

# Synapse development: still looking for the forest, still lost in the trees

Craig C. Garner · Clarissa L. Waites · Noam E. Ziv

Received: 7 June 2006 / Accepted: 8 June 2006 / Published online: 15 August 2006  
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**Abstract** Synapse development in the vertebrate central nervous system is a highly orchestrated process occurring not only during early stages of brain development, but also (to a lesser extent) in the mature nervous system. During development, the formation of synapses is intimately linked to the differentiation of neuronal cells, the extension of their axons and dendrites, and the course wiring of the nervous system. Subsequently, the stabilization, elimination, and strengthening of synaptic contacts is coupled to the refinement of axonal and dendritic arbors, to the establishment of functionally meaningful connections, and probably also to the day-to-day acquisition, storage, and retrieval of memories, higher order thought processes, and behavioral patterns.

**Keywords** Synapse · Synapse development · Axonal arbors · Dendritic arbors · Synapse assembly

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The authors acknowledge the support of the NIH (grant no. HD38760 DA016758) to C.C.G., the Ruth L. Kirchstein National Research Service Award (NRSA) to C.L.W., and the United States Israel Binational Science Foundation (grant no. 2003176) to C.C.G. and N.E.Z.

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C. C. Garner (✉) · C. L. Waites  
Department of Psychiatry and Behavioral Science,  
Nancy Pritzker Laboratory, Stanford University,  
Palo Alto, CA 94304-5485, USA  
e-mail: cgarner@stanford.edu

N. E. Ziv  
The Department of Physiology, Technion Faculty of Medicine,  
The Rappaport Family Institute for Research  
in the Medical Sciences,  
Haifa 31096, Israel

## Introduction

Structurally, synapses are specialized sites of cell-cell contact. They occur primarily between the axonal varicosities of one neuron and the dendrites or cell somas of other neurons, muscle fibers, or glands (Garner et al. 2002; Waites et al. 2005). Synapses in the central nervous system (CNS) can also form between pairs of dendrites or axons, referred to as dendro-dendritic and axo-axonic synapses, respectively. In general, CNS neurons make and receive tens of thousands of synapses, e.g., cerebellar Purkinje cells receive more than  $10^5$  synapses. The vast majority of CNS synapses are chemical, i.e., they release small molecules (neurotransmitters) to trigger a response in a postsynaptic cell. Less frequent but also formed between neuronal cells are electrical synapses. These synapses allow for a direct electrical coupling of nerve cells via gap-junctions (Bennett 2000) and are often found associated with and functionally coupled to chemical synapses (Pereda et al. 2003; see also, in this issue, Bruzzone and Dermietzel 2006). At present, little is known regarding the way that electrical synapses are formed, and this topic will not be discussed further (but see Bennett and Zukin 2004; Pereda et al. 2004).

Ultrastructurally, chemical synapses are asymmetric cellular junctions comprised of a presynaptic bouton, a synaptic cleft, and a postsynaptic specialization (Gray 1963). Presynaptic boutons have two characteristic features. First, they generally contain hundreds to thousands of 50-nm clear-centered vesicles filled with neurotransmitter. Second, these synaptic vesicles (SVs) dock, fuse, and release their contents into the synaptic cleft at a specialized region of the presynaptic plasma membrane called the active zone (Garner et al. 2002). As discussed more fully in the article by Schoch and Gundelfinger (2006, this issue), the active zone is functionally defined by a collection of

proteins that appear, by electron microscopy, as an electron-dense matrix or grid associated with the cytoplasmic face of the presynaptic membrane (see also Garner et al. 2002; Phillips et al. 2001; Zhai and Bellen 2004). Juxtaposed to the active zone, within the postsynaptic plasma membrane, is a second electron-dense structure referred to as the postsynaptic density (PSD). Similar to the active zone, the PSD is defined by a large collection of structural/scaffold proteins that serve to cluster neurotransmitter receptors and their signaling machinery at high density (Kennedy 2000; Kim and Sheng 2004; Montgomery et al. 2004). These two membrane specializations are held in register via a largely uncharacterized matrix of cell-adhesion molecules (CAMs) and extracellular matrix proteins (ECM) that span the synaptic cleft (Craig et al. 2006; Dean and Dresbach 2006; Waites et al. 2005; Yamagata et al. 2003; see, in this issue, Missler 2006; Dityatev and Schachner 2006). Although these basic elements are shared by all chemical synapses, significant variation in the size and morphology of synapses have been described. These features tend to correlate with the functional output properties of synapses. For example, highly reliable synapses, such as the neuromuscular junction (NMJ), are large and contain huge numbers of SVs (Kummer et al. 2006; Sanes and Lichtman 2001). In contrast, less reliable CNS synapses are smaller and contain only a few hundred SVs. Variations in the morphology of the PSD also exist; for example, whereas inhibitory synapses generally lack a recognizable PSD, excitatory synapses of the vertebrate CNS have prominent PSDs (Gray 1963) that are typically situated at the tips of dendritic spines, viz., small thorn-like protrusions along the lengths of dendrites (see Yuste and Bonhoeffer 2001, 2004). Although their role is actively debated, dendritic spines are thought to create a micro-environment that restricts information flow between adjacent synapses on the same dendrite (Tsay and Yuste 2004).

During the last decade, tremendous progress has been made in identifying the molecular components of synaptic junctions, particularly by using proteomic strategies (e.g., see Grant 2006; Jordan et al. 2004; Li et al. 2005; Peng et al. 2004), and the principles governing their assembly (Craig et al. 2006; Goda and Davis 2003; Waites et al. 2005; Ziv and Garner 2001, 2004). These studies have revealed that synapses are composed of hundreds of proteins, and that the specification of synaptic function, e.g., excitatory, inhibitory, or modulatory, is achieved via the recruitment and assembly of particular protein complexes. For example, the recruitment of  $\gamma$ -aminobutyric acid (GABA) and glycine receptors to inhibitory synapses is mediated by the scaffold protein gephrin (Craig et al. 2006). In contrast, glutamate receptors are directed to excitatory synapses by members of the MAGUK family of synaptic scaffold proteins (Montgomery et al. 2004).

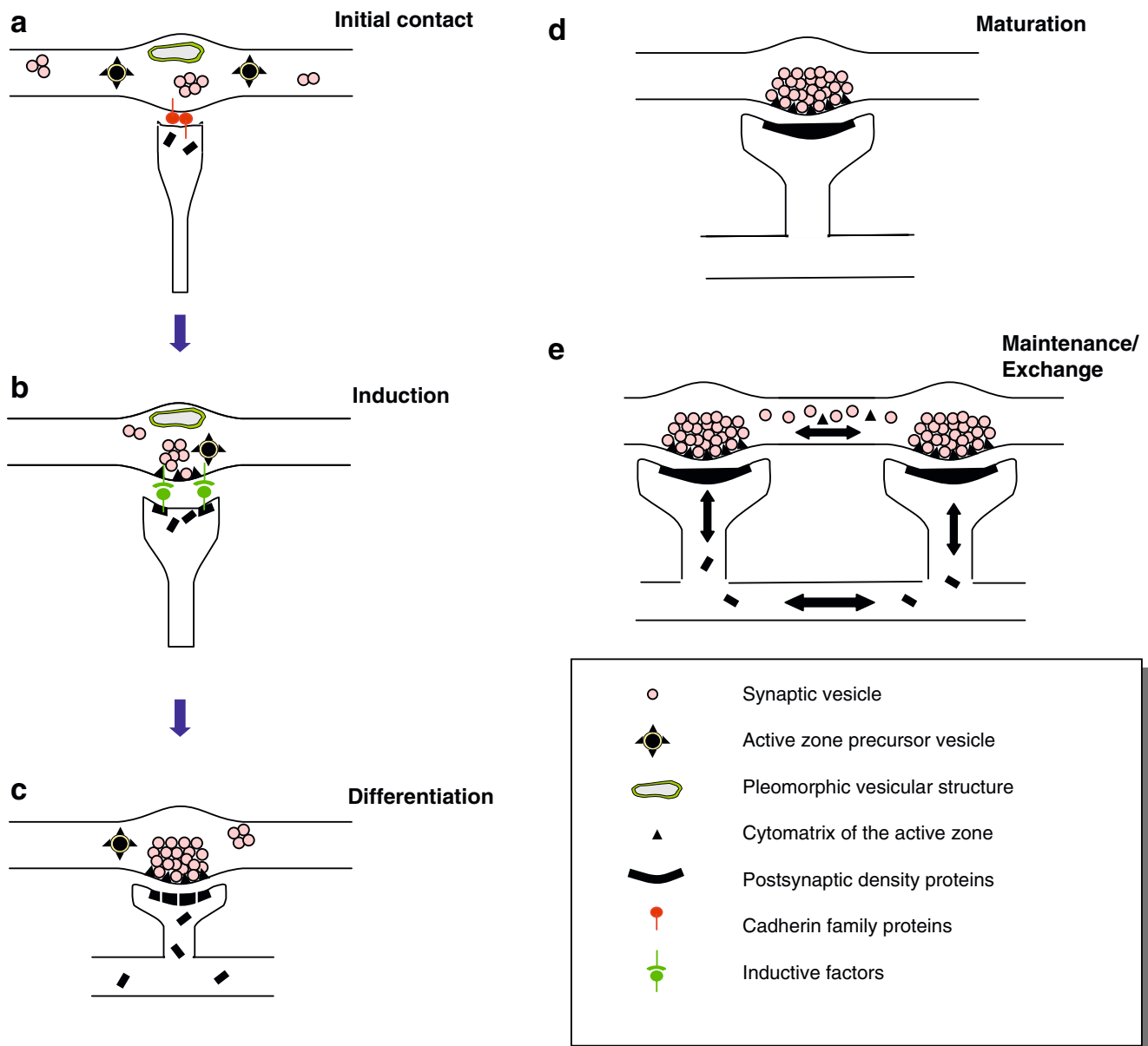
Clues as to the creation of active zones of different morphologies remain somewhat enigmatic, as most known active zone proteins are present at all types of synapses (Waites et al. 2005; Zhai and Bellen 2004; see, in this issue, Schoch and Gundelfinger 2006).

With regard to the induction and dynamics of synaptogenesis, significant progress has occurred in three areas: (1) the identification of putative inducers of synapse assembly; (2) an understanding of the cellular mechanisms of synapse assembly; (3) the characterization of the dynamics of synapse assembly and maintenance. In the current review, we focus on the major features and principles of synapse formation, highlighting the timing, dynamics, and induction of synaptogenesis and synaptic maturation and maintenance, rather than presenting an exhaustive summary of individual molecules and their hypothesized functions (see instead Craig et al. 2006; Dean and Dresbach 2006; Montgomery et al. 2004).

### Phases of synapse formation

Although synaptogenesis is a continuum from the time of initial axo-dendritic contact to the emergence of a fully functional synaptic junction, it is useful to divide it into five discrete steps (see Fig. 1). The first of these is the establishment of an initial contact site during which one neuronal process (axon or dendrite) recognizes the other as a potential and appropriate target. This initial step is thought to be governed primarily by several classes of CAMs including integrins, members of the cadherin and protocadherin family of calcium-dependent adhesion molecules, and members of the Ig superfamily of CAMs. The second is an inductive step triggering pre- and postsynaptic differentiation. As discussed later, this is believed to involve bidirectional signaling events that occur when subclasses of CAMs or ligand/receptor complexes are formed at the initial contact site (Craig et al. 2006; Waites et al. 2005). Induction is followed by a relatively protracted third step, lasting 1–2 h, during which vesicles and synaptic proteins accumulate within the presynaptic bouton and at the cytoplasmic face of the opposing postsynaptic membrane (Ahmari et al. 2000; Bresler et al. 2001, 2004; Ebihara et al. 2003; Friedman et al. 2000; Marrs et al. 2001; Niell et al. 2004; Okabe et al. 2001a; Prange and Murphy 2001). Importantly, these nascent synapses, which have yet to accumulate their full allotment of proteins, are capable of both releasing and detecting neurotransmitter within the first 30 mins of initial contact (see Buchanan et al. 1989; Ziv and Garner 2001).

This initial phase of synapse differentiation is followed by a fourth prolonged phase (hours to days) of structural and functional maturation (Fig. 1). For example, presynap-



**Fig. 1** The five steps of excitatory synapse formation. Synapse formation is a multi-step process involving numerous signaling cascades and molecules. Initial contact between axons, dendrites, and dendritic filopodia appears to be mediated by CAMs, including members of the cadherin family (a). The presence of inductive factors, including SynCAM,  $\beta$ -neurexin/neuroigin, Narp, and EphrinB/EphR at these contact sites is then thought to induce the formation of presynaptic active zones and postsynaptic densities by recruiting the proper protein components (b). During this process, cadherins and other CAMs probably work in synchrony with the inductive factors to stabilize the nascent synaptic junction. Following synaptic differenti-

ation, synapses are functional but do not yet contain their final composition of certain synaptic proteins, such as voltage-gated calcium channels or particular receptor subunit types (c). In addition, dendritic spines are not yet fully formed, and both pre- and postsynaptic elements are highly sensitive to cytoskeletal perturbations (c). Only after maturation do synapses attain their final protein compositions, stability, and mushroom-shaped dendritic spine morphologies (d). Finally, the replacement and exchange of pre- and postsynaptic proteins enables synapses to be maintained over long periods of time (e)

tic compartments of excitatory synapses that initially exhibit little ultrastructural specialization (Ahmari et al. 2000) evolve into highly differentiated presynaptic boutons with large well-defined SV pools (Mozhayeva et al. 2002). Similarly, the NMJ continues to mature for an additional

2 weeks after initial contact (Kummer et al. 2006; Sanes and Lichtman 2001). During this time, the presynaptic bouton becomes ensheathed by Schwann cells and the postsynaptic target muscle cell develops an elaborate set of folds, with each PSD (comprised of clusters of acetylcho-

line receptors) appearing at the apex of each fold, juxtaposed to a presynaptic active zone (see Sanes and Lichtman 2001). In these and other examples, synaptic maturation is accompanied by molecular changes in the composition of the active zones and PSD (see below).

In general, synaptic maturation is also associated with an increased stability of the junction and a resistance to disassembly. For example, nascent/immature synapses may only last for hours or less (Alsina et al. 2001; Jontes and Phillips 2006; Meyer and Smith 2006; Niell et al. 2004), are sensitive to actin depolymerizing drugs such as latrunculin A (Zhang and Benson 2001), and depend on the presence of “generic” cell adhesion molecules such as N-cadherin. Conversely, more mature synapses are insensitive to these drugs (Zhang and Benson 2001), do not depend on N-cadherin-based adhesion (Bozdagi et al. 2004), and appear to be much more stable (Ruthazer et al. 2006) lasting days, weeks, and even months (see Holtmaat et al. 2005; Meyer et al. 2003; Trachtenberg et al. 2002; Zuo et al. 2005).

The fifth step in the development of synaptic junctions involves the maintenance of mature synapse for hours, days, weeks, and possibly years (Fig. 1). Unfortunately, little is known concerning the molecular mechanisms underlying either the stabilization or the maintenance of mature synapses. Those aspects that are known will be discussed further below. However, when considering the mechanisms underlying synaptic stabilization, it is also worth keeping in mind that, beyond molecular mechanisms, changes in synaptic lifetime may also be affected by changes in the extent of mechanical forces that act to “tear apart” nascent junctions, forces generated by axons and dendrites as these extend through the developing neuropil, by cycles of extension and retraction of dendritic and axonal filopodia and, most likely, by additional developmental processes involving glia, vascularization, and so on. Thus, increased synaptic stability associated with maturation is likely to be attributable to both molecular mechanisms and to a reduction in developmental dynamics occurring over the same period.

### Formation of axodendritic contacts

One of the most intriguing aspects of brain development is the wiring of the nervous system. In most cases, this requires axons of differentiating neurons to extend great distances before finding the correct target cells with which to form synapses. These migrating axons ignore other potential targets until they reach their appropriate destination. This selectivity is thought to be regulated both by the surface molecules expressed on neuronal cells and by a temporal delay in their ability to form synapses. Studies in

dissociated neuronal cultures indicate that, whereas most axons exhibit the ability to make synapses soon after they emerge from the cell soma, dendrites require an additional 5–7 days before they become receptive (Fletcher et al. 1994). This latter phenomenon could prevent inappropriate synaptogenesis *in vivo*, by tightly coordinating axonal growth into a target area with dendritic maturation. Recent studies indicate that the regional release of growth factors such as FGF22 or thrombospondin (see below) may also facilitate the maturation of local populations of neuronal cells, thus allowing their competence to make synapses once afferent axon fibers arrive (Christopherson et al. 2005; Umemori et al. 2004).

Once axons arrive at an appropriate target area, what principles guide the point at which synapses form within the field? In the frog and chick optic tectum, at least two complementary systems come into play. The first, which creates a coarse map, utilizes an orthogonal gradient of guidance molecules (such as ephrin A and ephrin B), which steers axons to appropriate quadrants within the tectum (McLaughlin and O’Leary 2005). The second, which refines these maps, is controlled by neuronal activity and mediated by synaptic N-methyl-D-aspartate (NMDA) receptors (O’Leary and McLaughlin 2005; Ruthazer and Cline 2004). This latter mechanism has been shown to underlie the formation of ocular dominance columns in the visual system and barrel fields in the somatosensory system (Constantine-Paton et al. 1990; Foeller and Feldman 2004).

An unresolved question raised by these and other studies is what drives synaptogenesis: axons or dendrites. *In vivo* time-lapse imaging of axons and dendrites in the developing fish and frog visual systems has revealed that both contribute. For example, when labeled axons of retinal ganglion cells of *Xenopus* or zebrafish are followed for several days, one observes numerous axonal growth cones extending across the optic tectum (Ruthazer and Cline 2004). When the synaptic vesicle protein green fluorescent protein (GFP)-VAMP is expressed in these axons, bright fluorescent puncta, presumably presynaptic boutons, are seen to appear just behind the motile axonal growth cones (Meyer and Smith 2006; Ruthazer et al. 2006). These data indicate that many synaptogenic contacts are initiated by axons stochastically encountering dendrites. Interestingly, growing dendritic arbors exhibit similar features as they extend processes that elongate and bifurcate (Cline 2001; Jontes et al. 2000; Niell et al. 2004). Moreover, many fluorescent clusters of postsynaptic proteins, such as PSD-95, appear in the wake of dendritic growth cones (Bresler et al. 2001; Niell et al. 2004). In addition, numerous synaptic contacts form at the tips of motile dendritic filopodia (Dailey and Smith 1996; Fiala et al. 1998; Jontes et al. 2000; Korkotian and Segal 2001; Okabe et al. 2001a; Portera-Cailliau et al. 2003; Saito et al. 1997; Wong and

Wong 2000; Ziv and Smith 1996). Many of these nascent synapses have half-lives of mere minutes, acquiring longer half-lives as the filopodia stabilize and transform into dendritic spines. These data indicate that dendrites can also actively “seek out” axonal partners.

An interesting finding in this regard comes from the analysis of reconstructed pyramidal neurons and GABAergic interneurons (Stepanyants et al. 2004). This analysis has revealed that axons of GABAergic interneurons exhibit high tortuosity, small branch length, and trajectories that correlate well with the positions of postsynaptic neuron dendrites. Conversely, axons of pyramidal neurons usually take relatively straight courses through neuropil and show no correlation with the dendritic positions of their postsynaptic partners. One possible inference from these axonal growth patterns is that the formation of inhibitory synapses is mainly the outcome of axonal explorative behavior, whereas the formation of excitatory synapses may be mainly the outcome of dendritic explorative behavior. Perhaps this situation provides another explanation for the finding that most excitatory synapses (but not inhibitory synapses) are found on dendritic spines, as these structures are believed to represent explorative dendritic filopodia that have become stabilized following a successful encounter with a nearby axon.

Are axonal and dendritic growth coupled to synapse formation? Early electron-microscopic studies by James Vaughn gave rise to the idea that the growth of both axonal and dendritic arbors is mechanistically tightly coupled to synapse formation (Vaughn 1989; Vaughn et al. 1974; Vaughn and Sims 1978). More recently, support for this “synaptotrophic hypothesis” has come from real-time imaging studies in the developing zebrafish and *Xenopus* visual systems demonstrating that new axonal and dendritic branches nearly always sprout from synaptic sites. Moreover, a process almost never retracts beyond an existing synapse (Meyer and Smith 2006; Niell et al. 2004; Ruthazer et al. 2006). These data argue that the growth of axonal and dendritic arbors is largely dictated by the formation of stable synaptic contacts (Niell 2006).

### Induction of pre- and postsynaptic differentiation

As discussed above, initial axo-dendritic contact does not always lead to the formation of a synapse. When it does, the axonal and dendritic compartments at the contact site begin to differentiate into presynaptic boutons and postsynaptic specializations, respectively. Ultrastructurally, whereas the differentiation of presynaptic boutons includes the appearance of SV clusters and the formation of active zones, the differentiation of postsynaptic densities involves the accumulation of electron-dense material at a region of

the postsynaptic membrane that is juxtaposed to the active zone. Molecularly, this differentiation involves an accumulation of the proteins necessary to direct the regulated release of neurotransmitter from SVs in the presynaptic bouton and to detect and transduce this signal in the apposing postsynaptic dendrite.

An elusive goal in the study of synapse formation has been the characterization of signals that trigger pre- and postsynaptic differentiation. Studies concerning the formation of the vertebrate NMJ have led to the discovery of molecules secreted by motor neurons, such as agrin, that can induce postsynaptic differentiation (although it is now clear that the story here is much more complex; for recent reviews, see Kummer et al. 2006; also Witzemann 2006, this issue). Retrospective immunohistochemical analysis of nascent glutamatergic synapses forming in vitro (Friedman et al. 2000; Zhai et al. 2001) suggested that presynaptic differentiation often preceded postsynaptic differentiation, a finding later supported by another study in which spectrally separable variants of GFP were used to compare the accumulation of the presynaptic molecule synaptophysin with the accumulation of the postsynaptic molecules synapse-associated protein 90 (SAP90)/PSD-95 at new synaptic sites (Okabe et al. 2001a). This temporal order would be consistent with postsynaptic differentiation being induced by factors secreted by nascent presynaptic sites. However, as noted before by Ziv and Garner (2001), this cannot be the whole story, because dendrites possess an ability to induce the formation of presynaptic specializations along axonal shafts (as discussed above); thus, something (presumably on the dendritic membrane) has to trigger the formation of axonal SV release sites. Indeed, additional studies based on time-lapse microscopy of fluorescently tagged synaptic proteins have indicated that postsynaptic molecules are often observed to accumulate at presynaptic sites before presynaptic functionality can be detected at such sites (Bresler et al. 2001; Gerrow et al. 2006). Thus, the temporal order of synaptic assembly may not be as clear cut as initially thought, and synaptic differentiation may proceed nearly in parallel; this would be consistent with reciprocal signals crossing from the presynaptic to postsynaptic compartments and vice versa. These studies and others (Ahmari et al. 2000; Bresler et al. 2004; Ebihara et al. 2003; Marrs et al. 2001; Meyer and Smith 2006; Niell et al. 2004; Prange and Murphy 2001), however, have established that synapses can form within the order of 1–2 h from axo-dendritic contact.

What is the nature of such bidirectional signals that not only specify synaptic specialization but also dictate which type of synapse (excitatory, inhibitory, and so on) is to be formed? Answers to this question are beginning to come from a variety of venues, both genetic and cellular. Mutant screens in both *Drosophila* and *Caenorhabditis elegans*

point to the importance of CAMs in the inductive process. For example, in the developing visual system of the fly, members of the cadherin family of calcium-dependent adhesion molecules participate in the formation of synapses between photoreceptor cell axons and their targets (Lee et al. 2001; Prakash et al. 2005). Whether D-cadherin plays a role in target recognition, induction, or synapse stability is unclear. Interestingly in a recent study (Chen et al. 2006), Dscam, an adhesion molecule with an enormous number of alternative splicing variants, has been shown to be essential for appropriate neuronal wiring of mechanosensory neurons, although it seems to be primarily necessary for appropriate axon extension and targeting rather than for synaptogenesis per se.

At vertebrate synapses, classical cadherins appear to be important for the stabilization and plasticity of (nascent) synapses but not for synapse induction (Bozdagi et al. 2004; Scheiffele et al. 2000; Takeichi and Abe 2005). In *C. elegans*, genes encoding members of the Ig super family of CAMs (Syg1/Syg2) have been found to be essential for synapse formation between HSNL neurons (hermaphrodite-specific motorneurons) and their targets (Shen and Bargmann 2003; Shen et al. 2004). In this case, Syg2 expressed by guide-post cells of the vulva physically interacts with Syg1 expressed in the axon of the HSNL neuron, triggering the site-specific clustering of SVs and other active zone proteins (Shen et al. 2004). These data indicate that subclasses of CAMs expressed on postsynaptic targets can selectively trigger presynaptic differentiation. In vertebrate neurons, several classes of molecules appear to perform similar functions. The first is neuroligin, a member of the Ig super family of CAMs initially identified as a binding partner for the postsynaptic protein PSD-95 (Ichtchenko et al. 1995; Irie et al. 1997). Like Syg2, neuroligin can induce the formation of functional presynaptic active zones in heterologous cell systems (Scheiffele et al. 2000). Its presynaptic binding partners include members of the alpha and beta neurexin families of cell surface proteins (Boucard et al. 2005; Ichtchenko et al. 1995). Of these, the alpha-neurexins have been implicated in mediating the recruitment of voltage-gated calcium channels into the presynaptic active zone (Missler et al. 2003). This pair of trans-synaptic proteins has received a great deal of attention in the last couple of years (see Dean and Dresbach 2006). Studies have focused on addressing three issues. Are these proteins true inducers of presynaptic differentiation? Can they, through their heterophilic pairing, specify the formation of sub-types of synapses, e.g., excitatory versus inhibitory? Can neurexin binding to neuroligin influence postsynaptic differentiation? The answer to each of these questions is a resounding maybe.

As putative inducers, neuroligins clearly can induce the differentiation of presynaptic boutons (Scheiffele et al.

2000), and this activity appears to require beta-neurexin (Dean et al. 2003). Furthermore, the knockdown of neuroligin1–3 in cultured hippocampal neurons by using RNAi approaches has led to a reduction in the number of synapses (Chih et al. 2005). However, triple neuroligin1–3 knockout mice still make synapses in normal numbers (Varoqueaux et al. 2001; F. Varoqueaux and N. Brose, in preparation). This difference is difficult to explain but may imply that either other molecules can compensate for the loss of neuroligins in vivo (see below), and/or that they might have other roles in synapse stability and/or plasticity. The latter function is suggested by several studies showing that mutations in the neuroligin genes in humans can lead to autism (Chih et al. 2004; Dean and Dresbach 2006).

An interesting facet of the neuroligins is that they are differentially localized to different synapse types. For example, neuroligin1 is concentrated within PSDs of excitatory synapses (Song et al. 1999), whereas neuroligin2 is found at inhibitory synapses (Varoqueaux et al. 2004), suggesting that they play specific roles in directing the formation of synapses of different types. At present, there is not enough information concerning the spatial and temporal expression patterns of the neurexins or about their binding affinities for the neuroligins to consider this issue at length. Importantly, initial studies on beta-neurexin have shown that, in heterologous cell systems, it has the capacity to induce the partial differentiation of excitatory and inhibitory postsynaptic junctions (Graf et al. 2004, 2006). However, both GABAergic and glutamatergic PSD proteins become clustered at these sites, indicating that additional trans-synaptic signaling molecules may be needed to create specificity.

In addition to neuroligin/neurexin, several other molecules exhibit synaptogenic activities (see, in this issue, Missler 2006; Dityatev and Schachner 2006). These fall into two categories based on their putative functional roles. The first, which appears to function through cell-contact-mediated signaling at nascent synaptic sites, includes SynCAM, EphB/ephrinB, and Narp. SynCAM, a member of the Ig superfamily of CAMs (Biederer 2006; Biederer et al. 2002), shares certain features with neurexin and neuroligin (Sara et al. 2005). For example, it can induce presynaptic differentiation when expressed in a heterologous cell system. Here, induction is via a homotypic interaction, with SynCAM present on both the pre- and postsynaptic membranes. In contrast, activation of the EphB receptor by ephrinB or Narp promotes the postsynaptic clustering of NMDA or AMPA ( $\alpha$ -amino-3-hydroxy-5-methylisoxazole-4-propionic acid) receptors, respectively (Dalva et al. 2000; Martinez and Soriano 2005; O'Brien et al. 1999; Takasu et al. 2002). However, neither signaling pathway alone induces complete postsynaptic differentiation, again indicating that additional trans-synaptic signaling molecules remain to be discovered.

The second category of proteins, including FGF22, Wnt7, and thrombospondin, appears to function more indirectly to promote synapse formation (Christopherson et al. 2005; Hall et al. 2000; Umemori et al. 2004). Specifically, their simple addition to the media of cultured cells increases the number of synapses formed. As they do not appear to act within the confines of the synaptic junction, we interpret these results to imply that they can influence the maturation of neuronal cells, increasing their capacity to form synapses (Waites et al. 2005). As discussed previously, when temporally coupled to the arrival of axons at a target dendritic field, this property would help ensure the specificity, number, and appropriateness of synaptic connections.

In summary, we conclude that synaptic induction requires bi-directional signaling during the initial phases of axo-dendritic contact. Furthermore, presumably not just one inducer drives pre- and postsynaptic differentiation, but rather multiple molecules trigger cascades of signals that act both in series and in parallel. This concept is fundamental to a process that is imperfect at best and that requires enough flexibility and plasticity to realign connections until optimal connectivity is established (see also Jontes and Phillips 2006).

### Cellular mechanisms of synapse assembly

A major challenge facing synaptic biologists in the next decade is to unravel the way that synapses are physically assembled. Two types of scenario are possible. In the first, proteins made in the cell soma appear stochastically and independently at sites of axodendritic contact where they are subsequently assembled into the appropriate synaptic structures. In the second, proteins form complexes within the cell soma and are transported to contact sites en masse, allowing for the more rapid and efficient assembly of synapses. Neurons have found ways of integrating both these scenarios. As discussed above and in several recent reviews (Craig et al. 2006; Waites et al. 2005; Ziv and Garner 2001), presynaptic assembly appears to depend on vesicular intermediates that deliver preassembled protein complexes. These include SV precursors, which deliver a majority of the SV proteins to nascent synapses (Bauerfeind and Huttner 1993; Mundigl et al. 1993), and Piccolo-Bassoon transport vesicles (referred to as PTVs), which carry at least two presynaptic active zone structural proteins (Shapira et al. 2003; Zhai et al. 2001). In all likelihood, these represent just the tip of the iceberg with regard to the diversity of vesicles that contribute to nascent presynapse formation, particularly given the complexities of vesicle morphologies found in clouds around nascent synaptic sites (Ahmari et al. 2000).

Postsynaptic assembly may depend more on the gradual recruitment of individual proteins, as evidence for vesicular delivery of prefabricated postsynaptic protein complexes is inconclusive. As in axons, a rich repertoire of small and tubular vesicles have been observed in dendrites. These include endoplasmic reticulum (ER) and Golgi membranes and also vesicle pools carrying either NMDA or AMPA receptor subunits (Kennedy and Ehlers 2006). The former, together with the presence of dendritic ribosomes and mRNAs (Steward and Schuman 2001; Sutton and Schuman 2005), raise the possibility that both cytosolic and integral membrane proteins can be locally synthesized, processed, and inserted into nascent and mature synapses, a concept supported by several recent publications (Ju et al. 2004; Miyata et al. 2005). However, several studies have provided evidence that prefabricated protein complexes contribute to the assembly of the PSD. In one investigation, Hirokawa and colleagues (Setou et al. 2000) describe a class of vesicles that carry not only NMDA receptors, but several adaptor proteins including Veli/MALS, CASK, and the microtubule-dependent motor KIF17. A second investigation has demonstrated that the postsynaptic proteins PSD-95, GKAP, and Shank are found in non-synaptic clusters that move in an actin-dependent manner and often become incorporated at sites apposed to functional presynaptic active zones (Gerrow et al. 2006). A subset of these non-synaptic clusters also contains neuroligin1, is slow moving, and appears to induce the clustering of presynaptic SVs. These latter data suggest that clusters of postsynaptic scaffold proteins facilitate not only the patching of cell-surface CAMs, but also the formation of presynaptic boutons. Taking these two events together, one can imagine that an initial cluster of PSD proteins nucleates the aggregation of proteins (e.g., neuroligin/SynCAM) capable of inducing presynapse formation, and that additional units of postsynaptic material are added to the site as the synapse grows and matures.

One consistent observation concerning non-synaptic clusters of fluorescently tagged PSD molecules is that the mobility of these clusters is low and spatially restricted (Bresler et al. 2004; Gerrow et al. 2006; Marrs et al. 2001; Prange and Murphy 2001; Washbourne et al. 2002). Such mobility characteristics question the conclusion that non-synaptic clusters of fluorescently tagged PSD molecules constitute *bone fide* transport packets and thus raise questions as to their origin. Time-lapse imaging studies performed in dissociated hippocampal cultures indicate that PSD proteins accumulate gradually at nascent synapses with kinetics that have time constants in the range of 12 to 30 mins (Bresler et al. 2001, 2004). Although not well understood, the gradual clustering of these proteins indicates that PSD proteins are recruited to nascent synaptic sites from diffuse cytoplasmic or membranal pools or, in

some cases, are recruited to extrasynaptic sites where they form extrasynaptic clusters of these proteins. These clusters could then move laterally, merge with nearby synaptic clusters, or promote presynapse differentiation as explained above.

In evaluating these and other imaging studies (Marrs et al. 2001; Prange and Murphy 2001; Washbourne et al. 2002), we should consider whether mismatched pre- and postsynaptic elements observed in young neurons during synaptogenesis represent true intermediates in the assembly of synapses or are, indeed, artifacts of culture arising from a lack of appropriate partners, inefficient protein delivery, or recombinant protein overexpression. One strategy to overcome these issues is to examine nascent synapse formation in a setting in which the number of potential partners is not rate-limiting, and in which recombinant proteins are not in excess. In culture, this can be achieved by examining more mature neurons (>12 divisions) at higher culture densities. Under these conditions, non-synaptic clusters of PSD proteins are not as common, although they are observed occasionally (Bresler et al. 2001, 2004; Okabe et al. 2001a). In summary, until new methodologies are introduced that minimize these confounding factors, the importance of preformed clusters of postsynaptic molecules in PSD assembly remains unclear.

### Structural and functional maturation of synaptic contacts

The process following the initial formation of mostly transient synaptic connections is more protracted, whereby these connections are refined and stabilized. This transition from labile to stable involves numerous structural and functional changes. Morphologically, these include increases in the numbers of presynaptically clustered SVs, the appearance of pronounced postsynaptic densities, a shift from dendritic filopodia to mushroom and stubby spines, and a drastic decrease in spine mobility (Fig. 1; Yuste and Bonhoeffer 2004). Studies focused on changes in cytoskeletal dynamics have noted that, whereas immature synapses are highly sensitive to drugs that disrupt the actin cytoskeleton (in many cases disappearing upon treatment with latrunculin A), mature synapses are resistant to these drugs (Zhang and Benson 2001). Similarly, whereas disruption of the adhesive properties of the classical cadherins reduces the stability of immature synapses, the impact of their loss is more subtle at mature synapses, affecting their function rather than stability (Abe et al. 2004; Bozdagi et al. 2004; Takeichi and Abe 2005). These changes reflect a shift from an exploratory (i.e., search for appropriate partners) to a refinement/solidification (found an acceptable mate) phase of brain development. Moreover,

they mirror changes that occur in the axonal and dendritic cytoskeleton, such as the composition of microtubule-associated proteins (Matus 1990), and that favor stability over growth as the system matures. The molecular mechanisms that account for this reduction in developmental dynamics are not entirely clear; however, recent studies have provided many clues as to the molecules and signaling cascades involved in at least one form of synaptic stabilization, i.e., the maturation of dendritic spines (for a recent review, see Tada and Sheng 2006).

Concomitant with these structural changes is an array of functional changes that alter the firing properties of neurons as the nervous system matures. For example, whereas nascent presynaptic active zones in the hippocampus contain primarily N-type voltage-gated calcium channels, more mature active zones contain predominantly P/Q-type calcium channels (Pravettoni et al. 2000; Scholz and Miller 1995). Similarly, although the PSDs of immature excitatory synapses contain primarily NR2B subunits of the NMDA receptor, NR2A subunits appear in the second and third weeks of postnatal life as these synapses mature (Petralia et al. 2005; van Zundert et al. 2004). Not surprisingly, these alterations are associated with changes in SV release probability and the responsiveness of the postsynaptic neuron. In general, these changes are accompanied by a net decrease in the conduction of calcium into both the presynaptic bouton and the PSD in mature synapses.

One rather puzzling change concerns the neurotransmitter GABA. Intriguingly, during early development, GABA is an excitatory neurotransmitter leading to the depolarization of neurons rather than hyperpolarization (Ben-Ari 2002). Mechanistically, this is caused by the delayed expression of the outwardly directed  $K^+Cl^-$  co-transporter (KCC2) in the plasma membrane of young neurons. This situation is proposed to provide a mechanism for exciting developing neurons while avoiding the potentially toxic effects of a mismatch between GABA-mediated inhibition and glutamatergic excitation (Ben-Ari 2002) with the assumption in mind that activity plays an essential role in synapse formation and network maturation.

Is this assumption correct? Does synaptic or neuronal activity play a fundamental role in the wiring of the nervous system? This is a question that has fascinated neuroscientists since the time of Hebb. There is little doubt at this juncture that many regions of the brain, including the visual, olfactory, and somatosensory systems, require synaptic NMDA-dependent neurotransmission to set up proper wiring (Constantine-Paton et al. 1990; Foeller and Feldman 2004; Ruthazer and Cline 2004; Sullivan et al. 1995). However, the role of synaptic activity in influencing the formation and maturation of synapses is less clear. Studies of mice lacking Munc18 (Verhage et al. 2000) and Munc13 (Rosenmund et al. 2002), two proteins essential



for synaptic vesicle exocytosis, have shown that, in the absence of neurotransmission, the brain develops normally. These data suggest that the gross architecture of the nervous system is determined independently of synaptic transmission. Furthermore, these studies and many others performed in culture (see, for example, Harms and Craig 2005) indicate that, whereas transmission might play a secondary modulatory role during synapse formation (see below), it is dispensable for synapse formation per se. This raises the interesting issue of at what stage during synaptogenesis do synapses become functionally active. Are they functional from the moment of SV clustering and receptor accumulation, or are they initially silent, waiting for some signal to wake them up?

The term “silent synapse” has been used in recent years to describe several subpopulations of synapses present in the nervous system. The best characterized are postsynaptically silent synapses. These synapses have been found to contain NMDA-type, but not AMPA-type, glutamate receptors (Liao et al. 1999; Montgomery and Madison 2004). Moreover, they can be awakened by high frequency theta stimulation, indicating that neuronal activity can activate silent synapses and bring them online. By using this property (e.g., expression of NMDA receptors only), several groups have found that postsynaptically silent synapses are most abundant during early stages of neuronal differentiation (Liao et al. 1999; Malenka and Nicoll 1999), leading to the suggestion that nascent synapses are born “partially deaf”. However, not everyone agrees that this is indeed the case (Groc et al. 2006; Renger et al. 2001).

In addition to postsynaptically silent synapses, several groups have reported the existence of presynaptically silent synapses (Ma et al. 1999; Renger et al. 2001; Shen et al. 2006; for a review, see Voronin and Cherubini 2004). This population of synapses has been identified indirectly by taking advantage of the styryl dye FM1-43. Specifically, by using a loading and unloading paradigm, a significant number of new FM1-43 recycling sites (~15% of total in dissociated hippocampal cultures) has been demonstrated to appear between sequential loading experiments. This unsilencing can be induced either by a brief treatment of forskolin or a stimulating tetanus (Ma et al. 1999; Ryan et al. 1996; Shen et al. 2006). This form of presynaptic unsilencing has also been described electrophysiologically and is associated with an increase in mature excitatory postsynaptic current frequency (Reid et al. 2004; Shen et al. 2006). Whether this phenomenon involves the unsilencing of existing synapses or the formation of new synapses has not yet been resolved. Nonetheless, the concept that synapses can exist in states that neither “speak” nor “hear” remains a provocative idea that could govern the connectivity of physically connected neurons. Anecdotally, immature neuronal cultures have been shown to contain a larger

fraction of presynaptically silent synapses than mature cultures (Shen et al. 2006) suggesting that nascent synapses are initially presynaptically silent and require awakening.

### Maintenance of synaptic structure

In vivo imaging studies of neurons in mature somatosensory and visual cortex have shown that, whereas mature synapses do turn over, many remain stable over days, weeks, and even months (Holtmaat et al. 2005; Trachtenberg et al. 2002; Zuo et al. 2005). What cellular mechanisms govern synaptic stability? Are stable synapses molecularly different from those with shorter half-lives? One way to address these questions is to examine the exchange and turnover kinetics of individual protein components. For example, do longer-lived synapses have slower protein exchange rates than shorter-lived ones? If we assume that synaptic proteins are replaced over time, what is the source of new proteins and the fate of the old ones? Are proteins used once and then degraded or continually reused by the same or neighboring synapses?

Surprisingly, few studies have addressed these issues, at least as far as PSD molecules are concerned. In one study, the average turnover rate of synaptic proteins in a synaptosome preparation was found to be ~5 h, with individual components ranging from 4 to 15 h (Ehlers 2003). Notably, chronic changes in neuronal activity dramatically influenced the turnover of some of these. For example, although an increase in activity caused a faster turnover ( $t=2-10$  h) of NMDA receptor subunits, SAP102, protein phosphatase 1, and A-kinase-associated protein 75, a decrease in activity slowed their turnover ( $t=20-30$  h). However, such  $^{35}\text{S}$  pulse-chase studies do not resolve residency times or the exchange kinetics of proteins at individual synapses.

More direct imaging studies and investigations utilizing fluorescence recovery after photobleaching (FRAP) of individual boutons indicate that protein dynamics at individual synapses are much more substantial than pulse-chase studies imply. For example, pulse-chase studies of the SV-associated protein synapsin have indicated that its half-life at synapses is in the order of several weeks (Petrucci and Morrow 1991). On the other hand, imaging studies of GFP-tagged synapsin at individual presynaptic boutons have revealed that this molecule undergoes significant dispersion upon electrical stimulation and re-appears at these boutons within minutes after the cessation of the stimulus train (Chi et al. 2001). An intriguing question raised by such experiments is what happens to the Synapsin once it leaves a presynaptic bouton? Is it degraded, does it return to its bouton of origin, or is it free to go elsewhere? Clearly, a similar question applies to synaptic vesicles and

their proteins during their fusion with the plasma membrane, or after a stimulus train that uncouples them from the presynaptic matrix. Results from a growing number of studies indicate that SVs and perhaps even the machinery directing their fusion at active zone are free to wander off to adjacent presynaptic boutons (Darcy et al. 2006; Krueger et al. 2003; Matteoli et al. 2004). At present, however, there is little information regarding the kinetics of many key presynaptic molecules, although similar dynamics have been reported for presynaptic actin, clathrin, and Rab3 (Colicos et al. 2001; Mueller et al. 2004; Sankaranarayanan et al. 2003; Star et al. 2005).

Although the imaging of presynaptic proteins is helping to dispel the notion that synapses are essentially static structures, studies focused on the dynamics of a variety of postsynaptic proteins indicate that, here too, the story is far from simple. For example, the dynamics of acetylcholine receptors (AChR) at the NMJ are affected by two parameters. The first is the long half-life of the receptor, which is in the order of 9–14 days (Akaaboune et al. 1999; but see Gardner and Fambrough 1979). The second is the significantly faster exchange between existing pools of synaptic and extra-synaptic receptors; this has recently been calculated to be less than 8 h (Akaaboune et al. 2002). Perhaps, not surprisingly, these kinetics can be altered by changes in synaptic activity. Specially, a complete block of transmission can lead to increased turnover, a situation that has been proposed to be related to synaptic pruning during development (Akaaboune et al. 1999).

Analogous studies at central synapses indicate that both receptor and structural protein dynamics are significantly faster than at peripheral synapses, in their turnover and exchange rates, perhaps reflecting their more plastic nature. As mentioned above, estimates of protein turnover/half-life are in the range of a 3 to 20 h and dependent on the overall activity state of a neuronal network (Ehlers 2003) with slower times being associated with less activity, in contrast to the NMJ (Akaaboune et al. 1999). However, when considering the dynamics of individual postsynaptic proteins as revealed by imaging approaches, one is struck by the enormous volume and rate of exchange occurring within what appear to be stable structural junctions. For example, FRAP experiments suggest that many postsynaptic molecules (SAP90/PSD-95, SAP97, PSD-Zip45/Homer1c,  $\alpha$ -actinin; neurabin-1, actin, brain-enriched GK domain-associated protein, ProSAP1/Shank2, ProSAP2/Shank 3, AMPA receptor subunit GluR1, NMDA receptor subunit NR1, CaMKII, N-cadherin) are lost from and reincorporated into individual synapses at significant rates, some of which are dependent on activity (Bresler et al. 2004; Iki et al. 2005; Nakagawa et al. 2004a,b; Okabe et al. 2001b; Sharma et al. 2006; Yao et al. 2003). Collectively, these studies indicate that the multimolecular complexes

found at synapses are highly dynamic structures in which individual molecules are continuously exchanged with molecules in extrasynaptic pools (see also Inoue and Okabe 2003; Triller and Choquet 2005).

Nevertheless, if synaptic matrices are so labile, how do synapses retain their overall organization and function for long durations? Phrased differently, if synaptic molecules are in a dynamic equilibrium with extrasynaptic pools, what drives and maintains their high concentrations at synaptic locations? Perhaps certain synaptic matrix components are relatively stable and function as “nuclei” that define the location and perhaps the characteristics of synaptic scaffolds, serving metaphorically as “islands of stability in a sea of change”. At present, however, the existence of relatively stable core structures and their capacity to promote long-term synaptic persistence remain to be established. Regardless of the nature of such mechanisms, we can probably fairly state that the formation of a new synapse involves the establishment of a new site at which the equilibrium between the assembly of a multitude of molecules into multimolecular complexes and the dispersion of these multimolecular complexes is shifted toward net assembly (Bresler et al. 2004), and that these multimolecular complexes continue to remain in a state of dynamic equilibrium with extrasynaptic molecules throughout the life-span of the synapse. The challenge for the future is thus to gain an understanding, in molecular and biophysical terms, of the processes and molecular cascades that promote the formation and long-term maintenance of these assemblies at the particular locations of synaptic junctions.

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