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The Formation of Synapses in the Central Nervous System

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Abstract

Interneuronal synapses are specialized contact zones formed between the transmitting pole of one neuron, usually an axon, and the receptive pole of another nerve cell, usually a dendritic process or the soma. The formation of these synaptic contacts is the result of cellular events related to neurite elongation, the establishment of polarity, axon guidance, and target recognition. A series of morphological rearrangements takes place once synaptic targets establish their initial contact. These changes include the clustering of synaptic vesicles in the presynaptic element and the formation of a specialized area capable of signal transduction at the postsynaptic target. The present review discusses the role of different synaptic proteins in the cellular events leading to the formation of synapses among neurons in the central nervous system.

Index Entries: interneuronal synapses; synapsins; agrin; cadherins; neurotransmitter receptor clustering.

Introduction

Synaptic contacts are asymmetric and highly specialized contact zones established between the transmitting pole (the axon) of a presynaptic neuron and the receptive pole (either the dendrites or the soma) of the target nerve cells. Synaptogenesis, the final step in neuronal development, is the result of cellular events related to neurite elongation, the establishment of polarity, axon guidance, and target recognition *(1–3)*. The formation of these specific connections underlies normal functioning of the nervous system, and, therefore, has been the subject of numerous studies over the past three decades. Most of these studies have used the neuromuscular junction as a model system, both for its simplicity and its accessibility to experimental manipulations. A growing body of evidence indicates that, at the neuromuscular junction, the initial contact of an axonal growth cone with a muscle cell initiates a series

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of changes at the postsynaptic site. These changes include the synthesis and clustering of acetylcholine receptors (AChRs) at the synaptic site followed by their disappearance from the extrasynaptic membrane. Factors released by the presynaptic element are capable of inducing either AChR synthesis, i.e., ARIA (acetylcholine receptor-inducing activity), or the clustering of preexisting receptors, i.e., agrin (reviewed in ref. *4*). The formation of interneuronal synapses in the central nervous system (CNS), on the other hand, is the result of the transformation of axonal varicosities or axonal growth cones into the presynaptic elements and the rearrangement of areas of the dendritic shaft or dendritic spines into postsynaptic specializations. The cellular mechanisms underlying these morphological changes are largely unknown. However, recent time-lapse recordings of living neurons, coupled with vital fluorescent labeling methods, have analyzed the short-range interactions between synaptic partners and have begun to shed light on the sequence of events that leads to the formation of interneuronal synapses. These studies show that the formation of the initial synaptic contact is the result of the dynamic protrusive activity of dendritic filopodia that actively seek out synaptic partners (*5,6;* reviewed in ref. *7*). In addition, they suggest that individual synapses can form rapidly after axodendritic contact (within 1–2 h) and that presynaptic differentiation often precedes postsynaptic differentiation *(8–10).* Evidence suggests that presynaptic molecules are carried to the junction in preassembled complexes of synaptic components (*11;* reviewed in ref. *12*). The use of pre-assembled packets is consistent with the ability of new contact sites to rapidly differentiate. It is possible that a similar strategy may be used to assemble postsynaptic components, although a consensus has not been achieved as yet *(13,14).*

This review discusses the role of different molecular components of interneuronal synapses during the differentiation of both the presynaptic terminal and the postsynaptic apparatus, as well as during the formation of the synaptic cleft.

The Formation of the Presynaptic Terminal

One of the first detectable morphological changes associated with synapse formation at the presynaptic element is the formation of synaptic vesicle clusters in close apposition to the contact zones (reviewed in *15–18; see also* ref. *19*). The synapsins seem to play an important role in this event. The synapsins are a family of very abundant neuron-specific phosphoproteins encoded by three distinct genes: synapsin I, synapsin II, and synapsin III *(20–27).* Both the subcellular localization of these proteins and their pattern of expression suggest their participation in the organization of the presynaptic terminals. The synapsins are localized in the presynaptic compartment, where they associate with synaptic vesicles *(28–37).* In addition, an increase in synapsin I and synapsin II parallels the establishment of synaptic contacts in different brain areas both *in situ* and in cultured neurons *(38–40).* Studies of "gain or loss of function" have confirmed the participation of the synapsins in the process of synapse formation. When synapsin II was overexpressed in a neuroblastoma/glioma cell line, cells developed varicosities with ultrastructural characteristics that resembled the ones observed in the autonomic nervous system *(41).* More recently, several studies have analyzed the ability of neurons depleted of synapsins, either by homologous recombination techniques or antisense oligonucleotides, to establish synaptic contacts. In synapsin I knockout mice, there is a decrease in both the size of synaptic terminals and the number of synaptic vesicles per terminal *(42–44).* Abnormal synapse formation was also detected when hippocampal neurons obtained from synapsin-mutant mice were placed in culture. While synapsin I-, synapsin II-, and synapsin I/II-deficient hippocampal neurons were able to form synaptic contacts, they formed fewer synapses. In addition, synaptogenesis was delayed for almost a week in the absence of either synapsin I or synapsin II *(45–47).* Synapsin II also seems

to be required for synapse maintenance. When synapsin II was suppressed after synaptogenesis had already occurred, many of the preexisting synapses were lost *(47)*.

The synapsins might induce the formation of a presynaptic terminal through different mechanisms. High-affinity binding of synapsin I and synapsin II to the membrane of synaptic vesicles is mediated by multiple sites located in the N-terminal and middle portions of the synapsins *(32,48–50)*. Thus, the accumulation of the synapsins in "hot spots" at sites of contact between synaptic partners could result in the recruitment of synaptic vesicles to that site. The synapsins might also participate in the regulation of other proteins involved in synaptogenesis. When synapsin IIb was overexpressed in neuroblastoma/glioma cells, there was an increase in the expression of other presynaptic proteins including synapsin I and synaptophysin *(41)*. On the other hand, the depletion of synapsin I and/or II resulted in a concomitant decrease in synaptophysin, synaptotagmin, synaptoporin, synaptobrevin, and syntaxin *(44,51)*.

The signals that trigger the clustering of synaptic vesicles are unknown. Both soluble factors and cell adhesion molecules are probably involved. Signaling molecules that have been shown to induce at least some aspects of presynaptic differentiation may be target-derived, like WNT-7 and the brain-derived neurotrophic factor (BDNF), or released by the axons themselves, like agrin *(4,52–55)*. Additional aspects of presynaptic differentiation are probably mediated by cell-cell contact (*see* below). Regardless of the mechanisms, synapsin I and synapsin II appear to serve as upstream intermediates in the synapse formation pathway.

The Formation of the Postsynaptic Apparatus

The postsynaptic apparatus is a specialization of the plasma membrane that lies opposite transmitter release sites. It is characterized by the presence of clusters of glutamate receptors in excitatory synapses and γ-aminobutyric acid (GABA) or glycine (Gly) receptors in inhibitory synapses. These neurotransmitter receptor clusters are closely associated with multiple molecules whose functions range from a role in receptor localization and trafficking to signal transduction and cytoskeletal anchoring. One of the first cellular events associated with the differentiation of the postsynaptic apparatus is the redistribution of neurotransmitter receptors and their accumulation at contact sites. In hippocampal neurons in culture, neurotransmitter receptors are uniformly distributed at very early stages of development *(56)*. Upon contact with a synaptic partner, these receptors form well-defined clusters in the postsynaptic membrane. When motor neurons are co-cultured with GABAergic or glycinergic neurons, $GABA_A$ receptors and Gly receptors selectively accumulate opposite GABAergic and glycinergic terminals, respectively. Neither GABA nor Gly receptors cluster at the autaptic cholinergic synapses *(57)*. Similarly, glutamate and GABA receptors cluster opposite terminals releasing the corresponding neurotransmitter in cultured hippocampal neurons *(58)*. The mechanisms that trigger the clustering of neurotransmitter receptors in central synapses are not completely known. However, it is tempting to speculate that, if present, presynaptic factors that induce the formation of acetylcholine receptor clusters at the neuromuscular junction might be also necessary to induce receptor recruitment at interneuronal synaptic sites. This hypothesis prompted studies on the potential role of agrin, the key player in the assembly of the neuromuscular junction, during synaptogenesis in the CNS. Agrin, an extracellular matrix protein released by motor neurons, is encoded by one gene that gives rise to multiple isoforms through alternative splicing *(59,60)*. Agrin isoforms with an insert at the Z splice site (z+ agrin) are the critical nerve-derived inducer of postsynaptic differentiation at the neuromuscular junction *(61)*. Multiple lines of evidence have suggested that agrin may be implicated in some aspects of brain development and central synaptogenesis. Agrin is widely distributed in

the CNS *(62).* Upon synapse formation, agrin colocalizes with synaptic markers both in the retina and in cultured hippocampal neurons *(63,64).* Furthermore, its levels of expression peak during stages of active synapse formation in neurons that develop either *in situ* or in culture *(62,63).* Recently, several studies have attempted to address the role of agrin in the formation of synapses in the CNS by analyzing the agrin-null phenotype. The chronic suppression of agrin expression by homologous recombination techniques did not affect the time course of synapse formation. In addition, the synapses formed in the absence of agrin were morphologically and functionally indistinguishable from the ones formed by wild-type controls *(65–67).* These results seem to rule out a potential role of agrin in the formation of the postsynaptic apparatus in central synapses. However, it is possible that molecular redundancy with other agrin isoforms may be responsible for the lack of phenotype at interneuronal synapses of mutant mice. This explanation is supported by experiments in which agrin expression was suppressed by means of specific antisense oligonucleotides. The acute suppression of agrin expression by this method resulted in a decrease in the number of synapses formed by cultured hippocampal neurons *(63,68).* This decrease in the number of synapses was accompanied by impaired clustering of GABA receptors *(63).* On the other hand, no defects in glutamate receptor clustering were detected in agrin-depleted neurons *(63).*

The selective effect of agrin on the clustering of GABA receptors suggests the involvement of other factors capable of triggering the clustering of glutamate receptors. Recent evidence suggests that ephrinB ligands could have such a role. EphB receptors are tyrosine kinase receptors that are enriched at the postsynaptic apparatus of hippocampal neurons *(69).* They are activated by ephrinB ligands, transmembrane proteins that have been shown to influence synapse formation and function in vitro (reviewed in ref. *70*). EphrinB stimulation of cultured neurons leads to the recruitment of glutamate receptors to an EphB receptor complex and increases the number of both pre- and postsynaptic specializations *(71).*

Once the initial clustering of neurotransmitter receptors takes places, specific anchoring proteins need to be recruited to the postsynaptic apparatus to limit the lateral movement of these receptors. Several proteins that could play such a role have been identified. Glutamate receptors have been found to interact with different members of a growing superfamily of proteins containing PDZ domains. PDZ domains consist of ~90 amino acid residues that bind to short peptide sequences located on the C-terminal tails of the interacting protein (reviewed in ref. *72*). The interactions of specific receptor subunits with different PDZ domains may account for the regulation of both NMDA (N-methyl-D-aspartate) and AMPA (α-amino-3-hydroxy-5-methyl-4-isoxazolepropionic acid) glutamate receptors during development and synaptic plasticity. The NR2 subunit of glutamate NMDA receptors interacts specifically with the PDZ domain-containing protein PSD-95/SAP90 (Post-Synaptic Density protein 95 kDa/ Synapse Associated Protein 90 kDa) *(73).* On the other hand, the GluRl subunit of AMPA receptors binds to SAP97 (a member of the Synapse-Associated Protein family) and GluR2/GluR3 subunits bind to GRIP/ABP proteins (glutamate receptor interacting protein/AMPA receptor binding protein) and PICK1 (protein interacting with C kinase) *(74–77).* PDZ domain-containing proteins seem to have a role in glutamate receptor targeting and clustering. In cultured hippocampal neurons, PSD-95 was found to cluster opposite presynaptic terminals before either NMDA or AMPA receptors clustered at these presumptive postsynaptic sites *(78).* This observation, along with the fact that synaptic NMDA receptor clusters were always found to contain PSD-95, suggested that the formation of a postsynaptic scaffold containing PSD-95 is an early step in the differentiation of the postsynaptic apparatus and may direct receptor targeting to the synapse *(78).* In addition, in vitro studies showed that expression of PICK 1 in heterologous cells *(77)* and PSD-95

overexpression in hippocampal neurons *(79)* lead to receptor clustering. Surprisingly, the deletion of PSD-95 in mice did not affect the synaptic localization of NMDA receptors *(80).* However, it is possible that molecular redundancy among PDZ domain-containing proteins may compensate for the loss of PSD-95, obscuring the physiological role of this molecule during development.

The targeting and clustering of both GABA and glycine receptors, on the other hand, seems to be mediated by the peripheral membrane protein gephyrin. Gephyrin colocalizes with Gly and GABA receptors in different areas of the CNS *(81–87).* The potential role of gephyrin in the clustering of these receptors was confirmed by studying the gephyrin-null phenotype. The formation of Gly and GABA receptor clusters was prevented in neurons in which gephyrin expression was suppressed by means of either antisense oligonucleotides or homologous recombination techniques *(88–91).* Gephyrin, as well as PSD-95, not only mediates the clustering of neurotransmitter receptors but also serves to cross-link these receptors to different components of the cytoskeleton (i.e., tubulin, microtubule-associated proteins), cell adhesion molecules (i.e., neuroligin), and a variety of other molecules involved in signal transduction (reviewed in ref. *72*).

As reviewed earlier, the major molecular components of the postsynaptic apparatus have already been identified and characterized. However, a number of questions regarding the triggering and regulation of the mechanisms underlying the differentiation of the postsynaptic element remain open and await further investigation.

The Synaptic Cleft

During the past decade, a lot of attention has been directed to analyzing the composition of the synaptic cleft in interneuronal synapses. The clefts between synaptic partners in the CNS differ considerably from those at neuromuscular junctions. The basal lamina in the postsynaptic element and the dense matrix formed by laminin and collagen are characteristic of the neuromuscular junction but are not present in the interneuronal cleft. Synaptic clefts in interneuronal synapses are rich in cell adhesion molecules including cadherins, neurexins, and neuroligins. Cadherins are a family of cell-adhesion molecules that mediate cell-cell interactions through a homophilic Ca^{2+}-dependent mechanism *(92).* One member of this family, N-cadherin, has been localized to the synaptic cleft in central neurons. Immunoelectron microscopy and confocal laser-scanning microscopy have shown that N-cadherin is associated to the active sites in synaptic complexes *(93–96).* These results were confirmed by Western blot analysis of forebrain homogenates, which identified N-cadherin as the major glycoprotein in postsynaptic density preparations *(93).* More recently, studies using dissociated hippocampal neurons have shown that N-cadherin is restricted to glutamatergic synapses *(97).* These results suggested that other cadherins could be localized in synaptic contacts containing different neurotransmitters. This view has been reinforced by the identification of several members of the cadherin family including R-cadherin, cadherin-6, cadherin-6B, and cadherin-7 in synaptic contacts in the cerebellum, the optic tectum, and the retina *(92,98–101).* Based on these results, it has been proposed that the cadherins may provide the molecular basis for the formation of specific synapses in the brain *(95,96).* However, the actual role of the cadherins in the formation of specific synapses has yet to be determined.

Several studies have also localized dystroglycans at synaptic clefts in the central nervous system. Dystroglycans are highly glycosylated peripheral membrane proteins. The proteolytic cleavage of a precursor produces two isoforms: α and β dystroglycans *(102,103).* Both isoforms are known to be involved in the clustering of acetylcholine receptors at the neuromuscular junction. α and β dystroglycans are highly expressed in cortical neurons, pyramidal hippocampal neurons, and Purkinje cells *(104,105).* Ultrastructural analysis demonstrated that these proteins are highly concentrated at

postsynaptic specializations in most of the brain areas studied. Although the role of dystroglycans in central synapses is largely unknown, it is tempting to speculate that they are involved in the clustering of neurotransmitter receptors. This hypothesis seems to be supported by a recent study showing that the clustering of $GABA_A$ receptors is reduced in mice lacking dystrophin *(106).* Dystroglycans can also participate in synapse formation through their interactions with neurexins. The formation of the dystroglycan-neurexin complex in the brain has recently been characterized *(107).* Neurexins are cell-surface proteins encoded by three genes that produce two primary transcripts each, the α and β isoforms (reviewed in *108*). These six primary transcripts are alternatively spliced to generate numerous isoforms. The neurexins are present exclusively in neurons and their expression is developmentally regulated *(109–111).* Analysis of the structure of these proteins predicted that they could function as receptors that modulate cell-cell interactions and could play a key role in the establishment of specific connections. These functions could be mediated, at least in part, by their interaction with dystroglycans. However, the presence of many neurexin isoforms prompted the search for additional ligands. A few years ago, neuroligins were identified as ligands for the β isoform of the neurexins. Neuroligins are neuron-specific cell adhesion molecules highly concentrated at postsynaptic sites in excitatory synapses *(112,113).* Neuroligins mediate cell adhesion to β neurexins through their extracellular domain and to PSD-95 through their cytoplasmic C-terminal tail *(114).* The localization of neuroligins at postsynaptic sites, as well as their role as cell-adhesion molecules, suggested that they could be involved in the formation of synapses in central neurons. Recently, it has been shown that the heterologous expression of neuroligin in epithelial cells, Chinese Hamster ovary (CHO) cells, or fibroblasts can induce the formation of presynaptic specializations (*115,* reviewed in ref. *116*). Thus, the contact of pontine axons with epithelial cells transfected with neuroligin induced the formation of large flattened areas of contact where the plasma membranes were closely apposed. Ultrastructural analysis showed the presence of clusters of synaptic vesicles in close proximity to these areas of contact. In addition, these synaptic vesicles were capable of undergoing depolarization-dependent exocytosis and endocytosis (*115,* reviewed in ref. *116*). The mechanisms by which neuroligins induce the clustering of synaptic vesicles are not known. Neither is known whether neuroligin could induce the clustering of neurotransmitter receptors at the postsynaptic element. However, this study provided important insights into the role of neuroligins as potential signaling molecules capable of triggering synapse formation in the CNS.

The cell-adhesion molecules described earlier may play an important role in the stabilization of the initial axodendritic contact and in triggering downstream signaling pathways in both the pre- and the postsynaptic terminals during early phases of synaptogenesis. In addition, the differential expression patterns of each member of a given family of adhesion molecules may account, at least in part, for the remarkable specificity of synaptic contacts in the CNS (reviewed in ref. *117*).

Concluding Remarks

The numerous studies reviewed above have resulted in the identification of key players in synapse formation in the CNS. However, the full elucidation of the mechanisms involved in the formation and maintenance of synaptic contacts between central neurons will likely require a much broader understanding of the biology of both pre- and postsynaptic elements.

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