

# Mechanisms of Vertebrate Synaptogenesis

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## Key Words

synapse, active zone, postsynaptic density, membrane trafficking, cytoskeleton

## Abstract

The formation of synapses in the vertebrate central nervous system is a complex process that occurs over a protracted period of development. Recent work has begun to unravel the mysteries of synaptogenesis, demonstrating the existence of multiple molecules that influence not only when and where synapses form but also synaptic specificity and stability. Some of these molecules act at a distance, steering axons to their correct receptive fields and promoting neuronal differentiation and maturation, whereas others act at the time of contact, providing positional information about the appropriateness of targets and/or inductive signals that trigger the cascade of events leading to synapse formation. In addition, correlated synaptic activity provides critical information about the appropriateness of synaptic connections, thereby influencing synapse stability and elimination. Although synapse formation and elimination are hallmarks of early development, these processes are also fundamental to learning, memory, and cognition in the mature brain.

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**Axon:** a long, thin neuronal process that carries electrical signals from the cell soma to presynaptic boutons

**Dendrite:** a tapered neuronal process onto which presynaptic boutons form synapses. Signals from these dendritic synapses are propagated back to the cell soma, summed, and used to trigger an axonal action potential

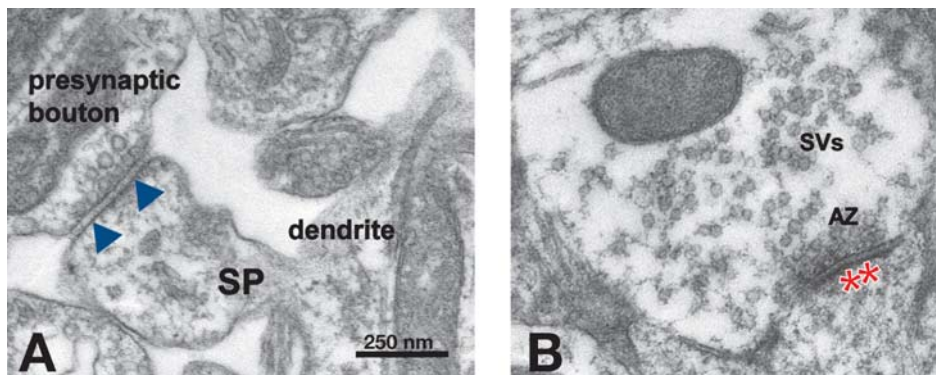
**Synapse:** a site of contact between neurons where electrochemical signaling occurs

## INTRODUCTION

The human brain is an amazingly complex organ composed of trillions of neurons. Every idea, emotion, and lofty thought we produce is created as a series of electrical and chemical signals transmitted through connected networks of neurons. Neurons transmit these signals to one another at specialized sites of contact called synapses. In the vertebrate nervous system, most neurons communicate via chemical synapses. As the name implies, chemical synapses function by converting electrical signals, in the form of action potentials racing down axons and invading presynaptic boutons, into chemical signals and then back to electrical impulses within the postsynaptic dendrite. Synapses perform this task by releasing neurotransmitters from the presynaptic neuron that bind and activate

neurotransmitter-gated ion channels on the postsynaptic cell.

Chemical synapses are asymmetric cellular junctions formed between neurons and their targets, including other neurons, muscles, and glands. Ultrastructurally, synapses are composed of several specialized domains (Gray 1963, Palay 1956) (**Figure 1**). The most prominent is the presynaptic bouton. These small axonal varicosities, ~1 micron in size, stud the length of axons and establish contacts with one or more postsynaptic cells. Each bouton is filled with hundreds to thousands of small ~50-nm clear-centered synaptic vesicles carrying neurotransmitter molecules. A depolarizing action potential invading the bouton causes synaptic vesicles docked at the plasma membrane to fuse and release their neurotransmitter into the synaptic cleft, a small space between the pre- and postsynaptic cells. Synaptic vesicle fusion does not occur randomly at the presynaptic plasma membrane but within another specialized domain called the active zone. Active zones are characterized by the presence of an electron-dense meshwork of proteins, also known as the presynaptic web, and synaptic vesicles that are both embedded within this matrix and docked at the plasma membrane (Burns & Augustine 1995, Hirokawa et al. 1989, Landis 1988, Phillips et al. 2001). Directly apposed to the active zone on the postsynaptic side is a third domain that functions to receive information sent by the presynaptic neuron. Similar to the active zone, this postsynaptic membrane specialization is characterized by the presence of an electron-dense meshwork of proteins that extends across the synaptic cleft to the active zone as well as into the cytoplasm of the postsynaptic cell (Garner et al. 2002, Palay 1956, Sheng 2001). This structure, referred to as the postsynaptic density (PSD), serves to cluster neurotransmitter receptors, voltage-gated ion channels, and various second-messenger signaling molecules at high density directly across from the active zone (Garner et al. 2002, Palay 1956, Sheng 2001). Filamentous proteins extending across the synaptic cleft are



**Figure 1**

Ultrastructure of excitatory glutamatergic synapses. Electron micrographs of two synapses formed between hippocampal neurons grown for 15 days in culture. The synapse in (A) is clearly onto a dendritic spine (SP), indicating that it is excitatory in nature. Docked synaptic vesicles can be seen along a prominent synaptic cleft (*arrowheads*). The synapse in (B) has many of the classic features of chemical synapses, including a presynaptic bouton containing  $\sim 50$ -nm synaptic vesicles (SVs), an active zone (AZ) characterized by an electron-dense meshwork of proteins and clusters of docked synaptic vesicles, and a prominent postsynaptic density (*asterisks*). Micrographs were taken and generously provided by J. Buchanan, Stanford University.

thought to hold the active zone and PSD in register. Although these features are shared by synapses throughout the CNS, variation in the size and organization of presynaptic active zones, as well as in the thickness of the PSD, have been documented and have been correlated with (a) synaptic type, e.g., the type of transmitter released by a given bouton; (b) synaptic function, e.g., whether the synapse is excitatory, inhibitory, or modulatory; and (c) synaptic efficacy, e.g., whether a synapse fires reliably or unreliably, continuously or sporadically. In this review, we restrict our comments to excitatory glutamatergic synapses because most of the insights into the molecular mechanisms of synaptogenesis have come from studies on these synapses.

The formation of synapses in vertebrate organisms occurs over a protracted period of development, beginning in the embryo and extending well into early postnatal life. As discussed below, synapse formation also occurs in adults, where it is thought to contribute significantly to learning and memory. During development, synaptogenesis is tightly coupled to neuronal

differentiation and the establishment of neuronal circuitry. For example, shortly after neurons differentiate and extend axonal and dendritic processes, many of the genes encoding synaptic proteins are turned on, resulting in the formation, accumulation, and directional trafficking of vesicles carrying pre- and postsynaptic protein complexes. During this time, the specification of correct neuronal connections is determined, as axons and dendrites make contact and establish initial, often transient, synapses. This courtship involves a myriad of secreted factors, receptors, and signaling molecules that make neurons receptive to form synapses. It also requires interactions between sets of cell-surface adhesion molecules (CAMs) that are involved in cell-cell recognition, as well as inductive signals that trigger the initial stages of synapse formation, including the assembly of pre- and postsynaptic specializations from their component proteins and membranes. Finally, synaptic activity determines whether these synapses will be stabilized or eliminated, both during development and in the mature brain. In this review, we discuss each of these

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**Active zone:** a specialized region of the presynaptic bouton where synaptic vesicles dock and fuse with the plasma membrane

**Postsynaptic density:** a specialized region of the postsynapse where neurotransmitter receptors and signaling molecules cluster at high density

**Synaptogenesis:** the complex process by which functional synapses form between neurons

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**Synaptic activity:**

correlated or uncorrelated electrochemical signaling between neurons

**Glutamate:** an amino acid that functions as an excitatory neurotransmitter

**Glia:** a population of cells in the brain that do not engage in synaptic transmission but are important for neuron survival

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issues with a special emphasis on how different classes of molecules appear to guide these events.

## **SPECIFICATION AND INDUCTION OF SYNAPSE FORMATION**

An important aspect of synaptogenesis is target recognition, i.e., the ability of axons from different brain regions to grow into their respective target fields and synapse with the correct cell type. In many cases, axons must navigate across large distances and complex terrains before encountering their target cells. Intriguingly, although they come into contact with a multitude of neurons during their journeys, they refrain from synapsing onto inappropriate target cells. For example, retinal ganglion cell axons traverse long distances from the eye into the lateral geniculate nucleus of the thalamus before synapsing onto thalamic cell dendrites (Shatz 1996, 1997). Similarly, motor neuron axons from the ventral horn of the spinal cord delay synapse formation until they innervate muscle fibers some distance away (Burden 2002, Sanes & Lichtman 2001). In these examples not only is synapse formation delayed until axons reach specific target regions, but even within these target regions there are lag times of days to weeks before synapses form (Lund 1972, Pfrieger & Barres 1996).

What types of factors/cues regulate this temporal and spatial specificity of synaptogenesis? In principle, temporal events can be regulated by intrinsic, genetically encoded programs such as those used in cell-fate determination during development. In the case of neuronal cells, these intrinsic signals could be part of an intracellular clock guiding neuronal growth and differentiation. Studies of dissociated cultures of hippocampal neurons support this idea. In these cultures, axons from E18 rat hippocampal neurons are immediately competent to function as presynaptic partners, whereas dendrites must mature for several days to become competent

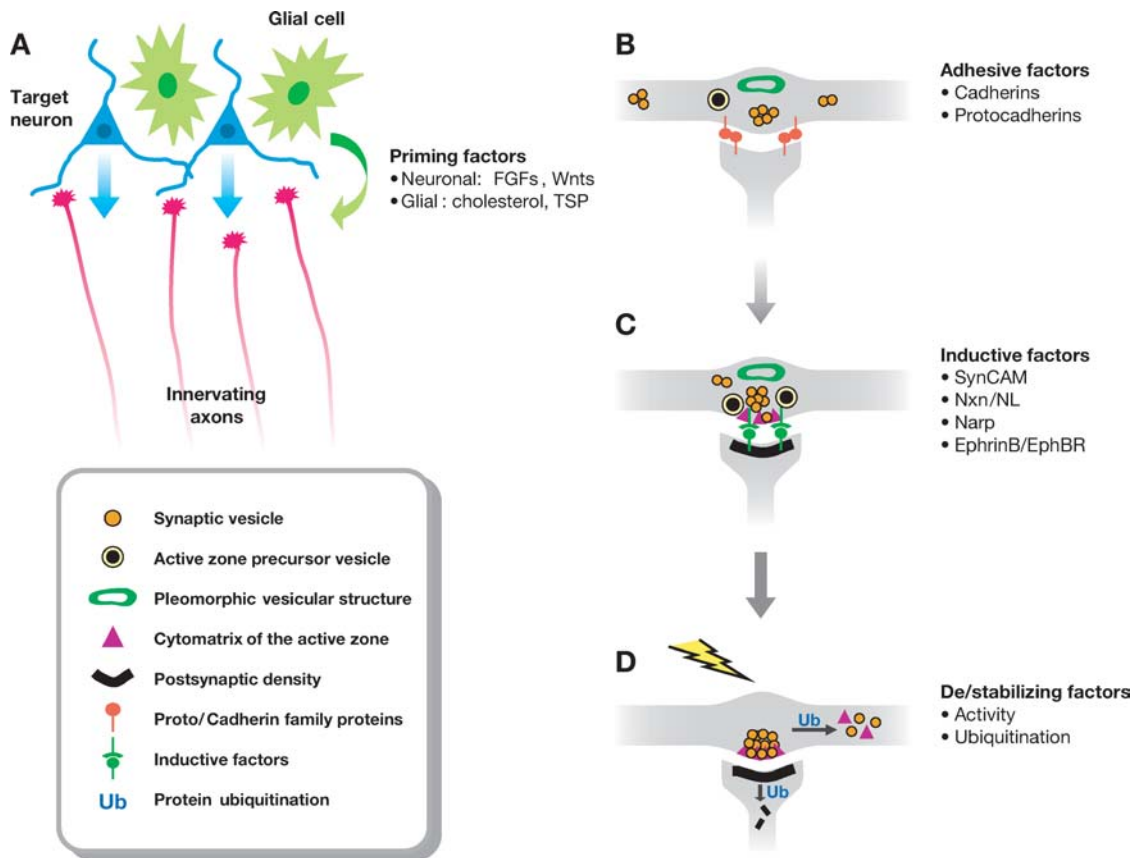
postsynaptic partners (Fletcher et al. 1994). Alternately, cues for regulating temporal and spatial specificity could be molecules derived from target neurons that act either indirectly, i.e., by activating transcription factors that allow neurons to become synaptogenesis-competent, or directly on axons to induce presynaptic differentiation (Ullian et al. 2004, Umemori et al. 2004).

Although intrinsic signals are a conceptually simple and attractive mechanism for temporally regulating synaptogenesis, very little progress has been made in identifying these important molecules. In contrast, significant progress has been made in the identification of target-derived factors that either accelerate neuronal maturation or directly induce synapse formation. We refer to the former as “priming molecules,” because they seem to prime neurons and make them competent to undergo synaptogenesis, and the latter as “inducing molecules,” because they appear to trigger synaptogenesis (see **Figure 2**). As discussed below, these molecules can be divided into several classes on the basis of when in the cascade of synaptogenesis they appear to function, and they include diffusible, target-derived factors as well as cell-surface adhesion molecules.

### **Diffusible Target-Derived Factors Guiding Synapse Specificity**

One class of proteins with synaptogenic activity is diffusible factors that are synthesized either by target neurons or the surrounding glia. These molecules have a range of activities, including abilities to guide axonal projections to their correct targets, stimulate local arborization, promote neuronal differentiation and maturation, and create a permissive environment wherein axo-dendritic contact leads to the formation of functional synaptic junctions.

A prominent group of target-derived molecules known to guide axons into reciprocally connected brain areas are those involved in growth cone guidance, including netrins,



**Figure 2**

Signaling pathways involved in regulating vertebrate synaptogenesis. Synaptogenesis is a multistep process involving a myriad of signaling molecules. Prior to synapse formation, secreted molecules such as netrins and semaphorins guide axons to their targets. These axons then encounter priming factors secreted by target neurons and the surrounding glia, including fibroblast growth factor (FGFs), Wnts, cholesterol, and thrombospondin (TSP), that act to promote axonal and dendritic maturation and facilitate the ability of these processes to initiate synapse formation (*A*). Recognition that axons are in the correct receptive field is corroborated by CAMs, including members of the cadherin and protocadherin superfamilies, during initial contact between axons and dendrites (*B*). The presence of a second group of CAMs, such as SynCAM and Neuroligin (NL), at these contact sites is then thought to induce the formation of presynaptic active zones (*C*). In this regard, the adhesive CAMs are likely to work in synchrony with the inductive CAMs to stabilize the nascent synaptic junction. The neuroligin binding partner  $\beta$ -neurexin (Nxn) as well as Narp and EphrinB promote the recruitment of glutamate receptors and postsynaptic scaffolding proteins (*C*). Neuronal activity, although not essential for synapse formation, has been strongly implicated in regulating synapse stability (*D*). Intracellular signaling pathways sensitive to the activity states of synapses, including ubiquitin-mediated degradation, not only regulate the turnover of synaptic components but also promote synapse elimination (*D*).

semaphorins, and ephrinA (Bagri & Tessier-Lavigne 2002, Pascual et al. 2004, Tessier-Lavigne 1995). Because these molecules have no demonstrated role in synaptogenesis, we do not discuss them further in this review.

A second group of target-derived molecules include members of the Wnt and fibroblast growth factor (FGF) families. These proteins are secreted by certain subpopulations of neurons and have been shown to induce

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**Cell-adhesion molecules:**

cell-surface proteins that mediate close contact between two cells

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regional axon arborization and/or accumulation of recycling synaptic vesicles in innervating axons (Scheiffele 2003). Such properties could serve to spatially restrict synaptogenesis. For example, Wnt-3 secreted by motor neuron dendrites in the spinal cord induces the arborization of innervating sensory axons (Krylova et al. 2002), whereas Wnt-7a, secreted by cerebellar granule cells, induces clustering of the synaptic vesicle-associated protein synapsin 1 in innervating mossy fiber terminals (Hall et al. 2000). A second factor secreted by cerebellar granule cells, FGF22, also promotes the formation of presynaptic active zones in innervating mossy fiber axons (Umemori et al. 2004). This action depends on expression of the FGF2 receptor by mossy fiber axons and FGF22 secreted from granule cell neurons. FGF7 and -10 are expressed by other subpopulations of neurons and have similar synaptogenic properties (Umemori et al. 2004). Neurotrophins can also promote neuronal maturation, including regional axon and dendrite arborization. BDNF in particular has been shown to directly regulate the density of synaptic innervation (Alsina et al. 2001), and thus may be considered a synaptogenic “priming molecule.”

In addition to neuronally derived synaptogenic factors, several studies indicate that glial-derived factors may also regulate the timing of synapse formation (Ullian et al. 2004). These studies have noted that synaptogenesis in the central nervous system coincides with the birth of astrocytes and that astrocytes or astrocyte-conditioned media dramatically enhance synapse formation in certain populations of neurons (Nagler et al. 2001; Pfrieger & Barres 1996, 1997; Ullian et al. 2001, 2004). Two glial-derived factors shown to promote synapse formation include cholesterol bound to apolipoprotein E (Mauch et al. 2001) and thrombospondin-1 (TSP1) (Ullian et al. 2004). Presumably, these factors act indirectly to facilitate the maturation of both target neurons and incoming axons.

As a final note, it is important to make a distinction between these target-derived synaptogenic molecules and the inductive molecules discussed later. In particular, the target-derived molecules appear to act diffusely from local sources, like the netrins and semaphorins. Further, when added to purified populations of cultured neurons, they cause a global increase in the number of synapses, even between neurons that normally do not form synapses among themselves. These features suggest that these molecules are not acting focally to induce synapse formation, but rather serve to promote neuronal maturation and make neurons competent to proceed with synaptogenesis. Understanding whether they act to change gene transcription and/or protein synthesis would be an excellent way to confirm a role for these proteins in synaptogenic priming.

### **Cell-Adhesion Molecules Guiding Synapse Specificity**

Several classes of CAMs have also been implicated in target recognition and the initial formation of synapses. Candidates include members of the cadherin family of calcium-dependent cell-adhesion molecules, including cadherins and protocadherins (Shapiro & Colman 1999, Takai et al. 2003). With regard to the classical cadherins, there are ~20 members expressed in the CNS (Yagi & Takeichi 2000). Not only are these homotypic CAMs localized to synapses at early stages of synapse formation (Fannon & Colman 1996, Shapiro & Colman 1999, Uchida et al. 1996, Yamagata et al. 1995), but also they exhibit distinct yet complementary expression patterns with respect to subgroups of neurons and their targets. For example, cadherin-6 is expressed in functionally connected groups of neurons involved in audition (Bekirov et al. 2002). Similarly, barrel field pyramidal cells and septal granule cells in the somatosensory cortex, together with their corresponding thalamic inputs, express N-cadherin and

cadherin-8, respectively (Gil et al. 2002). As such, the classical cadherins are ideally suited to help guide subclasses of axons to their targets. With regard to synapse formation, individual cadherins not only localize to the pre- and postsynaptic plasma membranes in a variety of synaptic types (Benson et al. 2001, Takai et al. 2003), but also are found at initial axo-dendritic contact sites (Benson & Tanaka 1998). However, cellular expression and reverse genetic studies indicate that classical cadherins are not directly involved in triggering synapse formation. For example, introduction of N-cadherin blocking antibodies in the developing chick optic tectum causes retinal ganglion cell axons to overshoot their targets and to form exuberant synapses but does not inhibit synapse formation per se (Inoue & Sanes 1997). Similarly, axons originating from photoreceptor cells in the *Drosophila* ommatidium are mistargeted when they lack N-cadherin, but synapse formation itself is not disrupted (Lee et al. 2001). Thus, these data support a role for cadherins in target specification and perhaps stabilization of early synaptic contact sites but not in the induction of synapse formation.

A second class of CAMs that may be involved in target recognition and synapse specificity is the protocadherins. These molecules are encoded by a huge family of genes that undergo alternative splicing and have region-specific expression patterns in the developing brain (Hirano et al. 2002; Kohmura et al. 1998; Phillips et al. 2003; Wang et al. 2002a,b; Wu & Maniatis 1999). As such, they are theoretically capable of providing the spatial specificity required for target recognition. Similar to classical cadherins, protocadherin-gammas partially localize to synaptic sites (Phillips et al. 2003), and genetic studies in *Drosophila* indicate that they are involved in target recognition rather than synapse formation (Lee et al. 2003). Studies of protocadherin gamma knockout mice support this conclusion and indicate that these CAMs are not essential for neuronal differentiation

or synapse formation but rather for neuronal survival (Wang et al. 2002b).

## Inducers of Synapse Formation

In contrast to the cadherins and protocadherins, several classes of molecules are capable of directly inducing various aspects of synapse formation. These include Narp and Ephrin B1, two secreted proteins capable of clustering subsets of postsynaptic proteins, and SynCAM and Neuroligin, two CAMs that can trigger the formation of functional presynaptic boutons (Biederer et al. 2002, Dalva et al. 2000, O'Brien et al. 1999, Scheiffele et al. 2000).

One of the first molecules demonstrated to have synaptogenic activity was Neuronal activity-regulated pentraxin (Narp) (O'Brien et al. 1999). Narp was first identified as an activity-induced transcript in the hippocampus (Tsui et al. 1996). It is a member of the pentraxin family of secreted proteins that not only localizes to synapses, but also binds to the extracellular domains of subunits of the  $\alpha$ -amino-3-hydroxy-5-methylisoxazole-4-propionic acid (AMPA)-type glutamate receptor (O'Brien et al. 1999). Intriguingly, when overexpressed in spinal cord neurons, Narp increases the synaptic clustering of AMPA receptors (O'Brien et al. 1999). This activity is blocked by a dominant-negative form of Narp that interferes with its secretion at synapses (O'Brien et al. 2002). Further evidence for Narp's direct role in the synaptic clustering of AMPA receptors came from studies in which Narp was overexpressed in HEK293 cells. This not only induced the clustering of surface AMPA receptors coexpressed with Narp, but also the ectopic clustering of neuronal AMPA receptors on spinal cord neurons cocultured with these Narp-expressing HEK293 cells (O'Brien et al. 2002). These data clearly indicate that Narp has a potent AMPA receptor-clustering activity. Furthermore, Narp promotes the clustering of NMDA as well as AMPA

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**Hippocampus:** a region of the mammalian brain implicated in learning and memory and containing many glutamatergic synapses

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**FM1-43:** a dye that is taken up by recycling synaptic vesicles and used as a marker of functional presynaptic active zones

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receptors in certain classes of interneurons (Mi et al. 2002). However, Narp's clustering activity is primarily restricted to glutamatergic synapses forming on inhibitory interneurons and does not influence synapse assembly on pyramidal neurons (Mi et al. 2002).

A second molecule with synaptogenic activity, EphrinB, is a member of the Ephrin family of axonal growth cone guidance molecules. EphrinB family members promote the clustering of subunits of the N-methyl-D-aspartate (NMDA) type of glutamate receptor (Dalva et al. 2000). This activity was initially identified when investigators discovered that the extracellular domain of the EphrinB receptor, EphB, interacts directly with the extracellular domain of the NMDA receptor subunit NR1 (Dalva et al. 2000). Furthermore, EphrinB-mediated aggregation of EphB receptors leads to coaggregation of NMDA receptors. Independent evidence also strongly implicates ephrins and Eph receptors in dendritic spine development (Murai et al. 2003). Activation of postsynaptic EphBs with clustered EphrinB1 promotes spine maturation (Penzes et al. 2003), and triple knockout of EphB1, -2, and -3 results in mice that are viable but have defects in hippocampal spine morphology (Henkemeyer et al. 2003). Interestingly, EphrinB-mediated aggregation of EphB receptors does not induce coaggregation of other postsynaptic components such as PSD-95 family proteins, scaffolding proteins that normally aggregate at synapses prior to or coincident with NMDA receptors. Thus, as with Narp, EphrinB-EphBs act as specialized regulators of certain aspects of postsynaptic differentiation, e.g., the clustering of NMDA receptors and spine morphology. This limited data suggests that the induction of postsynaptic differentiation may involve multiple signaling pathways acting combinatorially. Such a possibility would not be unexpected given the greater molecular heterogeneity of postsynaptic specializations compared with presynaptic specializations (Craig & Boudin 2001).

In contrast to the restricted activities of Narp and Ephrin, molecules that in-

duce presynaptic differentiation cause a more complete functional differentiation of the presynaptic active zone. These synaptogenic molecules fall into two classes: secreted proteins, such as Wnts and FGFs (discussed above), and cell-surface adhesion proteins, such as SynCAM and neuroligin (see below). Whereas the secreted molecules function at a distance, perhaps as synaptogenic "priming molecules," the two CAMs, SynCAM and neuroligin, directly induce presynaptic differentiation through axo-dendritic contact.

The first to be identified was neuroligin, a postsynaptic single-pass transmembrane protein capable of inducing presynaptic differentiation when expressed in HEK293 cells (Scheiffele et al. 2000). The presynaptic receptor for neuroligin is a second single-pass transmembrane protein called  $\beta$ -neurexin (Dean et al. 2003, Scheiffele et al. 2000). The interacting domains of  $\beta$ -neurexin and neuroligin are laminin-G (LG) and acetylcholine esterase (AChE)-like domains, respectively (Dean et al. 2003, Scheiffele et al. 2000). The LG motif was initially characterized in laminin and agrin (Rudenko et al. 1999), molecules important for the differentiation of the neuromuscular junction (Sanes & Lichtman 1999). Remarkably, the binding affinity and biological activity of both agrin and neurexins is regulated by alternative splicing at specific conserved sites within the LG domain (Rudenko et al. 1999). Although structurally similar to AChE, the AChE domain in neuroligin is not catalytically active (Dean et al. 2003). Neuroligins cluster  $\beta$ -neurexin, and this clustering is associated with the formation of functional active zones as assessed by the ability of synaptic vesicles at these sites to recycle the styryl dye FM1-43 (Dean et al. 2003).

A second cell-surface adhesion molecule capable of inducing presynaptic differentiation is a protein called SynCAM (synaptic cell-adhesion molecule). SynCAM is a member of the Ig superfamily of adhesion molecules. It is a homophilic CAM expressed on both sides of the synapse (Biederer et al. 2002, Scheiffele



2003). SynCAM1 overexpression in cultured neurons promoted synapse formation, whereas overexpression in nonneuronal cells rapidly induced the formation of functional presynaptic active zones in axons contacting these cells (Biederer et al. 2002). Furthermore, neurons expressing a dominant-interfering construct against SynCAMs and neuroligins exhibit compromised presynaptic differentiation (Biederer et al. 2002). These studies suggest that SynCAM1 is a potent inducer of presynaptic differentiation. Intriguingly, there are three other genes encoding SynCAMs, and heterophilic adhesion between the various SynCAM isoforms can occur (Shingai et al. 2003). However, it is unclear whether this diversity also contributes to synaptic specification.

A very recent study (Graf et al. 2004) reported reciprocal signaling by one of these pairs of inducers. Whereas neuroligin induces presynaptic differentiation in contacting axons, its binding partner  $\beta$ -neuroligin induces postsynaptic differentiation in contacting dendrites.  $\beta$ -Neuroligin induced local clustering of PSD-95 and NMDA receptors, but not AMPA receptors, by binding to dendritic neuroligins (Graf et al. 2004). Furthermore,  $\beta$ -neuroligin induces local dendritic clustering of GABA as well as glutamate postsynaptic proteins, apparently via different neuroligins. This finding highlights the question of how matching of appropriate pre- and postsynaptic specializations is achieved. It remains to be determined whether SynCAMs or Ephrin/EphB also signal bidirectionally.

Obviously, SynCAM, neuroligin, and neuroligin are exciting molecules because of their potent hemi-synapse-inducing activities. However, many details about how they function at a molecular level remain to be explored. For example, are they solely involved in triggering pre-/postsynaptic differentiation, or are they also involved in synapse specification via selective cell adhesion? How are their signals transduced within neurons? More globally, how many of these priming, adhesive, and inducing molecules function to-

gether to build a given synapse, and how many are essential? Demonstration of the inducing activity of many of these molecules has been performed in cultured neurons. Rigorous analysis of combinatorial conditional knockout mice is warranted to determine the precise *in vivo* roles of each of these synaptogenic proteins.

Thus, an emerging picture of the specification and induction of synaptogenesis demonstrates that these complex processes involve many molecules and signaling pathways (Figure 2). For example, a series of hierarchical interactions between sets of secreted and cell-surface adhesion molecules seems to be required. Some of these, such as Wnts, FGFs, and TSP1, are target- or glia-derived and facilitate neuronal maturation, allowing neurons to undergo synaptogenesis in the correct spatial-temporal window. Others, such as cadherins and protocadherins, may serve to specify appropriate axodendritic connections and stabilize sites of early contact. A third class, including SynCAM, neuroligin/neuroligin, Narp, and EphrinB/EphB, seem to trigger various aspects of synapse formation locally. Mechanistically, it is unclear whether pre- and postsynaptic partners engage in bidirectional signaling at the time of contact or in the continuous exchange of factors/signals back and forth between the nascent pre- and postsynaptic compartments. Still less is known about the second messenger signaling pathways that participate in this early inductive stage of synaptogenesis. Much of what we do know has come from genetic screens in worms and flies (for a review see Jin 2002).

## CELLULAR MECHANISMS OF SYNAPSE ASSEMBLY

Subsequent to induction and prior to the appearance of a fully functional synapse is the molecular assembly of the synaptic junction and the delivery of pre- and postsynaptic components. How are the inductive signals discussed above translated into the

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**PSD-95:** a well-characterized modular protein of the postsynaptic density involved in receptor anchoring and clustering

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site-specific recruitment of pre- and postsynaptic molecules, and what cellular mechanisms are responsible for their correct targeting? Of particular interest is whether synapse assembly involves the sequential recruitment of individual synaptic proteins or whether sets of proteins are delivered as preassembled complexes.

### Membrane Trafficking in Presynaptic Assembly

Although local recruitment of individual molecules surely contributes to presynaptic assembly, studies coming from a number of laboratories strongly suggest that the vesicular delivery of proteins plays a critical role. For example, during the assembly of presynaptic boutons, clusters of pleiomorphic vesicles are observed at newly forming synapses (Ahmari et al. 2000). These include small, clear-centered vesicles, tubulovesicular structures, and 80-nm dense core vesicles. The exact composition and characteristics of these morphologically distinct vesicle types remain to be investigated, but vesicles within these clusters seem to be somewhat specified. For example, the small clear-centered vesicles appear to be synaptic vesicle precursors, carrying primarily synaptic vesicle proteins (Hannah et al. 1999, Huttner et al. 1995, Matteoli et al. 1992). The 80-nm dense core vesicles contain numerous multidomain scaffold proteins of the active zone, such as Piccolo, Bassoon, and Rab3 interacting molecule (RIM) (Lee et al. 2003, Ohtsuka et al. 2002, Shapira et al. 2003) as well as components of the synaptic vesicle exocytotic machinery including syntaxin, SNAP25, and N-type voltage-gated calcium channels (Shapira et al. 2003, Zhai et al. 2001). Finally, the tubulovesicular structures may represent either post-Golgi membranes of mixed protein composition and/or endosomal intermediates. The former presumably carry newly synthesized proteins and the latter recycled membrane proteins.

The presence of different vesicle types at nascent synapses suggests that active zone formation is driven by the vesicular delivery of proteins. An exciting but unresolved set of questions relates to the hierarchy of protein delivery. Assuming that proteins such as neuroligin and SynCAM are important inducers of presynaptic active zone assembly, it is reasonable to conclude that their delivery to the plasma membrane and clustering at nascent axodendritic contact sites is one of the earliest events. At present, it is unclear whether these molecules are already in the plasma membrane and are simply induced to cluster at contact sites through lateral movement, or whether the contact site itself triggers the directed delivery and fusion of neuroligin and SynCAM-containing vesicles.

On the basis of temporal studies, the fusion of 80-nm dense core vesicles carrying structural components of the active zone such as Piccolo, Bassoon, and RIM is likely to occur shortly thereafter (Ziv & Garner 2004). Presumably, delivery of these proteins allows for the rapid establishment of functional synaptic vesicle docking and fusion sites and provides a platform for the subsequent delivery of additional presynaptic proteins and synaptic vesicle precursors (Ziv & Garner 2004). The biogenesis and clustering of mature synaptic vesicles is likely to occur after these initial events. Although this model of active zone formation is consistent with most available data, it is nonetheless quite speculative and cannot account for all observations. For example, recent data on the dynamics of synaptic vesicle recycling demonstrate the existence of functional active zones lacking postsynaptic partners (Takao-Rikitsu et al. 2004). At present, it is unclear whether these so-called "orphan active zones," which retain the ability to recycle synaptic vesicles in an activity-dependent manner, represent an early event in nascent active zone formation, are an artifact of low-density dissociated hippocampal cultures or are remnants of synapses undergoing elimination.

## Membrane Trafficking in Postsynaptic Assembly

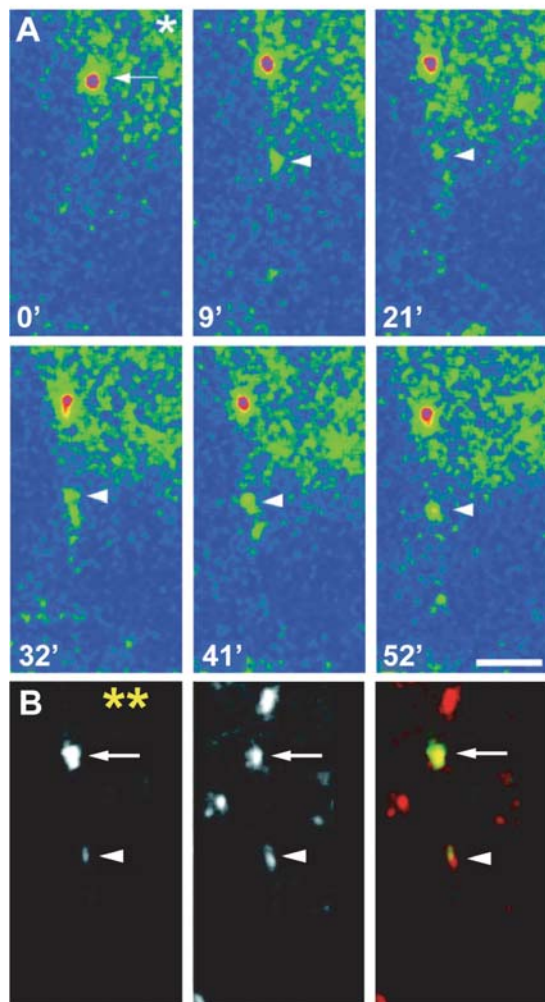
In contrast to the presynaptic active zone, where delivery of integer numbers of transport vesicles provides sufficient material for synapse formation (Shapira et al. 2003), assembly of the postsynaptic density appears to occur primarily by gradual accumulation of molecules (Bresler et al. 2004, Ziv & Garner 2004) (**Figure 3**). One of the earliest events in postsynaptic differentiation is the recruitment of scaffolding proteins of the PSD-95 family. These molecules are present at synapses in postnatal day 2 hippocampus (Sans et al. 2000) and detectable within 20 min of axodendritic contact in culture (Bresler et al. 2001, Friedman et al. 2000, Okabe et al. 2001). Although some investigators have reported modular transport of recombinant PSD-95 clusters during synaptogenesis (Prange & Murphy 2001), other studies have observed more gradual, nonquantal accumulation of PSD-95 at nascent synapses (Bresler et al. 2001, Marrs et al. 2001). Gradual accumulation of PSD-95 could occur by local trapping of diffuse plasma membrane pools or by sequential local fusion of numerous vesicles, each carrying only small numbers of PSD-95.

Closely following the synaptic recruitment of PSD-95 is recruitment of NMDA-type and AMPA-type glutamate receptors, which are independently regulated. As described above for PSD-95, modular transport of NMDA receptor clusters during synaptogenesis has been reported (Washbourne et al. 2002), but other studies have observed more gradual accumulation at nascent synapses (Bresler et al. 2004). Nonsynaptic clusters of endogenous PSD-95 and NMDA receptors are present early in development (Rao et al. 1998) but are rare and unlikely to represent prefabricated postsynaptic elements. For AMPA receptors, evidence exists to support both local insertion of receptor-containing vesicles near the synapse and insertion over the bulk of the dendritic plasma membrane, followed by diffusion and trapping at the synapse

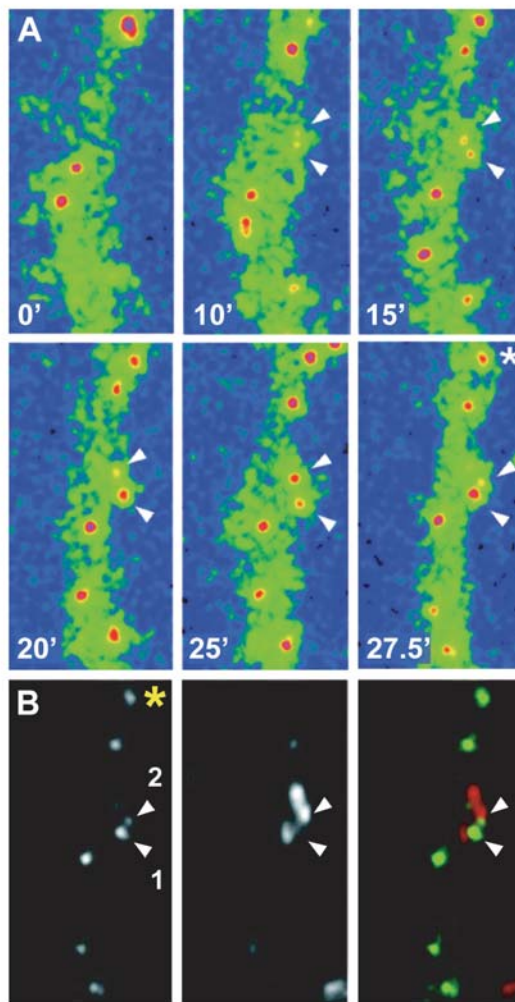
(Borgdorff & Choquet 2002, Passafaro et al. 2001). Interestingly, the mechanisms may differ depending on the AMPA receptor subunit composition, GluR2 being more locally inserted than GluR1 (Passafaro et al. 2001). In the past few years, much effort has focused on how synaptic delivery of AMPA and NMDA receptors is controlled by interacting proteins (Bredt & Nicoll 2003, LeVay et al. 1980, Malinow & Malenka 2002, Wenthold et al. 2003). Both receptor classes interact with different sets of PDZ domain-containing proteins, chaperones, endocytic adaptors, and cytoskeletal proteins. These interactions can regulate exit from the endoplasmic reticulum, transport to the synapse, local receptor stabilization, endocytosis, recycling, and degradation. An interesting example is the linkage to molecular motors. Whereas NR2B-containing NMDA receptors link to the kinesin family motor KIF17 (Setou et al. 2000), GluR2/3-containing AMPA receptors link to the kinesin family motors KIF5 and KIF1A through a different scaffolding complex (Setou et al. 2002, Shin et al. 2003). These NMDA and AMPA receptor complexes lack PSD-95, further suggesting independent synaptic recruitment of PSD-95, NMDA receptors, and AMPA receptors. But there is not universal agreement, as other researchers suggest that NMDA receptors may traffic with PSD-95 family proteins to the synapse (Wenthold et al. 2003).

Accumulation of other postsynaptic components also occurs by individual recruitment. For example, the most intensively studied such component, calcium calmodulin-dependent protein kinase II (CaMKII), accumulates on the cytoplasmic face of the postsynaptic density (Petersen et al. 2003), apparently by regulated trapping of local pools (Shen & Meyer 1999) rather than by active vesicular transport. Regulated synaptic accumulation of the scaffolding proteins Homer 1c and Shank2/3 also occurs by gradual accumulation from a cytosolic pool (Bresler et al. 2004, Okabe et al. 2001). Local protein

## I. GFP-Bassoon



## II. GFP-ProSAP1



**Figure 3**

Different mechanisms of protein recruitment at nascent pre- versus postsynaptic sites. Many presynaptic proteins, such as Bassoon (pictured here in *I*), are delivered to nascent active zones via integer numbers of transport vesicles. In contrast, postsynaptic proteins, such as ProSAP1 (pictured here in *II*), appear to be gradually recruited to nascent postsynaptic densities from cytosolic pools. (*IA*) Time-lapse sequence of an axon expressing GFP-Bassoon. At the beginning of the time lapse, a large and static GFP-Bassoon cluster is seen (arrow). A new GFP-Bassoon cluster then appears (arrowhead) and stabilizes over the course of the next 52 min. (*IB*) FM4-64 labeling at the end of this experiment (middle panel) shows that the new GFP-Bassoon cluster (left panel) colocalizes with recycling synaptic vesicles (right panel), indicating that it is a functional presynaptic active zone. Quantitative measurements of the fluorescence changes at this site indicate that it contains 2–3 unitary amounts of GFP-Bassoon. All times are given in minutes. Scale bar, 5  $\mu\text{m}$ . (*IIA*) Time-lapse sequence of a dendrite expressing GFP-ProSAP1, a PSD protein. The formation of two new GFP-ProSAP1 clusters (arrowheads) over the course of 27.5 min is shown. (*IIB*) FM4-64 loading performed at the end of the experiment (middle panel) revealed that the new GFP-ProSAP1 clusters (left panel) colocalize with functional presynaptic active zones (right panel), which suggests that new synapses had formed at these sites. Note the gradual increase in fluorescence intensity of these two clusters. All times are given in minutes. Adapted from figures 6 and 11 in Bresler et al. 2004.

synthesis may also contribute to synapse assembly, particularly in cases where the mRNA is abundant in dendrites, such as for CaMKII $\alpha$ , Shank, NR1, and GluR1/2 (Bockers et al. 2004, Ju et al. 2004, Steward & Schuman 2001). However, this possibility has not been well explored.

Considering the extensive heterogeneity in postsynaptic composition, even among glutamatergic synapses, and the ability of activity and kinases to separately regulate the density of different components such as PSD-95, NMDA receptors, AMPA receptors, and CaMKII, one might expect the delivery of each of these components to occur independently. Thus, the existence of preassembled mobile postsynaptic transport packets ready to mediate fusion of numerous postsynaptic proteins, as with presynaptic packets, remains an open possibility but one with little experimental support (**Figure 3**) (Bresler et al. 2004). Further biochemical characterization of postsynaptic transport intermediates, high sensitivity ultrastructural localization, and simultaneous time-lapse imaging of multiple tagged postsynaptic proteins may help resolve these issues.

### Synaptic Maturation

A general feature of synaptic development is a prolonged maturation phase. During this phase, synapses expand in size; for example, the number of synaptic vesicles per terminal increases two- to threefold over the first month of cortical development (Vaughn 1989). Remarkably, pre- and postsynaptic elements develop in a coordinated fashion, maintaining a correlation among the size of different components: bouton volume, number of total synaptic vesicles, docked vesicles, active zone area, postsynaptic density area, and spine head volume (Harris & Stevens 1989, Pierce & Mendell 1993, Schikorski & Stevens 1997). This finding of correlated size suggests that the cell-adhesion complexes that span the cleft, and perhaps associated secreted factors, signal to each partner to define

the area of associated cytomatrix and synapse volume.

Perhaps the most dramatic maturational change in synapses is the change in postsynaptic form. Glutamatergic synapses initially form on filopodia or dendrite shafts that develop over time into dendritic spines. Both are motile actin-based structures (Fischer et al. 1998), filopodia are typically  $>2 \mu\text{m}$  long, of thin diameter, and have a half-life of several minutes, whereas spines are typically  $<2 \mu\text{m}$  long, have a bulbous head, and have a half-life of days or more (Grutzendler et al. 2002, Sorra & Harris 2000). Mature spines can be of mushroom, branched, thin, or stubby morphology. Live cell-imaging studies suggest that most synapses form on dendritic filopodia and then transform directly to more stable spines (Okabe et al. 2001, Ziv & Smith 1996). Conversion of shaft synapses into spine synapses has also been inferred on the basis of the percentage of synapses of different morphological classes in developing hippocampus (Fiala et al. 1998). Dendritic spine morphogenesis is regulated by numerous mechanisms including cell adhesion via the cadherin, syndecan, and ephrin systems. These in turn signal through proteins that regulate Rho and Ras family GTPases, actin-binding proteins, and calcium regulatory mechanisms (Hering & Sheng 2001, Tashiro & Yuste 2003).

The functional properties of synapses also change with development. As hippocampal synapses mature, the probability of transmitter release decreases (Bolshakov & Siegelbaum 1995, Chavis & Westbrook 2001), and the reserve pool of vesicles increases. Quantal size shows an initial rapid increase and then remains constant (De Simoni et al. 2003, Liu & Tsien 1995, Mohrmann et al. 2003). The kinetics of synaptic responses change concomitantly with developmental changes in receptor subunit composition. For example, expression of the NR2A subunit of the NMDA receptor and its selective incorporation into synaptic NMDA receptors, partially replacing NR2B subunits, mediate a decrease in hippocampal and neocortical

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**Filopodia:** dynamic protrusions found on axons and dendrites, particularly at growth cones

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NMDA current duration (Sorra & Harris 2000, Tovar & Westbrook 1999). Many developing brain regions exhibit “silent synapses” characterized by functional NMDA but not AMPA currents (Durand et al. 1996, Isaac et al. 1997). Such silent synapses lack surface AMPA receptors, and great variability in AMPA receptor content of hippocampal synapses is indeed observed (Matsuzaki et al. 2001, Nusser et al. 1998, Takumi et al. 1999). NMDA receptor activation can unsilence these synapses, presumably by recruitment of AMPA receptors to the postsynaptic plasma membrane. Furthermore, receptor content and spine morphology are correlated with larger spines selectively bearing more AMPA receptors. However, other explanations for these silent synapses have been proposed. Alterations in modes of vesicle fusion may activate NMDA receptors while failing to activate AMPA receptors (Choi et al. 2000, Renger et al. 2001). Furthermore, functional AMPA synapses develop normally in genetically targeted neurons lacking functional NMDA receptors (Cottrell et al. 2000, Li et al. 1994). Thus, although not an obligatory step in development, most synapses develop functional NMDA receptors prior to AMPA receptors, and NMDA receptor activity may instruct insertion of AMPA receptors into the plasma membrane.

### **ACTIVITY-DEPENDENT REGULATION OF SYNAPTOGENESIS**

The bulk of synaptogenesis occurs during early postnatal development, but synapses can also form in the mature brain. At both stages, activity sculpts neuronal arbor growth and synapse formation (Knott et al. 2002, Maletic-Savatic et al. 1999, Rajan & Cline 1998, Schmidt et al. 2004, Trachtenberg et al. 2002, Wong & Wong 2001, Hua & Smith 2004). However, several studies have demonstrated that neuronal activity is not required for synapse formation during development (Varoqueaux et al. 2002, Verhage

et al. 2000). For example, in hippocampal cultures, synaptogenesis occurs normally in the presence of glutamate receptor blockers that prevent action potentials (Rao & Craig 1997). Moreover, Munc-18 and Munc-13 knockout mice lacking neurotransmitter release exhibit normal synapse morphology and density, although synapses appear less stable over time (Varoqueaux et al. 2002, Verhage et al. 2000). These findings suggest a more nuanced role for activity in synaptogenesis, presumably one involving the regulation of synapse stability and elimination rather than formation per se. Mechanisms by which activity regulates synapse stability are explored in the following sections.

### **Synapse Elimination**

Although synapse formation is the focus of this review, synapse elimination is arguably an equally important developmental process. For example, the initial number of synapses formed in the brain is far greater than the number retained, which suggests that synapse elimination is a crucial step in normal brain development (Hashimoto & Kano 2003, Huttenlocher et al. 1982, Lichtman & Colman 2000, Rakic et al. 1986). Indeed, activity-dependent pruning of synapses appears to underlie many critical aspects of nervous system development, including formation of ocular dominance columns in the visual cortex (LeVay et al. 1980, Shatz & Stryker 1978) and innervation of muscles by neurons originating in the spinal cord (Lichtman & Colman 2000).

In the mature brain, synapse elimination is probably also an important mechanism for removing inappropriate or ineffective connections to fine-tune networks. Evidence that activity regulates synapse elimination, as well as synapse formation, between mature neurons has come from two recent studies of rodent barrel cortex, an area of somatosensory cortex that receives sensory input from the whiskers (Knott et al. 2002, Trachtenberg et al. 2002). In one study, Trachtenberg et al.

(2002) performed *in vivo* imaging of dendritic arbors and observed high rates of turnover of dendritic protrusions (17% persisting for one day, 23% for only 2–3 days). The authors correlated the appearance of new protrusions with the formation of new synapses and showed that activity affected their stability, as sensory deprivation by whisker trimming increased the ratio of transient-to-stable protrusions. Knott et al. (2002) stimulated a single rodent whisker in a freely moving animal for 24 h then performed serial section electron microscopy to examine synapse density in the region of barrel cortex receiving input from the whisker. Amazingly, after this brief stimulation period, the authors observed a 35% increase in synapse density and a 25% increase in spine density. This increase was largely reversible as excitatory synapse density returned to prestimulation levels several days after stimulation ceased. These studies demonstrate that synapses between mature neurons can be formed and eliminated rapidly and that activity, in the form of whisker stimulation or trimming, regulates these processes.

### Ubiquitin Regulation of Synapse Stability

As demonstrated by the studies described above, activity seems to play a fundamental role in synapse formation, stability, and elimination. By which molecular mechanisms does activity regulate these processes? As discussed above, activation of NMDA receptors induces a number of changes, including insertion of AMPA receptors and changes in dendritic spine morphology, that lead to synapse stability and maturation. Activity may also regulate the stability of synaptic proteins via ubiquitination. A recent and intriguing study by Ehlers (2003) demonstrated that large groups of postsynaptic proteins are up- or down-regulated together in response to sustained changes in neuronal network activity. Ehlers showed that ubiquitin-mediated protein degradation was responsible for this activity-dependent protein turnover and that only a

few postsynaptic density proteins are ubiquitinated, including ProSAP/Shank, GKAP, and AKAP79/150. Thus, activity-dependent ubiquitination of these few proteins could lead to the rapid destabilization and degradation of large postsynaptic protein complexes by the proteasome, providing neurons with a mechanism for regulating synapse stability via activity. Other studies also indicate that ubiquitin-mediated protein degradation is an important mechanism for controlling synapse stability and function. For example, Burbea et al. (2002) showed that expression of the *C. elegans* postsynaptic glutamate receptor GLR-1 is regulated by ubiquitination (Burbea et al. 2002). Mutations that prevent ubiquitination increase GLR-1 abundance at synapses and, concomitantly, synaptic strength, whereas overexpression of ubiquitin decreases GLR-1 levels and the density of GLR-1-containing synapses. In the vertebrate hippocampus, activity-dependent internalization of homologous AMPA receptors was also shown to be regulated by ubiquitination (Colledge et al. 2003). Taken together, these studies indicate that ubiquitination of postsynaptic glutamate receptors can regulate synaptic strength and stability.

Ubiquitin-dependent proteolysis of synaptic proteins also regulates presynaptic function. At the *Drosophila* neuromuscular junction, application of proteasome inhibitors induced a rapid strengthening of synaptic transmission owing to a 50% increase in the number of synaptic vesicles released (Aravamudan & Broadie 2003, Speese et al. 2003). Along with increased vesicle release, these authors observed a doubling of Dunc-13 (the *Drosophila* homolog of Munc-13), a protein that regulates synaptic vesicle priming/release and is ubiquitinated *in vivo* (Aravamudan & Broadie 2003). This study suggests that Dunc-13/Munc-13 may be a substrate for the regulation of neurotransmitter release by ubiquitin-mediated protein degradation.

Another example of ubiquitin-mediated regulation of presynaptic assembly and

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**Ubiquitination:** a chemical modification to proteins that targets them to the proteasome for degradation

**AChE:** acetylcholine esterase

**AKAP79/150:** A-kinase anchoring protein

**AMPA receptor:**  $\alpha$ -amino-3-hydroxy-5-methylisoxazole-4-propionic acid type glutamate receptor

**AZ:** active zone

**CAMs:** cell-adhesion molecules

**CaMKII:** calcium calmodulin-dependent protein kinase II

**EphB:** EphrinB receptor

**FGF:** fibroblast growth factor

**GFP:** green fluorescent protein

**GKAP:** guanylate kinase domain-associated protein

**GLR-1:** *C. elegans* postsynaptic glutamate receptor

**GluR2, GluR1:** subunits of the AMPA receptor

**KIF5, KIF17:** kinesin super-family

**LG:** laminin-G

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**Munc-18:** mouse homolog of the *C. elegans* uncoordinated mutant unc-18

**Munc-13, Dunc-13:** mouse and *Drosophila* homologs of the *C. elegans* uncoordinated mutant unc-13

**NL:** neuroligin

**Narp:** neuronal activity-regulated pentraxin

**NMDA:** N-methyl-D-aspartate type of glutamate receptor

**NR1, NR2A,**

**NR2B:** subunits of the NMDA receptor

**Nxn:**  $\beta$ -neurexin

**PDZ:** PSD-95/DLG/ZO1 homology domain

**ProSAP1:** proline rich synapse-associated protein 1

**PSD:** postsynaptic density

**PSD-95:** postsynaptic density protein-95

**RIM:** Rab3 interacting molecule

**SP:** dendritic spine

**SynCAM:** synaptic cell-adhesion molecule

**SVs:** synaptic vesicles

**TSP:** thrombospondin

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function involves the *C. elegans* gene *rpm-1*, the *Drosophila* homolog highwire and the mouse homolog Phr1. In *rpm-1* loss-of-function mutants, synapses either fail to form or are highly disorganized, often lacking active zones and SV clusters (Schaefer et al. 2000, Zhen et al. 2000). *Drosophila highwire* mutants have excessive numbers of synapses that are smaller than normal, whereas mice lacking Phr 1 also exhibit abnormal synaptic morphology (Burgess et al. 2004, Wan et al. 2000). On the basis of the deduced amino acid sequence of *rpm-1*/highwire/Phr 1, these genes appear to be involved in ubiquitin-mediated protein degradation because they have domains exhibiting sequence homology with E3 ubiquitin ligases (Jin 2002, Wan et al. 2000, Zhen et al. 2000). These data suggest that ubiquitination in general and these proteins in particular have an important role in regulating presynaptic active zone size and organization. Perplexingly, *rpm-1* is a periaxonal zonal protein (Wan et al. 2000, Zhen et al. 2000), which raises some questions about how proteins outside the active zone can influence its formation. One possibility is that these molecules regulate proteins of the periaxonal zonal plasma membrane that normally serve to delineate active zone size. Thus, a growing body of data indicate that ubiquitination of both pre- and postsynaptic proteins, perhaps in an activity-dependent manner, is an important mechanism for regulating both synapse formation and stability.

## CONCLUDING REMARKS

In recent years, considerable progress has been made in understanding the cellular and molecular mechanisms of vertebrate synaptogenesis. By all accounts, it is a complex process that begins before axonal projections reach their targets and involves a series of hierarchical signals. These signals include secreted factors and cell-adhesion molecules that both guide axons to their correct targets and regulate their maturation, insuring that synap-

togenesis occurs only once the proper neurons are encountered. These initial priming signals are thought to work in synchrony with subsequent contact-initiated inductive signals to stabilize nascent axodendritic contacts and trigger the assembly of synapses. Assembly of pre- and postsynaptic compartments then occurs through a combination of vesicle trafficking and local recruitment of synaptic proteins. Finally, correlated or uncorrelated synaptic activity leads to synaptic strengthening and stabilization, or weakening and elimination. Although recent studies have begun to unravel the intricacies of synaptogenesis, many important questions remain. For example, what makes some contacts productive for synapse formation and others not? Is there a minimal contact time and/or set of local molecular players needed? What is the distribution of the inductive factors prior to synaptogenesis? How are the inductive signals initiated by axon-dendrite contact translated into the complex, and distinct, processes of pre- and postsynaptic assembly? Specifically, which second-messenger signaling pathways translate the interactions of cell-surface CAMs into the vesicular delivery of proteins on either side of the synapse? What is the temporal sequence of pre- and postsynaptic protein recruitment during synaptogenesis? What are the morphological characteristics and precise molecular compositions of the vesicles that deliver proteins to nascent synapses, and which mechanisms regulate their timely delivery? What are the signals that mediate prolonged maturational changes and synapse-specific features? How stable are synapses during periods of peak synaptogenesis versus in the mature brain? Are the same cellular and molecular mechanisms responsible for synapse formation during development also responsible for this process in the mature brain? By which molecular mechanisms does neuronal activity regulate synapse stability and elimination? These questions are likely to emerge as quintessential issues for developmental neuroscientists in the coming years.



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