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Review

Synaptogenesis in hippocampal cultures

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Abstract. Hippocampal cultures offer unique advantages for the study of neuronal development and synaptogenesis. Studies performed on this model enabled dissection of the temporal sequence of events which lead to the differentiation of pre- and postsynaptic compartments.

Key words. Synaptogenesis; hippocampal neurons; synaptic vesicles; glutamate receptors.

Introduction

Synapses represent highly specialized and asymmetrical intercellular junctions, characterized by specific morphological features. These include the close apposition of the presynaptic and postsynaptic plasmalemma via an intervening extracellular matrix which is thought to contain unique proteins, a postsynaptic density extending from the plasma membrane to the cytoplasm and the presence of clusters of clear vesicles adjacent to the presynaptic membrane (synaptic vesicles, SVs). The presynaptic compartments are specialized for regulated secretion of neurotransmitters, and the postsynaptic sites are specialized for reception and integration of signals.

Whereas the mechanisms underlying the flow of information mediated by chemical signals released from the presynaptic terminal have been clarified to a relatively large extent, much less is known about the signalling which is required for the formation of a mature synapse. This type of signalling is likely to be a bidirectional one, with anterograde signals directing the organization of the postsynaptic membrane and retrograde signals regulating vesicle clustering and dynamics in the presynaptic terminal.

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The molecular mechanisms mediating reciprocal recognition of pre- and postsynaptic elements and the following steps, which lead to the formation of distinct, highly specialized pre- and postsynaptic domains, are at present largely unknown. Most of the knowledge in this field comes from studies of neuromuscular junction development in culture, where the focus has been on events participating in the differentiation of the postsynaptic domain in the muscle cell [1]. The complexity of the central nervous sytem (CNS) has largely precluded analysis of the cellular and molecular events underlying synapse formation. Moreover, the lack of suitable experimental models has strongly impaired the study of the mechanisms by which neurons control the localization of synaptic components.

Cultured hippocampal neurons, an experimental system thoroughly characterized in recent years, present unique advantages for the study of neuronal development and synaptogenesis. Hippocampal neurons grown in vitro extend axons and dendrites by a stereotyped sequence of developmental events [2], even though the exact time course of synaptogenesis may vary depending on the density of plating and, consequently, on the axon target distance [3]. Shortly after the cells have attached to the substratum, they form highly motile lamellipodia

which then condense to originate minor processes with similar lengths (stage 2). Polarity becomes evident when one of these processes starts elongating rapidly and differentiates as an axon (stage 3). After a few days, the shorter processes start growing and acquire morphological and biochemical pattern of proteins typical of dendrites (stage 4). Eventually, neuronal processes become interconnected (fig. 1), and axosomatic or axodendritic, functional synaptic contacts become formed in a rather synchronous fashion [4] (fig. 2A). Synaptogenesis is followed by a further maturation of synaptic contacts and by the proper integration of individual neurons into a functional synaptic network [5–7].

Neurite extension

The extension of neuronal processes proceeds preferentially by addition of new membrane patches at the growth cone [8]. A number of studies have examined the effects of disrupting the synthesis and the intracellular transport of membrane components necessary to support neurite extension. In cultured hippocampal neurons, axonal outgrowth and branching are impaired by inhibitors of sphingolipid synthesis [9, 10] and by treatments affecting the molecular motors involved in vesicular transport. Indeed, antisense oligonucleotides to kinesin, a microtubule-based motor protein, inhibit process elongation [11].

Interestingly, proteins involved in the exo-endocytotic recycling of SVs have also been shown to control neurite extension. Axon formation is inhibited in hippocampal cultures after depletion by antisense oligonucleotides for synapsin II [12, 13], a SV-associated phosphoprotein which interacts with the actin cytoskeleton [14]. Similar results were obtained in neuronal cultures from knock-out mice. In particular, a delay in axon formation or a retard in the formation of synaptic contacts was observed in cultures from mice lacking synapsin II or I, respectively [13].

Amphiphysin I and dynamin I, two partner proteins which play a key role in the endocytic reaction of SV membranes [15], appear to be actively involved in the process of neuritogenesis, as demonstrated by the findings that suppression of dynamin I [16] or amphiphysin I [17] by antisense oligonucleotides produces potent inhibition of neurite formation and collapse of growth cones in cultured hippocampal neurons. Even though the mechanisms by which these two proteins regulate neurite outgrowth remains unclear, it is tempting to speculate that suppression of process extension in dynamin I- or amphiphysin I-depleted neurons may reflect at least in part a role of these molecules in the function of the actin cytoskeleton [17 and references therein]. It is interesting to note that depletion of synapsin II from neuronal cells is associated with abnormal distribution of intracellular filamentous actin (F-actin) [12], thus supporting the idea that perturbation of the actin-based cytoskeleton during the early stages of nerve cell devel-

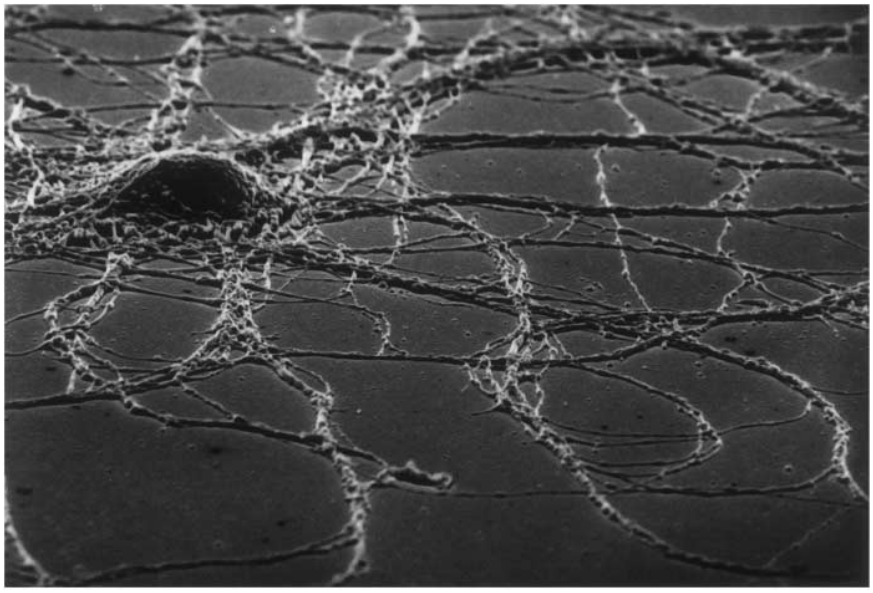


Figure 1. Scanning electron microscopy of a fully differentiated hippocampal neuron forming an interconnected network of processes.

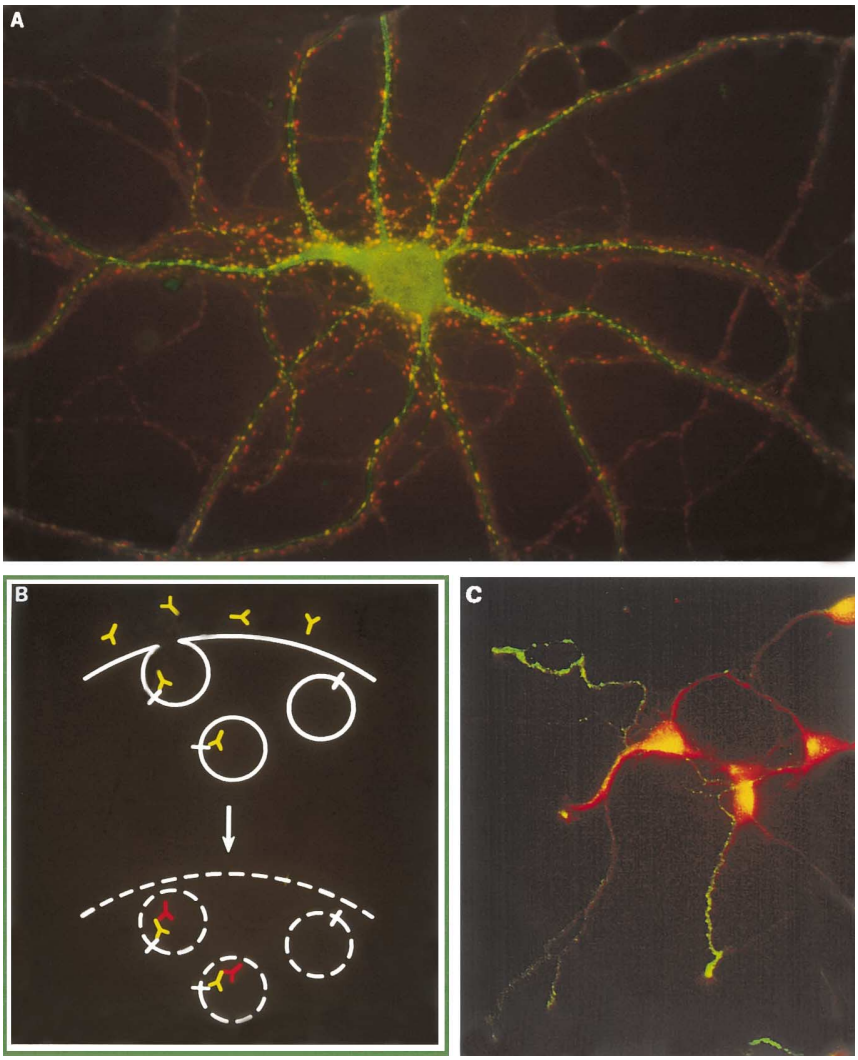


Figure 2. (*A*) Synaptic contacts formed by cultured hippocampal neurons. Clusters of synaptic vesicles (SVs) are revealed by immunolabelling with antibodies against synaptotagmin (red). Neuronal cell body and dendrites are visualized with antibodies directed against the cytoskeletal protein MAP2 (green) [Matteoli, Banker, Sudhof and De Camilli, with permission of Cell/Neuron **72**, 1993, © Cell Press, MA, USA]. (*B*) Cartoon representing the immunocytochemical assay to monitor the exoendocytotic recycling of SVs. Antibodies against the intravesicular domain of the SV protein synaptotagmin (Syt-ecto Abs) bind to the protein and become internalized inside the lumen of vesicles when they fuse with the plasma membrane. The internalized antibodies are visualized, after fixation and permeabilization, by using fluorochrome-conjugated secondary antibodies. (*C*) Distribution of SVs, as revealed by antibodies directed against the SV protein synaptobrevin/VAMP2, in hippocampal neurons before synaptogenesis. SVs are preferentially localized in the axons. Double-labelling with antibodies directed against the neuronal glutamate transporter EAAC1 (red), shows a somatodendritic localization of this glutamate carrier at early developmental stages.

opment may result in alterations of the process extension.

Indeed, small guanosine-5′-triphosphate (GTP)-binding proteins of the Rho family, which are known to be involved in the organization of the actin-based cytoskeleton, have been implicated in cytoskeletal reorganization during neuritogenesis [18]. A new neural-specific member of the Rho family (Rac1B) has recently been described [19] and has been found to induce enhanced neuritogenesis and neurite branching in primary neurons [20]. The small GTPases Rac and Cdc-42 may be differently involved in the generation either of the axon or the dendritic tree. Indeed, perturbation of Rac1 activity has been shown to block axonal outgrowth but not dendritic extension in Purkinje cells of mouse cerebellum, although the size and number of

dendritic spines were found to be altered [21]. The morphological differentiation of axon terminals into mature synapses also involves a protein homologous to guanosine-5′-triphosphate (GTP)-guanosine-5′-diphosphate (GDP) exchangers (still life, cloned in *Drosophila*) which, by activating Rho-like GTPases, regulates synaptic differentiation through the organization of the actin cytoskeleton [22]. In cultured hippocampal neurons, the application of antisense oligonucleotides to Rab GDP-dissociation inhibitors (GDI) was found to block axonal but not dendritic outgrowth [23, 24], further suggesting that distinct proteins playing in the GTP-GDP cycle may be differently involved in the elaboration of axon and dendrites [21].

Maturation of the presynaptic compartment

Specific features of SVs include a unique membrane protein composition, an extremely short latency between the stimulus and the exocytotic response, the occurrence of their exocytosis at highly specialized plasmalemma sites and their property to undergo exo-endocytotic recycling using specific molecular machinery. At each exo-endocytotic cycle SVs are refilled locally with neurotransmitters [15, 25–31].

Whereas most of these properties have been established for SVs in mature presynaptic nerve endings, much less was known about SVs or SV precursor organelles at stages of neuronal development preceding synaptogenesis. The presence of SV proteins (synapsin, synaptophysin, rab3A, synaptotagmin, synaptobrevin/VAMP) has been described in processes of developing neurons prior to the formation of synaptic contacts [32–35]. Interestingly, the plasmamembrane proteins involved in SV fusion (SNAP 25 and syntaxin I) are also expressed from early developmental stages [35, 36, 68]. SV proteins increase their expression during neuronal development in vitro [32, 35, 68] and undergo regional redistribution in parallel with the establishment of neuronal polarity and synaptogenesis. Indeed, whereas at early developmental stages SV proteins are uniformly distributed throughout the neuronal cytoplasm, they become sorted to the axon as soon as this process forms and starts elongating (fig. 2C). Low levels of SV proteins are detectable in the somatodendritic compartment [32–34]. Eventually, in parallel with the formation of synaptic contacts, SVs undergo clustering at presynaptic sites [4, 32–34] (fig. 2A).

Given the presence of SV proteins already at stages preceding synapse formation, the question arises whether the formation of presynaptic specialization represents a prerequisite for SV fusion, or whether synapse formation simply correlates with a relocation of sites of SV fusion to selected regions of the axonal plasma membrane and/or with the activation of specific mechanisms regulating SV exocytosis. We report here the results of studies aimed at clarifying these issues.

Synaptic vesicle dynamics in living hippocampal neurons

To evaluate the dynamics of SVs during synaptogenesis and to quantify the rate of SV exocytosis independent of neurotransmitter secretion, we have extensively used an immunocytochemical assay based on the use of rabbit polyclonal antibodies (Syt_{lum}-Abs) directed against the lumenal N-terminus of the SV protein synaptotagmin I (Syt I) [34, 37, 38]. These antibodies become internalized in the lumen of SVs as a result of their fusion with the plasmalemma (fig. 2B). In addition, Syt_{lum}-Abs are not immediately targeted to the late-endosomal/lysosomal pathway for degradation after internalization, but recycle in parallel with SVs [34]. This makes Syt_{lum}-Abs an extremely powerful tool to study SV traffic and synaptogenesis in living neurons, bearing several advantages over other probes which are used to monitor morphologically SV dynamics in living cells. The most common of these probes, FM1-43, which binds to phospholipid bilayers with very fast kinetics, suffers the limitations that it binds nonspecifically to all membranes and that it is unloaded from the vesicle in parallel with exocytosis. For these reasons it cannot be used to reliably label SVs at sites where internalization of SV membranes does not represent the predominant endocytic traffic, for example developing axons. Moreover, it cannot be used for long-lasting labelling of SVs.

Addition of Syt_{lum}-Abs to neuronal cultures resulted in their rapid and specific internalization not only at synaptic sites but also in isolated processes of developing neurons [34]. Following endocytosis, Syt_{lum}-Abs were rapidly reexposed to the cell surface, indicating the occurence of exo-endocytotic recycling. These data indicated that the presence of typical presynaptic specialization is not crucial for SV exocytosis to take place, and that the ability to undergo exo-endocytosis is a general characteristic of SVs.

Not only is the ability to undergo fusion with the plasma membrane an intrinsic feature of SVs, but the property of aggregating to form clusters is as well. Indeed, videomicroscopy using Syt_{lum}-Abs directly conjugated to the fluorochrome CY3 (CY3-Syt_{lum}-Abs), in combination with electron microscopy, revealed the presence in immature isolated axons and in their filopodia of SVs arranged in highly motile clusters which move in a bulk in an anterograde and retrograde direction [37]. The ability to undergo bidirectional movement inside axons is also shared by trans Golgi network (TGN)-derived tubulovesicular organelles,

which have been shown to represent a general transport machinery for newly synthesized plasma membrane and SV proteins in dorsal root ganglia cultured neurons [39]. In hippocampal neurons, the immobilization of SV clusters to the cell surface and their coalescence into larger clusters were found to take place only on contact with the postsynaptic cell [37], provided this has reached a critical stage of maturation [40]. Indeed, SVs present in growing axons of hippocampal neurons have been shown to form large presynaptic clusters already after 1 day in culture if they are plated over mature neurons which provide appropriate, differentiated postsynaptic targets [40].

The ability of SVs to undergo exo-endocyotic recycling already at stages preceding synapse formation is in line with the results of earlier studies carried out by electrophysiological techniques on motor neurons in primary culture, showing that acetylcholine (ACh) is released from developing axons prior to the formation of synaptic contacts [41–44]. ACh secretion from growing axons is mediated by locally recycling SVs [45]. In cultured hippocampal neurons, release of glutamate in the extracellular space also occurs at stages preceding synaptogenesis [46]. The ability of growing axons to recycle SVs and to release neurotransmitters may account for the observation that, even though the formation and maturation of synaptic contacts may take some time (days or even weeks in the case of the neuromuscular junction), physical and chemical interactions between the two partner cells start immediately after the cell-cell contact [44]. The preexistence of SV packages, which can be recruited quickly at the site of cell contact, together with early activation of the secretory machinery at stages preceding synapse formation, may be instrumental for the rapid establishment of a functional synaptic contact. Moreover, exocytosis of neurotransmitters before synapse formation may have an important role in the ontogenesis of the mature nervous system. Released neurotransmitters may play a role as chemoattractants, regulating the interactions between the growth cone and its immediate environment [47], and influencing the formation of the cytoarchitecture of the brain [48, 49].

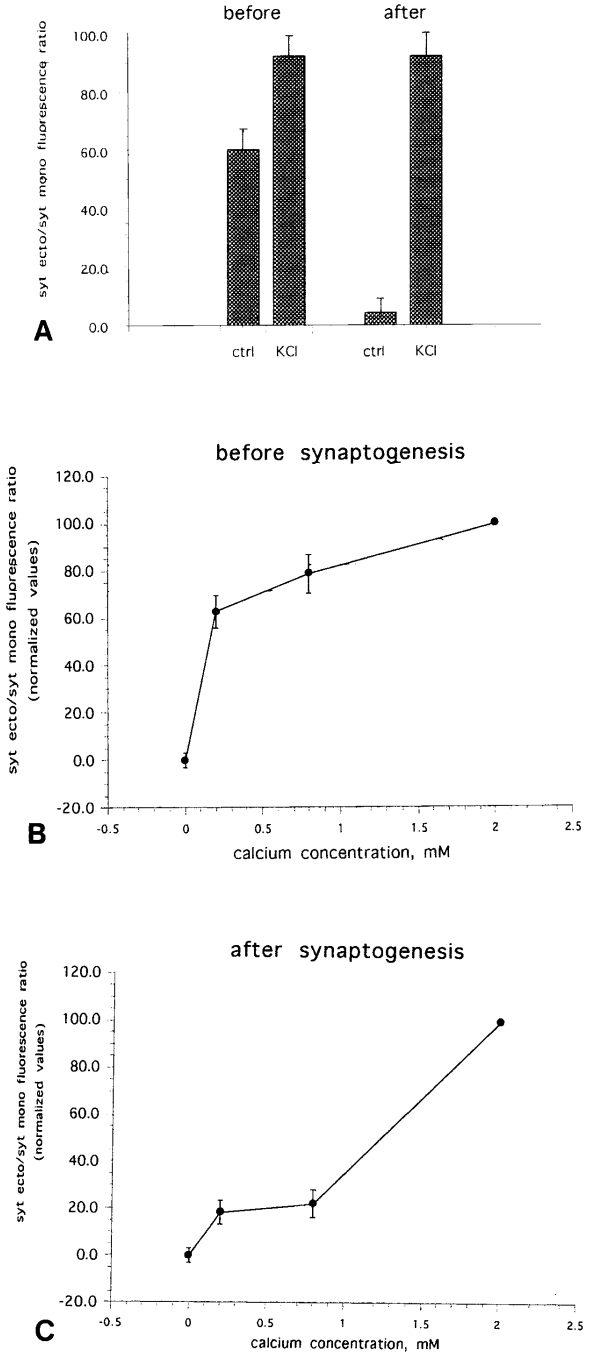


Figure 3. Calcium-dependent exocytosis monitored by the immunocytochemical assay in hippocampal neurons before and after synaptogenesis. Quantitative analysis of Syt-ecto Ab internalization in neurons exposed for 5 min to Syt-ecto Abs, both in the presence and in the absence of high KCl (*A*). Note the higher levels of Syt-ecto Ab internalization occurring in basal conditions in neurons before synaptogenesis. (*B* and *C*): Quantitative analysis of Syt-ecto Ab internalization in neurons stimulated with 50 mM KCl at different external calcium concentrations. A massive internalization of the antibodies is driven by KCl already at low calcium concentrations (< 0.8 mM) in neurons before synaptogenesis (2–4 DIV) (*B*). In neurons after synaptogenesis (12–14 DIV), a more substantial increase in the efficiency of internalization is produced by the incubation of neurons in calcium concentrations > 0.8 mM (*C*) (modified from Coco et al. [38] with permission of J. Neurochem. **71:** 1987–1992).

Regulation of synaptic vesicle exocytosis at different developmental stages

Mature synaptic contacts are characterized by high spatial precision, speed and great fidelity, which are critically dependent on SV exocytosis being triggered and turned off over extremely short time scales, depending on intraterminal calcium levels [50]. It is therefore conceivable that, though SVs are able to recycle and to release neurotransmitters already before their clustering at synaptic sites, the formation of synaptic contacts may coincide with the activation of specific mechanisms that control the exocytotic process.

A first difference detected in SV dynamics before and after synapse formation is the occurrence of a higher rate of spontaneous SV recycling in isolated axons [37, 38] (fig. 3A). Synaptogenesis thus appears to correlate with downregulation of basal SV exocytosis, possibly via strengthening of a mechanism that prevents vesicle recycling [51–53]. A second feature characterizing SV dynamics at distinct developmental stages is represented by the different dependence of the exo-endocytosis rate on external calcium concentrations. Indeed, whereas in neurons before synaptogenesis a massive exocytosis is triggered by depolarizing agents already at low calcium concentrations (fig. 3B), the larger increase in the SV fusion rate takes place at mature synaptic contacts only when external calcium concentration is higher than 0.8 mM [38] (fig. 3C). This opens the possibility that the downregulation of basal SV recycling after synaptogenesis is related to exocytosis being triggered by higher increases in calcium concentrations.

It is interesting to note that two distinct components of transmitter release, similar to those previously detected at the neuromuscular junction [50, 54], have been demonstrated at synapses of cultured hippocampal neurons: a fast, synchronous component and a slower, asynchronous component of release [55]. The presence of these two components is compatible with the existence of two calcium sensors, a low-affinity sensor which mediates an efficient release when calcium concentrations in the presynaptic terminal are very high, and a high-affinity sensor sustaining release at low calcium concentrations [55]. It appears therefore that the component activated by low calcium concentrations is turned on in neurons at stages preceding synaptogenesis, whereas formation of synapses coincides with the activation of the component activated by high calcium concentrations.

The fusion machinery for synaptic vesicle exocytosis

The molecular mechanisms which control SV exocytosis at mature synaptic sites have been widely investigated, and several protein components of the neuroexocytosis apparatus have been now identified [28, 29, 31, 56–58]. These studies have demonstrated that SV exocytosis implicates the interaction of the synaptic vesicle membrane proteins synaptobrevin/VAMP 1 and 2 (v-SNAREs) with the plasma membrane proteins syntaxin and SNAP-25 (t-SNAREs). Synaptic SNARE proteins have been shown to represent specific substrates of the proteolytic action of clostridial tetanus (TeNT) and botulinum (BONT) neurotoxins, which act as potent blockers of exocytosis from nerve terminals: synaptobrevin/VAMP is the substrate of TeNT and of BoNT/B, BoNT/D, BoNT/F and BoNT/G; syntaxin and SNAP-25 are specifically cleaved by BoNT/C and by BoNT/C, BoNT/A and BoNT/E respectively [59–64].

The three SNARE proteins synaptobrevin/VAMP2, SNAP25 and syntaxin I are already expressed by cultured hippocampal neurons at early developmental stages [35, 68], as well as in the embryonic brain [65, 68], although it has been reported that synaptobrevin/VAMP2 differently associates with other SV proteins during brain development [66]. The early expression of SNARE proteins opens the possibility that the machinery that controls SV exocytosis at the synapse may be operative already before synaptogenesis. Taking advantage of the ability of clostridial toxins to enter hippocampal neurons already at early developmental stages [67], we have tested whether toxin treatment already impairs SV recycling before synaptogenesis. Results indicate that, although internalized in neurons already before synapse formation, TeNT does not significantly impair the exocytotic process before synaptogenesis [68]. On the other hand, SNAP25 and syntaxin appear to be substantially involved in controlling SV recycling in immature neurons, as indicated by the fact that BoNT/A and BoNT/C strongly inhibit SV recycling before synaptogenesis [68]. Interestingly, exocytotic events mediating neurite elongation have been shown to proceed in the presence of TeNT, though impaired by BoNT/A and C (for review see [69]). These data indicate a specific role of synaptobrevin/VAMP2 at the mature synaptic contacts and a more general role of syntaxin and SNAP25 in two fusion processes (SV recycling and neurite extension) occurring in developing neurons.

Calcium channels

Although SV recycling before synaptogenesis takes place at a high basal rate, the process is already stimulated by depolarizing treatments [37, 38]. This indicates that the machinery responsible for the evoked release is at least partially activated at early developmental stages. Interestingly, however, when exocytosis was induced by stimulation from hippocampal cultures before and after synaptogenesis, a different relative contribu-

tion of distinct types of calcium channels was found to support glutamate release. In particular, whereas in mature cultures rich in synaptic contacts ω-Aga-IVA-sensitive channels (P/Q type channels) mainly appeared to control glutamate release, in developing axons prior to synaptogenesis the contribution of ω-Cgtx-sensitive calcium channels (N-type channels) was found to be predominant [46]. The switch from a still immature to a fully mature synapse has been found to be accompanied by further involvement of ω-Aga-IVA-sensitive channels in supporting neurotransmitter release [70]. Thus distinct classes of calcium channels begin to participate in transmitter release at different times during synapse development and maturation.

The different role of ω-CTx-GVIA- or ω-Aga-IVA-sensitive calcium channels in supporting evoked glutamate release at different developmental stages may have several explanations, including different levels of expression of the two classes of channels or changes in the topological relationship between the sites of SV exocytosis and the localization of these channels. In mature neurons, multiple subtypes of voltage-gated calcium channels have been shown to be differentiallly localized in distinct submembrane domains, suggesting that they serve different roles in neuronal excitation and signalling [71–73]. Changes in the expression of different subtypes of calcium channels also appear to take place during neuronal differentiation. In neurons at early developmental stages, N-type calcium channels appear to be present throughout the neuronal surface, including the axonal membrane [74, 75], the compartment where SVs are also segregated. This may account for the relevant role of N-type channels in supporting glutamate release before synaptogenesis [46]. In parallel with synaptogenesis, N-channels disappear from the axonal membrane and accumulate in the somatodendritic region [75, 76]. N-type calcium channels also appear to redistribute to the presynaptic terminal [74, 77]. Synaptic contacts, however, also contain P/Q-type channels [77], and this population of channels appears to play a relevant role in supporting glutamate release at the mature synapse [46, 70, 78, 79]. These findings thus support the possibility that the different role of distinct types of calcium channels in glutamate release during neuronal development in culture may result from a regional redistribution of these channels.

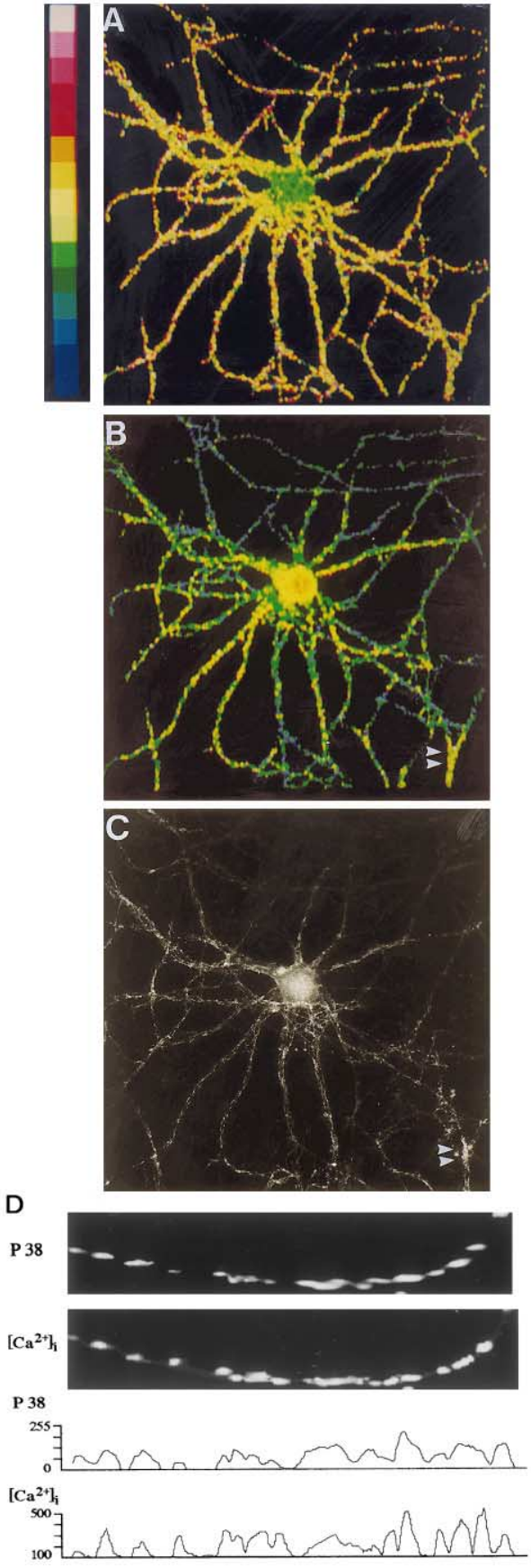
Maturation of the postsynaptic compartment

The postsynaptic site is generally represented by a portion of the dendrite or perikaryal plasma membrane which contains an almost crystalline array of ionotropic receptors, the neurotransmitter-gated ion channels, and is lined at its cytoplasmic side by specialized submembranous cytoskeleton. Most of our knowledge about the biological events underlying the differentiation of a postsynaptic compartment has derived from studies carried out at the neuromuscular junction. Here, cellular and molecular mechanisms responsible for aggregation of ACh receptors underneath presynaptic membrane have been described in some detail. An association between the 43K protein and actin has been proposed as a mechanism for immobilizing ACh receptors at the postsynaptic site [80]. In addition, a search for molecules that direct the accumulation of ACh receptors at nerve-muscle synapses led to the discovery of agrin [81]. Agrin activates the muscle-specific tyrosine kinase receptor MuSK, initiating a process requiring rapsyn and possibly also receptor phosphorylation [82].

Anchoring proteins for GABA and glutamate receptors

The finding that in the CNS a functional analogue of 43K, gephyrin, promotes and sustains clustering of the glycine receptors in cultured spinal neurons by mediating the interaction between receptors and cytoskeleton [83, 84] has suggested the general concept that a peripheral membrane protein may link neurotransmitter receptors to the submembrane cytoskeleton. Interestingly, gephyrin has been detected also in hippocampal cell cultures, where it changes its distribution from predominantly axonal to more dendritic with time in culture, eventually anchoring γ-aminobutyric acid (GABA) and glycine receptors, but not glutamate receptors, at postsynaptic sites in mature GABAergic synapses [85].

The mechanisms which govern the targeting, clustering and stabilization of glutamate receptors in the postsynaptic membrane are at present widely investigated. Glutamate acts via the activation of ionotropic and metabotropic receptors. Ionotropic N-methyl-D-aspartic acid (NMDA) and non-NMDA (α-amino-3-hydroxy-5-methyl-4-isoxazolepropionic acid (AMPA) and kainate) receptors, in addition to conveying fast excitatory neurotransmission, appear to play a major role in neuronal differentiation and CNS development, as well as in those processes that give rise to long-term potentiation and memory formation. Similar to the ACh receptors at the neuromuscular junction, glutamate receptors appear to be concentrated at postsynaptic sites. Recently, a number of proteins that specifically interact with the C-terminal of glutamate receptor subunits have been identified. These proteins have been shown to contain a PDZ protein interaction domain which appears to be responsible for localizing the ligands to specific submembrane domains. The metabotropic glutamate receptors, NMDA receptors and AMPA receptors bind to specific PDZ domains of the proteins Homer, PSD-95 and GRIP, respectively [86, 87]. The



existence of different proteins involved in the postsynaptic clustering of specific subtypes of glutamate receptors allows the differential distribution of these receptors among different synapses and even within the same glutamatergic synapse.

Expression of glutamate receptors before synaptogenesis

Glutamate receptors are expressed by hippocampal neurons from very early stages of neuronal development, and a progression in the distribution of glutamate receptors to synaptic clusters takes place in parallel with culture maturation. This was established by classical immunocytochemical procedures using antibodies directed against specific subunits of glutamate receptors [88–90], and by a calcium-imaging approach, which provided information about distribution of functional glutamate receptors [91] (fig. 4). Functional non-NMDA and NMDA glutamate receptors are expressed by hippocampal neurons as early as 1 day after plating, when they are present throughout the entire neuronal membrane [88, 89, 91]. The observation that functional glutamate receptors are expressed by hippocampal neurons at stages preceding synapse formation, together with the finding that the NMDA receptor subunit NR2B is expressed by immature hippocampal neurons in axonal growth cones and processes [92], suggests that glutamate receptors may play a role in the regulation of neurite outgrowth as well as in the interactions between the growth cone and its immediate environment [48, 49]. A role of neurotransmitters as chemoattractants for the guidance of neuronal processes has been demonstrated in isolated embryonic spinal neurons in *Xenopus* cell cultures [47]. The presence of functional glutamate receptors, together with the occurrence of SV recycling and glutamate release before synapse formation, may have an important role in the ontogenesis of the mature nervous system. Since neuronal development is characterized by a concerted action of trophic and degenerative processes, release of glutamate from the very early

Figure 4. Localization of NMDA functional glutamate receptors in hippocampal neurons. Pseudocolor representation of $[Ca^{2+}]_i$ changes induced by 50 mM KCl (*A*) or 30 μM NMDA (*B*) in the same fura-2-loaded 16-day-old neuron. $[Ca^{2+}]_i$ increases produced by KCl occurr throughout all neuronal processes (*A*), whereas $[Ca^{2+}]_i$ increases produced by NMDA are restricted to a subset of processes (*B*). (*C*) Immunostaining of the same neuron with antibodies to the synaptic vesicle protein synaptophysin. Bright dots represent sites of synaptic contacts. (*D*) Detail of a process stimulated with NMDA and stained for synaptophysin (P38). Note that the intradendritic distribution of functional NMDA receptors matches with the presence of synaptic contacts (arrowheads in *B* and *C*, and *D*). Graphs in *D* represent the density profiles of synaptophysin and calcium signals in the same dendrite. (Modified from Verderio et al. [91], with permission of J. Cell Biol.)

stages of development may influence ontogenesis and plasticity of the cytoarchitecture of the brain.

Synaptic localization of glutamate receptors

When neurons establish a clear axonal and dendritic polarity, glutamate receptors segregate in the somatodendritic membrane (fig. 4B, C), and eventually localize to synapse-enriched portions of dendrites [88, 89, 91, 93] (fig. 4D). Binding and immobilization of the receptors at postsynaptic sites appear to be dependent on the putative anchoring proteins postsynaptic density (PSD)-95, chapsyn and cGMP-dependent-proteinkinase anchoring protein (GKAP). These proteins have been shown to redistribute during synaptogenesis, following a pattern which is consistent with a role in glutamatergic synapse formation [89].

The extent of colocalization of different glutamate receptors at a same synapse appears to be particularly relevant in phenomena such as synaptic plasticity. It has been proposed that pure NMDA receptor-containing synapses are present at early stages of development, and start expressing functional AMPA receptors later on, or at least only after the synapses become fully functional and able to generate long-term synaptic plasticity. This process is referred to as 'spine AMPAfication' [94–97]. The insertion of functional AMPA receptors allows the synapse to be active at resting membrane potentials, whereas pure NMDA synapses, the so-called silent synapses, cannot be heard at resting potentials due to the Mg^{2+} block of the NMDA receptors [98]. The elucidation of those mechanisms which are involved in the localization of glutamate receptors in specific areas of neuronal cells during development is therefore essential for understanding the basis of glutamatergic functionality in the CNS, and primary cultures of hippocampal neurons have been used to investigate this aspect.

NMDA and non-NMDA receptor localization has been suggested to be regulated by different mechanisms, based on the immunocytochemical demonstration that they cluster at synaptic sites at different times in culture, even though the developmental sequence of clustering is still a matter of debate. Indeed, whereas Rao et al. [89] report that the NMDA receptor subunit NR1 is recruited at postsynaptic sites at 4–5 weeks in culture, Liao et al. [90] have recently shown that most of excitatory synapses contain NMDA receptors already at earlier developmental stages. Similarly, the synaptic clustering of AMPA receptors during neuronal development remains controversial. A diffuse staining throughout the young neurons and the presence of NMDA clusters which do not contain AMPA receptors even after 3 weeks in culture [90] appears to be in contrast with previous reports indicating a clustering of AMPA receptors even at early stages of synaptogenesis [89]. Differences in the staining protocols as well as in culture conditions may account for these discrepancies [90]. Interestingly, functional studies support the idea that a significant proportion of functional NMDA receptors is localized at postsynaptic sites already before 4–5 weeks in culture. Indeed, NMDA-induced calcium transients have been shown to significantly colocalize with presynaptic SV clusters already after 2 weeks in culture [91 and figs. 4 and 5]. Moreover, recent electrophysiological studies have demonstrated the persistence of a population of synapses containing clustered NMDA but not AMPA receptors in cultured hippocampal neurons [99], thus providing anatomical and physiological evidence for the existence of silent synapses in cultured hippocampal neurons [90, 99].

The glutamate transporter EAAC1

Rapid reuptake is essential for terminating synaptic transmission by most neurotransmitters in the nervous system. Specific carriers transport neurotransmitters across the plasma membrane of neuronal cells and surrounding glial cells, allowing the clearance of the neurotransmitter from the synaptic cleft and its further metabolic processing [100, 101]. Among the different high-affinity glutamate transporters, excitatory amino acid carrier 1 (EAAC1) is neuron-specific, and it is widely distributed throughout the brain [102, 103]. The localization of EAAC1 during neuronal development has been investigated in hippocampal cultures [104]. This study has provided further support for the idea that this neuronal glutamate carrier is not localized in the presynaptic membrane [102]. EAAC1 was found to be expressed at stages preceding the formation of synaptic contacts, and, unlike glutamate receptors, it appeared to be segregated in the somatodendritic compartment of hippocampal neurons from 1 day after plating (fig. 2C). At later developmental stages, EAAC1 was found to be localized along the dendritic shaft and the spine neck, being undetectable in the postsynaptic membrane [104]. The presence of EAAC1 on the spine neck suggests a strategic role of EAAC1 in lowering glutamate concentrations during the diffusion process and in restricting transmitter spillover to the nearby areas. Not only is EAAC1 expressed in glutamatergic neurons before synapse formation but it is also present in GABAergic neurons [102, 104] with the same distribution pattern. These data, together with the observation that EAAC1 is expressed at high levels already at embryonic stages in the hippocampus in vivo, support the idea that the neuronal glutamate carrier also plays nonsynaptic functions, e.g. in brain development and synaptogenesis [104].

Stabilization of synaptic contacts

The formation of intercellular junctions requires multiple interactions between membranous and cytosolic proteins. However, the precise mechanisms regulating the assembly of the pre- and postsynaptic compartments have not been completely clarified. Formation and stabilization of contacts between neurons have been described as involving different sets of scaffolding proteins, including cadherins, homophilic adhesion proteins mediating cell-to-cell contact [105–107]. N-cadherin has been reported to be expressed in devel-

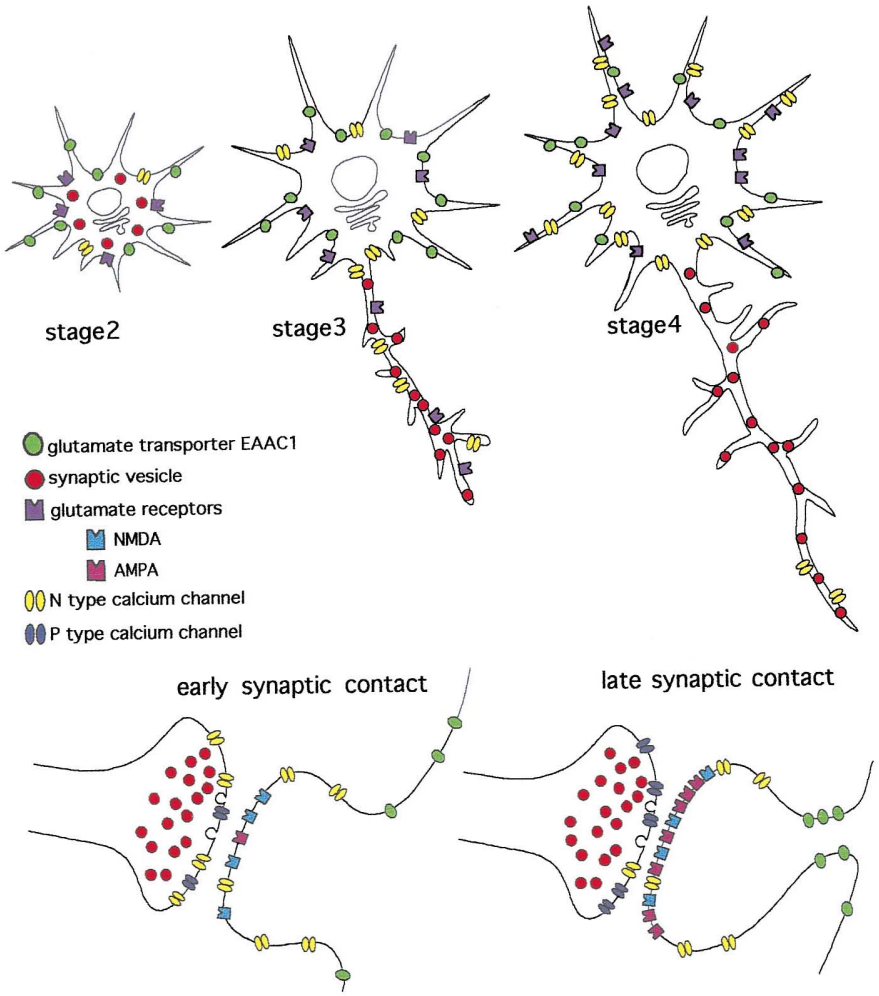


Figure 5. Scheme representing the redistribution of some synaptic components during the establishment of neuronal polarity and synaptogenesis in cultured hippocampal neurons. Most of synaptic components are expressed by hippocampal neurons at stage 2. At this time in culture, glutamate receptors, glutamate transporters and calcium channels appear to be uniformly distributed throughout neuronal membrane [88, 91, 104]. As soon as the axon forms and starts elongating (stage 3), synaptic vesicles become sorted to this compartment [32, 34]. Glutamate receptors [88, 91] and N-type calcium channels [74, 75] are present along the entire neuronal surface, whereas the glutamate transporter EAAC1 appears to be excluded from the axonal surface [104]. At stage 4, a clear compartmentalization of all these synaptic components is established, with glutamate receptors, transporters and N-type calcium channels being enriched in the somatodendritic region [74, 76, 88, 91, 104] and synaptic vesicles being selectively localized in the axon [32–34]. The possible presence of N-type calcium channels at the growing tip of the axon is depicted. No data regarding the distribution of P-type calcium channels at these developmental stages are available at present. When the synaptic contact is formed, synaptic vesicles become clustered in the presynaptic ending [32–34]. At the same time, glutamate receptors concentrate in the postsynaptic membrane [88, 91]. Recent data suggest that NMDA glutamate receptors are preferentially present at the early synapse and that AMPA receptors become inserted at later stages, when the synapse has become fully functional [90, 99]. N-type channels are present in the postsynaptic, somatodendritic compartment [71, 75, 76], even though they appear to be expressed at a certain extent also at the presynaptic level [70, 73, 130]. The maturation of the synapse coincides with an enrichment of P-type calcium channels at the presynapse [46, 70]. At the mature synapse, the glutamate transporter EAAC1 is localized to the spine neck [104].

oping axons and dendrites of hippocampal neurons as a recruitable pool of molecules that can be rapidly assembled into synaptic junctions at both sides after cell-to-cell contact [108, 107]. Both excitatory and inhibitory early synaptic contacts are associated with N-cadherin, but after maturation of synapses, N-cadherin becomes restricted only at excitatory synaptic junctions [107]. This finding suggests that different members of the cadherin family could mediate the stabilization of excitatory versus inhibitory synapses [107].

In addition to the homophilic interactions mediated by N-cadherins, heterophilic interactions between cell surface proteins may play a role in the assembly of the synaptic junction. A complex of three proteins containing PDZ domains (CASK, Mint1 and Veli), in the presynaptic terminal, has been proposed to couple proteins involved in SV exocytosis to proteins mediating the assembly of the synaptic junction [109]. In particular, a model has been proposed in which the cell surface protein β-neurexin (coated on the presumptive presynaptic side by CASK) and the cell surface protein neuroligin (coated on the presumptive postsynaptic side by PSD-95) may interact as heterophilic cell adhesion molecules [110], keeping the pre- and postsynaptic membranes in register [109]. Thus PDZ-containing proteins appear to be involved in the organization of the synaptic cytoarchitecture [30] not only at the postsynaptic level but even at the presynapse, eventually keeping together pre- and postsynaptic membranes via heterophilic adhesion of cell membrane proteins.

Role of glial cells and neurotrophins during synaptogenesis

Glial cells make up a large percentage of the cell population in the brain. In the past, the main role of glial cells was thought to be that of providing structural and trophic support to neurons, keeping them stuck together and providing them with factors essential for their survival. On the other hand, the function of transmitting and processing information was attributed exclusively to neurons. This concept has deeply changed in recent years. It is now widely believed that glial cells and neurons, which are intimately juxtaposed throughout the nervous system, are functionally interacting. It is also clear that the key site of neuron-glia interactions is the synapse, which is the most specialized structure responsible for transmitting and processing information between neurons. Recently reported data suggest the existence of a close dialogue between neurons and glia, which appears to influence synaptic functionality [111–113].

Glial cells also appear to influence the time course of synapse maturation. A role of glial cells in promoting synaptogenesis has been suggested by data obtained on primary cultures of cortical and retinal neurons [114, 115], using functional approaches such as electrophysiology and calcium imaging, to detect the establishment of fully differentiated synapses. A different efficiency in the formation of functional synaptic contacts was found to take place in cultured hippocampal neurons growing in the absence of glial cells, or cocultured with hippocampal or cortical astrocytes. Whereas most of the neurons grown in contact with astrocyte monolayers displayed a full synaptic functionality after 3–5 days in culture, neurons growing without neighbouring glial cells displayed no or few miniature synaptic events [Bacci et al., unpublished data]. A strict proximity between neurons and astrocytes appeared to be relevant in supporting the enhanced synaptogenesis, possibly by facilitating the delivery of trophic factors by astrocytes to neurons.

The nature of the factors involved in fastening synapse formation is still unknown. We have known for several years that astrocytes release diffusible neurotrophic factors which promote neuronal survival and development [116–118]. Interestingly, a neurotrophic factor secreted by a glial-cell line (GDNF) [119] has been found to regulate neuronal survival, differentiation and formation of synapses in at least two diverse neuronal populations, including dopaminergic neurons and motoneurons (for review see [120]). More recently, it has been shown that a low molecular weight fraction, containing high levels of L-serine and present in a glial-conditioned medium, displays neurotrophic activity. When added to hippocampal neurons, L-serine was found to improve neuronal survival and neurite outgrowth [121].

Different neurotrophins have recently been found to be important in the formation of synapses between cultured hippocampal neurons. Neurotrophin-3 (NT-3) enhances neurite outgrowth and branching in low-density hippocampal neurons in culture [122]. This neurotrophic factor also accelerates the development of neuronal polarity through an accumulation of bundles of looped microtubules within axonal growth cones [122]. In a recent paper, Vicario-Abejon et al. [123] exposed hippocampal cultures to brain-derived neurotrophic factor (BDNF) and NT-3. They found that neurons obtained from E-16 embryos displayed low levels of spontaneous and evoked synaptic activity even after two weeks in culture, at variance with cultures prepared from E-18 embryos. BDNF and NT-3 exposure to E-16 neurons for 24–72 h induced synaptic responses, and the major effect of these neuro-

trophic factors seemed to be an increase in the number of functional synapses rather than a strengthening of those already existing. They also found an increase in the mean miniature excitatory postsynaptic current (mEPSC) frequency, suggesting that the neurotrophins act presynaptically to promote the maturation of neurotransmitter release mechanisms. Taken together, these data suggest that although the basic patterns of neuronal maturation may be endogenously determined, critical aspects of their morphology and physiology are strongly modulated by neurotrophic factors. Neurotrophins also regulate the physiology of the mature synapse, inducing an enhancement of synaptic transmission produced by the action both at the presynaptic [124] and postsynaptic sites [125–127].

Conclusions

The cellular machinery responsible for cell-to-cell communication reaches its maximum efficiency and spatial complexity in neurons. In these cells, mechanisms for both transmission and reception of chemical signals coexist, and in most cases, these two functions are carried out by distinct neuronal districts, the axon and the dendrites. From the data available at the moment, it appears that most, if not all, synaptic components are expressed by neurons at early developmental stages. SVs are present in the axon and are already able to undergo exo-endocytotic recycling; proteins of the SV fusion machinery and voltage-activated calcium channels are also expressed. Neurotransmittter receptors are inserted in the neuronal membrane and appear to be already functional at very early stages of development in vitro. Formation of synaptic contacts coincides with a regional redistribution of SVs, receptors and channels, and this reorganization leads to the formation of highly specialized surface and cytoplasmic domains, which define the pre- and postsynaptic compartments. Formation and stabilization of contacts between neurons appear to involve different sets of scaffolding proteins, including proteins anchoring neurotransmitter receptors and homophilic and heterophilic adhesion proteins mediating cell-to-cell adhesion. In addition to an endogenously determined program of neuronal maturation, extrinsic players, such as astrocytes, also modulate the process of neuronal synaptogenesis.

During synaptogenesis, some functional properties of neurotransmitter release appear to be specifically activated: downregulation of SV exo-endocytotic recycling takes place, and a putative low-affinity calcium sensor, which allows SV exocytosis to be triggered in response to large increases in the levels of intracellular calcium, becomes activated. At the same time, SV recycling acquires the sensitivity to toxins which are known to impair neurotransmitter release at a mature synapse. Although the mechanisms which induce exocytosis to switch from an immature to a mature form are at present far from being clarified, it seems reasonable to hypothesize that the presence of an appropriate postsynaptic target may play a role in inducing this switch. This hypothesis is in line with data recently obtained in different experimental systems, suggesting that maturation of presynaptic structure and function is affected by signals from the postsynaptic cell [40, 128–133]. The possibility that control of postsynaptic activity on the presynaptic mechanisms which control SV fusion may be actively involved during synaptogenesis between neurons in the mammalian CNS thus becomes likely.

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