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Review

The synapsins: beyond the regulation of neurotransmitter release

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Abstract. The synapsins are a family of five closely related neuron-specific phosphoproteins associated with the membranes of synaptic vesicles. The synapsins have been implicated in the regulation of neurotransmitter release. They tether synaptic vesicles to actin filaments in a phosphorylation-dependent manner, controlling the number of vesicles available for release at the nerve terminus. A growing body of evidence suggests that the synapsins play a broad role during neuronal development. They participate in the formation and maintenance of synaptic contacts among central neurons. In addition, each synapsin has a specific role during the elongation of undifferentiated processes and their posterior differentiation into axons and dendrites. In this review, we focus on these novel roles of synapsins during the early stages of development.

Key words. Synapsins; synaptogenesis; neurite elongation; axonal differentiation; mental diseases.

The synapsin gene family

The central nervous system is characterized by the presence of specialized contact zones or 'synapses' where neurons communicate with one another. Information is passed along either through the propagation of electrical stimuli or, more frequently, via the release of chemical substances. The formation of these connections, which are the basis for the normal function of the nervous system, could be considered the result of events related to neurite elongation and differentiation, target recognition and the 'locking in' of the pre- and postsynaptic membranes [1–3]. In the past 25 years, a great deal of attention has been devoted to establishing how these specific synaptic contacts are formed. One of the experimental approaches used to obtain insights into the mechanisms underlying synapse formation and synaptic function has been to identify the molecular components of synaptic contacts. A family of phosphoproteins, the synapsins, was identified as the result of those studies. The first member of this family, synapsin I, was discovered in 1972 as a major substrate for cyclic AMP (cAMP)-dependent protein kinase and Ca^{2+}/calmodulin-dependent protein kinase [4, 5]. Since then, more members of this family have come to light, and today no fewer than five distinct synapsin isoforms are known to exist [6–10]. Taken together, the synapsins represent one of the most abundant families of synaptic proteins, comprising ~1% of the total protein in the brain [11–13]. They are expressed only in neurons and are specifically localized in the presynaptic compartment of the synapses [14–16]. The synapsins are encoded by three distinct genes: synapsin I, synapsin II and synapsin III [7–10]. Localization of these genes

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has been the focus of several studies. These reports indicate that the synapsin genes are located on three different chromosomes. The synapsin I gene has been assigned to the X chromosome at band Xp11 in humans and at band XA1-A4 in mice, using in situ hybridization techniques followed by Southern blot analysis [7]. The synapsin II gene has been localized to chromosome 3 [10] and the synapsin III gene has been localized to chromosome 22q12.3 [8, 10]. Alternative splicing of the primary transcripts encoded by these genes yields the five known synapsins: Ia, Ib, IIa, IIb and IIIa. The differences between the 'a' and 'b' isoforms seem to be restricted to the C-terminal region. In the case of synapsin I, the difference between the isoforms is due to the inclusion of a 38-nucleotide exon. In the case of synapsin II, the differences between isoforms are generated by the inclusion of two exons in the C-terminal end of the molecule [17]. Only one synapsin III isoform, synapsin IIIa, has been characterized up to now [8, 10].

The structure of the synapsins incorporates both conserved and variable domains assembled in various combinations to form the different members of the family. Eight different protein domains exist (A–H) [18]. The most conserved of these protein domains are A and C, which are present in all of the synapsins [17, 19]. Also relatively conserved is the E domain, which is a component of synapsin Ia, synapsin IIa and synapsin IIIa isoforms [8, 18].

The protein domains, besides providing different structural elements to the various proteins, also confer different functional properties. For example, the A domain has been shown to control the interaction of synapsins with vesicles [20]. This domain contains the only phosphorylation site observed in all of the known synapsins. This site is a substrate for both protein kinase A (PKA) and Ca^{2+}/calmodulin-dependent (CaM) kinase I [8, 18, 20]. In synaptosomes separated into cytosolic and membrane-bound fractions by high-speed centrifugation, only a small amount of the total synapsin I content was found in the cytosolic fraction [20]. However, nearly all of the phosphorylated synapsin I was found in the cytosolic fraction, and almost no phosphorylated synapsin I was found associated with vesicle membrane [20]. Increasing the level of phosphorylation of domain A using an analog of cAMP to activate PKA also led to dissociation of synapsins from synaptic vesicles [20]. In addition, using GST fusion proteins of the A, B and C regions of all of the synapsins, only the A domain, either alone or in combination with the other domains, interacted strongly with phospholipids [20].

Region C is another highly conserved synapsin domain. Interestingly, the crystal structure of the C domain is very similar to those of bacterial ATP-dependent synthetases, suggesting that it may have an enzymatic function [21]. Support for this idea comes from the observation that the C domain is a site for ATP binding in all synapsins [18, 22]. However, no enzymatic role has been established for the synapsins to date. In addition to binding ATP with high affinity, the C domain also binds to other synapsin C domains, mediating the formation of both homo- and heterodimers [23].

Although much has been learned about these proteins in terms of genetics and structure, their complete range of functions is still far from clear. Since the synapsins are associated with the cytoplasmic surface of neurotransmitter-containing vesicles, early studies focused on their potential role as regulators of neurotransmitter release. A growing body of evidence supports a model in which the synapsins tether synaptic vesicles to actin filaments in a phosphorylation-dependent manner, controlling the number of vesicles available for release at the nerve terminus. The role of the synapsins as regulators of neurotransmitter release has been the subject of numerous reviews and will not be reviewed herein [24–34]. Instead, we focus on the novel roles of the synapsins during neuronal development.

The role of synapsins in the formation and maintenance of synaptic contacts

One of the ultrastructural hallmarks of chemical synapses is the presence of clusters of synaptic vesicles in the presynaptic terminals [35]. Prior to synapse formation, synaptic vesicles distribute uniformly along the axons. Upon contact with synaptic partners, there is a redistribution of neurotransmitter-containing vesicles, which organize into two pools. One of these pools contains vesicles docked to the active zone, ready to be released (reviewed in [35]; see also [36]). The other is a reserve pool, which can be recruited in response to increased activity (reviewed in [35]; see also [36]). The mechanisms that trigger the formation of these synaptic vesicle clusters are not completely understood. However, the synapsins seem to play an important role in these mechanisms. Several lines of evidence support this view:

1) Studies of the expression of the synapsins revealed that an increase in synapsin I and synapsin II levels parallels the establishment of synaptic contacts in different brain areas both in situ and in cultured neurons [37–39].

2) Immunoelectron microscopy studies showed that within the nerve terminals, the synapsins are concentrated in areas occupied by synaptic vesicles [17, 18, 40–42]. In addition, subcellular fractionation studies showed that the synapsins are enriched in fractions containing synaptic vesicles [8, 43, 44]. Later studies determined that the 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 molecules. These sites bind specifi-

cally to acidic phospholipids [18, 45, 46]. The presence of multiple binding sites in each synapsin molecule permits one to envision a model in which the selective localization of these proteins in a given axonal area could result in the clustering of synaptic vesicles that is characteristic of the presynaptic terminals.

3) Studies of gain or loss of function confirmed the participation of the synapsins during synapse formation. The first direct evidence indicating that the synapsins could induce the formation of a presynaptic terminal came from analysis of the phenotype generated by the expression of synapsin IIb in a neuroblastoma/glioma hybrid cell line [47]. Neuroblastoma/glioma cells overexpressing synapsin IIb developed many varicosities containing small clear vesicles, large dense-core vesicles, bundles of cytoskeletal elements, coated vesicles, mitochondria and smooth endoplasmic reticulum. The ultrastructure of these varicosities resembled the ones observed in the autonomic nervous system [47]. Additional evidence supporting the role of the synapsins in synapse formation was obtained with an alternative gain-of-function experimental approach. The injection of synapsin I or synapsin II into embryonic spinal neurons of *Xenopus* embryos resulted in the acceleration of both the morphological and functional development of neuromuscular synapses [48–50].

The role of the synapsins in the formation of synaptic contacts has also been investigated by generating synapsin I, synapsin II or synapsin I/II null mutations by homologous recombination techniques. Studies independently carried out in different laboratories reported that in synapsin I knockout mice, there is a decrease in the size of synaptic terminals and in the number of synaptic vesicles per terminal [51–53]. Abnormal synapse formation was also detected when hippocampal neurons obtained from synapsin-mutant mice were placed in culture. Whereas 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 [54, 55]. Using cultured hippocampal neurons, it has been possible to determine whether this delay in synaptogenesis is attributable to defects in the pre- or postsynaptic elements. The experimental paradigm used involved the establishment of heterochronic cocultures of neurons from wild-type and synapsin I-deficient mice [56]. This study suggested that the decrease in the number of synaptic contacts observed in synapsin I-deficient hippocampal neurons is due to a defect in the presynaptic element. Surprisingly, this study showed that the loss of synapsin I expression also affects the maturation of the postsynaptic element [56]. Taken collectively, these data suggest a broad role for synapsin I in the structural development of the synapse, participating directly or indirectly in the maturation of both presynaptic and postsynaptic sites.

The defects in synapse formation observed in synapsin I, II and I/II deficient hippocampal neurons during the initial phases of development were corrected upon further development, suggesting that compensatory mechanisms could overcome the absence of synapsin I, synapsin II or both [54, 56]. Deficits in these synapsins might be compensated by the overexpression of other members of this family of phosphoproteins. However, it seems unlikely that synapsin III could compensate for the lack of either synapsin I or synapsin II since it has distinct effects on neuronal development. These data suggest that other unknown members of the synapsin family and/or related proteins might have a redundant function on synaptogenesis. This functional redundancy could explain why the effects of the chronic loss of synapsin I or synapsin II could be either masked or overcome in adult mutant mice. Defects in synapse formation during early stages of development, however, cannot be completely ruled out since only adult mutant animals have been analyzed.

An alternative experimental approach has been extensively used to generate a loss-of-function paradigm in central neurons. This approach relies on the acute suppression of the expression of a given protein by means of specific antisense oligonucleotides. Using this technique, it was possible to block the expression of synapsin II either before or after synaptogenesis had taken place. The suppression of synapsin II by antisense oligonucleotides after axon outgrowth, but prior to synapse formation, resulted in inhibition of synaptogenesis in hippocampal neurons [57]. When synapsin II was suppressed after synapses were formed, the neurons lost most of their synapses. These results suggest that synapsin II is required not only for the initial formation of synaptic contacts but also for synapse maintenance [57].

The nature of the mechanism by which synapsin I and synapsin II regulate synaptogenesis is likely to be complex. In addition to their role as organizers of the distribution of synaptic vesicles in the presynaptic terminal, the synapsins might also participate in the regulation of other proteins involved in synaptogenesis. Evidence for this comes from experiments in which the synapsins were either overexpressed or depleted in neuronal cells. 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 [47]. On the other hand, the depletion of synapsin I and or II by either homologous recombination techniques or the use of antisense oligonucleotides resulted in a concomitant decrease in synaptophysin, synaptotagmin, synaptoporin, synaptobrevin and syntaxin [53, 58]. The mechanisms by which the synapsins might regulate the expression of these proteins are not completely understood. One explanation is that the synapsins regulate the synthesis of other synaptic vesicle proteins. However, the possibility that the synapsins act as transcriptional

factors seems unlikely due to their cytoplasmic localization. An alternative explanation is that the synapsins prevent the degradation of these proteins by stabilizing the synaptic vesicles through their binding to actin filaments. Regardless of the mechanisms, synapsin I and synapsin II appear to serve as upstream intermediates in the synapse formation pathway.

The data reviewed above clearly indicate that synapsin I and synapsin II have similar roles during the formation of synaptic contacts. Surprisingly, the newest member of the synapsin family, synapsin III, seems to have a different function. In a recent report we showed that synapsin III was localized in extrasynaptic sites along the axons of cultured hippocampal neurons and that its highest level of expression did not correlate with the period of active synapse formation in these cells. Moreover, no changes in either the time course or the extent of synapse formation were detected when synapsin III was suppressed [39]. The elucidation of the role of synapsin III in synaptogenesis will require further investigation.

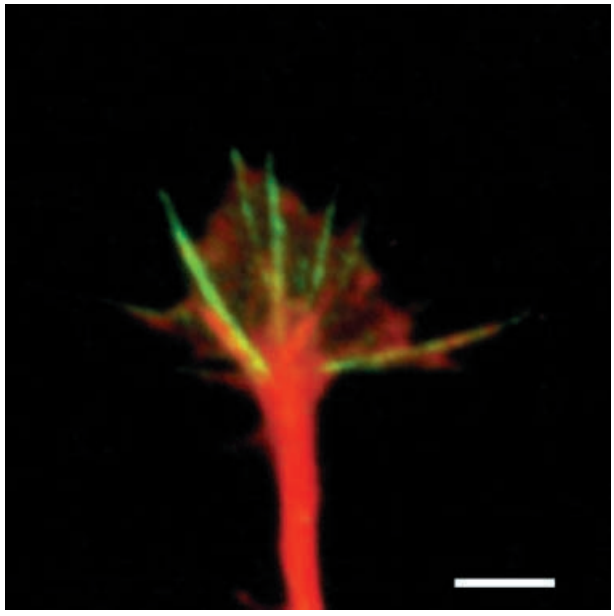

Figure 1. Synapsin III is localized in growth cones of cultured hippocampal neurons.Cultured hippocampal neurons were double-stained with tubulin (red) and synapsin III (green) antibodies. Note the localization of synapsin III in the growth cone. Sacle bar, 20 µm.

The synapsins as regulators of neurite elongation

One of the novel roles of the synapsins that has received attention in the last decade is their involvement in neurite elongation. Studies of the potential role of the synapsins in neurite outgrowth were prompted by reports suggesting that the synapsins are expressed well before synapse formation. In situ hybridization histochemistry has been used to study the expression of the synapsins throughout the central and peripheral nervous systems during embryonic and postnatal development [38]. Synapsin I, for example, has been detected as early as embryonic day 12 in both the central and peripheral nervous systems. In addition, immunocytochemical studies have detected the presence of the synapsins in cell compartments associated with active elongation, including the growth cones of cerebellar granule cells [59] and the distal third of axonal processes and growth cones of hippocampal neurons [39, 42, 58]. This pattern of expression and subcellular localization seems to be shared by all members of the synapsin family. However, a comparative study determined that synapsin III is the one that is most concentrated in growth cones in hippocampal neurons (fig. 1) and is also the one most highly expressed during early stages of development [39].

Studies using cultured hippocampal neurons have been performed to better understand the temporal relationship between the expression of the synapsins and neurite elongation and differentiation. This model system has been chosen because (i) these cells represent a relatively homogeneous population of neurons, and (ii) they differentiate in a reproducible fashion through a series of well-characterized morphological changes [60, 61]. Immediately after dissociation, neurons are round and partially or totally surrounded by lamellipodial veils (stage 1). The consolidation of these veils into short and undifferentiated processes (stage 2) takes place as early as 4 h after plating. These processes show no net elongation for almost a day, at which time one of them begins to elongate at a rapid rate and becomes the axon (stage 3). After 4 days in culture, the remaining minor processes start to elongate and differentiate into dendrites (stage 4) (fig. 2). Synapses are first detected in stage 4 cells [42, 57]. This sequence of events is distinctly altered by the suppression of synapsin I, synapsin II or synapsin III (fig. 2). Hippocampal neurons obtained from synapsin I knockout mice elongated and differentiated an axon and several minor processes. However, these neurites were significantly shorter than their wild-type counterparts [54]. After 3 days in culture, the difference in length between axons of wild-type and synapsin I-deficient neurons increased due to the lack of axonal branching in the mutant cells [54]. These results indicate that synapsin I is involved in the progression of stage 3 (fig. 2).

The depletion of synapsin II by either homologous recombination techniques or by antisense oligonucleotides affects the initial phases of neurite outgrowth. Some synapsin II-depleted hippocampal neurons were unable to elongate processes (20% of cells). Many grew aberrant neurites (i.e. flattened, wide processes surrounded by lamellipodial veils; 80% of cells) but failed to elongate one of those neurites into an axon during the first 48 h in

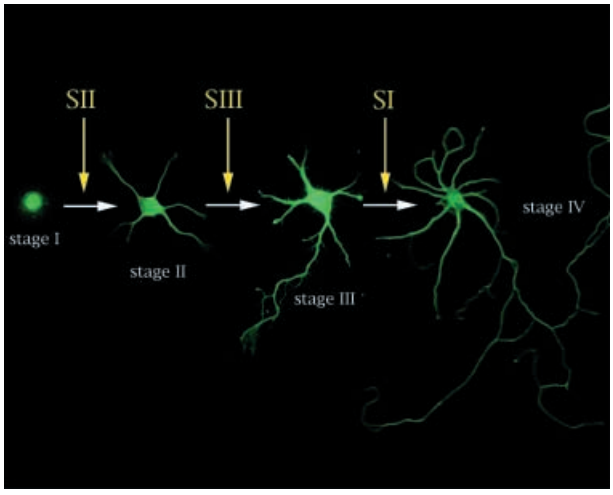

Figure 2. Scheme showing the morphological changes leading the establishment of polarity in hippocampal neurons that develop in culture. Each synapsin is involved at different stages of development as indicated by the arrows (yellow).

culture [55, 58]. Therefore, synapsin II seems to be involved in the transition stage 1 to stage 2 (fig. 2).

On the other hand, hippocampal neurons depleted of synapsin III were capable of extending normal minor processes but failed to elongate and differentiate their axons. These results suggest that synapsin III plays a role in the transition between stage 2 and stage 3 [39] (fig. 2). These studies suggest that each synapsin has a distinct role in the cellular events leading to the elongation and differentiation of axons and dendrites.

The mechanisms by which the synapsins regulate neurite elongation and the establishment of polarity in central neurons are not known. However, insights into these mechanisms have been obtained by overexpressing synapsin II in nonneural cells. The ectopic expression of synapsin I or synapsin II in a rat skin fibroblast cell line provoked changes in cytoskeletal organization and elicited the formation of highly elongated cellular processes [62]. In the past few years, significant progress has been made analyzing the changes in the cytoskeleton leading to axonal differentiation. These studies suggest that the selective stabilization of microtubules in one of the undifferentiated processes could account for its selective net elongation [63, 64]. This selective stabilization could be achieved through different mechanisms, including the binding of microtubule-associated proteins to microtubules and/or by the interaction of microtubules with actin filaments and the plasma membrane [65]. In addition, it has been suggested that the local instability of actin filaments in a given growth cone could act as a physiological signal triggering axonal differentiation [66]. The synapsins could play a role in these mechanisms since it has been shown that they interact with different components of the cytoskeleton, including F-actin, microtubules and neurofilaments [67–70]. The interaction of synapsin I and synapsin II with actin has been extensively analyzed. Both synapsins have been implicated in the polymerization and bundling of actin filaments [68–70]. Although no direct evidence is available regarding the interaction of synapsin III with actin, its high degree of homology with the actin-binding domain of synapsin I [67] suggests that synapsin III also plays a role in the organization of the actin cytoskeleton. These interactions of the synapsins with components of the cytoskeleton could explain, at least in part, their role in neurite elongation and in the establishment of polarity in central neurons. However, a more detailed analysis of these functions is still needed.

Concluding remarks

The data reviewed above seem to indicate that that the synapsins participate in several important developmental events in central neurons, including neurite elongation, the establishment of neuronal polarity, synapse formation and synapse maintenance. Unlike their role as regulators of transmitter release, the mechanisms by which the synapsins participate in these developmental milestones are not completely known. Further investigation of these mechanisms will lead to a better understanding of basic mechanisms underlying neuronal differentiation and degeneration in the central nervous system.

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