD-dependent synapse formation might also apply for glutamatergic spine synapses. In particular, in vitro experiments provided evidence that the NTD of the AMPA-receptor subunit GluA2 promotes the differentiation of spine synapses in hippocampal neurons (Passafaro et al. 2003). This function may be mediated through direct extracellular coupling between GluA2 and the adhesion molecule N-cadherin (Nuriya & Huganir 2006, Saglietti et al. 2007). The exact role for these interactions in regulating synaptic structure awaits further study because, surprisingly, synaptic morphology is largely unperturbed in cells that lack GluA2 or all GluA receptor isoforms in vivo (Lu et al. 2009).

Importantly, NTD-mediated synaptic differentiation signals are not unique to AMPA-type glutamate receptors but are emerging as a general principle for postsynaptic neurotransmitter recruitment in the CNS. LIVBP-like N-terminal domains are also contained in the GluN1 and GluN2 subunits of NMDA-type glutamate receptors. In vitro studies identified the GluN1 NTD as a direct interaction site with EphB-receptor tyrosine kinase receptors (Dalva et al. 2000). Whereas EphB-receptors and their ephrinB ligands have been primarily characterized as signaling molecules in axonal guidance, ligands and receptors are expressed during synapse formation and in the adult CNS (Klein 2001). Presynaptic ephrinB-ligands may cluster postsynaptic EphB receptors, which in turn nucleate a tripartite complex of ephrinB, EphB-receptor, and the NMDA-receptor complex. The functional relevance of the GluN1 NTD in the synaptic recruitment of receptors has not been tested in vivo. However, the ablation of full-length EphB2 expression in mice results in reduced NMDA-receptor synaptic localization and function (Grunwald et al. 2001, Henderson et al. 2001), which is consistent with a critical role for EphB-receptors in NMDA-receptor incorporation in glutamatergic synapses. Moreover, synapse density and dendritic spine development are decreased in knockout mice that lack multiple EphB receptors, which further emphasizes an

important role for EphB tyrosine kinase receptors in synapse development (Henkemeyer et al. 2003, Kayser et al. 2008).

Finally, the GluD2 receptor is probably the most remarkable example of the importance of NTD-interactions with neurotransmitter receptors in synaptic differentiation. GluD2 shares the same domain organization with AMPA- and NMDA-type receptors but, owing to amino acid alterations in the pore, it does not mediate currents (Yuzaki 2003). GluD2 is selectively expressed in cerebellar Purkinje cells. Mice that lack GluD2 suffer from severe ataxia and exhibit two major phenotypes on the synaptic level: First, parallel fiber synapses form between cerebellar granule cells and Purkinje cells detach from Purkinje cell dendritic spines, which indicates a severe weakening of trans-synaptic interactions; second, the remaining parallel fiber synapses exhibit diminished long-term depression. Both phenotypes can be rescued by the reintroduction of full-length GluD2, but GluD2 that lacks the NTD only restores LTD (Hirano et al. 1994; Kakegawa et al. 2008, 2009). In cultured cerebellar granule cells, the GluD2 NTD is sufficient to trigger the assembly of presynaptic terminals, which suggests that it is part of a trans-synaptic signaling complex (Kakegawa et al. 2009, Uemura & Mishina 2008). A hint at the identity of the presynaptic ligand for the GluD2 NTD comes from an analysis of mutant mice that lack cerebellin-1 (Cbln1), a small, secreted protein that is part of the C1q/TNFalpha superfamily (Yuzaki 2008). Cbln1 mutant mice closely phenocopy the GluD2 knockout phenotype (Hirai et al. 2005, Kurihara et al. 1997). Whereas the GluD2 receptor is accumulated in Purkinje cell dendritic spines, Cbln1 is secreted from cerebellar granule cells and concentrates at parallel fiber synapses with Purkinje cells. Therefore, Cbln1 may bind directly to the GluD2 NTD and thereby exert its synaptogenic activity in the cerebellum (Kakegawa et al. 2009).

Recently, a similar mechanism has also been identified for the synaptic concentration of AChRs in *C. elegans* in which a complement control-like protein interacts directly with the extracellular domain of a neurotransmitter receptor subunit (Gendrel et al. 2009). These examples illustrate an alternative mechanism to the receptor recruitment via cytoplasmic scaffolding molecules. Neurotransmitter NTD interactions may drive the equilibrium of extrasynaptic, dispersed receptors towards the clustering of receptors at synaptic sites. The focal presentation of axon-derived differentiation signals at sites of cell contact and the direct action onto receptor extracellular domains represent a particularly effective and elegant way to achieve a rapid synaptic accumulation. In this model, cytoplasmic scaffolding molecules act downstream of neurotransmitter receptors by enlarging and stabilizing postsynaptic assemblies, and neurotransmitter receptors serve a structural role in synapse assembly that is independent from their ionotropic action.

Wingless as an Anterograde Signal in *Drosophila* Neuromuscular Junction Formation

Another anterograde regulator was discovered through studies of glutamatergic NMJs in *Drosophila*. Wnt-1 wingless (wg) was found at presynaptic terminals, and its receptor Frizzled (DFz) is concentrated at the postsynaptic membrane as well as on the presynaptic side. Wg secreted from the presynaptic terminals is critical for the development of fly NMJs. The loss of wg causes reduced bouton size and the incomplete development of boutons, whereas the overexpression of Wg leads to enhanced bouton proliferation (Packard et al. 2002). Interestingly, the cytoplasmic region of DFz is cleaved by proteases that lead to the generation of an 8kd polypeptide fragment. Wg activation of DFz appears to promote the translocation of this fragment into the nucleus, which then regulates the differentiation of postsynaptic development (Mathew et al. 2005).

Wg loss-of-function mutants also display presynaptic defects. Mutant boutons have enhanced unbundled microtubules, which may explain bouton proliferation defects because MT dynamics affect the proliferation of boutons (Roos et al. 2000, Ruiz-Canada & Budnik

2006). This hints that Wg can act not only as an anterograde inducer to regulate postsynaptic development but also might act to modify presynaptic development through an autocrine loop because the DFz receptor is also found on the presynaptic membrane. Presynaptic effects may be caused by a retrograde inducer whose activity is regulated by Wg. Further experiments are required to distinguish these possibilities.

The growth of the *Drosophila* NMJ is stimulated by neuronal activity, and Wg might mediate this effect. To support this notion, Wg secretion was enhanced by activity and coincided with rapid activity-dependent NMJ growth. Furthermore, heterozygous wingless mutants suppressed activity-dependent synaptic growth; the overexpression of Wg reduces the strength of electric stimuli that are required to reach a certain level of growth (Ataman et al. 2008).

Wnts have also been implicated as modifiers of agrin-induced AChR clusters in vertebrate neuromuscular junctions. Stimulatory and inhibitory actions of wnts have been reported in the literature. In some contexts, the readout of the trans-synaptic Wnt signal might depend on the activation of canonical or noncanonical wnt signaling pathways (Davis et al. 2008). A detailed discussion on this topic can be found in a recent review (Korkut & Budnik 2009). Moreover, in the vertebrate CNS, wnts act as retrograde signals, which are discussed below.

RETROGRADE ORGANIZERS

Retrograde organizers are factors that are secreted from postsynaptic cells that influence the differentiation and maturation of presynaptic terminals (Figure 2c). The precise apposition of synaptic membranes argues that synaptogenesis involves bidirectional communication between synaptic partners. Moreover, as animals grow in size, the dimension and number of synapses grow accordingly so that the presynaptic neuron can efficiently excite postsynaptic cells (Davis 2006). This homeostasis process strongly suggests that the activation state of the postsynaptic cell must be sensed by presynaptic terminals, which is then converted to a growth signal to regulate the size and function of presynaptic terminals. Therefore, understanding the action of retrograde signals will not only provide insight into how synapses form but also how synapses grow. Indeed, genetic analyses of different synapses identified several retrograde signals.

The Transforming Growth Factor Beta Family Member *gbb* Activates Neuromuscular Junction Growth in *Drosophila*

Forward genetic screens in the *Drosophila* NMJ mutant led to the isolation of the Wishful thinking (Wit) mutant, which exhibits reduced numbers of synaptic boutons and abnormalities in ultrastructure that include the detachment of pre- and postsynaptic membranes and floating T-bars. Electrophysiologically, these mutant synapses showed drastically reduced quantal content and frequency of spontaneous release, consistent with a predominantly presynaptic defect (Aberle et al. 2002, Marques et al. 2002). Wit encodes the *Drosophila* ortholog of the human BMP type II receptor, is expressed in the axon, and is required for the activation of the BMP downstream signaling pathway (Marques et al. 2002, 2003). These observations argue that Wit receptors on axon terminals receive a ligand that stimulates the growth and maturation of the NMJ.

The search for the ligand for Wit resulted in the discovery of BMP-7 glass boat bottom (Gbb), which is expressed in muscles. Gbb binds to Wit and activates its signaling pathway in the axon (McCabe et al. 2003). The loss of *gbb* results in a reduced number of synapses and defective synaptic transmission, similar to phenotypes found in Wit mutants. The loss of other components of the BMP pathway, which include type I receptors (Tkv and Sax), the R-smad (Mad), and the co-Smad (Med), causes similar synaptic-growth phenotypes

(McCabe et al. 2004, Rawson et al. 2003). Moreover, these signal transduction genes appear to be required in the motor neuron for their function in synaptic growth, which further argues that the neuron is the receiving end of the *gbb* pathway. Therefore, as animals grow in size, muscles secrete the TGF family protein *gbb* to promote the growth of synapses and to scale with the increase of muscle mass through the activation of the Wit receptor on nerve terminals.

Growth Factors as Synaptic Differentiation Signals in the Cerebellum

In many parts of the nervous system, synapses are organized into specialized synaptic complexes. For example, the cerebellar glomerular rosette is a multisynaptic structure that is formed between a glutamatergic mossy fiber axon, dendrites from numerous cerebellar granule cells, and Golgi cell terminals that form inhibitory synapses onto granule cell dendrites (Altman & Bayer 1997). The large size of this specialized synapse makes it an excellent model synapse to examine the mechanisms of synaptic differentiation in the vertebrate CNS. During development, upon contacting its postsynaptic targets, the typical developing mossy fiber axon spreads out into a fan-like structure that is eventually converted into the synaptic rosette with multiple pre- and postsynaptic elements (Mason et al. 1997). Signaling through several growth factors has been implicated in the development of this synaptic complex. The first to be identified was *Wnt7a*, which is released from postsynaptic granule cells at the time of synapse formation. The addition of recombinant *Wnt7a* is sufficient to induce changes in growth-cone morphology in mossy fiber explant culture, whereas blocking endogenous Wnts inhibits the maturation process (Hall et al. 2000). In *Wnt7a* knockout mice, rosette formation is delayed, which suggests that *Wnt7a* is an important player in this process and that there might be additional partially redundant inducers besides *Wnt7a*. Several Wnt downstream molecules have been implicated in mediating the action of *Wnt7a* in this context. For example, *Dvl* single mutants exhibit defects in mossy fiber synapses that are similar to those in *Wnt7a* mutants. *Dvl* and *Wnt7* double mutants exhibit more severe phenotypes compared with single mutants (Ahmad-Annuar et al. 2006). Moreover, inhibitors of GSK3 β mimic the effects of wnt in vitro (Hall et al. 2000, 2002). Taken together, this indicates that target-derived *Wnt7a* induces changes in the presynaptic terminal through a *Dvl*- and GSK3 β -mediated signaling pathway.

A second granule cell-derived retrograde signal is the fibroblast growth factor FGF22. Similar to *Wnt7a*, FGF22 and its close homologs induce morphological changes and the accumulation of synaptic vesicles in mossy fiber axons in vitro (Umemori et al. 2004). Blocking FGF22 signaling with recombinant proteins or genetic ablation of its receptor FGFR2c results in a reduction in synaptic vesicle accumulation in mossy fiber rosettes, whereas the synaptic structures themselves appear to persist. This indicates that trans-synaptic FGF-signaling controls selectively presynaptic vesicle accumulation at mossy synapses in the cerebellum. The similarity of *Wnt7a* and FGF22 activities on mossy fiber terminals raises the question of whether these two signals have redundant function or whether they each control specific aspects of mossy fiber differentiation. *Wnt7a* signaling has been proposed to alter microtubule stability. In contrast, FGF signaling has been primarily linked to the distribution of synaptic vesicles in axons. However, the delineation of cytoplasmic mediators of FGF signaling at synapses is required to understand whether FGF and Wnt signaling regulate presynaptic assembly through parallel pathways or whether they converge in common effectors. Notably, the integration of FGF and Wnt signaling is observed during cell fate decisions in earlier development (Fuentelba et al. 2007), and it remains to be investigated whether an analogous integration occurs during trans-synaptic signaling in the central nervous system.

Within the glomerular structure, *Wnt7a* and FGF22 trans-synaptic signaling may act specifically on glutamatergic mossy fiber terminals. What are the signals that control the

formation of inhibitory Golgi cell terminals within the same glomerular structure? Mutant mice that lack the neurotrophin receptor TrkB in the cerebellum and cerebellar afferents exhibit a strong reduction in the number of GABAergic Golgi cell synapses in cerebellar glomeruli (Rico et al. 2002). Notably, the density of asymmetric synapses within the glomerulus is not altered, which indicates that TrkB signaling selectively regulates GABAergic synapse assembly in this structure. Although the source and role for the TrkB ligand BDNF in this system remains to be determined, BDNF signaling has been linked to the maturation of GABAergic synapses in other CNS circuits (Vicario-Abejon et al. 2002). In summary, these studies highlight the importance of multiple retrograde growth factors in the differentiation of specific synaptic structures in the cerebellar glomerulus.

Laminin $\beta 2$ Organizes Active Zones in the Vertebrate Neuromuscular Junction

Although the essential roles of agrin and ACh in patterning postsynaptic AChRs argue strongly that anterograde axonal signals play the lead role in NMJ formation, there is also experimental evidence that supports instructive roles for muscle-derived retrograde signals in synapse formation. For example, Burden and colleagues showed that the overexpression of MuSK leads to ectopic AChR clusters outside of the normal synaptic region on the muscle surface. This manipulation also leads to abnormal axon branching and the formation of ectopic synapses (Kim & Burden 2008). However, the nature of the retrograde inducer is not clear yet.

During the maturation phase of NMJ formation, the axon terminal and muscle continue to interact with each other. One retrograde factor involved is an extracellular matrix protein, laminin $\beta 2$. Laminins are secreted glycoproteins and major components of the basal lamina. Laminin $\beta 2$ is made by the muscle and localizes to small stretches of basal lamina that extend through the synaptic cleft at vertebrate neuromuscular junctions (Patton et al. 1997). The loss of laminin $\beta 2$ causes multiple presynaptic defects that include a reduced number of active zones and an abnormal distribution of synaptic vesicles, which suggests that laminin $\beta 2$ is essential for the maturation of presynaptic terminals (Noakes et al. 1995). One interesting question is how muscle-secreted laminin $\beta 2$ clusters synaptic vesicles and builds active zones near a postsynaptic specialization. A critical issue in our understanding of laminin's function at synapses is to define its receptor. Laminin $\beta 2$ binds directly to the voltage-gated calcium channels, a component of the active zone (Nishimune et al. 2004). More importantly, the perturbation of this interaction in vivo results in the disassembly of neurotransmitter release sites. Artificial beads coated with laminin $\beta 2$ are sufficient to cluster calcium channels at a point of contact on the axon, as well as to accumulate other active zone proteins and synaptic vesicles. These data suggest that laminin $\beta 2$ is secreted by muscle cells and deposited at the synaptic basal lamina where it induces presynaptic differentiation through direct binding to the voltage-gated calcium channels. One outstanding question is how laminin $\beta 2$ achieves its specific localization at the synaptic cleft.

SYNAPSE ELIMINATION

A major mode of cellular behavior that contributes critically to connection specificity is synapse elimination. Anatomical experiments on the developing nervous system in vertebrate animals demonstrate many examples of synapse elimination and axon pruning (Lichtman & Colman 2000). Axon pruning can lead to the loss of major axon projections or local branches (Luo & O'Leary 2005), both of which might be intimately related to synapse elimination. However, how synapse disassembly leads to the retraction of axons is unclear.

In principle, there are two classes of synapse elimination processes: one in which the stoichiometry of innervation is reduced and one in which the cell type or subcellular

specificity of synaptic connections is refined. The best understood example of synapse elimination that results in a change in innervation stoichiometry is found in the maturation of the vertebrate neuromuscular junction. In immature NMJs, multiple axons innervate each myotube. As development proceeds, all but one of the axons are eliminated, which results in the mature mono-innervation pattern between axons and muscles (Lichtman & Colman 2000). This elimination process appears to depend on neuronal activity in which active axons are more likely to win in the competition among innervating axons. Other examples of synapse elimination with axon loss include climbing fiber-Purkinje neuron connections (Crepel et al. 1976), thalamocortical axon-layer 4 neuron synapses (Hubel et al. 1977), and infrapyramidal mossy fiber axon-CA3 synapses in the hippocampus (Liu et al. 2005).

Although the phenomenon of activity-dependent synapse elimination is well established in the vertebrate, the molecular mechanism of synapse disassembly is poorly understood. Invertebrate nervous systems also show stereotyped synapse elimination. The most prominent case is the pruning of axons during metamorphosis (Luo & O'Leary 2005). There are also examples of synapse elimination without the loss of axons in *C. elegans* in which most of the synaptic connections are made en passant. Below we discuss recent work that is starting to elucidate the molecular mechanisms that underlie the stereotyped synapse elimination programs.

Mono-Innervation of Vertebrate Neuromuscular Junctions

Synapse elimination is a critical step in building the mono-innervated, mature neuromuscular junction. This is a protracted process with many inputs that gradually decrease in synaptic strength as a single input increases its strength (Colman et al. 1997). Genetic manipulations that generate situations in which active axons compete with inactive axons showed that active axons always win the competition, which suggests that axons must be effective in depolarizing the muscle in order to be maintained (Buffelli et al. 2003). Furthermore, the blockade of synaptic activity throughout an entire junction prevents synapse elimination, whereas increasing activity accelerates the process (Sanes & Lichtman 1999). Forced synchronous activity in all the fibers that innervate a single NMJ prevents synapse elimination, which indicates that the unequal ability of axons to depolarize muscle is the driving force for synapse elimination in the vertebrate NMJ (Busetto et al. 2000).

Synapse Elimination Through the Complement Cascade in the Central Nervous System

As discussed above, synapse elimination at the NMJ is critical to generate the mature one neuron—one fiber innervation pattern. Similarly, synapse elimination is important during the maturation of the visual circuits. The visualization of developing circuits in the visual systems of *Xenopus* and zebrafish provides a picture of how mature synaptic connections are established. Live imaging studies revealed the highly dynamic neuronal arbors that undergo frequent phases of synapse formation and synapse elimination (Hua & Smith 2004). In immature circuits of the mouse, each dorsal lateral geniculate nucleus (dLGN) neuron receives synaptic input from multiple retinal ganglion cell (RGC) axons. As the circuit matures, each dLGN neuron receives stable input from only one or two RGC axons, owing to synapse elimination (Hooks & Chen 2006). Unexpectedly, the glia-derived complement pathway proteins C1q and C3 are required for this synapse elimination event. C1q and C3 are found locally at developing synapses but are absent from mature circuits. In C1q mutants, the eye-specific segregation of RGC axons in LGN is defective, and LGN neurons remain multiply innervated at a mature age. Similar defects were also observed in C3-deficient mice (Stevens et al. 2007). These results strongly suggest that synapse elimination is a critical process in the peripheral nervous system as well as the central nervous system.

Synaptic Pruning Mediated by Semaphorins

The infrapyramidal bundle (IPB) of the mossy fiber pathway in the hippocampus is present in newborn animals and significantly shortened by P30. This pruning is mediated by repulsive axon guidance receptors, plexin A3 and neuropilin-2, because IPB persists in plexinA3- and neuropilin-2-deficient mice. Semaphorin 3F, a ligand for the plexinA3 and neuropilin-2 receptor, appears to be expressed in the right place at the right time to trigger the pruning of IPB (Yaron et al. 2005). This genetic evidence strongly suggests that sema3F and its receptors prevent the maintenance of the IPB. However, the evidence does not offer cell biological insight into what cellular process initiates and underlies the pruning event. Further studies using electron microscopy showed that IPB pruning is intimately associated with synapse elimination. Cheng and colleagues found that IPB axon collaterals form transient synaptic complexes with basal dendrites of CA3 pyramidal cells in the early postnatal mouse hippocampus. At later postnatal ages, these synaptic complexes stop maturing and are removed before stereotyped pruning. In knockout mice that lack plexin-A3 signaling, the synaptic complexes continue to mature, and, as a result, the collaterals are not pruned. Thus, intact plexin-A3 signaling triggers axon pruning by promoting synaptic elimination (Liu et al. 2005). The same ligand and receptors also mediate the pruning of corticospinal axons from the visual cortex (Low et al. 2008).

Recent studies further elucidated functions of semaphorins in specifying synaptic connectivity in vertebrate neurons. Overexpression and knockdown studies in hippocampal neurons demonstrated that Sema5B reduces synapse numbers, presumably by destabilizing presynaptic terminals that are associated with a Sema5B-expressing postsynaptic cell (O'Connor et al. 2009). In mouse cortex, Tran and colleagues found that Sema3F negatively regulated the number and size of dendritic spines in dentate gyrus (DG), granule cell (GC), and cortical layer V pyramidal neurons, possibly by promoting the loss of spines and synapses. In contrast, a distinct Sema3A pathway controls basal dendritic arborization in layer V cortical neurons. These disparate effects of secreted semaphorins are reflected in the restricted dendritic localization of Npn-2 (a Sema3F receptor) to apical dendrites and of Npn-1 (a Sema3A receptor) to all dendrites of cortical pyramidal neurons (Tran et al. 2009).

Molecular Insights into Synapse Elimination from *C. elegans*

The extracellular ligand-receptor interaction provides the logic for the specificity and timing of synaptic elimination. Only certain specific synapses are eliminated at particular developmental stages because the expression of ligands and receptors is spatially and temporally controlled. The next questions are to understand how the synapse elimination program is coordinated with the development of the organism and how receptor signaling leads to the disassembly of the synaptic structure. Two studies in *C. elegans*, discussed below, are starting to provide some insights into these mechanisms.

In *C. elegans*, DD-type GABAergic motor neurons (DDs) remodel their synaptic circuits during larvae development. During the first larvae stage, DDs eliminate their embryonic presynaptic terminals and form new en passant synapses without changing their cell shape. Although the molecular mechanism of this remodeling is not yet clear, its coordination with organismal development is mediated by the heterochronic gene, LIN-14, which was previously known to specify the timing of cell division patterns in the development of non-neuronal tissues (Ambros & Moss 1994). In *lin-14* mutants, synapse elimination occurs precociously—a phenotype that can be rescued by LIN-14 expression in DD neurons, which suggests that LIN-14 functions cell autonomously to prevent synapse elimination (Hallam & Jin 1998). Consistent with this notion, the expression level of LIN-14 is drastically reduced right before the synapse elimination starts. Hence, the controlled expression of a heterochronic gene drives the elimination process.

Transient presynaptic terminals are also found in egg-laying motor neuron HSNs. Synapse formation of this neuron takes place in the L4 stage, whereas HSN only becomes functionally active several hours later in the adult stage (Desai et al. 1988). In early L4 animals, numerous en passant synapses form in a segment of the axon near the vulva organ. As animals mature, stereotyped synapse elimination converts this immature pattern of synapses to a more restricted, mature distribution pattern. Immature synapses at the anterior location are always eliminated, whereas synapses at the center of the vulval area persist and grow. Thus, the decision of synapse elimination is executed by the SYG-1 and SYG-2 family of immunoglobulin superfamily proteins (Shen & Bargmann 2003, Shen et al. 2004). First, in *syg-1* or *syg-2* mutants, the synapse elimination process is defective, which leads to persistent anterior synapses. Second, SYG-1 functions cell autonomously in HSN to execute synapse elimination and is specifically localized to the center of the vulval region. Synapses that colocalize with SYG-1 are spared from elimination, whereas synapses that fall out of the SYG-1 zone are doomed to disappear. Third, the overexpression of SYG-1 leads to the precocious elimination of anterior synapses. Therefore, the decision of where to eliminate synapses is made by the SYG-1 protein in HSN (Ding et al. 2007).

Then, how can SYG-1 locally control the fate of synapses? The search for SYG-1 interaction proteins led to the discovery of SKR-1, an ortholog of SKP1, which is a central component of the SKP-Cullin-F-box (SCF) ubiquitin ligase complex. The SCF complexes are E3 ubiquitin ligases that ubiquitinate protein substrates destined for degradation (Cardozo & Pagano 2004). The loss of function of SKR-1, Cullin, or F-box protein sel-10 leads to delayed and incomplete synapse elimination in HSN, which suggests that the SCF complex is required for this process. Interestingly, SYG-1 binding to SKR-1 disrupts the assembly of the SCF complex, which indicates that SYG-1 protects synapses by inhibiting the synapse elimination mediated by the SCF complex (Ding et al. 2007). Taken together, these studies suggest that synapse elimination activity is distributed across the entire axon, whereas synapse protection activity is localized by specific recognition events between synaptic partners or guidepost cells. The balance between synapse elimination and assembly at different subcellular locations leads to the mature synapse pattern.

TRANSCRIPTIONAL CONTROL OF SYNAPTIC CONNECTIVITY

The emergence of molecular signaling systems that either promote or inhibit the assembly and/or stability of synaptic junctions raises the question of how the expression of such components is controlled during neuronal development. Afferent neurons undergo substantial transcriptional changes upon entry in the target area; moreover, dendritic growth is regulated by transcriptional programs (Diaz et al. 2002, Polleux et al. 2007). In recent studies, transcriptional regulators have been identified that control synapse formation programs at two levels: First, there are cell-specific transcriptional programs that are linked to the fate determination of the cell that also control key wiring decisions at later developmental stages. Thus, many aspects of axonal trajectories and synaptic interactions are prespecified by the expression of guidance and specificity factors in a cell-specific manner. Second, the transcription of signaling factors can be regulated dynamically depending on neuronal activity and signals from the environment. This second mechanism has been primarily implicated in the regulation of the number of synaptic connections that are formed by a cell, whereas the first mechanism is primarily involved in tying cells into specific neuronal circuits.

Transcriptional Control of Wiring Specificity

The remarkable reproducibility of neuronal connectivity between individuals and the substantial preservation of connection specificity in the absence of neuronal activity suggest that many aspects of neuronal connectivity are genetically encoded. Recent studies have

provided several examples that single transcription factors can directly control the expression of individual surface receptors that endow cells with specific responsiveness to molecular cues for migration, axon guidance, and synaptic partner selection. The best-established examples for this type of regulation are found in axonal guidance, specifically, the response of axonal projections to midline-derived guidance cues. The zinc-finger transcription factor *Zic2* binds directly to promoter elements of the axon guidance receptor *EphB1* and thereby directs the ipsilateral growth of retinal ganglion cell axons at the optic chiasm (Herrera et al. 2003). Similarly, in the mouse spinal cord, the LIM homeodomain transcription factor *Lhx2* is required for the expression of the Robo-receptor *RIG1* and the immunoglobulin superfamily member *TAG-1* in a population of commissural interneurons (Wilson et al. 2008). The loss of *Lhx2* results in a loss of *RIG1* and thereby the ipsilateral misprojection of axons in mutant mice. Importantly, *RIG1* expression in other populations of commissural interneurons does not depend on *Lhx2*. That means, there is not simply one transcriptional program that defines the *RIG1*-dependent commissural trajectory; instead, the interpretation of transcriptional programs is complex and occurs in a cell-type-specific manner.

Also, other binary choices in axonal trajectories are directly regulated by transcription factors. The LIM homeodomain transcription factor *Lhx3* regulates FGF-receptor 1 expression in medial-class spinal motor neurons. In the absence of FGF receptor 1, axons fail to respond to target-derived FGF signals from the dermomyotome and misproject to limb muscles (Shirasaki et al. 2006). Another LIM-homeodomain transcription factor, *Lhx1*, instructs the outgrowth of motor axons towards dorsal limb muscles through regulating the expression of Eph-receptors (Kania et al. 2000, Kania & Jessell 2003).

These examples of transcriptional regulation of axon guidance decisions exemplify how single transcription factors directly regulate the expression of individual cell surface receptors, which in turn mediate growth decisions at choice points of the neuronal trajectory. There is emerging evidence that the same principle of transcriptionally controlled wiring programs also underlies key aspects of synaptic target selection. For example, the synaptic layer specificity of two closely related groups of *Drosophila* photoreceptor neurons, R7 and R8, is controlled by the interplay of the transcriptional regulators *senseless*, *prospero*, and *NF-Y*. Here, the R8-specific transcription factor *senseless* directly regulates the cell surface receptor *Capricious*, a leucine-rich repeat protein that is essential for R8 receptor targeting (Shinza-Kameda et al. 2006). The R8-specific targeting program is repressed in R7 neurons by *NF-Y*, and the loss of *NF-Y* results in *senseless*-dependent mistargeting of R7 neurons to the R8 target lamina. Notably, *NF-Y* mutant R7 neurons continue to express many R7 markers, and targeting defects only occur at a late stage of R7 development, which indicates that the loss of *NF-Y* does not result in a complete fate switch of the neurons but only a selective defect in their targeting specificity (Morey et al. 2008).

In vertebrate systems, the best evidence for a transcriptionally-controlled wiring mechanism is derived from studies on sensory-motor reflex circuits, in which the analysis of target specificity is greatly facilitated by transcriptional markers for specific motor neuron pools and highly selective labeling approaches through dye injections into muscle targets. Combinations of Hox gene transcription factors expressed in motor neurons specify motor neuron pools and provide a transcriptional framework for connection specificity (Dasen et al. 2003, 2005). The Hox code restricts the competence of cell pools to activate downstream wiring programs. Interestingly, surface molecules that control synaptic specificity are often not directly under Hox transcription factor control and, therefore, not immediately expressed upon the specification of neuronal populations. Instead, these wiring molecules are controlled by transcriptional regulators that depend on target-derived signals, similar to ETS transcription factors *Pea3* and *Er81* (Lin et al. 1998). Target-derived GDNF is required for

the upregulation of *Pea3* (Haase et al. 2002), whereas a yet unknown target-area-derived signal leads to the upregulation of *Er81* in motor neurons. Motor neurons in *Pea3*-deficient mice exhibit alterations in several critical aspects of terminal differentiation: cell body positioning, dendrite morphology, and the pattern of sensory-motor connectivity (Vrieseling & Arber 2006). Specifically, *Pea3* is selectively expressed in a pool of motor neurons that innervate the cutaneous maximus muscle (CM). These CM motor neurons are devoid of monosynaptic input from proprioceptive sensory neurons. In contrast, other motor neurons that are *Pea3*-negative receive direct mono-synaptic proprioceptive inputs. In *Pea3* knockout mice, proprioceptive sensory neurons form aberrant monosynaptic connections with CM neurons, which indicates the loss of a signaling system that controls synaptic specificity in this reflex arc (Vrieseling & Arber 2006). The repellent signal *Sema3e* and its receptor *PlexinD1* are selectively expressed in the CM reflex arc, with *Sema3e* present in the *Pea3*-positive CM motor neurons and *PlexinD1* in the proprioceptive sensory neurons that innervate CM. Notably, *Sema3e* expression is lost in *Pea3* mutants. The genetic ablation of either *Sema3e* or *PlexinD1* phenocopies the connectivity defect seen in *Pea3* mutant mice, with CM motor neurons that receive direct, monosynaptic proprioceptive input (Pecho-Vrieseling et al. 2009). These findings establish a repellent *Sema3e*-*PlexinD1* signaling system and its control through the transcription factor *Pea3* in the establishment of synaptic specificity in this CNS circuit. Notably, the ablation of *Sema3e* does not result in a complete loss of specificity because proprioceptive inputs remain restricted to CM motor neurons and do not innervate inappropriate motor neuron pools. This highlights the fact that, in this system, synaptic specificity is encoded by multiple signals that each control specific aspects of the connectivity program.

In summary, analyses of transcriptional programs for neuronal wiring specificity support a model of layered specificity in which a hierarchy of transcription factors regulate different aspects of connectivity. Transcriptional programs are established in a step-wise fashion with some intermediate transcription factors that are activated downstream of an initial transcription factor code and others that are regulated by target-derived signals. Initial programs direct the growth trajectory of neuronal processes, whereas later aspects of neuronal connectivity can be modulated by neuronal activity or target-derived signals.

The Control of Synapse Numbers by Transcriptional Programs

The transcriptional regulation of synaptic connectivity controls not only the selectivity of axonal trajectories and synaptic partners but also the numbers of synapses formed by a single cell. The function of neuronal circuits critically depends on precisely balanced numbers of synapses formed on a target cell. In several systems, homeostatic mechanisms have been described that result in an adaptation of synapse numbers as well as synaptic function in response to acute alterations in neuronal activity (Davis 2006). These adaptive processes may rely on transcription factors that are directly controlled by cellular depolarization. In contrast to the cell type-specific transcriptional programs described above, these adaptive programs may be common to many neuronal cell populations and activate common programs that drive the formation or elimination of synapses. Over the past years, several activity-regulated transcription factors have been identified (Polleux et al. 2007); two recent examples are discussed below.

Work by the Greenberg laboratory identified myocyte enhancer factor 2 (MEF2A-D) as a family of transcription factors that are regulated by calcium signaling as negative regulators of synapse number (Flavell 2006). Calcium-dependent dephosphorylation of MEF2 leads to its activation and transcription of target genes. Gain- and loss-of-function studies on MEF2 in cultured hippocampal neurons revealed that the suppression of MEF2 results in an increase in glutamatergic synapse numbers, whereas MEF2 activation or overexpression results in decreased synapse numbers. Cowan and colleagues demonstrated that the

suppression and overactivation of MEF2 in the nucleus accumbens in vivo result in an increase and decrease, respectively, of dendritic spine density and thereby presumably synapse numbers (Pulipparacharuvi et al. 2008). A genome-wide search for MEF2 targets revealed several genes that encode proteins that are implicated in the destabilization of glutamatergic synapses, such as *arc*, *homer1a*, and *delta-protocadherins* (Flavell & Greenberg 2008). Targets identified in the nucleus accumbens appear to differ significantly from those identified in hippocampal cells (Pulipparacharuvi et al. 2008). This might imply that although MEF2-mediated transcription in both systems results in a reduction in synapse numbers, it might not mediate those effects through the same transcriptional targets. Interestingly, the activity of MEF2 might be further regulated by sumoylation, a small ubiquitin-like covalent modification. Whereas nonsumoylated MEF2A acts as a transcriptional activator, the conjugation of a sumo-residue to an arginine residue converts MEF2A to a transcriptional repressor. This function is critical for the formation of dendritic claws in cerebellar granule cells, a specific aspect of post-synaptic differentiation in these cells (Shalizi et al. 2006).

Another example of the regulation of synapse numbers at the transcriptional level is the basic-helix-loop-helix transcription factor Npas4 (Lin et al. 2008). Although MEF2 is primarily regulated by posttranslational modifications, Npas4 expression is strongly increased in response to neuronal activity. Increased Npas4 expression stimulates the formation of GABAergic synapses on Npas4-expressing cells (Lin et al. 2008). A genome-wide analysis of Npas4 target genes identified 270 candidate targets that include brain-derived neurotrophic factor (BDNF), which has been implicated in promoting the development of GABAergic synapses. The suppression of BDNF expression in Npas4-expressing cells attenuates the increase in GABAergic innervation downstream of Npas4. This suggests that the upregulation of BDNF represents one of the relevant target genes that mediate a Npas4-induced increase in GABAergic synapse formation.

In summary, recent studies have provided a first glimpse at transcriptional programs that dynamically regulate glutamatergic and GABAergic synapse numbers. Although these transcription factors do not appear to be essential for the formation of synapses during development, loss-of-function phenotypes reveal important functions specifically in altering synapse numbers in response to acute stimulation or long-lasting changes in network activity. Given that several trans-synaptic signaling systems have been identified that can act as positive and negative regulators of synapse formation, it will be interesting to see which of these regulators are controlled through factors such as MEF2 and Npas4. Another unresolved question is whether there are specific, rate-limiting synaptic proteins that are common to all neuronal populations or whether different cell types and synapses formed with select synaptic partners have evolved unique molecular mechanisms that differ between neuronal and synapse populations.

MOLECULAR DIVERSITY BEYOND ANATOMICALLY DEFINED CELL TYPES

Recent efforts to unravel the molecular mechanism of synaptic specificity have also brought up renewed questions regarding the diversity of neurons in the nervous system (Masland 2004, Nelson et al. 2006). Classically, neuronal cell types have been defined by morphological criteria. More recently, additional criteria for the characterization of neuronal subpopulations have been added, which include: specific electrophysiological properties, responsiveness to specific stimuli (such as direction selectivity of cells in the retina), specific connectivity, and molecular markers that have become a key approach for classifying neuronal populations.

Although some studies on individual genes or gene families have highlighted molecular heterogeneity within cell populations, primarily, genome-scale studies have revealed new aspects of cellular diversification. Two key technologies are the combination of single-cell transcript analysis with microarray technology as well as genome-wide in situ hybridization studies with cellular resolution (Thompson et al. 2008, Tietjen et al. 2003). A surprising finding from these studies is that apparently morphologically identical neurons can express highly divergent molecular markers. These findings are notable for several reasons: First, they reveal a previously unappreciated diversity of neuronal populations; second, the identification of regulatory elements of genes with highly selective expression patterns will facilitate the selective genetic manipulation of these populations; and third, some of the products encoded by differentially expressed genes may contribute to selective wiring patterns or cell-specific functional properties. We discuss several examples below.

The Molecular Diversity of *Drosophila* Dscam1 Encodes Self-Recognition

Arguably, the most impressive example of molecular diversity is provided by the immunoglobulin superfamily protein Dscam1 in *Drosophila*. The combination of alternative exons at three positions in dscam mRNA generates Dscam1 variants with 19,000 different extracellular domains. Above, we have discussed the functions of vertebrate Dscam and Dscam-like-1 (DscamL), which control laminar specificity in the chick retina through homophilic adhesive interactions (Yamagata & Sanes 2008). In systematic biochemical studies of *Drosophila* Dscam1, Zipursky and colleagues uncovered exclusively homophilic binding between Dscam1 variants—this means alternative splicing enables a single gene to encode 19,000 unique recognition events (Sawaya et al. 2008; Wojtowicz et al. 2004, 2007). A detailed analysis of Dscam1 variant expression in different cell populations and a comparison of the Dscam1 content of single photoreceptor cells leads to two important conclusions: First, single cells express multiple (14–50) distinct Dscam1 isoforms; and second, splice isoform choice is apparently stochastic, with single photoreceptor cells of the same type that express different Dscam1 isoforms (Neves et al. 2004). Given the large number of potential isoforms, the stochastic expression of variants will leave every cell with a unique signature of Dscam1 variants. In mutant flies that lack five of the twelve possible alternative exons at one of the alternative splicing sites, the branching pattern of mechanosensory neurons was severely disrupted, which highlights that even a modest reduction of Dscam1 diversity results in anatomical phenotypes (Chen et al. 2006). Whether this function of Dscam1 involves trans-synaptic interactions between molecules located on axons and their target cells or primarily axo-axonal interactions between Dscam1 molecules on the neuronal processes of a single cell remains unknown.

A second process that critically requires Dscam1 function is in the dendrite development of *Drosophila* sensory neurons. Here, homophilic Dscam1 interactions mediate self-recognition in individual sensory neurons that cover the body walls of *Drosophila* larvae. Self-recognition between two branches of a given dendrite is converted into repulsion between the two dendritic processes and thereby ensures the spreading of dendrites to evenly cover a field of body-wall surface (Hughes et al. 2007, Matthews et al. 2007, Soba et al. 2007). This self-avoidance function is conserved in mouse retina where Dscam is expressed in a subset of amacrine cells that use the transmitter dopamine. In mice with a spontaneous loss-of-function mutation in Dscam, dendrites of these dopaminergic amacrine cells exhibit an unusual degree of self-crossings, which subsequently develop into large fascicles with amacrine cell bodies that aggregate into clumps (Fuerst et al. 2008). Importantly, these defects in self-avoidance are specific to this Dscam-positive population of cells because other amacrine cell populations develop their normal mosaic spacing within the retina. This indicates that, similar to *Drosophila* Dscam1, vertebrate Dscam is required for iso-neuronal self-avoidance.

The Molecular Diversity of Synaptic Proteins in Vertebrates

One protein family that has provided an early example of expression in neuronal subpopulations is the group of clustered protocadherins (Morishita & Yagi 2007). These proteins are encoded in the mouse genome within three closely related protocadherin gene clusters (α , β , and γ) that generate 58 protocadherin variants that differ in their extracellular domains. Each beta-protocadherin protein is encoded by a single exon, whereas the molecular complexity of alpha- and gamma-protocadherins is achieved by the combination of variable exons, which encode the extracellular domain of proteins, with constant exons that encode the cytoplasmic tail (Wu & Maniatis 1999).

Although alpha protocadherin isoforms (originally termed cadherin neuronal receptors) are expressed in grossly similar patterns in the nervous system, individual isoforms are not detected in all neurons of one cell type but in divergent subpopulations (Kohmura et al. 1998). A single-cell analysis of individual Purkinje cells in the mouse cerebellum revealed that single cells within this morphologically homogeneous population express multiple but divergent variants of the protocadherin family of surface receptors (Esumi et al. 2005). Similarly, neighboring periglomerular cells in the olfactory bulb express different combinations of protocadherin exons (Kohmura et al. 1998). The alpha- and gamma-protocadherin proteins are localized to synapses, although they are not restricted to synaptic sites (Kohmura et al. 1998, Phillips et al. 2003, Wang et al. 2002). Moreover, similar to classical cadherins, protocadherin variants were proposed to mediate isoform-specific homophilic interactions (Fernandez-Monreal et al. 2008). The ablation of the entire protocadherin-gamma cluster, which encodes 22 of the described protocadherin isoforms, did not result in dramatic changes in neuronal migration and process outgrowth but uncovered a requirement for these proteins in neuronal survival (Wang et al. 2002). When apoptotic cell death in protocadherin-gamma neurons was prevented in double mutants that lacked the proapoptotic gene Bax, synapse numbers were still reduced (Weiner et al. 2005). This suggests that gamma-protocadherins are indeed required for some aspect of synaptic differentiation. Interestingly, this synaptic function appears to involve multiple modes of cellular interactions because gamma-protocadherin proteins are not only concentrated at neuron-neuron junctions but also contribute to synapse formation at neuron-glia junctions, adjacent to synaptic sites (Garrett & Weiner 2009). Further studies on neuronal connectivity in the retinae of conditional gamma-protocadherin knockout mice did not reveal alterations in the specificity of laminar connectivity but primarily suggest a function for gamma-protocadherins in coordinating the numbers of specific cell populations in the retina (Lefebvre et al. 2008). In contrast, mutant mice in which the constant region of the alpha-protocadherin gene cluster was ablated appear to show wiring-specificity defects. In these mice, olfactory sensory neurons form small, ectopic glomerular structures rather than coalescing into one larger glomerular structure per hemisphere as seen in wild-type mice (Hasegawa et al. 2008). Although currently available data support important functions for clustered protocadherins at synapses as well as in cell survival, the relevance of their molecular diversity has remained obscure and requires further study.

Other examples of molecular diverse synaptic proteins are neuroligins, synaptic cell surface receptors that have potent synapse-organizing functions (discussed above), and takusans (a Japanese word that means many), which are cytoplasmic scaffolding proteins at glutamatergic synapses. For neuroligins, more than 3900 variants are predicted to be generated from three genes (NRXN1, -2, and -3), through the expression from an alternative promoter and alternatively splicing at five sites (Missler & Sudhof 1998, Ushkaryov et al. 1992). Intriguingly, similar to Dscams and protocadherin family members, the molecular diversity of neuroligins is restricted to the extracellular domain of the protein, which provides another example of a cell surface receptor that might connect diverse extracellular interactions into a common intracellular signaling pathway or structure. Some biochemical

isoform-specific interaction partners and functions have been identified (Boucard et al. 2005, Chih et al. 2006, de Wit et al. 2009, Graf et al. 2006, Ko et al. 2009), but the relevance of neurexin diversity for synapse formation and function remains largely unexplored.

Takusans further add to the perplexing complexity of synaptic components. This gene family consists of more than 400 variants of a cytoplasmic protein, which were identified on the basis of their upregulation in mouse brains with decreased NMDA-receptor function (Tu et al. 2007). Some Takusan isoforms interact directly with the glutamatergic scaffolding protein PSD95 and regulate the surface transport of GluA1-containing AMPA-type glutamate receptors. Single, cortical pyramidal cells exhibit significantly divergent contents of Takusan splice variants. Therefore, individual cells might fine tune synaptic AMPA-receptor function through the expression of different Takusan isoforms.

Notably, alternative splicing is only one of many gene expression mechanisms to achieve genetically preprogrammed or stochastic selection of molecular repertoires (see review by Muotri & Gage 2006). Many neuronal gene products underlie complex transcriptional regulation from multiple alternative promoters, but gene regulation may also occur through epigenetic and posttranscriptional mechanisms.

Although the functional relevance of molecular diversity in neuronal proteins is only beginning to emerge, current examples allow us to advance hypotheses on how molecular diversity in cellular subpopulations underlies specific connectivity or functional properties. First, molecularly diverse proteins might contribute to wiring and tie specific cellular subpopulations into functional circuits. Such a model would be most conceivable for homophilic cell adhesion molecules in which subpopulations of cells that express a given isoform are preferentially connected. Second, in other cases, the molecular diversification might provide a mechanism for self-recognition, through homophilic signaling similar to Dscam1, or through coupling to heterophilic ligands for the cell-autonomous functional regulation of single cells. Third, the molecular heterogeneity of splice variants of cell surface or cytoplasmic proteins in neuronal cell populations might represent the fine tuning of functional properties, which sets excitability and plasticity properties of cells within a network. Notably, electrophysiological studies reveal considerable heterogeneity in mRNA expression for ion channels, even in apparently identical neurons (Schulz et al. 2006, 2007). In the latter case, the molecular diversification would enable cells to achieve certain electrophysiological properties through a well-balanced but variable expression of different components.

CONCLUSIONS

The extensive literature cited in this review reveals two overarching features of the molecular programs for synaptogenesis. First, diverse molecular and cellular mechanisms encode the specificity of synaptic connections, which not only testifies to the complexity of the brain but also affords the flexibility for any particular neural circuit to achieve precise synaptic connections. None of the mechanisms are mutually exclusive, which makes it likely that the sequential recruitment of positive and negative selections may be used to achieve specificity in a step-wise manner. Second, multiple redundant pathways exist to ensure the completion of the synapse formation process. Despite an extensive search for the agrin-like molecule for central synapses, no genetic manipulation of a single molecule or several molecules eliminates synapses. Interestingly, convincing evidence shows that several synaptic adhesion molecules are sufficient to drive synapse formation *in vitro*. Together, these findings highlight the possibility that multiple, redundant trans-synaptic pathways cooperate *in vivo*, although each of them has the capacity to support an extensive program for synapse formation.

Our current knowledge on synapse specificity and synapse formation raises many new questions and begs for more definitive answers. The diversity of molecular strategies employed at different populations of synapses emphasizes the need to focus loss-of-function analyses on specific synapses between reproducibly identifiable partners. A dissection of redundant synaptogenetic programs in vivo should be feasible by the combination of genetic knockouts and viral silencing methods that target multiple trans-synaptic signaling systems and will provide us with an opportunity to test whether synaptogenesis can be completely blocked. Using simple model organisms such as flies and worms, we might be able study particular model synapses to gain a full understanding of the interplay of extracellular signals that mediate synaptic target choices and determine subcellular synaptic localization, as well as the intracellular events that eventually assemble the pre- and postsynaptic apparatus.

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Figure 1.

Model mechanisms for synaptic specificity during development. Interactions with appropriate synaptic partners can be accomplished by mutual attraction through positive regulators (*green*), or selective repulsion through negative regulators (*red*) derived from an inappropriate target cell or released by other cell types in the target territory in the form of a morphogenetic gradient. The synapse formation competence of afferents can be locally controlled by priming factors that are released in the target territory. During development, synaptic specificity can emerge through the elimination of contacts with inappropriate targets. The formation of transient synapses with guidepost cells (*squares*) provide a means of prepatterned synaptic structures before final target cells have arrived or matured in the target area. In some systems, guidepost cells are transient populations that are eliminated by cell death once the final wiring pattern has been accomplished.

Figure 2.

Synapse organizing signals. Differentiation of pre- and postsynaptic domains, recruitment of synaptic vesicles, and pre- and postsynaptic receptors and scaffolds can be driven by multiple trans-synaptic signals, derived from either a synaptic partner or astroglia that flank synaptic sites.