

Vesicle turnover in developing neurons: how to build a presynaptic terminal

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Over the past decade, evidence has accumulated indicating that, during development, the construction of synapses – the sites of communication between neurons – might rely on the utilization of preassembled sets of synaptic proteins, which have already accumulated in the axon and are highly mobile, before getting recruited to the sites of contact with the postsynaptic neuron. In this review, we discuss evidence from most recent publications pointing to the existence of active vesicle traffic and turnover in developing neurons, which lead to the construction of new synaptic sites.

SYNAPSES (see Glossary) are specialized and asymmetrical intercellular junctions, characterized by the close apposition of the presynaptic and postsynaptic plasmalemma, which are separated by the SYNAPTIC CLEFT. The presynaptic compartment contains clusters of vesicles filled with neurotransmitter called SYNAPTIC VESICLES. Through regulated fusion with specialized portions of the presynaptic plasma membrane called ACTIVE ZONES, synaptic vesicles release their content into the synaptic cleft. The postsynaptic compartment is characterized by the accumulation of neurotransmitter receptors and the presence of a submembranous electron-dense scaffold termed the POSTSYNAPTIC DENSITY (PSD). In the most typical case, the presynaptic compartment is located in the ‘outpocketing’ of an axonal branch and the postsynaptic compartment is at the surface of the cell body or a dendrite. In many cortical synapses, the postsynaptic site is localized in small protrusions of the postsynaptic cell called spines, which are generally present in peripheral dendrites [1].

In recent years, the study of synaptogenesis (the formation of synaptic contacts) has taken great advantage of a unique cellular system – the hippocampal neurons in primary culture, which develop *in vitro* through a stereotyped sequence of events [2]. Shortly after they have attached to the substrate, neurons form highly motile lamellipodia (stage 1), which then condense to form minor processes with similar lengths (stage 2). Polarity becomes evident when one of these processes begins elongating faster and differentiating into axon (stage 3). After a few days, the shorter processes acquire morphological and biochemical features typical of dendrites (stage 4).

Eventually, neuronal processes become interconnected and functional synapses are formed (Figure 1a). The process of synaptogenesis is initiated by motile axonal FILOPODIA, which search for potential targets. Cell

Glossary

Active Zone: Portion of the presynaptic membrane located opposite the postsynaptic density across the synaptic cleft. The active zone is the site of synaptic vesicle docking and neurotransmitter release. At central synapses, it is characterized by the presence of a dense matrix consisting of proteins such as RIM, Piccolo and Bassoon, also called active zone proteins.

Dense Core Vesicle: Vesicle (diameter 70–200 nm) with an electron-dense core that store and release peptide neurotransmitters. They are assembled in the area surrounding the *trans*-Golgi network, thus the availability of vesicles loaded with peptides at the nerve ending depends on vesicle supply from the perikaryal region.

Depolarization: Change in the differential charge across the neuronal membrane, produced by ionic fluxes through the plasma membrane and resulting in a net accumulation of positive charge on the inside of the cell. Depolarization of the nerve terminal opens voltage-dependent calcium channels, which trigger fusion of synaptic vesicles and neurotransmitter release.

Filopodia: Cylindrical projections of neurites playing an inductive role in synapse formation.

Growth Cone: Specialized region at the tip of a growing axons responsible for sensing the local environment and moving towards the target cell of the neuron.

Pleiomorphic Vesicles And Tubulovesicular Structures: Organelles with variable morphology, ranging in shape from spherical to tubular, structure, and lengths of up to 1 μ m that function as carrier vesicles.

Postsynaptic Density (PSD): Dense matrix adjacent to the cytoplasmic face of the postsynaptic membrane. It is a scaffold of modular proteins that mediate receptor clustering, regulate receptor function, control receptor internalization and link the postsynaptic receptors to the cytoskeleton; they also have signaling function.

Synapse: Specialized intercellular junction between neurons, or between neurons and other excitable cells, where signals are propagated from one cell to another with high spatial precision and speed. It is formed by a presynaptic terminal and a postsynaptic compartment.

Synaptic Cleft: Intercellular space between the pre and postsynaptic compartments.

Synaptic Vesicles: Clear vesicles (diameter 35–50 nm), which store and release classical nonpeptide neurotransmitters. At the presynaptic terminal, they undergo a local exo-endocytic recycling and at each cycle they are reloaded with neurotransmitter molecules.

Synaptic Proteins: proteins specifically localized in the presynaptic compartment. Proteins mentioned in the present review include:

- **SNAREs:** Protein complex consisting of the synaptic vesicle protein synaptobrevin/VAMP, and the two proteins of the plasma membrane, syntaxin and SNAP25. These three proteins form a complex that is necessary for the exocytosis of synaptic vesicle.
- **Synaptophysin:** Synaptic-vesicle protein characterized by a single transmembrane region. By interacting with synaptobrevin/VAMP, it regulates its availability for the formation of the SNARE complex.
- **Sec1/Munc18:** Protein able to bind to syntaxin, involved in the regulation of SNARE assembly.
- **Munc13/UNC-13:** Protein of the active zone, required for the calcium-triggered exocytosis.

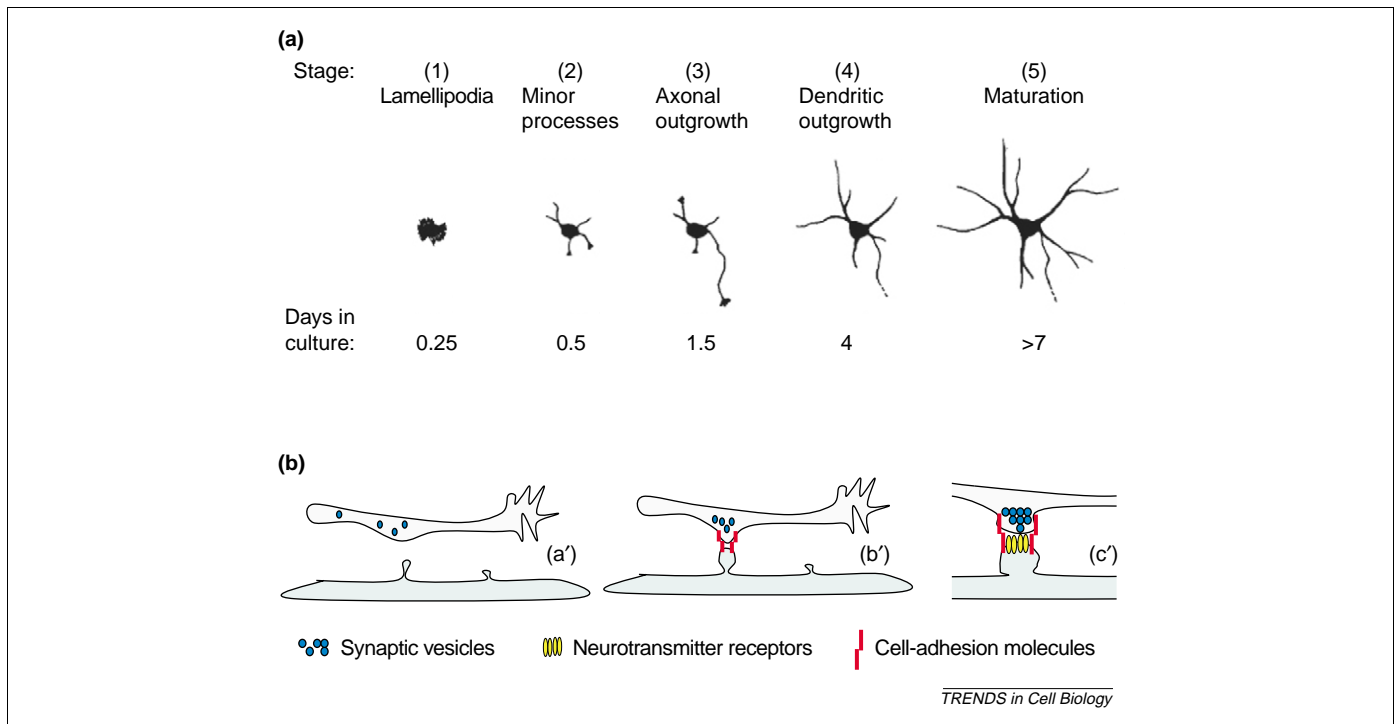


Figure 1. Stages of neuronal development and of synapse formation. (a) Stages in the development of hippocampal neurons in culture. Approximate times for each stage of development are shown at the bottom. Reproduced from [2]. (b) Key steps in synaptogenesis. Motile filopodia search for potential partners (a'). Cell adhesion molecules stabilize the sites of cell contact (b'). Synaptic vesicles cluster at presynaptic sites, whereas neurotransmitter receptors accumulate in the postsynaptic membrane (c').

adhesion molecules then direct and stabilize the sites of cell contact (Figure 1b; Box 1). After the contact, active-zone proteins and synaptic vesicles accumulate at the presynaptic site, whereas neurotransmitter receptors and scaffolding proteins assemble on the postsynaptic membrane (Figure 1b).

One of the most interesting aspects of the formation of synaptic contacts is the ability of axons to establish functional synaptic transmission shortly after contact with the target cell [3]. The rapidity of synapse formation suggests that, within axonal GROWTH CONES and filopodia, a fully competent neurotransmitter-secretory machinery, together with an efficient mechanism for coupling of excitation and secretion, exists even before their contact with a target cell; in the past decade, supporting evidence for this hypothesis has accumulated.

The scope of this review allows summarizing evidence from recent publications showing that developing neurons (i.e. immature, undifferentiated neurons in the process of acquiring their mature features) possess pools of vesicles that undergo active traffic and turnover and lead to the construction of new synaptic sites.

Methods for monitoring synaptic-vesicle recycling and traffic

The first evidence for the existence of actively recycling vesicles in developing neurons dates back to the beginning of 1990s, when morphological methods for studying vesicle turnover in neuronal cells became available (Figure 2). Cloning of synaptic-vesicle proteins made it possible to generate antibodies specifically raised against intravesicular epitopes of such proteins. The first intravesicular antibody of the synaptic vesicle was raised against the

intravesicular domain of the synaptic-vesicle protein synaptotagmin (Syt-ecto abs) [4]. Upon fusion of synaptic vesicles with the plasma membrane, the intravesicular domain of synaptotagmin becomes exposed to the neuronal surface, allowing the binding of Syt-ecto abs and their subsequent internalization through the endocytosis of synaptic vesicle (Figure 2a) [4]. An alternative approach for studying vesicle turnover in living neurons is based on the development of lipophilic dyes. The first of these dyes was FM1-43 [5,6], which labels endocytic vesicles very effectively and dissociates from membranes upon re-exposure of the vesicle lumen to the extracellular medium during exocytosis (Figure 2b). At presynaptic sites, where the membranes of synaptic vesicles are primarily involved in exo-endocytotic recycling, the exocytosis of these vesicle can be monitored by studying the loading and unloading of the dye [5-8]. In the past decade, the extensive use of FM dyes to monitor the exocytosis of synaptic vesicles has established them as the tools of choice for elucidating the mechanisms underlying activity-dependent secretion, which occur at mature synapses. Although Syt-ecto abs are less effective at monitoring the kinetics of synaptic-vesicle exocytosis with close temporal fidelity, they show a high specificity of synaptic-vesicle labeling, which allows their use at sites where synaptic vesicle turnover might not be predominant over other types of recycling. One of these situations is mimicked by developing isolated axons. The recent introduction of synapto-pHluorins (pH-sensitive green fluorescent protein fused to a synaptic vesicle protein) [8-10], might further improve the measurement of the kinetics of exo-endocytotic recycling in developing neurons by

Box 1. Retrograde signals in synapse formation

Synapse formation occurs by a coordinated development of pre and postsynaptic compartments, requiring a continuous exchange of information between the two partner cells. Recent work from Colicos [50] and Nikonenko [51] laboratories indicate that the extension of presynaptic filopodial protrusions and the formation of new terminals depend upon activation of postsynaptic AMPA and NMDA receptors. These data suggest that the dynamics of presynaptic terminal not only are affected by the action of released glutamate on presynaptic AMPA and kainate receptors but also involve postsynaptic feedback mechanisms that would be important for coordinating the formation of a new functional synapse [52]. Because the description of retrograde signaling is beyond the scope of this review, only the main mechanisms that are thought to be involved in synapse formation and maturation, and without taking into account the retrograde signals involved in synaptic remodeling and plasticity, are summarized here.

There are three classes of retrograde signals [53]: membrane-permeable soluble molecules, such as nitric oxide (NO); membrane-impermeable soluble molecules, such as neurotrophins; and direct signaling mediated by physical interaction of membrane-bound proteins and/or transmembrane proteins. These signals appear to be differentially involved at different stages of synapse formation. Before synaptic contact, Salinas and colleagues [54] showed that presynaptic differentiation of the growth cone is induced by soluble retrograde signals released from the target cell. WNT-7a, a factor secreted by granule cells in the cerebellum, induced the remodeling of growth-cone microtubules [54]. Other potential soluble retrograde signals involved in synaptogenesis are neurotrophins, which support both survival and differentiation of neurons, as well as synaptic maturation [53]. During initial cell-cell contact and recognition, signaling possibly depends upon specific molecules present on the neuronal surfaces. Intercellular interactions are most probably mediated by cell adhesion molecules, which are membrane-anchored proteins the extracellular domains of which directly interact to help with holding the membranes of the two

cells together. Recent functional studies suggest that adhesion molecules could be involved not only in the recognition of the appropriate target by an arriving axon, but also (i) in the formation and alignment of the pre and postsynaptic compartments and (ii) in the regulation of synaptic structure and function. The latter step is important for both synapse maturation and synaptic plasticity, which is believed to involve changes in synaptic size [55].

Four major groups of adhesion molecules have been identified at synapses: classical cadherin, cadherin-like and cadherin-related neuronal receptors, members of the immunoglobulin-like (Ig) superfamily and the neuroligin-neurexin system [56]. Overall, it seems possible that all synapses bear at least one cadherin-like adhesion molecule and at least one immunoglobulin superfamily member [55]. Classical cadherin-catenin complexes are required for the early stage of synapse formation, where they regulate the organization of pre and postsynaptic structures [57]. Perturbation of cadherin function significantly affects spine morphology of hippocampal neurons and, in turn, the morphology of presynaptic terminals [58]. It has recently been shown that complexes of neuroligins and neuroligin-1 and -2 [59] and of SynCAM-1 [60] play a role in the differentiation of presynaptic specializations. Expression of these adhesion molecules in non-neuronal cells induces cocultured hippocampal neurons to differentiate presynaptic compartments at sites of contact with the transfected cells, thus indicating that adhesive interactions could be sufficient for at least some aspects of synapse formations [55]. In addition to cadherins, neuroligins and neuroligins, other proteins, such as ephrins and Eph receptors and integrins, also appear to be involved in synapse formation. Ephrin-Eph receptor interaction appears to be important for not only axon guidance but also the formation and maintenance of synapses, possibly through the clustering of NMDA receptors [55,61]. Integrins are involved in the maturation of synapses, triggered by increased synaptic activity through a switch in the composition of NMDA subunit [37].

quantitative live imaging, combining the advantages of both methods.

Vesicle turnover and neurotransmitter release before synaptogenesis

Using Syt-ecto abs, the existence of pleiomorphic clear vesicles, many of which were in the same size range as mature synaptic vesicles and able to undergo repetitive cycles of fusion with the plasma membrane, was demonstrated in hippocampal neurons at stages preceding synaptogenesis (2–4 days *in vitro*) [4]. These vesicles were immunopositive for different markers of synaptic vesicles, including SYNAPTOPHYSIN and SYNAPTOBREVIN/VAMP2 [4,11,12]. Recently, the vesicular glutamate transporter v-Glut1 was detected in recycling vesicles present in axonal growth cones ([13]; U. Schenk and M. Matteoli, unpublished); this supports the hypothesis, previously raised by functional assays [14,15], that recycling of vesicles in immature neurons of the central nervous system might already be associated with neurotransmitter release.

Recent evidence for regulation of the motility of axonal and dendritic filopodia by neurotransmitters suggest that recycling of synaptic vesicles in axons could play an 'instructive' role in the establishment of new synaptic contacts during early development. Studies by De Paola and colleagues [16] show that glutamate promotes the formation of dynamic extensions of axonal filopodia, through activation of AMPA receptors. Furthermore, low

concentrations of glutamate increased filopodial motility by binding to kainate receptors [17], whereas high glutamate concentrations inhibited the mobility of axonal filopodia [17,18], as well as that of the entire growth cone [19], through activating AMPA [18,19] and Kainate [17,18] receptors. Similarly, glutamate blocked spine motility by binding to postsynaptic AMPA receptors [20]. Thus, glutamate released during repetitive fusion of vesicles might modulate synaptogenesis by binding to its receptors present on both pre and postsynaptic compartments.

Motile clusters of vesicles are recruited to the nascent synapse

Time-lapse videomicroscopy, using fluorochrome-CY3-conjugated Syt-ecto abs and complemented by electron microscopical detection of the internalized antibodies, revealed that recycling vesicles present in isolated axons of hippocampal neurons were arranged in highly motile clusters (Figure 3a,b) [11] that, similar to synaptic vesicles at synaptic sites [21], were disrupted by exposure to okadaic acid (a specific inhibitor of phosphoserine and/or phosphothreonine protein phosphatase 1 and 2a). Motile clusters present in nonsynaptic regions moved in anterograde and retrograde directions and coalesced into larger aggregates that, upon contact with the target neuron, became immobilized to form typical presynaptic terminals [11]. Electron microscopical analysis confirmed that the motile packages were formed mainly by clear vesicles, with the sporadic presence of larger vesicles, sometimes with a

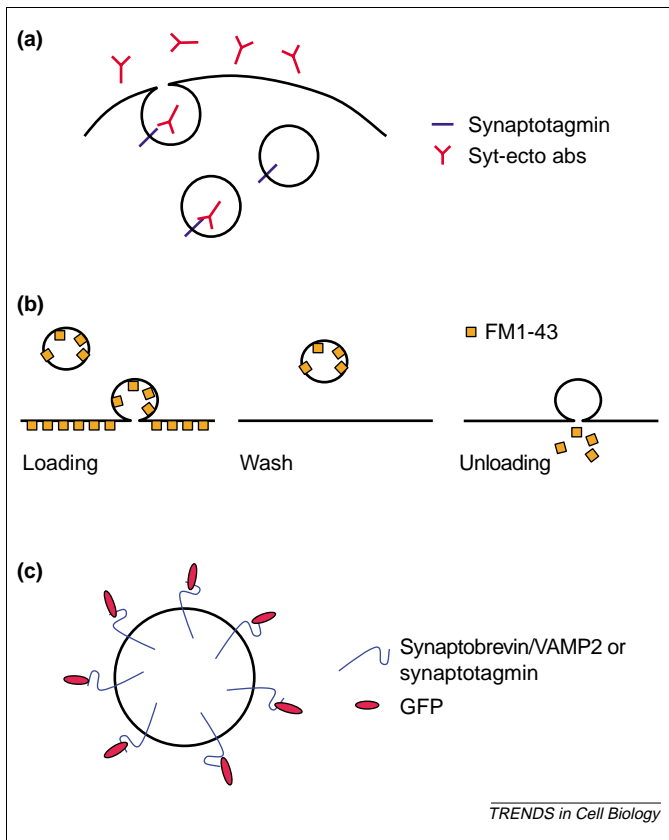


Figure 2. Methods to monitor the recycling (a and b) and traffic (a-c) of synaptic vesicles in developing neurons. (a) Use of antibodies against the intravesicular domain of the synaptic vesicle protein synaptotagmin (Syt-ecto abs). Upon fusion of synaptic vesicles with the plasma membrane, the intravesicular domain of synaptotagmin becomes exposed to the neuronal surface, allowing the binding of Syt-ecto abs and their subsequent internalization through synaptic-vesicle endocytosis. (b) Use of the lipophilic dye FM1-43, which very effectively labels endocytic vesicles (loading and wash) and dissociates from membranes upon re-exposure of the vesicle lumen to the extracellular medium during exocytosis (unloading). (c) Use of green fluorescent protein (GFP) to follow exogenously expressed proteins of synaptic vesicles in living cells.

dense core (DENSE CORE VESICLES) (Figure 3c–e) [11]. These data demonstrate that presynaptic compartments might form through the recruitment of preassembled clusters of synaptic vesicle to the sites of cell contact. Later studies revealed that the sites of immobilization could possibly be specified by the exocyst complex [22] – a set of proteins that determine the sites of vesicle targeting in yeast and epithelial cells [23] (also see below).

Similar to the observation in hippocampal neurons, a local recycling of vesicles within axons has also been demonstrated in spinal-cord neurons of *Xenopus* labeled with FM1-43 [24,25]. Loading of the dye into cultured *Xenopus* neurons upon DEPOLARIZATION resulted in a punctate fluorescent staining along the entire neurite, matching the labeling with synaptic-vesicle markers [24,25]. FM1-43-labeled spots were highly motile and coalesced into nascent presynaptic nerve terminals upon contact with a postsynaptic target [24]. Using patches of membranes rich in acetylcholine (ACh) receptors and attached to a recording electrode, to sense locally released neurotransmitters, it was shown that recycling vesicles from isolated axons released ACh [25–27]. After the formation of synapses, neurons showed reduced capability for neurotransmitter release along the neurite, suggesting

that ‘packages’ of ACh molecules, which are available for release before the formation of nerve–muscle synapse, were depleted from extrasynaptic regions [28].

More recently, the use of green fluorescent protein (GFP) to follow exogenously expressed proteins in living cells allowed the revisiting of vesicle traffic in developing neurons with new methodologies (Figure 2c). The study by Ahmari and colleagues [29] confirmed the existence in developing neurons of motile clusters of vesicles that are employed to deliver molecular components to the nascent synapse (Figure 3f,g). The authors reported the existence of highly motile transport packages of synaptobrevin/VAMP–GFP that, in addition to some proteins of synaptic vesicles, were found to carry other synaptic components, including subunits of voltage-dependent calcium channels and proteins of the endocytotic machinery [29]. The results of this analysis indicate that several components required for the formation of a presynaptic site could be present in the same mobile transport package. The authors also noted that, in addition to PLEIOMORPHIC VESICLES AND TUBULOVESICULAR STRUCTURES, these packages contained mainly dense core vesicles (Figure 3i,h), indicating that motile packages of vesicles in isolated axons are formed by heterogeneous structures.

Is fusion with the plasma membrane required for vesicle maturation?

During neuronal development, a process of remodeling could be necessary to produce vesicles of uniform size that are typical of active zones. How such a process could occur? It is conceivable to hypothesize that the previously described repetitive cycles of fusion with the plasma membrane [4,11] could play a role in vesicle maturation, allowing a progressive redistribution of molecular components between the vesicle and the plasma membrane. This process could lead to the generation of a homogeneous pool of vesicles from the pleiomorphic population (Figure 4). A recent study by Murthy and colleagues [30] provides further support to this hypothesis. The authors investigated the fate of newly synthesized synaptotagmin–GFP in *Drosophila* neurons lacking *sec5*, a component of the exocyst complex, which comprises a set of proteins conserved from yeast to humans and implicated in trafficking to the cell surface. Murthy and colleagues [30] showed that, in *Drosophila* neurons, *sec5* mutations impaired membrane insertion of newly synthesized proteins, without affecting the fusion of synaptic vesicles with the nerve terminal. The authors also reported that newly synthesized synaptotagmin was unable to concentrate at synaptic sites, despite being efficiently transported along the axon; in other words, synaptotagmin transport vesicles that had not fused with the plasma membrane were not matured into synaptic vesicles and were not delivered to presynaptic terminal, remaining instead in the axon or returning to the soma by retrograde transport. The requirement for the fusion of vesicles with the plasma membrane to achieve their maturation, as demonstrated by the Murthy laboratory, is in line with current models of the biogenesis of synaptic vesicles. These models suggest that transport vesicles containing newly synthesized proteins of synaptic vesicles fuse with the plasma

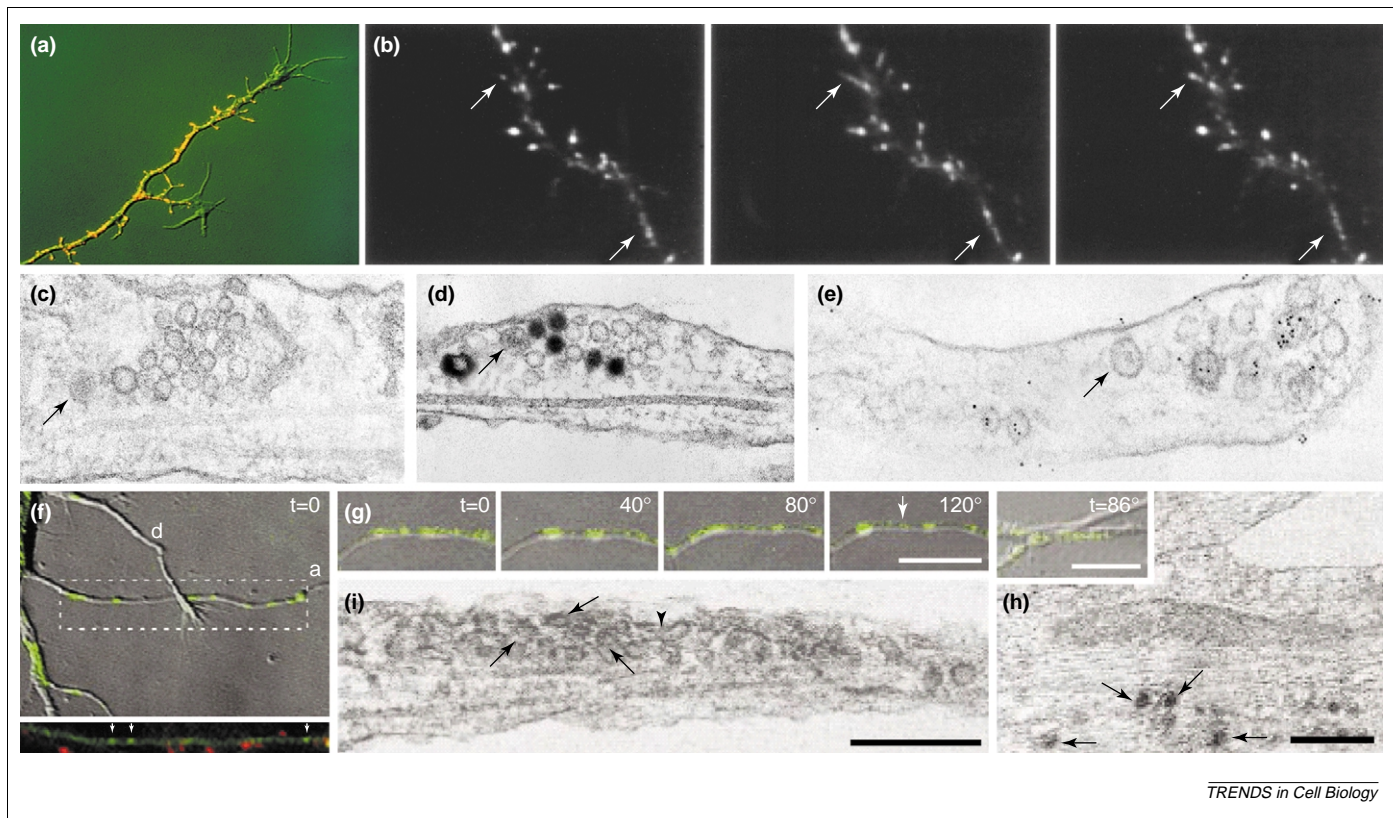


Figure 3. Mobile clusters of vesicles in developing hippocampal neurons. Packages of recycling vesicles stained with Syt-ecto abs (a) move along the axon and filopodia (b). Arrows in B indicate motile clusters of vesicles positively stained with Syt-ecto abs. Images are taken at 2 min intervals. Packages are mainly formed by clear vesicles and a minor proportion of large dense-core vesicles, arrows in (c-e). When cultures were incubated for 1 h before fixation with the fluid-phase marker horseradish peroxidase (HRP), a significant number of vesicles were labeled with HRP reaction product, indicating that they undergo exo-endocytosis (d). (e) Vesicles are labeled by Syt-ecto abs and then with gold particles, further confirming their ability to undergo repetitive cycles of fusion with the plasma membrane. Packages of VAMP-GFP vesicles (f) move along the axon (g). Packages of vesicles are not labeled by FM1-43 (inset in (f), arrowheads). The white arrow in (g) indicates the location of retrospective electron microscopy. Packages are formed by dense-core vesicles (h) and clusters of vesicles with heterogeneous shapes and sizes (i). (a-e) are reproduced from [11] and (f-i) from [29]. Bars: 15 μm (g); 250 nm (i); 250 nm (h).

membrane through the constitutive pathway of exocytosis from the *trans*-Golgi network and are then re-internalized and sorted in endosomes to generate mature synaptic vesicles [31,32].

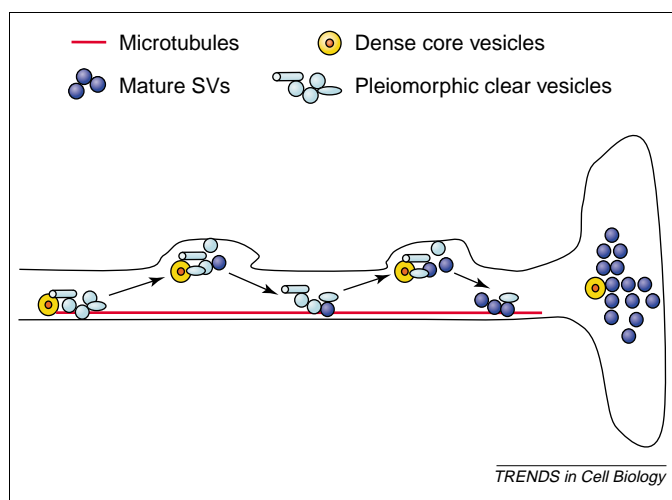


Figure 4. Vesicle turnover in developing axons. The cartoon depicts a model for vesicle turnover in relation to the construction of presynaptic terminals. Packages formed by heterogeneous vesicles, such as clear vesicles, tubulovesicular structures and large dense-core vesicles, travel along the axon. Repetitive cycles of fusion with the plasma membrane lead to the generation of mature synaptic vesicles, which are then recruited to the nascent synapse.

Regulatory mechanisms of vesicle fusion in immature neurons and at mature nerve terminals

Regulatory mechanisms of vesicle fusion in developing hippocampal axons differ from those operating at mature synapses. At early developmental stages (i.e. before synaptogenesis), vesicle recycling in neurons occurs spontaneously, at a high rate and increases only slightly upon depolarization [11]. Furthermore, the exocytotic machinery is different from that used for neurotransmitter release at mature nerve terminals. The former is mediated by a tetanus-toxin-resistant isoform of synaptobrevin/VAMP2 [12] and lacks the modulatory control provided by the interaction between synaptobrevin/VAMP2 and synaptophysin [33,34].

Distinct exocytotic mechanisms were also detected in the developing motor axons when compared with the presynaptic nerve terminals. In particular, in contrast to the mature synapse, the release of ACh in immature axons was blocked by brefeldin A [25]; Brefeldin A is a specific inhibitor of the ADP ribosylation factor 1 (ARF1), a small GTP-binding protein that plays important roles in intracellular trafficking in animal and yeast cells. This evidence suggests that the system that causes immature release of neurotransmitters might utilize different adaptor proteins in vesicle budding. Interestingly, a brefeldin-A-sensitive vesicle-cycling mechanism persisted

at the adult neuromuscular junction of mice deficient in neural cell adhesion molecule (N-CAM), suggesting that N-CAM might be required for silencing the immature release process by mediating cell contact between pre and postsynaptic partners and/or by activating signal transduction pathways [35]. Although characterized by some distinct immaturity features, vesicle recycling and ACh release in developing motor axons are calcium- and stimulus-dependent and exhibit plasticity phenomenon, thereby sharing few similarities with the exocytosis of synaptic vesicles at mature neuromuscular synapses [25,27].

It has been proposed that a crosstalk between the pre and the postsynaptic elements could provide a signal for switching exocytosis from an immature to a mature state (Box 1) [12,34–37]. The most striking example of pre-synaptic regulation by a postsynaptic target is that reported by Ahmari and colleagues [29]. In this study, vesicles contained in packages were unexpectedly not labeled with FM1–43 and could undergo evoked recycling only upon cell–cell contact [29]. A recent report suggests the possibility of a more complex scenario that extends the process of functional maturation beyond the contribution of dendritic contact [38]. Krueger and colleagues [38] reported that, in hippocampal cultures, after the onset of synaptogenesis (10–21 days *in vitro*), ‘orphan’ clusters of vesicles lacking postsynaptic specializations but nonetheless able to undergo evoked exo–endocytotic recycling were visualized by FM1–43 [38]. Interestingly, the vesicles present at orphan release sites in mature cultures appeared to utilize the same mechanisms of synaptic-vesicle exocytosis and retrieval as those used at synapses (i.e. a mechanism sensitive to tetanus toxin and resistant to brefeldin A) [38]. Although a functional maturation of the exocytotic machinery occurred independent from the presence of a postsynaptic target, the apposition of a postsynaptic element further affected both the structure and the function of release sites. Indeed, in agreement with previous data [11,34,36,39], the dendritic contact increased the size of the synaptic-vesicle pool and influenced the coupling of calcium influx with neurotransmitter release in a retrograde manner (Box 1) [38]. A model emerges from the ensemble of these data, suggesting that vesicle recycling during neuronal development might undergo a functional maturation through at least two subsequent steps; the first step would not require the presence of a postsynaptic target and the second step would rely on dendritic contact [38]. This view might provide explanations for the reported variability in the vesicle composition of mobile packages, as well as in the general features of release properties, which could change depending on different stages of neuronal maturation. Another source of variability might be the different experimental methods used to monitor the recycling of synaptic vesicles. In particular, Syt-ecto abs, which do not dissociate from the vesicle upon fusion with the plasma membrane, allow the efficient monitoring of high-rate spontaneous exocytosis of synaptic vesicles; on the other hand, the property of FM dyes to dissociate from the vesicle membrane upon fusion with the neuronal surface might not allow the accumulation of the dye into spontaneously recycling vesicles at detectable levels, because

their rapid loading would be simultaneous with their unloading [35].

Recruitment of synaptic vesicles and active-zone proteins

The active zone is the site of synaptic-vesicle docking and neurotransmitter release. At central synapses, it is characterized by the presence of a dense matrix consisting of proteins, such as RIM, Piccolo and Bassoon, which might provide a scaffold for the localization of synaptic vesicles [40–42]. What temporal relation exists between the recruitment of synaptic vesicles and that of active-zone proteins during synaptogenesis? An early working model for synaptogenesis suggested that the clustering of synaptic vesicles in the ‘nascent’ synapse is preceded by the local deposition of presynaptic active-zone proteins [43]. The recruitment of cytomatrix active-zone proteins at synaptic sites occurs through vesicles called Piccolo–Bassoon transport vesicles, (PTVs), which are highly motile along the axon and carry a comprehensive set of essential active-zone molecules including Piccolo, Bassoon, RIM and the subunits of calcium channel, but not synaptic-vesicle proteins [41,42]. By contrast, Krueger and colleagues [38] demonstrated that, in mature hippocampal cultures, functional modules exist in which the active-zone protein Bassoon is tightly associated with clusters of synaptic vesicles. The authors proposed that these preassembled modules could be mobilized to rapidly establish a new functional presynaptic terminal in a single step. The existence of presynaptic particles containing active-zone proteins in tight association with components necessary for the retrieval of vesicle membrane proteins has also been reported [40]. The two models, however, might be not mutually exclusive but rather alternatively utilized to establish new synaptic contacts, either during early development or during synapse remodeling in the mature nervous system [38].

A different vesicle pathway is involved in neurite outgrowth

A final problem in synaptogenesis is the delivery of components required for the growth of the axon during neuronal development. It is unlikely that the exocytosis of vesicles present in axonal packages and PTVs mediates axonal growth. The outgrowth of axons requires massive transport of lipids and proteins from the Golgi complex to the plasma membrane, which must occur through a vesicular mechanism [44]. The recent report, demonstrating that *sec5* mutations in *Drosophila* neurons impair addition of newly synthesized proteins to plasma membrane without affecting the fusion of synaptic vesicles, directly indicate that neurite outgrowth and neurotransmission rely on different molecular machineries [30]. These results provide further support to previous data showing that, in mice deficient in either *SEC1/MUNC18* [45] or *MUNC13/UNC-13* [46] – two regulatory proteins for synaptic-vesicle fusion, complete loss of neurotransmitter secretion occurs but neuronal growth and normal brain assembly are not affected. Recently, a vesicular compartment mediating the insertion of new membrane during neurite outgrowth has been identified that, in terms of

molecular composition and subcellular localization, is distinct from the recycling vesicles mediating neurotransmitter release [47,48]. These vesicles are enriched at the tip of growth cones, lack the markers of synaptic vesicles [47], contain L1 (a cell adhesion molecule implicated in axonal elongation) [49] and are characterized by the presence of the v-SNARE tetanus-neurotoxin-insensitive vesicle-associated membrane protein (TI-VAMP) [47,48].

Although vesicles of axonal packages are not primarily involved in axonal outgrowth, one cannot exclude the possibility that, during the repetitive cycles of fusion, some components of their membranes could be retained at the neuronal surface. It has been recently suggested that vesicle recycling that occurs in filopodia of the growth cone could play a role in quickly delivering proteins, which might be relevant for axonal path finding or growth-factor signaling, to the neuronal surface [13]. Indeed, the demonstration that, in isolated axons, vesicle recycling mediates the insertion of glutamate receptors in the membranes of growth cones [19] supports the possibility that, in developing neurons, vesicle turnover might also be involved in dynamically regulating the protein composition of the plasma membrane, thereby modulating the neuronal responsiveness to external cues.

Concluding remarks

Within developing neurons, different pathways of exocytosis coexist. These pathways play distinct roles in mediating neurotransmitter release and the insertion of membrane proteins required for neuronal growth. Synaptic vesicles and their precursor organelles are preassembled in highly motile packages and already undergo recycling before dendritic contact. This recycling could represent an important aspect of vesicle maturation. Upon contact with a postsynaptic target, packages of synaptic vesicles and modules of active-zone proteins are recruited at the presynaptic terminal. Here, regulatory mechanisms might elaborate and refine the basic exocytotic machinery, possibly through retrograde signaling from the postsynaptic target. A future challenge for the neuroscientists will be to not only further define the cellular and molecular aspects of synapse development but also better understand the dynamic nature of the molecular associations that occur at both the pre and the postsynaptic compartments during synaptogenesis. Furthermore, it will be relevant to provide an overview of the pathways that operate in parallel or intersect one with each other to obtain a stereotyped organization of neuronal connectivity. Combining new live-imaging techniques with ultrastructural, molecular and genetic approaches will most probably allow reaching these goals.

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References

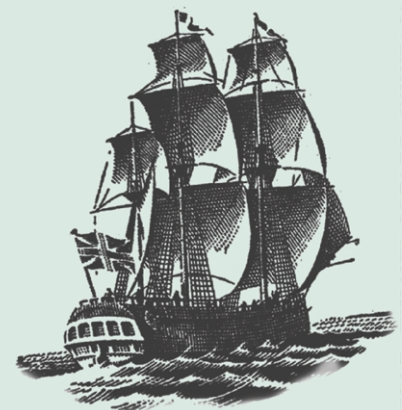
- De Camilli, P. *et al.* Synaptic vesicle endocytosis. In *Synapses* (Cowan WM, S.T.a.S.C., ed.), pp. 217–274
- Dotti, C. *et al.* (1988) The establishment of polarity by hippocampal neurons in culture. *J. Neurosci.* 8, 1454–1468
- Goda, Y. and Davis, G.W. (2003) Mechanisms of synapse assembly and disassembly. *Neuron* 40, 243–264
- Matteoli, M. *et al.* (1992) Exo-endocytotic recycling of synaptic vesicles in developing processes of cultured hippocampal neurons. *J. Cell Biol.* 117, 849–861
- Betz, W. and Bewick, G. (1992) Optical analysis of synaptic vesicle recycling at the frog neuromuscular junction. *Science* 255, 200–203
- Betz, W. *et al.* (1996) Imaging exocytosis and endocytosis. *Curr. Opin. Neurobiol.* 6, 365–371
- Ryan, T.A. *et al.* (1993) The kinetics of synaptic vesicle recycling measured at single presynaptic boutons. *Neuron* 11, 713–724
- Ryan, T. (2001) Presynaptic imaging techniques. *Curr. Opin. Neurobiol.* 11, 544–549
- Yuste, R. *et al.* (2000) Synapto-pHluorins: chimeras between pH-sensitive mutants of green fluorescent protein and synaptic vesicle membrane proteins as reporters of neurotransmitter release. *Methods Enzymol.* 327, 522–546
- Poskanzer, K. *et al.* (2003) Synaptotagmin I is necessary for compensatory synaptic vesicle endocytosis *in vivo*. *Nature* 426, 559–563
- Kraszewski, K. *et al.* (1995) Synaptic vesicle dynamics in living cultured hippocampal neurons visualized with CY3-conjugated antibodies directed against the luminal domain of synaptotagmin. *J. Neurosci.* 15, 4328–4342
- Verderio, C. *et al.* (1999) Tetanus toxin blocks the exocytosis of synaptic vesicles clustered at synapses but not of synaptic vesicles in isolated axons. *J. Neurosci.* 19, 6723–6732
- Sabo, S. and McAllister, A. (2003) Mobility and cycling of synaptic protein-containing vesicles in axonal growth cone filopodia. *Nat. Neurosci.* 6, 1264–1269
- Verderio, C. *et al.* (1995) Calcium-dependent glutamate release during neuronal development and synaptogenesis: different involvement of omega-agatoxin IVA- and omega-conotoxin GVIA-sensitive channels. *Proc. Natl. Acad. Sci. U. S. A.* 92, 6449–6453
- Soeda, H. *et al.* (1997) Neurotransmitter release from growth cones of rat dorsal root ganglion neurons in culture. *Neuroscience* 77, 1187–1199
- De Paola, V. *et al.* (2003) AMPA receptors regulate dynamic equilibrium of presynaptic terminals in mature hippocampal networks. *Nat. Neurosci.* 6, 491–500
- Tashiro, A. *et al.* (2003) Bidirectional regulation of hippocampal mossy fiber filopodial motility by kainate receptors: a two-step model of synaptogenesis. *Neuron* 38, 773–784
- Chang, S. and De Camilli, P. (2001) Glutamate regulates actin-based motility in axonal filopodia. *Nat. Neurosci.* 4, 787–793
- Schenk, U. *et al.* (2003) Regulated delivery of AMPA receptor subunits to the presynaptic membrane. *EMBO J.* 22, 558–568
- Fischer, M. *et al.* (2000) Glutamate receptors regulate actin-based plasticity in dendritic spines. *Nat. Neurosci.* 3, 887–894
- Betz, W. and Henkel, A. (1994) Okadaic acid disrupts clusters of synaptic vesicles in frog motor nerve terminals. *J. Cell Biol.* 124, 843–854
- Hazuka, C.D. *et al.* (1999) The sec6/8 complex is located at neurite outgrowth and axonal synapse-assembly domains. *J. Neurosci.* 19, 1324–1334
- Hsu, S. *et al.* (1999) Targeting vesicles to specific sites on the plasma membrane: the role of the sec6/8 complex. *Trends Cell Biol.* 9, 150–153
- Dai, Z. and Peng, H.B. (1996) Dynamics of synaptic vesicles in cultured spinal cord neurons in relationship to synaptogenesis. *Mol. Cell. Neurosci.* 7, 443–452
- Zakharenko, S. *et al.* (1999) Neurotransmitter secretion along growing nerve processes: comparison with synaptic vesicle exocytosis. *J. Cell Biol.* 144, 507–518
- Hume, R.I. *et al.* (1983) Acetylcholine release from growth cones detected with patches of acetylcholine receptor-rich membranes. *Nature* 305, 632–634
- Sun, Y.A. and Poo, M.M. (1987) Evoked release of acetylcholine from

- the growing embryonic neuron. *Proc. Natl. Acad. Sci. U. S. A.* 84, 2540–2544
- 28 Chow, I. and Poo, M.M. (1985) Release of acetylcholine from embryonic neurons upon contact with muscle cell. *J. Neurosci.* 5, 1076–1082
- 29 Ahmari, S. *et al.* (2000) Assembly of presynaptic active zones from cytoplasmic transport packets. *Nat. Neurosci.* 3, 445–451
- 30 Murthy, M. *et al.* (2003) Mutations in the exocyst component Sec5 disrupt neuronal membrane traffic, but neurotransmitter release persists. *Neuron* 37, 433–447
- 31 Calakos, N. and Scheller, R.H. (1996) Synaptic vesicle biogenesis, docking, and fusion: a molecular description. *Physiol. Rev.* 76, 1–29
- 32 Hannah, M.J. *et al.* (1999) Synaptic vesicle biogenesis. *Annu. Rev. Cell Dev. Biol.* 15, 733–798
- 33 Becher, A. *et al.* (1999) The synaptophysin–synaptobrevin complex is developmentally upregulated in cultivated neurons but is absent in neuroendocrine cells. *Eur. J. Cell Biol.* 78, 650–656
- 34 Bacci, A. *et al.* (2001) Chronic blockade of glutamate receptors enhances presynaptic release and downregulates the interaction between synaptophysin–synaptobrevin-vesicle-associated membrane protein 2. *J. Neurosci.* 21, 6588–6596
- 35 Polo-Parada, L. *et al.* (2001) Alterations in transmission, vesicle dynamics, and transmitter release machinery at NCAM-deficient neuromuscular junctions. *Neuron* 32, 815–828
- 36 Chavis, P. and Westbrook, G. (2001) Integrins mediate functional pre- and postsynaptic maturation at a hippocampal synapse. *Nature* 411, 317–321
- 37 Haghghi, A. *et al.* (2003) Retrograde control of synaptic transmission by postsynaptic CaMKII at the *Drosophila* neuromuscular junction. *Neuron* 39, 255–267
- 38 Krueger, S. *et al.* (2003) The presynaptic release apparatus is functional in the absence of dendritic contact and highly mobile within isolated axons. *Neuron* 40, 945–957
- 39 Coco, S. *et al.* (1998) Calcium dependence of synaptic vesicle recycling before and after synaptogenesis. *J. Neurochem.* 71, 1987–1992
- 40 Phillips, G. *et al.* (2001) The presynaptic particle web: ultrastructure, composition, dissolution, and reconstitution. *Neuron* 32, 63–77
- 41 Zhai, R. *et al.* (2001) Assembling the presynaptic active zone: a characterization of an active one precursor vesicle. *Neuron* 29, 131–143
- 42 Shapira, M. *et al.* (2003) Unitary assembly of presynaptic active zones from Piccolo–Bassoon transport vesicles. *Neuron* 38, 237–252
- 43 Friedman, H. *et al.* (2000) Assembly of new individual excitatory synapses: time course and temporal order of synaptic molecule recruitment. *Neuron* 27, 57–69
- 44 Pfenninger, K.H. and Friedman, L.B. (1993) Sites of plasmalemmal expansion in growth cones. *Brain Res. Dev. Brain Res.* 71, 181–192
- 45 Verhage, M. *et al.* (2000) Synaptic assembly of the brain in the absence of neurotransmitter secretion. *Science* 287, 864–869
- 46 Varoqueaux, F. *et al.* (2002) Total arrest of spontaneous and evoked synaptic transmission but normal synaptogenesis in the absence of Munc13-mediated vesicle priming. *Proc. Natl. Acad. Sci. U. S. A.* 99, 9037–9042
- 47 Coco, S. *et al.* (1999) Subcellular localization of tetanus neurotoxin-insensitive vesicle-associated membrane protein (VAMP)/VAMP7 in neuronal cells: evidence for a novel membrane compartment. *J. Neurosci.* 19, 9803–9812
- 48 Martinez-Arca, S. *et al.* (2001) A common exocytotic mechanism mediates axonal and dendritic outgrowth. *J. Neurosci.* 21, 3830–3838
- 49 Alberts, P. *et al.* (2003) Cross talk between tetanus neurotoxin-insensitive vesicle-associated membrane protein-mediated transport and L1-mediated adhesion. *Mol. Biol. Cell* 14, 4207–4220
- 50 Colicos, M. *et al.* (2001) Remodeling of synaptic actin induced by photoconductive stimulation. *Cell* 107, 605–616
- 51 Nikonenko, I. *et al.* (2003) Presynaptic remodeling contributes to activity-dependent synaptogenesis. *J. Neurosci.* 23, 8498–8505
- 52 Muller, D. and Nikonenko, I. (2003) Dynamic presynaptic varicosities: a role in activity-dependent synaptogenesis. *Trends Neurosci.* 26, 573–575
- 53 Tao, H. and Poo, M. (2001) Retrograde signaling at central synapses. *Proc. Natl. Acad. Sci. U. S. A.* 98, 11009–11015
- 54 Hall, A. *et al.* (2000) Axonal remodeling and synaptic differentiation in the cerebellum is regulated by WNT-7a signaling. *Cell* 100, 525–535
- 55 Yamagata, M. *et al.* (2003) Synaptic adhesion molecules. *Curr. Opin. Cell Biol.* 15, 621–632
- 56 Missler, M. (2003) Synaptic cell adhesion goes functional. *Trends Neurosci.* 26, 176–178
- 57 Thiery, J. (2003) Cell adhesion in development: a complex signaling network. *Curr. Opin. Genet. Dev.* 13, 365–371
- 58 Togashi, H. *et al.* (2002) Cadherin regulates dendritic spine morphogenesis. *Neuron* 35, 77–89
- 59 Scheiffele, P. *et al.* (2000) Neuroligin expressed in nonneuronal cells triggers presynaptic development in contacting axons. *Cell* 101, 657–669
- 60 Biederer, T. *et al.* (2002) SynCAM, a synaptic adhesion molecule that drives synapse assembly. *Science* 297, 1525–1531
- 61 Dalva, M. *et al.* (2000) EphB receptors interact with NMDA receptors and regulate excitatory synapse formation. *Cell* 103, 945–956



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