CELLULAR AND MOLECULAR MECHANISMS OF PRESYNAPTIC ASSEMBLY

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The formation of a presynaptic neurotransmitter secretion site involves the transformation of a patch of unspecialized plasma membrane into a complex structure that is highly specialized for sustained, depolarization-evoked synaptic vesicle exocytosis. This transformation is governed by dynamic intracellular and intercellular processes, and by global developmental processes. Recent work has shed light on the cellular processes that are responsible for transporting presynaptic molecules to nascent presynaptic sites and for the subsequent transformation of these sites into functional presynaptic structures. Other studies are providing clues to the identity of molecules that induce and initiate the transformation process.

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doi:10.1038/nrn1370

Chemical synapses are sites of cell–cell contact that are specialized for the transmission of signals between neurons and their targets, which include muscles, glands or, most commonly, other neurons. Synaptic transmission depends on the tightly regulated secretion of neurotransmitters by the presynaptic cell and the presence of specific receptors on the postsynaptic membrane.

The directional nature of synaptic transmission is manifested in the asymmetric ultrastructure and molecular composition of the pre- and postsynaptic compartments. Presynaptic compartments are characterized by the presence of hundreds to thousands of neurotransmitter-filled synaptic vesicles, and by active zones — specialized regions of the presynaptic plasma membrane where synaptic vesicles dock, fuse and release neurotransmitter into the synaptic cleft[1,2] (FIG. 1). The active zone is characterized by the presence of an electron-dense matrix of proteins, which show a lattice-like organization in which regular arrays of electron-dense tufts are linked together by fine filamentous material[3–6]. This structure, known as the cytoskeletal matrix (CAZ) or 'presynaptic grid'[7,8], is thought to facilitate the regulated translocation of synaptic vesicles to the active zone plasma membrane, and in turn define the site of synaptic vesicle docking and fusion. The postsynaptic reception apparatus is also characterized by an electron-dense thickening, which is referred to as the postsynaptic density (PSD)[1,2,9–11], the central function of which is to confine receptors of the appropriate type beneath the active zone. The PSD and CAZ seem to be held in register by trans-synaptic cell-adhesion molecules (CAMs) and extracellular matrix proteins (BOX 1).

Neurotransmitter secretion by presynaptic terminals has been the subject of intense research for over 60 years, initially by means of electrophysiological[12] and ultrastructural techniques[1,2,13], and more recently by imaging, molecular and genetic approaches[11,14,15]. Although a comprehensive discussion of this work is beyond the scope of the current review (see REFS 16–20), some of the salient features are summarized below and in BOX 1. Neurotransmitter secretion is a complicated multi-step process involving synaptic vesicle exocytosis and compensatory endocytosis, which are tightly coupled to form a cycle[16,18–21]. Each step in this cycle is catalyzed and regulated by specific molecules (BOX 1), many of which have been identified and characterized (TABLE 1). A large pool of synaptic vesicles, known as the reserve pool, is present within the presynaptic terminal. This pool can contain up to thousands of synaptic vesicles, but at any given moment only a few are 'tethered' or 'docked' at the active zone plasma membrane.

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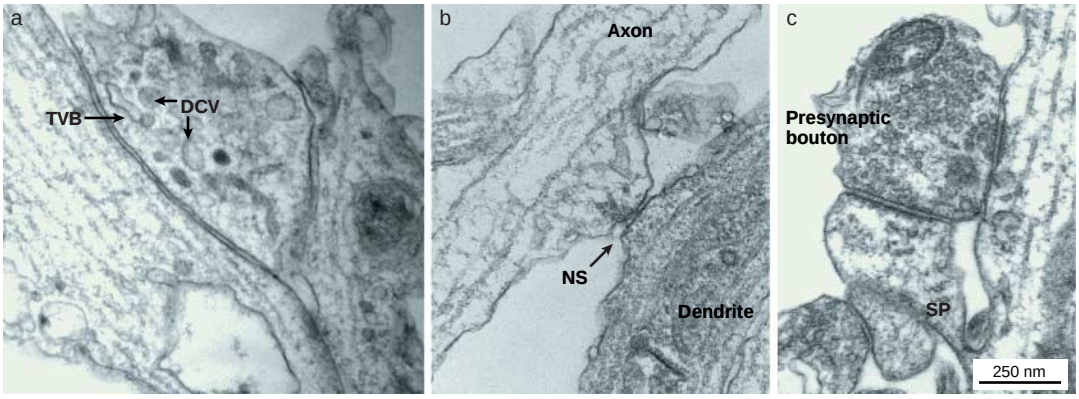

Figure 1 | **Ultrastructure of synapses at various stages of development.** Electron micrographs of an axonal varicosity (**a**), as well as a nascent synapse (**b**) and mature spiny synapse (**c**) formed between hippocampal neurons grown for 8 days (**a**, **b**) and 15 days (**c**) in culture. **a** | One characteristic of structures that seem to be involved in very early presynaptic formations is the aggregation of pleomorphic, tubulovesicular (TVB) and dense-core vesicles (DCV) in axonal varicosities. Certain DCVs have been shown to carry many structural proteins of the active zone, and they might represent active zone precursor vesicles that facilitate the rapid establishment of a functional neurotransmitter release site. However, as these formations lack obvious presynaptic ultrastructural features, there is some uncertainty as to their true identity. **b** | This state is thought to be followed by the appearance of flat parallel membranes, electron-dense membrane thickenings and a few synaptic vesicles at nascent synapses (NS). **c** | At more mature synapses, prominent active zones and postsynaptic densities are observed, with numerous synaptic vesicles clustered within the presynaptic bouton. As shown here, dendritic spines (SP) also appear at excitatory synapses. Micrographs were taken and kindly provided by JoAnn Buchanan, Stanford University.

The docked vesicles are known as the readily releasable pool of synaptic vesicles. A depolarizing action potential, and the consequential opening of voltage-dependent calcium channels, leads to an influx of calcium that triggers the fusion of docked and primed vesicles with the presynaptic plasma membrane. Synaptic vesicle membrane proteins are then retrieved by CLATHRIN-mediated endocytotic processes that are thought to occur outside the active zone[18,20], or possibly by direct retrieval of vesicles at active zones[22,23]. Endocytosed vesicles are refilled with neurotransmitter, possibly after passage through specialized endosomal compartments, and they are then returned to the reserve pool to become available for another round of exocytosis[11,18,20,21,24].

It follows that the formation of a new functional presynaptic site is a demanding process. Calcium channels must be recruited, active zones must be formed, molecules involved in docking, priming, fusion and endocytosis of synaptic vesicles must be recruited, synaptic vesicles must be obtained or formed, confinement mechanisms for the synaptic vesicle reserve pool must be set in place, and the endocytotic machinery for membrane retrieval and synaptic vesicle regeneration must be set up. In addition, the formation of the presynaptic site must be tightly coordinated with the assembly of the appropriate receptive apparatus on the postsynaptic side. Surprisingly, our current understanding of presynaptic assembly processes is quite limited in comparison to the wealth of information gained on presynaptic function. In this review, we will summarize the current state of knowledge on the elaborate cellular and molecular mechanisms that lead to the formation of presynaptic terminals and discuss some of the exciting questions that face researchers.

CLATHRIN
A major structural component of coated vesicles that are implicated in protein trafficking. Clathrin heavy and light chains form a triskelion, the main building element of clathrin coats.

Development of presynaptic sites

Most presynaptic sites are formed during development, as axons grow out and establish connections with their targets. Much of our understanding of presynapse formation comes from work on the development of the neuromuscular junction (NMJ), the synapse that is formed between motor neurons and skeletal muscle cells[25]. The formation of these synapses begins early in development, a few hours after the closure of the neural tube. Motor neuron axons, guided by growth cones at their tips, establish contacts with muscle targets that are in their final stages of differentiation. The axons grow a short distance down the midline of the differentiating muscle, giving rise to side branches in the process. Eventually, the growth cones develop into bulbous enlargements that possess a rudimentary capacity for spontaneous and evoked release of neurotransmitter. With time (hours to days), these structures differentiate into typical presynaptic terminals and gradually acquire mature functional characteristics.

Motor axons do not form synapses *en route* to their final destinations, and their presynaptic compartments are located near their terminal ends (giving rise to the name 'presynaptic terminals'). So, studies that have focused on presynaptic differentiation have tended to highlight the intimate relationships between axonal growth cones and emerging synaptic vesicle release sites. These relationships were emphasized by findings that growth cones of cultured *Xenopus*, chick, *Aplysia* and *Drosophila* neurons release neurotransmitter even before they contact their targets[26–31]. In the vertebrate CNS, however, many presynaptic sites are distributed along the length of axonal segments, like beads along a string, forming small swellings or varicosities known as presynaptic boutons. This arrangement allows one axon to form *en passant* synaptic connections with many

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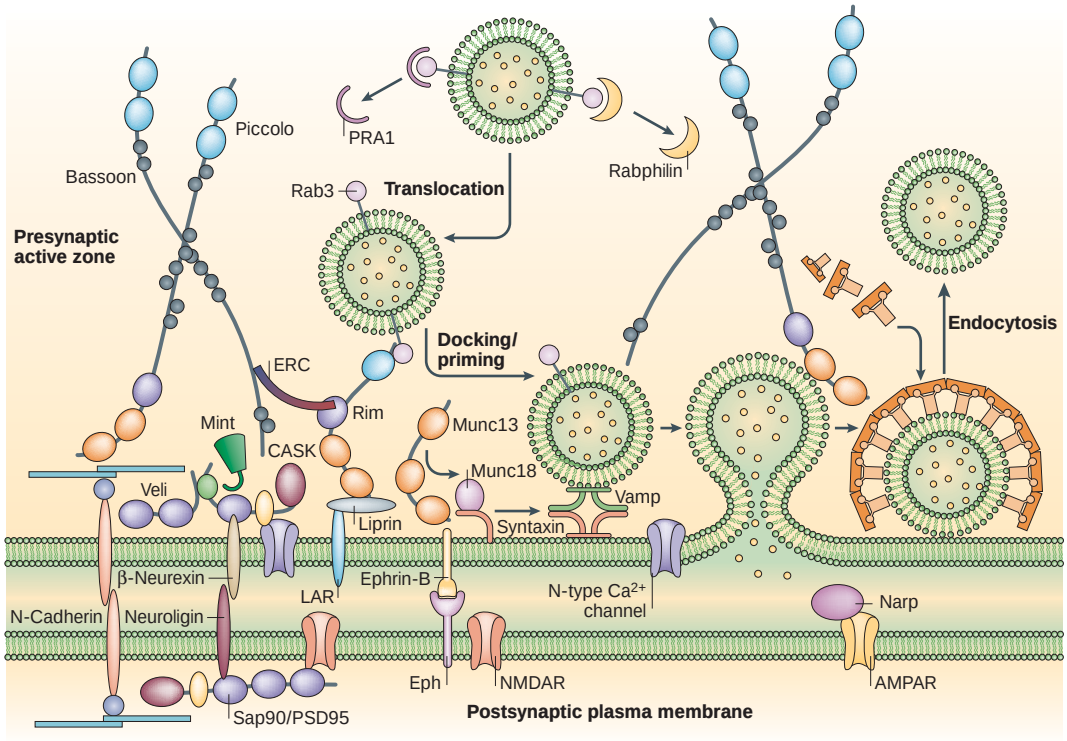
Box 1 | Molecular structure of CNS glutamatergic synapses



Current studies indicate that three distinct complexes help to define the active zone (see TABLE 1 for a list of key active zone proteins). The first complex is largely structural, and is thought to hold the active zone in register with the postsynaptic density (PSD) and clusters calcium channels within the active plasma membrane. It includes cell adhesion molecules (CAMs), including neuroligin, neurexin and N-cadherin, as well as synaptic CAM (SynCAM) and neuronal CAM (NCAM, not shown), and cytoskeletal proteins, such as piccolo, bassoon, ERC/Cast, liprin, calcium/calmodulin-dependent serine protein kinase (CASK), velis, mint and spectrin[10,12]. The second complex is involved in synaptic vesicle docking and fusion, and it contains components of the SNARE complex, including syntaxin and Snap25, as well as Rim, Rab3a, Munc13, Munc18 and N and P/Q type calcium channels[14,15]. Before fusion can occur, the synaptic vesicles must be primed, and molecules such as Rim, Munc13 and Munc18 are crucial for this step. Protein complexes that are involved in synaptic vesicle endocytosis include clathrin, dynamin and a family of SH3-domain-containing adaptor proteins[26,27].

Postsynaptically, PDZ-domain-containing proteins, such as SAP90/PSD95 (postsynaptic density protein 95), are abundant, and they are thought to build specialized protein complexes around specific subclasses of ionotropic glutamate receptors (NMDAR (*N*-methyl-D-aspartate), AMPAR (α-amino-3-hydroxy-5-methyl-4-isoxazole propionic acid receptor)) and metabotropic glutamate receptors (not shown)[18,19,20]. The tyrosine kinase receptor EphB is also tethered to the postsynaptic density, and it is an excellent candidate for regulating the recruitment of neurotransmitter receptors from endosomal pools. Proteins such as NARP and ephrin-B are thought to promote the clustering of AMPA and NMDA receptors, respectively[10].

SNARE
Soluble NSF (*N*-ethylmaleimide-sensitive fusion protein) accessory protein (SNAP) receptor.

NEUROPIL
A felt-like network that is interspersed between the cells of the grey matter in the CNS. It consists of neuronal and glial processes and synaptic terminals.

dendrites along its route. In principle, *en passant* presynaptic boutons could be formed behind advancing axonal growth cones as they weave their way through the developing NEUROPIL[32–35] (FIGS 2,3). There are indications, however, that this is not the entire story. Cultured *Xenopus* spinal neurons, rat hippocampal neurons and *Drosophila* neurons show some capacity for evoked synaptic vesicle recycling along entire axonal segments, even in the absence of muscle or dendritic targets[29,36–42]. Furthermore, molecules that are essential for synaptic vesicle release, such as syntaxin and Snap25, are found to be widely distributed along axons rather than concentrated at presynaptic sites[20,40,43,44]. These findings indicate that the potential to form presynaptic boutons might be distributed along the entire axonal membrane, at least in immature neurons. Indeed, the formation of functional

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Table 1 | Synaptic proteins involved in active zone formation and synaptic vesicle recycling

Protein	Function
Synaptic vesicle exocytosis	
Synaptobrevin/Vamp, Snap25, syntaxin	Components of SNARE complex involved in synaptic vesicle docking and fusion
NSF, α- and β-SNAPs	Dissociation of SNAREs
Synaptotagmins	Calcium sensors; interact with syntaxin and Rim
N- and P/Q-type calcium channels	Calcium influx
Munc18	Binds and negatively regulates syntaxin and synaptic vesicle fusion
Munc13	Involved in synaptic vesicle priming; interacts with Rim; displaces Munc18
Rim1α	Involved in synaptic vesicle priming; interacts with Munc13, RimBPs and synaptotagmin
Rab3A	Regulates synaptic vesicle cycle; interacts with Rabphilin, Doc2, Pra1 and Rim
Complexin	Binds and regulates SNARE complex
Structural molecules of active zones	
CASK	CaMKII domain-containing MAGUK; forms a complex with MINTs, veli and calcium channels, neurexin and SynCAM
MINTs	Munc18-interacting molecules; found in complex with CASK and veli
Veli	Found in complex with CASK, MINTs and calcium channels
Bassoon and piccolo	Large structural proteins of the CAZ that interact with Pra1, profilin, Abp1 and ERC
Synapsins	Anchoring of synaptic vesicles to actin; regulation of reserve pool of synaptic vesicles
α-Liprin	Scaffold proteins that bind Rim, ERC and LAR
Spectrin	Cortical cytoskeletal protein; interacts with actin, cell-adhesion molecules and receptors
ERC/Cast	CAZ proteins that interact with piccolo, bassoon, Rim and liprin
RimBP	Rim binding protein; component of the CAZ
Synaptic adhesion and signalling	
β-Neurexin	Presynaptic adhesion; interacts with CASK and neuroligins; can trigger active zone formation
α-Neurexin	Presynaptic adhesion; binds and localizes N-type calcium channels to active zones
SynCAM	Homophilic cell-adhesion molecule that can induce the formation of functional active zones
CNR	Cadherin-related cell-adhesion molecules
N-cadherin	Neuronal cell-adhesion molecule; interacts with catenins and the spectrin/actin cytoskeleton
β-Catenin	Binds the C-terminal tail of N-cadherin
Neuroligin	Postsynaptic cell-adhesion molecule; interacts with presynaptic β-neurexins; induces active zone formation
NCAM	Member of the IgG superfamily of adhesion molecules that binds spectrin
Narp	Neural activity-regulated pentraxin; a secreted molecule that promotes clustering of AMPARs
Ephrin-B	Synaptogenic factor that promotes clustering of NMDARs after binding the EphB receptors
EphB	Tyrosine kinase receptor for ephrin-B, binds and clusters NMDAR
LAR	Receptor protein tyrosine phosphatase; binds liprin and regulates active zone assembly
Synaptic vesicle endocytosis	
Clathrin	Involved in synaptic vesicle endocytosis; interacts with dynamin, AP2, amphiphysin and other molecules
Dynamin	GTPase involved in pinching off synaptic vesicles during endocytosis
Amphiphysin	Binds dynamin and is involved in synaptic vesicle endocytosis
Actin	5 nm cytoskeletal filament; found surrounding the active zone as well as within dendritic spines

AMPAR, α-amino-3-hydroxy-5-methyl-4-isoxazole propionic acid receptor; AP2, adaptor protein 2; CaMKII, calcium/calmodulin-dependent kinase II; CASK, calcium/calmodulin-dependent serine protein kinase; CAZ, cytoskeletal matrix assembled at active zones; IgG, immunoglobulin G; MAGUK, membrane-associated guanylate kinase-like proteins; NMDAR, *N*-methyl-D-aspartate receptor; RimBP, Rim base-pair; SNARE, soluble NSF (*N*-ethylmaleimide-sensitive fusion protein) accessory protein SNAP receptor.

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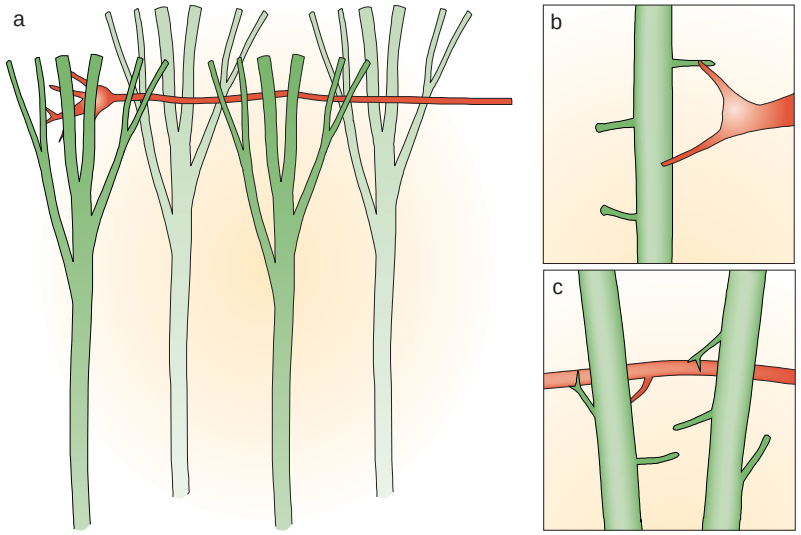

Figure 2 | **Formation of axodendritic contacts. a** | As elongating axons tipped by motile growth cones extend through the developing neuropil, presynaptic differentiation is induced at sites of contact with adjacent dendrites. **b**,**c** | Contacts with dendrites might occur at the leading edge of the elongating axon (**b**), or later on along well-formed axonal segments (**c**), by dendritic protrusive structures such as lateral filopodia and dendritic growth cones, and by lateral axonal filopodia.

synaptic vesicle release sites can be induced along axons by beads that are coated with various substances (such as polylysine, polyornithine and basic fibroblast growth factor)[39,45–49], or by manually manipulated spherical muscle cells termed myoballs[50].

The proposition that non-terminal axonal segments can differentiate into presynaptic boutons is consistent with studies that indicate that dendritic protrusive structures — growth cones at the tips of elongating dendritic branches and FILOPODIA that extend laterally from dendritic shafts — could induce the formation of presynaptic boutons at sites of contact with nearby axons (reviewed in REFS 51–53). This has been shown to occur in primary cultures of dissociated neurons[33,54,55] (see also FIG. 3) and there are strong indications that it also occurs *in vivo*[56–64], perhaps even during NMJ formation[65,66]. Axons also extend highly protrusive lateral structures that might also give rise to presynaptic boutons far behind leading-edge growth cones[38,59,67–70]. So, new presynaptic boutons can be formed from axonal growth cones, immediately behind advancing axonal growth cones and along established axonal segments.

The differentiation of growth cones or patches of axonal membrane into well-formed functional presynaptic compartments is associated with important structural changes (as exemplified in great detail by Yoshihara and colleagues[71]). Unfortunately, most of these structural changes are beyond the resolving power of light microscopy, including the cutting-edge microscopy techniques that have been so informative in documenting the choreography of synaptogenesis. Electron microscopy (EM) has therefore been used to examine the fine structure of synapses in fixed samples from developing tissue, in an attempt to determine the sequence of structural rearrangements that takes place as nascent contacts evolve into mature presynaptic specializations. However, the main advantage of EM has also been its stumbling block. Synapses are recognized at the EM level by the presence of particular ultrastructural characteristics; that is, apposed pre- and postsynaptic plasma membranes, a synaptic cleft, structural specializations of the pre- and postsynaptic membranes, and the presence of synaptic vesicles (FIG. 1). It is reasonable to assume that new synapses might lack most of these structural characteristics. So, in the absence of additional information, the identification of new synapses in EM micrographs has presented a formidable problem[72].

EM-based studies on the development and maturation of CNS synapses have yielded conflicting conclusions (see REF. 51 for an insightful review), but there is some consensus on several findings. Initially, CNS synapses have parallel, apposed membranes, with varying degrees of structural specializations (electron-dense thickenings). Their presynaptic compartments are simple, essentially composed of a patch of electron-dense membrane (perhaps an active zone) associated with only a small number (2–3) of synaptic vesicles. Reserve synaptic vesicle pools are almost non-existent, as are the mitochondria that are commonly observed in mature presynapses. On the other hand, PLEOMORPHIC vesicular structures, dense-core vesicles, some with SPICULES projecting from their surface, as well as coated vesicles are often observed at nascent presynaptic sites (FIG. 1a). With development, synaptic vesicle numbers increase and boutons become larger, and the presynaptic membrane becomes more complex (FIG. 1). Curiously, the length of juxtaposed pre- and postsynaptic thickenings in single EM sections (and, presumably, active zone area) does not seem to change markedly with development, at least for some types of CNS synapses[73–76]. It is worth reiterating, however, that these characterizations of nascent presynaptic structures were somewhat circular in nature, as the identification of nascent synapses was based on the presence of recognizable membrane specializations and synaptic vesicles. So, the earliest stages of presynaptic differentiation could have been missed. Furthermore, as the structures that were examined had formed at variable times before tissue fixation, the relationships between particular structural characteristics and synaptic 'age' were not known.

In addition to changes in structural characteristics, presynaptic maturation is associated with changes in functional characteristics. These include changes in the types and subunit composition of voltage-dependent calcium channels that are involved in evoked neurotransmitter release[77–86], increased sensitivity to TETANUS TOXIN[79], reduced sensitivity to the vesicle-budding inhibitor Brefeldin A (REF. 41), and changes in the probability of neurotransmitter release[76,87–94]. The details of these maturational changes differ from one type of synapse to another and from one organism to another, making it difficult to provide a single all-encompassing description of presynaptic functional maturation. It is of note, however, that the changes in functional characteristics are protracted and take place over days or even weeks. Unfortunately, the relationships between the structural and functional maturation processes are not well understood.

FILOPODIA
Long, thin protrusions that are present at the periphery of migrating cells and growth cones. They are composed largely of F-actin bundles.

PLEOMORPHIC
Existing in more than one form.

SPICULES
Needle-like structures.

TETANUS TOXIN
Protein derived from *Clostridium tetani* that can block transmitter release owing to its ability to degrade synaptobrevin. Tetanus toxin is the causative agent of tetanus.

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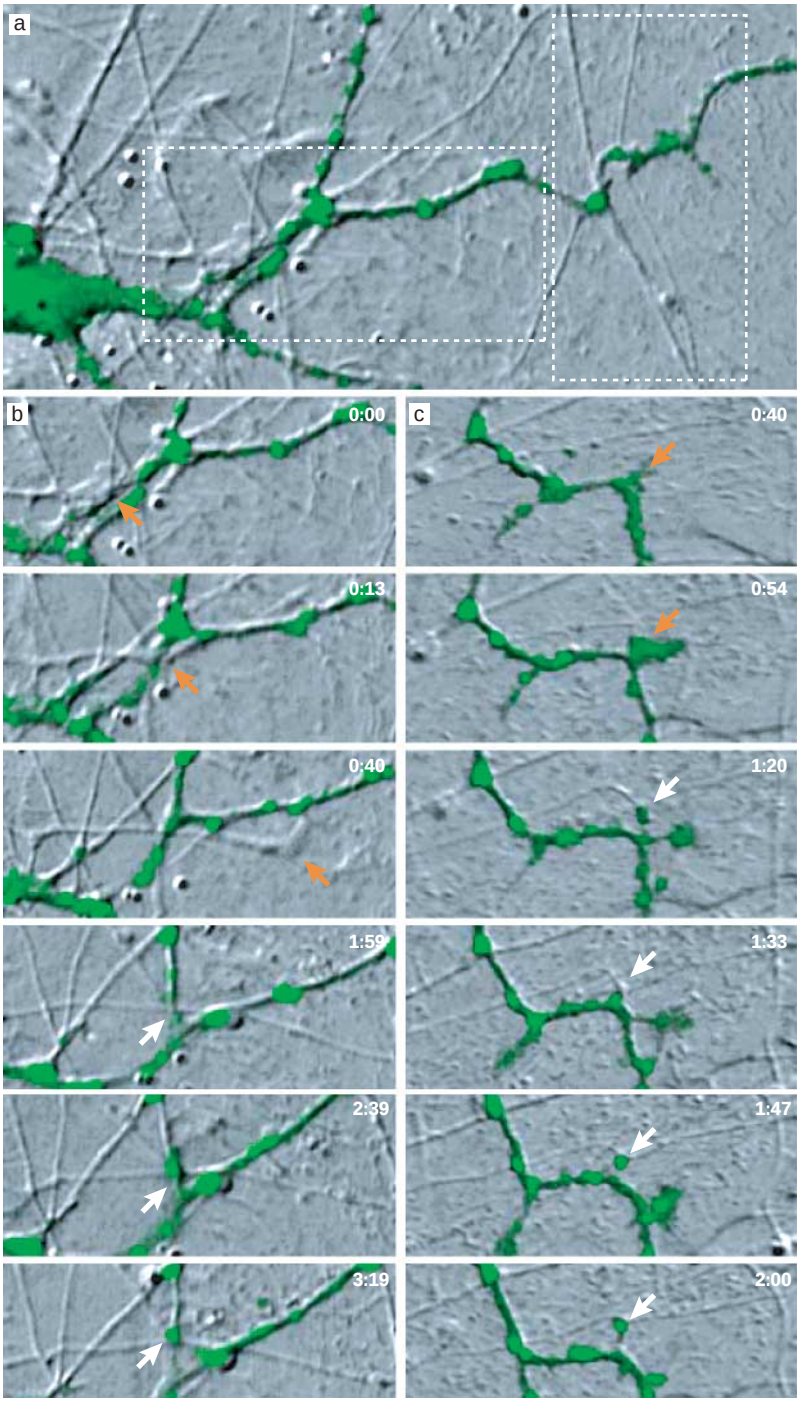

Figure 3 | **Formation of axodendritic contacts in primary cultures of rat hippocampal neurons. a** | An image of a neuron expressing a green fluorescent protein (GFP)-tagged variant of the postsynaptic density (PSD) molecule ProSAP2/Shank3 (GFP:ProSAP2; green) overlaying a differential interference contrast (DIC) image of the same field. **b** | A time-lapse sequence showing an axodendritic contact initiated by an axonal growth cone. An axonal grown cone was observed to grow from the left side of the field to the right (orange arrows). At time (t) = 0:13, the growth cone contacted a dendritic segment at the site indicated by a white arrow. About two and half hours later, a PSD had formed at the intersection site. **c** | A time-lapse sequence showing an axodendritic contact initiated by a lateral dendritic protrusive structure. A small dendritic structure was observed to extend laterally (orange arrows) and contact a nearby axon (white arrow). Subsequently, the retracting dendritic structure pulled the axon back towards the dendritic shaft, and eventually developed into a dendritic spine-like structure. Note the degree to which the relative positions of axons, dendrites and synapses changed with time, which is suggestive of the mechanical forces at play, and the forces that nascent axodendritic junctions encounter during synapse formation. Images recorded by Hagit Vardinon-Friedman, Technion Faculty of Medicine, Israel.

Ideally, one would like to monitor the formation of individual synapses, determine their functional characteristics and then examine their ultrastructure by EM. The development of *in vitro* co-culture preparations of motor neurons and muscle cells[95,96] has made such experiments possible. One such system — *Xenopus* spinal neurons co-cultured with muscle cells — revealed that excitatory postsynaptic potentials, as well as increases in intra-axonal calcium concentrations, can be recorded within seconds to minutes of nerve–muscle contact[36,50,97–99]. During the next few hours, marked increases were observed in the frequency of spontaneous synaptic currents and in the amplitude of both spontaneous and evoked synaptic currents[100,101]. Surprisingly, almost no well-formed synaptic specializations were found when the same nerve–muscle contact sites were scrutinized by EM, in spite of their marked capacity for synaptic transmission[101,102]. In particular, no obvious active-zone-like structures were detected. Instead, presynaptic regions contained scattered synaptic vesicles, some dense-core vesicles and endocytotic profiles. More mature-like presynaptic specializations were observed only a day or so after nerve–muscle contact.

More recently, Ahmari and colleagues[33] examined the ultrastructure of nascent CNS synapses that were formed between hippocampal neurons in primary culture. The formation of axodendritic contacts was recorded by time-lapse light microscopy, and retrospective EM was then used to examine the ultrastructure of the same sites. Analysis of complete sets of serial sections did not reveal well-formed active zones or numerous typical synaptic vesicles at nascent presynaptic sites within 2–3 hours of their formation, even though a capacity for stimulation-evoked vesicle recycling was demonstrated at the same sites before fixation. Instead, numerous pleomorphic and tubulovesicular structures, as well as dense-core vesicles, were observed. Although the possibility that minute presynaptic specializations escaped detection cannot be completely ruled out, these findings indicate that the ultrastructure of nascent CNS presynaptic sites might be significantly different from that of mature presynaptic boutons (FIG. 1a,b).

The findings described so far seem to indicate that presynaptic sites form according to the following temporal sequence. Axonal growth cones, shafts and protrusive structures possess an inherent capacity for rudimentary synaptic vesicle recycling. Following contact with a prospective postsynaptic target, the vesicle recycling machinery is concentrated at the site of membrane apposition, which develops some capacity for synaptic transmission. Later on (hours or days), active zones, which are associated with a small number of docked vesicles, form at the contact sites. Finally, synaptic vesicles accumulate at presynaptic sites, forming the large reserve pools that are observed at mature presynaptic sites.

This account of presynaptic formation is in agreement with a recent study on the functional and structural maturation of presynaptic boutons that were formed in cultures of rat hippocampal neurons[103]. Here, optical methods (recycling of STYRYL DYES) and electrophysiological recordings were used to examine how functional

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properties of presynaptic boutons change with time in culture. These experiments indicated that presynaptic boutons pass through three distinct functional states. At the onset of detectable synaptic function (about five days after plating), synapses lack readily releasable vesicles, although they possess a pool of recycling vesicles that can release neurotransmitters under strong stimulation. Over the next two days, pools of functionally docked, readily releasable vesicles begin to appear (presumably reflecting the formation of active zones). Subsequently, reserve pools of synaptic vesicles start forming, increasing their size concomitantly with an increase in the readily releasable pool size over the rest of the maturation period (several more days). Interestingly, although the identities and sizes of the various pools were determined solely on the basis of functional parameters (exocytosis kinetics, susceptibility to hyper-osmotic shock-induced neurotransmitter release), EM analysis of age-matched preparations confirmed the maturational changes in the various synaptic vesicle pools.

It would be nice to stop here, but unfortunately, this rather simple account of presynaptic differentiation has some basic shortcomings. One is an implicit assumption that synapses in the systems that were examined above form more or less synchronously and mature together over the subsequent days and weeks. However, even in culture, individual synapses do not form simultaneously. During periods of synaptogenesis that can last days, weeks or even months (depending on the organism), axons and dendrites grow vigorously, establishing and breaking synaptic contacts in the process[35,42,55–57,62,104,105]. This means that at any given time during these periods, the population of presynaptic sites is heterogeneous in terms of synaptic age. It is therefore unlikely that this synchronous and protracted progression through well-defined developmental stages represents the developmental history of all individual presynaptic sites. It is more likely that much of this developmental process reflects genetically programmed global changes in neuronal maturational states. The maturational state would dictate the availability and cellular distribution of synaptic building blocks, the competence of axons and dendrites to evolve into mature synaptic structures, and the functionality of molecular pathways that are involved in coordinating and assembling mature presynaptic structures.

This point is illustrated by several examples. *Xenopus* spinal neurons form rudimentary synapses with co-cultured muscle cells within one day in culture, as described above. However, synapsin I, a prominent presynaptic molecule[106], is not detected before day three in culture, nor is it detected *in vivo* during early phases of synaptogenesis[107]. It was therefore suggested that the onset of presynaptic maturation is causally related to the onset of synapsin I expression[107], a suggestion that is supported by findings that experimentally elevated levels of synapsin I accelerate presynaptic maturation in this preparation[108,109]. Another interesting example concerns the expression of dynamin and amphiphysin, two molecules that have crucial roles in synaptic vesicle endocytosis[17,20]. The expression of these molecules is delayed (beginning around postnatal days 7–9 in rats) in comparison to synaptic vesicle proteins such as synaptotagmin and SV2 (synaptic vesicle glycoprotein 2) (REFS 110–113, see also REFS 114,115). Given their roles in presynaptic endocytosis, it is conceivable that the delayed expression of these molecules could be related to the late formation of the reserve pools of synaptic vesicles at new presynaptic sites.

One final example concerns the consistent observation that functional synapses are first observed in primary cultures of rat hippocampal neurons only several days after contacts begin to form. It was suggested that this delayed onset might be related to the delayed expression of presynaptic-specific calcium channels[77], low affinity calcium sensors[81] or as yet undefined dendritic maturation factors[116] (see also REF. 117). In summary, although relationships between the onset of expression of particular synaptic proteins and synaptic maturation are still speculative, it is important to keep in mind that presynaptic development is a combination of global, cell-autonomous maturation processes and individual synapse-specific assembly processes.

Presynaptic assembly: a cellular perspective

Our understanding of the processes involved in the formation of individual new synapses has benefited enormously from the development of live imaging microscopy techniques[118,119]. Studies based on these techniques indicated that new individual presynaptic boutons can form within an hour or less of axodendritic contact[33,34,54,120,121], and they have led to the realization that the formation of new individual presynaptic boutons is governed by highly dynamic processes. Some of these processes seem to be stochastic, and are often reversible[122]. Furthermore, the predominance of various processes seems to change with development and from one experimental system to another. So, it becomes difficult to provide a single simple, linear account of the formation of individual presynaptic boutons. Instead, we will describe various processes that are thought to occur and suggest how these might be involved in the formation of new presynaptic sites. We will focus on CNS synapses, which have been the subject of many recent live imaging studies.

As mentioned above, axons of immature neurons show a capacity for evoked recycling of synaptic vesicles along axonal segments, even in the absence of target cells[29,36–42]. Closer examination has revealed the presence of mobile packets of vesicles or vesicular material that travel along axons at rates of up to 0.1–1 μm sec^{-1} (REFS 33,38,39,123–128), sometimes entering the filopodia that extend from growth cones[129] and axonal shafts[38,51]. These packets move intermittently in both directions, often stopping for a while, sometimes splitting into smaller packets or merging into larger clusters. Some of these packets show a capacity for depolarization-evoked exocytosis and endocytosis that is similar in some respects to that observed at bona fide synapses, but differs in others, such as altered dependence on extracellular calcium (REF. 81), reduced sensitivity to tetanus toxin[79] and

STYRYL DYES
Fluorescent cationic dyes that are commonly used to visualize membrane trafficking/recycling.

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a sensitivity to Brefeldin A (REF. 41). A time-lapse study in cultured hippocampal neurons revealed that mobile packets labelled with a GREEN FLUORESCENT PROTEIN (GFP)-tagged variant of the synaptic vesicle molecule Vamp2/synaptobrevin II accumulated rapidly at new axodendritic contacts sites[33]. Furthermore, a capacity for stimulation-evoked synaptic vesicle recycling was subsequently demonstrated at these sites as soon as one hour after axodendritic contact, indicating that functional presynaptic sites had formed at these locations. Intriguingly, retrospective immunohistochemical analysis revealed that these packets were associated with additional presynaptic molecules, including voltage-dependent calcium channels, Sv2, synapsin I and amphiphysin. These findings have led to suggestions that the mobile packets observed in axons might be precursors for the formation of true presynaptic structures[38] or 'prototerminals'[130] — preassembled precursors of presynaptic release sites[130].

The concept that vesicular structures might be used for presynaptic assembly was originally put forward by Vaughn[51]. As mentioned above, electron micrographs of developing spinal cord synapses revealed the presence of dense-core vesicles with spicules projecting from their surface. On the basis of their structural similarity to active zone material and their proximity to presynaptic membranes, Vaughn suggested that these might be involved in the delivery of materials that are necessary for presynaptic differentiation. In agreement with this idea, a previously unknown 80 nm dense-core vesicle was shown to specifically contain multiple active zone components, including the CAZ scaffolding molecules bassoon, piccolo and Rim[131,132], but not characteristic synaptic vesicle molecules (synaptophysin, Vamp2/synaptobrevin II). These 80 nm vesicles, named piccolo/bassoon transport vesicles (PTVs), were suggested to constitute 'active zone precursor vesicles' that could lead to the rapid formation of new active zones on fusion with the presynaptic plasma membrane.

Intriguingly, quantitative immunocytochemical analysis indicated that the bassoon, piccolo and Rim content of presynaptic boutons could be accounted for by their recruitment from integer multiples of the bassoon, piccolo and Rim contents of PTVs, indicating that presynaptic sites might be assembled in unitary fashion from small numbers of precursor particles[132]. Indeed, time-lapse imaging of PTVs labelled with GFP-tagged variants of bassoon revealed that they are transported rapidly along axons[132], and that the formation of new functional synaptic vesicle release sites is preceded by the recruitment of 2–5 PTVs to these sites[121]. Biochemical studies indicate that PTVs also carry other active zone proteins that have been implicated in synaptic vesicle exocytosis, such as Munc13, Munc18, syntaxin, Snap25 and N-type calcium channels[132], as well as α-liprin[133] and ERC (ELKS, Rab6-interacting protein 2 and CAST)[134], which have been implicated in linking together components of the active zone cytoskeletal matrix. It is not known whether PTVs are the sole source for these and other active zone proteins, or whether additional vesicular intermediates carry them to nascent and maturing synapses.

A theme that is emerging from these studies is the use of modular transport packets for presynaptic assembly. Interestingly, all putative transport packets that have been examined so far have shown similar mobility characteristics, namely intermittent travel in both directions, merging into larger clusters or splitting into smaller clusters[33,38,39,121,125,132,135]. However, not all of the mobile particles seem to be part of the same 'prototerminal' population. For example, mobile PTVs do not seem to be tightly associated with synaptic vesicles or with the presynaptic molecule synapsin I, unlike the transport packets described by Ahmari and co-workers[33].

A recent study has described another class of mobile presynaptic structure[42]. Time-lapse microscopy of synaptic vesicles labelled with the styryl dye FM1-43 revealed that functional release sites occasionally gave rise to small mobile vesicle clusters that move along axons, sometimes merging with nearby presynaptic sites. Furthermore, entire FM1-43 puncta were sometimes observed to move from one location to another (see also REFS 34,126). These mobile clusters displayed a capacity for synaptic vesicle release, with functional characteristics similar to those observed at mature presynaptic sites, including increased sensitivity to tetanus toxin and reduced sensitivity to Brefeldin A. Surprisingly, many of these mobile clusters were not juxtaposed to postsynaptic structures, or even to dendrites, but they were associated with the CAZ molecule bassoon. It was therefore suggested that these mobile structures might be well-formed synaptic vesicle release sites that do not have postsynaptic counterparts, and they were termed 'orphan' release sites. The recycling synaptic vesicle pool of orphan release sites was smaller than that of synaptic release sites, but quantitative analysis indicated that synaptic release sites might contain integer multiples of unitary amounts of recycling synaptic vesicle pools that are associated with orphan release sites. Intriguingly, orphan release sites were sometimes observed to give rise to new presynaptic sites at axodendritic contacts, indicating that new presynaptic compartments might be assembled from 'units' of synaptic vesicle release machinery that 'bud off' pre-existing synapses. Although intriguing, these studies raise many questions. What is the origin of these orphan release sites? Are they a consequence of excess presynaptic protein in relation to postsynaptic target availability? Do they result from synaptic destabilization? Does the absence of glia, which are known to profoundly affect neuronal differentiation and synapse formation in culture, make these synapses prone to fall apart[136]? Do orphan release sites occur *in vivo* as well?

If presynaptic sites are formed from mobile precursors, what induces the recruitment of the precursor structures to nascent presynaptic sites? Before delving into the molecular details, we wish to consider the constraints that would be placed on such processes by the dynamics of axodendritic contact establishment. As mentioned above, many axodendritic contacts might be established by dynamic filopodia, which extend from axonal and dendritic growth cones and shafts. Typically, such filopodia have a limited lifespan (in the order of

GREEN FLUORESCENT PROTEIN
Fluorescent protein cloned from the jellyfish *Aequoria victoria*. The most frequently used mutant, EGFP, is excited at 488 nm and has an emission maximum at 510 nm.

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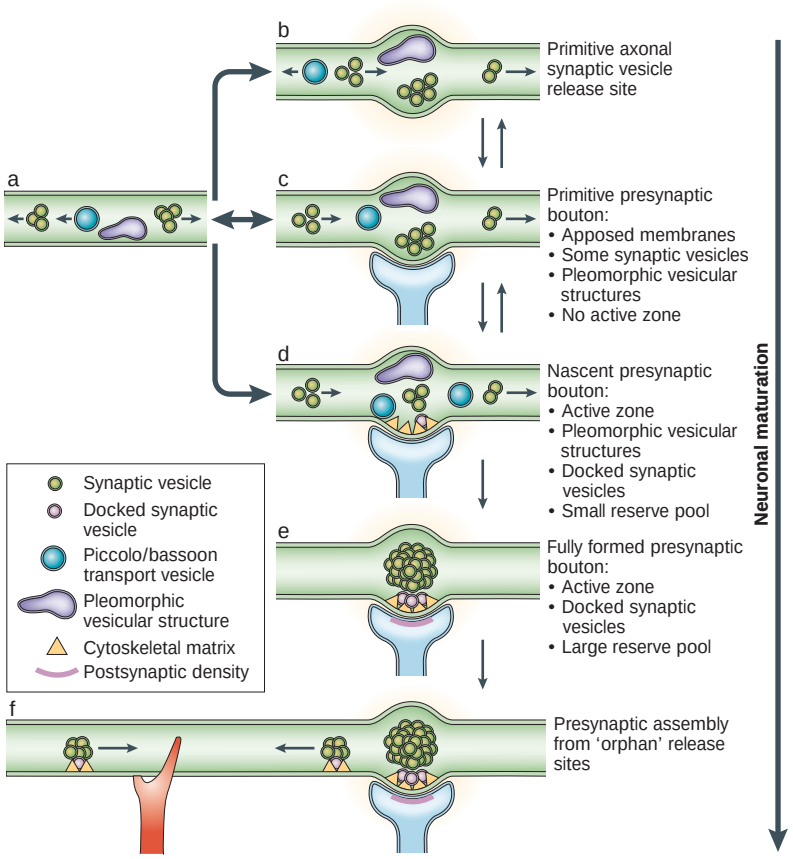

Figure 4 | **A model for presynaptic assembly.** Presynaptic assembly is envisioned to occur by multiple processes that take place over several timescales. **a** | The axons of developing neurons contain several types of transport packets that are used for the assembly of nascent presynaptic structures. These include synaptic vesicle packets, pleomorphic tubulovesicular structures and active zone precursor vesicles such as piccolo/bassoon transport vesicles (PTVs). **b** | In immature axons, primitive sites of synaptic vesicle recycling form 'spontaneously' along axonal segments. **c** | Contacts between axons and targets lead to the accumulation of various transport packets at contact sites, resulting in the formation of 'primitive' presynaptic boutons. These sites display some capacity for synaptic vesicle recycling, but lack the ultrastructural features of mature presynaptic boutons. **d** | Alternatively, the establishment of an axodendritic contact might lead to the rapid formation of an active zone by the fusion of active zone precursor vesicles (such as PTVs) and the subsequent recruitment of synaptic vesicles. It is possible that this scenario becomes more common as neurons mature, but this is unknown. **e** | With time (days), new synapses form large reserve pools of synaptic vesicles, and acquire the structural and functional characteristics of mature synapses. **f** | Once presynaptic boutons have formed, units of active zone material and cognate synaptic vesicle clusters ('orphan' release sites) can 'bud' from established presynaptic sites and wander away, sometimes giving rise to new presynaptic sites. It should be noted that there is much uncertainty with regard to many details of this scheme. For example, it is not known whether presynaptic sites form with a precise temporal order of events. Primitive presynaptic sites (**c**) could form first, followed by active zone formation (arrow from **c** to **d**), or primitive sites could fall apart (arrow from **c** to **a**) and reform later according to scheme **d** (arrow from **a** to **d**). Axonal release sites (**b**) might form first and then become synaptic (arrow from **b** to **c**), or eventually fall apart (arrow from **b** to **a**). Are the transitions shown here reversible? Also, is a particular type of presynaptic structure always formed according to one of these scenarios, or do most scenarios occur for all types of structure, but with a prevalence that depends on the developmental and maturational state of the organism?

10 minutes)[54,64], during which they elongate, extend and subsequently retract. Although it is thought that filopodia can be stabilized by contacts with suitable counterparts, time-lapse recordings repeatedly indicate that the overwhelming majority, even those that contact nearby axons or dendrites, are not stabilized and eventually retract[54,55,118].

These observations might lead to two tentative conclusions. First, molecular cascades involved in presynaptic differentiation often have a narrow time window to stabilize nascent contacts before they are severed by filopodia retraction. Second, the formation of a synaptic junction at a transient axodendritic contact site is a low probability event[34]. The likelihood that a transient contact will develop into a functional presynaptic site might be highly regulated, for example by the degree of matching between specific recognition molecules on both membranes (REFS 122,137,138 and see below). Alternatively, contact stabilization and presynaptic differentiation might be stochastic processes[105] whose outcome is determined by the extent of destabilizing mechanical forces (such as filopodia retraction) acting at any particular moment, the local abundance of stabilizing cell surface molecules, and the presence of appropriate transport packets at the right time and place. Regardless of the exact reasons, it is worth remembering that the molecular processes that underlie presynaptic differentiation are governed to varying degrees by temporal, mechanical and perhaps probabilistic constraints.

At this point we propose a tentative portrayal of the cellular events that are involved in presynapse formation (FIG. 4). This portrayal is admittedly imperfect but is useful for identifying findings that can be consolidated, and questions for which no satisfactory answers are available yet. During early stages of neuronal differentiation, as axons are growing towards their targets, there is an inherent capacity for rudimentary synaptic vesicle recycling at axonal growth cones and along the axonal plasma membrane. Mobile packets of synaptic vesicles, synaptic vesicle precursors and active zone precursors travel up and down axons. As axons reach their target regions, fleeting axodendritic contacts that are initiated by axonal and dendritic growth cones and filopodia induce the clustering of such mobile packets at contact sites. Most of these contacts break up, and the transport packets disperse and renew their migration. As the neurons mature, an increasing proportion of transient contacts do not break up, but develop into functional presynaptic boutons, presumably by fusion of PTVs with the presynaptic membrane, recruitment of tubulovesicular synaptic vesicle precursor transport packets, synaptic vesicle packets and perhaps additional molecules from vesicular or other sources. The new presynaptic sites are not completely stable, however, and occasionally 'orphan release sites' are uncoupled from their nascent postsynaptic partner and migrate to adjacent presynaptic sites or participate in the formation of new ones, presumably at sites of contact with dendritic structures. As neurons mature, protrusive activity of axonal and dendritic filopodia subsides[54,58,62,64,139]. In the adult, protrusive activity becomes relatively rare, but the likelihood that new axodendritic contacts will induce presynaptic release site formation increases[63,124,140].

This portrayal raises many additional questions. For example, where do the mobile synaptic vesicle packets come from? Where are synaptic vesicle precursors[125] processed into prototypical synaptic vesicles? Why do nascent synaptic sites have so few well-formed synaptic

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vesicles and active zones[33] (but see REFS 141,142)? Is the formation of presynaptic release sites preceded by the formation of active zones, or do active zones stabilize 'primitive' release sites? Can orphan release sites form spontaneously, or do they always bud from existing presynaptic sites? Do presynaptic sites form only in the brief time that follows the formation of a new axodendritic contact site, or can presynaptic sites eventually form at existing axodendritic adhesion sites? Does presynaptic bouton formation in the adult rely on the same mechanisms as during development? What induces the recruitment and confinement of presynaptic components to nascent axodendritic contact sites, and what determines whether nascent structures are stabilized? In the next section, we will discuss how answers to some of these questions are emerging from the study of the molecular mechanisms of presynaptic differentiation.

Synapse assembly: a molecular perspective

In parallel to the progress that has been made in elucidating the cellular mechanisms of presynaptic differentiation, considerable progress has been made in identifying the types of molecules and molecular pathways that are involved[143]. As might be expected for such a young field, much is still unknown, and relationships between cellular events and molecular mechanisms are not well understood. Nevertheless, provocative clues are emerging[144].

When considering which molecules might have a role in triggering presynaptic differentiation, CAMs are obvious candidates. Some of the first to be studied were members of the cadherin superfamily of calcium-dependent CAMs[145]. This family, which also includes the protocadherins and the cadherin-related neuronal receptors (CNR), is an attractive candidate[146,147], especially given its central role in cell–cell adhesion between non-neural cells. The best characterized member of this family with regard to synaptogenesis is N-cadherin, which seems to span the synaptic cleft that surrounds the active zone and postsynaptic density[148,149]. These CAMs are linked to the actin cytoskeleton through β-catenin and the actin-binding protein α-catenin[150].

The roles of the cadherins in synaptogenesis are not entirely clear; for example, they do not seem to be essential for maintaining the integrity of some synaptic junctions[142,151–153]. However, a recent study, in which two methods were used to perturb cadherin function, provided some interesting clues[154]. The first method was based on the expression of a dominant-negative variant of N-cadherin that lacked a functional extracellular domain, but was still capable of competing with endogenous cadherins for intracellular binding partners. The second was based on a mutation in αN-catenin (a nervous system-specific form of α-catenin) that interfered with the downstream interactions of cadherins with the actin cytoskeleton. Expression of the dominant-negative N-cadherin variant markedly reduced the number of functional presynaptic boutons that were formed on dendrites, and in particular on lateral dendritic protrusions, which retained their immature, filopodia-like morphology. By contrast, blocking the downstream pathways that are mediated by αN-catenin did not strongly affect presynaptic formation. These findings indicate that the primary functions of cadherins during synaptogenesis might be related to their adhesive properties, and their downstream effector functions might be secondary. Intriguingly, the effects of the dominant-negative perturbation were reported to decline with neuronal maturation, but were still evident where filopodial/spine tips were concerned.

Taken together, these findings indicate that the main role of cadherins, and perhaps other adhesion molecules, is to stabilize short-lived, dynamic axodendritic contacts just long enough to allow more specialized membrane proteins to interact with their counterparts and activate specific intracellular cascades that induce pre- (and post-) synaptic differentiation. This permissive model of cadherin function fits well with the observation that N-cadherin alone cannot induce the formation of presynaptic release sites[155], and that the genetic elimination of β-catenin does not reduce presynaptic release site number or drastically affect their structural properties[156]. Furthermore, the diminishing requirement for cadherins in older networks is in agreement with the reduced levels of cellular dynamics associated with neuronal maturation, and a reduced need for adhesion-based stabilization of labile contacts. This permissive model is attractive because it predicts that the probability that a nascent axodendritic contact will induce presynaptic (and postsynaptic) differentiation would depend to a large extent on the specific adhesion molecules that are present on axonal and dendritic membranes and on the degree to which these molecules adhere to each other[122,137,138,157]. It also allows presynaptic differentiation to occur through common molecular mechanisms that rely on limited numbers of highly specialized molecules (see below). This mode of action is reminiscent of the proposed roles for adhesion molecules during the formation of the 'immunological synapse' — the interface that is formed between T cells and antigen presenting cells (APCs) of the immune system[158].

The maturation-associated reduced sensitivity to perturbations that affect N-cadherin adhesiveness is paralleled by a similar reduction in the sensitivity to drugs that depolymerize actin[142]. For example, exposure of immature cultured neurons (5–6 days *in vitro*) to latrunculin A, an actin depolymerizing drug, for 4–8 hours leads to loss of presynaptic sites and dispersal of synaptic vesicles. As the neurons mature, presynaptic sites become increasingly resistant to this manipulation, and by 18–20 days in culture they do not seem to be affected structurally or functionally by this treatment (although interestingly, exposure to latrunculin A is associated with a nearly complete loss of N-cadherin from presynaptic sites). These findings indicate that during early stages of neuronal differentiation, the actin-based cytoskeleton is important for the establishment and maintenance of presynaptic sites, and that the dependence on actin (and N-cadherin) for presynaptic integrity decreases with neuronal maturation.

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At mature synapses, polymerized actin seems to form a perimeter around the presynaptic clusters of vesicles[159–162]. Intriguingly, electrical stimulation leads to actin polymerization at presynaptic terminals[120,160,161], and it has been suggested that this polymerization might be necessary for synaptic vesicle endocytosis and rerouting to the reserve pool[160], or for keeping diffusible regulatory proteins such as synapsin at presynaptic sites[161]. However, actin depolymerization does not lead to synaptic vesicle dispersal in mature synapses[142,160,161,163]. So, what maintains the reserve pool of synaptic vesicles, anchors it to active zones and facilitates the translocation of synaptic vesicles from the reserve pool to the presynaptic membrane and back? This has long been thought to involve proteins of the synapsin family, on the basis of evidence from a myriad of studies showing that synapsin binds synaptic vesicles as well as actin filaments[164], and that in its absence the reserve pool is reduced[165–167]. The association of synapsin with synaptic vesicles and microfilaments is regulated by synaptic activity through the activation of calcium/calmodulin-dependent kinase II (CaMKII)[106] and mitogen-activated kinase (MAP)[168,169], which directly phosphorylate synapsin. It is argued that synapsins tether synaptic vesicles to cytoskeletal elements or each other at presynaptic sites, until stimulation-induced phosphorylation releases these tethers and allows vesicle mobilization to the plasma membrane. Indeed, activity was shown to cause a transient phosphorylation-dependent dispersal of GFP-tagged synapsin I that slightly precedes synaptic vesicle exocytosis[170,171]. However, as dispersion of actin filaments does not lead to synaptic vesicle dispersal[161], it seems unlikely that synapsin-based tethering of synaptic vesicles to filamentous actin (F-actin) provides the sole mechanism of synaptic vesicle retention at presynaptic boutons[172].

Somewhat unexpectedly, synaptic vesicle retention at presynaptic boutons was recently shown to depend on β-catenin[156]. A conditional ablation was used to suppress the expression of β-catenin in pyramidal neurons in the mouse hippocampus. Although synapse formation *per se* and presynaptic ultrastructure were not grossly affected, the number of synaptic vesicles that clustered at presynaptic boutons was significantly reduced. Further analysis revealed that the vesicles were more mobile and much less restricted to presynaptic sites, indicating that β-catenin was somehow involved in the clustering and tethering of synaptic vesicles at presynaptic sites. Interestingly, these effects were not dependent on α-catenin-mediated interactions with the actin cytoskeleton, but depended on interactions with unidentified molecules through the carboxyl (C) terminus PDZ binding motif of β-catenin.

A similar role was recently proposed for NCAM (neural CAM)[135]. Live imaging indicated that intracellular organelles of trans-golgi network origin are linked to clusters of extracellular-facing NCAM, and that these complexes travel together along axons until they are trapped at axodendritic contacts sites. The authors suggested that NCAM might mediate the anchoring of intracellular organelles at synaptic sites. Mice lacking NCAM form NMJs whose structure and molecular architecture are nearly normal, although they are slightly smaller and their differentiation is somewhat delayed[173,174]. But on closer examination, a large fraction of synaptic vesicle recycling seems to occur at sites that are not juxtaposed to postsynaptic receptors. Intriguingly, these extrasynaptic sites retain certain functional characteristics that are typical of immature synaptic vesicle recycling sites, namely sensitivity to Brefeldin A and to L-type calcium channel blockers[175]. One possible interpretation of these findings is that NCAM is important for targeting, clustering and confining synaptic vesicles, synaptic vesicular precursors and perhaps other presynaptic molecules to bona fide presynaptic sites. But it is important to keep in mind that presynaptic sites ultimately form in NCAM-deficient mice, as in α-/β-catenin-deficient mice and in the presence of DOMINANT-NEGATIVE forms of N-cadherin.

The lack of solid evidence for an instructive role for classical adhesion molecules in inducing presynaptic differentiation has led several groups to develop assays to test the ability of particular molecules to trigger the formation of synaptic vesicle release sites. These involve expressing candidate molecules in heterologous cells, such as human embryonic kidney (HEK293) cells, and then evaluating whether neurons growing over these cells form 'hemi-synapses' — presynaptic boutons without postsynaptic counterparts. N-cadherin and other candidate molecules, including ephrin-B1, agrin, TAG1 and L1, could not induce presynaptic differentiation in these assays. However, the PSD molecules neuroligin 1 and 2 (REF. 155), and the immunoglobulin domain-containing protein SynCAM (synaptic CAM)[176], showed a remarkable capacity to induce synaptic vesicle clustering along overlying axons. The hemi-synapses that formed on neuroligin-expressing cells contained the presynaptic molecule synapsin I, showed a capacity for depolarization-dependent synaptic vesicle recycling, and displayed ultrastructural characteristics that are typical of presynaptic boutons[155]. Similarly, functional measurements of synaptic vesicle recycling indicated that the functional properties of hemi-synapses that formed on SynCAM-expressing cells were similar to those of true presynaptic boutons[176].

SynCAM is a homophilic calcium-independent CAM that is found on both sides of the synapse[176]. By contrast, neuroligin is thought to act heterotypically in a calcium-dependent manner[177] through presynaptically-expressed β-neurexin[155,178,179]. Indeed, clustering of β-neurexin is observed in cultured hippocampal neurons at sites of axonal contact with neuroligin-coated beads[180]. Furthermore, clustering of β-neurexin by neuroligin-coated beads or by other means leads to the formation of synaptic vesicle clusters that have a capacity for activity-induced recycling[180]. Finally, soluble β-neurexin was shown to inhibit vesicle clustering that was induced by postsynaptic target cells[155]. Taken together, these findings strongly indicate that the clustering of β-neurexin can induce the formation of synaptic vesicle release sites. Therefore, the neuroligin–β-neurexin interaction might be a key molecular step in the induction of presynaptic differentiation.

DOMINANT NEGATIVE
A mutant molecule that can form a heteromeric complex with the normal molecule, knocking out the activity of the entire complex.

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It is worth noting, however, that preliminary analysis of mice deficient in neuroligin 1, 2 and 3 indicates that synapse formation is not drastically altered (F. Varoqueaux and N. Brose, personal communication).

Many molecules that are proposed to be involved in presynaptic differentiation are thought to reside on axons and dendrites alike and act through homophilic interactions. This property raises a conceptual problem — how would axons avoid the formation of presynaptic boutons at axo-axonic contact sites? Although there are axo-axonic synapses, most synapses are axodendritic. This implies that some of the molecules involved in triggering presynaptic differentiation act through heterophilic interactions, with axons and dendrites expressing different but complementary pairs of such molecules. In this regard, the dendritic neuroligins and axonal neurexins are attractive candidates.

SynCAM and β-neurexin have similar C-terminal tails that include PDZ BINDING MOTIFS, indicating that they might trigger active zone formation through similar signalling pathways. One PDZ DOMAIN-containing protein that can bind the C-terminal tail of both molecules is calcium/calmodulin-dependent serine protein kinase (CASK)[176,181]. CASK is a multi-domain scaffold protein that, together with protein 4.1 and spectrin, supports the polymerization of actin on neurexins[182]. At present, it is unclear whether these interactions help to stabilize the adhesion site, promote clustering or participate in signalling cascades that trigger active zone formation. One interesting finding concerning CASK is its ability to form a tripartite complex with the ADAPTOR PROTEINS veli and Mint (Munc18-interacting protein)[183,184] and N-type calcium channels[185]. One prediction of such a cascade of molecular interactions is that neuroligin-induced clustering of β-neurexin[180] should lead to the recruitment not only of CASK, but also N-type voltage-gated calcium channels. Although pseudogenetic studies — for example, using RNA INTERFERENCE — support a role for CASK in this pathway[186], data on SynCAM and β-neurexin are lacking. Intriguingly, mouse knockout data on α-neurexin, a longer version of β-neurexin that is transcribed from the same gene[187], demonstrate that this form of neurexin is required for the synaptic targeting of voltage-gated calcium channels[188]. In contrast to β-neurexin, which binds neuroligin[178], α-neurexin interacts through its extracellular domain with α-latrotoxin[189] and neurexophilin[190]. Importantly, both α- and β-neurexin have identical intracellular domains that can bind CASK[181], indicating that they might be linked in a similar manner to the intracellular cortical cytoskeleton. The neurexins also bind Mint[191] and synaptotagmin[192], providing molecular links not only to the calcium channels but also the synaptic vesicle fusion machinery. Although the main components of the CAZ, including piccolo, bassoon, Rim, ERCs, liprins and Munc13, are tightly associated with the synaptic junction, how the aforementioned SynCAM, neurexin and CASK complex are attached at a molecular level is not known. The mapping of the domains that anchor each to the active zone and their interacting proteins should help to resolve this question.

There seems to be a hierarchy in the assembly of the CAZ. The Munc13 protein, which is involved in synaptic vesicle priming, seems to require Rim1α for its synaptic localization[193], but Rim1α, a Rab3a interacting protein[194], is not required for the synaptic localization of its other binding partners, liprin-1α, ERCs/Cast1, Rim binding proteins or synaptotagmin[193]. By contrast, Rim1α requires its PDZ domain and its interactions with the C-terminal tail of ERC2/Cast1 for its own synaptic localization[134,195]. ERC2/Cast1, an active zone protein containing primarily COILED-COIL DOMAINS[134,195], was also found to promote the synaptic localization of liprin-1α[196]. This recruitment required the amino (N) terminus of ERC2/Cast1, a region that was recently found to bind the third coiled-coil domain of both piccolo and bassoon but not to influence the synaptic recruitment of these large active zone proteins[197]. These data indicate that piccolo and bassoon might be the lynchpins in the molecular assembly of the CAZ. However, knockout data on bassoon[198] do not support this conclusion, and similar data on piccolo are currently unavailable.

As we have discussed, there is evidence that active zones and presynaptic boutons are at least partially assembled from vesicular intermediates and modular transport packets. So, many of the associations described above could initially occur within the endoplasmic reticulum and/or Golgi apparatus, which might act as platforms for protein complex formation and generation of highly specialized transport organelles. At present, however, an understanding of these issues in molecular terms is still lacking. How many types of vesicular intermediates are involved? What molecules and molecular complexes are carried by each type? What molecules are involved in the axonal targeting and transport of these intermediates? What causes these transport packets to stop at particular sites? Do these vesicular intermediates fuse with the axonal membrane, and if so, what triggers these fusion events? How are these processes related to the molecular interactions that are thought to trigger presynaptic differentiation? Although considerable progress has been made in the identification of molecules involved in presynaptic differentiation, further work is required to bridge the gap between our understanding of presynaptic differentiation in terms of molecules and of cellular physiology.

Concluding remarks

The formation of presynaptic sites is a dynamic and elaborate process that is governed by various forces and mechanisms acting over various timescales. At present, however, our understanding of the relationships between the formation of individual active zones and global developmental processes, between structural maturation and functional maturation, and between molecular cascades and cellular mechanisms of presynaptic assembly, are still vague.

We have attempted to find unifying themes within the myriad of data from several decades of work, but the consolidation of all data into a canonical model of presynaptic assembly seems difficult at present. It might be incorrect to expect that presynaptic terminal formation

PDZ BINDING MOTIF
A peptide sequence that binds to a PDZ domain.

PDZ DOMAIN
A peptide-binding domain that is important for the organization of membrane proteins, particularly at cell–cell junctions, including synapses. It can bind to the carboxyl termini of proteins or can form dimers with other PDZ domains. PDZ domains are named after the proteins in which these sequence motifs were originally identified (PSD95, Discs large, zona occludens 1).

ADAPTOR PROTEINS
Proteins that contributes to cellular function by recruiting other proteins to a complex. Such molecules often contain several protein–protein interaction domains.

RNA INTERFERENCE
(RNAi). A method by which double-stranded RNA that is encoded on an exogenous vector can be used to interfere with normal RNA processing, causing rapid degradation of the endogenous RNA and thereby precluding translation. This provides a simple way of studying the effects of the absence of a gene product in simple organisms and in cells.

COILED-COIL DOMAIN
A protein domain that forms a bundle of two or three α-helices. Short coiled-coil domains are involved in protein interactions, whereas long coiled-coil domains, which form long rods, occur in structural or motor proteins.

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at the invertebrate NMJ will resemble the formation of presynaptic boutons on dendritic spines in the primate cortex, or that the formation of presynaptic sites early in development will be identical to the formation of presynaptic sites in the adult[63,124,140]. So, there might be several right answers to any one question.

It is also important to remember that the formation of a presynaptic terminal/bouton is only part of the processes by which the entire synapse is formed. So, presynaptic differentiation involves many reciprocal signals that pass between pre- and postsynaptic sides, some of which have been addressed in recent reviews on synapse formation[9,10,14,25,122,138,144,199,200]. Other related issues, such as the sources of presynaptic structural and functional heterogeneity[201], the nature of presynaptic sites that form but do not function ('mute' synapses) and the roles of glia[202] have not been addressed here, but a good comprehension of these topics will probably be important for obtaining a satisfactory understanding of presynaptic assembly. It is hoped that future findings will lead to the convergence of disparate facts into a relatively simple and coherent picture of presynaptic assembly.

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Competing interests statement

The authors declare that they have no competing interests.

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At-a-glance

- Presynaptic compartments are characterized by the presence of hundreds to thousands of neurotransmitter-filled synaptic vesicles, and by active zones — specialized regions of the presynaptic plasma membrane where synaptic vesicles dock, fuse and release neurotransmitter into the synaptic cleft.
- Much of our understanding of presynapse formation comes from work on the neuromuscular junction. Motor axons do not form synapses *en route* to their final destinations, so these studies have tended to highlight the intimate relationships between axonal growth cones and emerging synaptic vesicle release sites.
- By contrast, in the vertebrate CNS, presynaptic sites are distributed along the length of axonal segments. So, the potential to form presynaptic boutons might be distributed along the entire axonal membrane, at least in immature neurons.
- One emerging theme is the use of modular transport packets for presynaptic assembly. A dense-core vesicle has been identified that contains active zone components, including the cytoskeletal matrix scaffolding molecules bassoon, piccolo and Rim. These vesicles, named piccolo/bassoon transport vesicles (PTVs) were suggested to constitute 'active zone precursor vesicles'. The endoplasmic reticulum and Golgi apparatus might act as platforms for protein complex formation and generation of specialized transport organelles.
- Cell adhesion molecules (CAMs) are obvious candidates for a role in triggering presynaptic differentiation. The best-characterized member of this family with regard to synaptogenesis is N-cadherin. It is suggested that the main role of cadherins is to stabilize dynamic axodendritic contacts just long enough to allow more specialized membrane proteins to activate specific intracellular cascades that induce pre- and postsynaptic differentiation.
- Several groups have developed assays to test the ability of particular molecules to trigger the formation of synaptic vesicle release sites. These involve expressing candidate molecules in heterologous cells, such as human embryonic kidney cells, then evaluating whether neurons growing over these cells form 'hemi-synapses'; that is, presynaptic boutons without postsynaptic counterparts.
- The postsynaptic density molecules neuroligin 1 and 2, and the immunoglobulin domain-containing protein SynCAM, show a remarkable capacity to induce synaptic vesicle clustering along overlying axons. SynCAM is an adhesion molecule that is found on both sides of the synapse, whereas neuroligin is thought to act heterotopically by inducing the clustering of presynaptically-expressed β-neurexin.
- The formation of presynaptic sites is a dynamic and elaborate process that is governed by various forces and mechanisms that act over various time scales. We still have much to learn about the relationships between the formation of individual active zones and global developmental processes, structural maturation and functional maturation, and molecular cascades and cellular mechanisms of presynaptic assembly.

Biographies

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