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# Synapsins as regulators of neurotransmitter release

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One of the crucial issues in understanding neuronal transmission is to define the role(s) of the numerous proteins that are localized within presynaptic terminals and are thought to participate in the regulation of the synaptic vesicle life cycle. Synapsins are a multigene family of neuron-specific phosphoproteins and are the most abundant proteins on synaptic vesicles. Synapsins are able to interact *in vitro* with lipid and protein components of synaptic vesicles and with various cytoskeletal proteins, including actin. These and other studies have led to a model in which synapsins, by tethering synaptic vesicles to each other and to an actin-based cytoskeletal meshwork, maintain a reserve pool of vesicles in the vicinity of the active zone. Perturbation of synapsin function in a variety of preparations led to a selective disruption of this reserve pool and to an increase in synaptic depression, suggesting that the synapsin-dependent cluster of vesicles is required to sustain release of neurotransmitter in response to high levels of neuronal activity. In a recent study performed at the squid giant synapse, perturbation of synapsin function resulted in a selective disruption of the reserve pool of vesicles and in addition, led to an inhibition and slowing of the kinetics of neurotransmitter release, indicating a second role for synapsins downstream from vesicle docking. These data suggest that synapsins are involved in two distinct reactions which are crucial for exocytosis in presynaptic nerve terminals. This review describes our current understanding of the molecular mechanisms by which synapsins modulate synaptic transmission, while the increasingly well-documented role of the synapsins in synapse formation and stabilization lies beyond the scope of this review.

**Keywords:** synapsins; synaptic vesicle; exocytosis; neurotransmitter release; synaptic depression; synaptic plasticity

## 1. INTRODUCTION

Neurotransmitter release occurs by the exocytotic fusion of discrete, uniformly sized packets or quanta of transmitter, which are stored in individual small synaptic vesicles (del Castillo & Katz 1954; Palade & Palay 1954; Ceccarelli & Hurlbut 1980). Synaptic vesicles undergo a local cycle of trafficking at the active zone of a presynaptic terminal (Ceccarelli *et al.* 1972; Heuser & Reese 1973; for reviews, see Greengard *et al.* 1993; Rothman 1994; Südhof 1995; Augustine *et al.* 1996; Hanson *et al.* 1997; Hay & Scheller 1997; Brodin *et al.* 1997). A large portion of the total number of vesicles within a presynaptic terminal, called the reserve pool of vesicles, is tethered in a cluster away from the plasma membrane (Greengard *et al.* 1993). A subset of vesicles, referred to as the releasable pool, is docked, or physically linked to components associated with the plasma membrane. Upon docking, some synaptic vesicles are thought to undergo a series of priming reactions, which make them competent for fusion. In response to $Ca^{2+}$ influx through voltage-gated $Ca^{2+}$ channels during an action potential, a subset of the docked, fusion-competent vesicles fuse with the plasma membrane, releasing their contents into the synaptic cleft. Vesicle membrane components are then retrieved from the plasma membrane via clathrin-mediated endocytosis (Heuser & Reese 1973; Takei *et al.* 1996; Shupliakov *et al.* 1997). Upon maturation, most recycled vesicles, rather than directly redocking at the plasma membrane, are incorporated into the reserve pool of vesicles. This synaptic vesicle cycle can account for the features specific to regulated, neuronal exocytosis, as compared to constitutive secretion in non-neuronal cells: (i) the rapid and $Ca^{2+}$-dependent exocytosis of synaptic vesicles derives from the colocalization of voltage-dependent $Ca^{2+}$ channels with fusion-competent vesicles at the active zone, which are held there until $Ca^{2+}$ entry allows vesicles to fuse with the plasma membrane; and (ii) the reserve pool provides a strategically localized source of synaptic vesicles to allow synapses to operate over a wide range of levels of activity. Regulation of the synaptic vesicle cycle at any of the above-mentioned steps is likely to underlie at least some forms of synaptic plasticity. For example, a dynamic

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equilibrium between the reserve and the releasable pool of vesicles is thought to represent one possible mechanism by which nerve cells can regulate the amount of neurotransmitter released. Therefore, proteins involved in the formation and maintenance of the reserve pool of vesicles are prime candidates for modulating the efficiency of synaptic transmission.

## 2. THE SYNAPSIN PROTEIN FAMILY

Synapsins are a family of phosphoproteins specifically associated with the cytoplasmic surface of the synaptic vesicle membrane (Huttner *et al.* 1983). In vertebrates, they are encoded by three distinct genes (termed synapsins I, II and III) (Südhof *et al.* 1989; Kao *et al.* 1998; Hosaka & Südhof 1998*b*). Alternative splicing of each synapsin gene generates distinct isoforms (termed a, b and b-like (H.-T. Kao and P. Greengard, unpublished observations), with the primary structural differences between these isoforms restricted to the COOH-terminal part of the molecule. The $NH_2$-terminal portions of all synapsin isoforms are highly conserved. In the two invertebrate species studied to date, multiple synapsin isoforms, which differ in their COOH-terminal regions, seem to be encoded by a single gene (Klagges *et al.* 1996; Hilfiker *et al.* 1998).

Alignment of the primary structures of the synapsins reveals a pattern of sequence identity and dissimilarity that suggests a domain model for the protein family (figure 1). Three common regions are found among vertebrate and invertebrate synapsins. At the $NH_2$-terminus, all synapsins share a stretch of homology within domain A encompassing residues which represent a consensus phosphorylation site for protein kinase A and $Ca^{2+}$/calmodulin-dependent protein kinase I (CaM kinase I), suggesting that phosphorylation of this site is likely to be an important, evolutionarily conserved regulatory feature. The most extensive homology is found within domain C, a large region (around 300 amino acids) representing the central core of synapsins. An amino acid identity of over 50% between vertebrate and invertebrate synapsins predicts that domain C is likely to be crucial for many of the conserved functions of the synapsins. Although derived from three distinct genes by distinct alternative splicing mechanisms, the vertebrate a-type synapsin isoforms share a COOH-terminal region termed domain E. This domain is conserved in selected invertebrate synapsins, supporting the finding that this region is of functional significance (see below) (figure 1).

The region in all synapsins between domains A and C (domain B), as well as the isoform-specific regions between domains C and the COOH-terminal domains (domains D, G, H and J) show little primary sequence identity between vertebrate and invertebrate synapsins. However, certain features within these domains are maintained, such as their similar amino acid compositions rich in prolines, glutamines, alanines and serines, and the presence of consensus sites for phosphorylation by mitogen-activated protein kinase (MAP kinase) as well as $Ca^{2+}$/calmodulin-dependent protein kinase II (CaM kinase II). Finally, the short, COOH-terminal domains specific to the b-type synapsin isoforms (domains F and I) are not conserved in invertebrate synapsins, and the significance of these domains for synapsin function has not been established. The conservation of a general domain structure and of consensus phosphorylation sites among vertebrate and invertebrate synapsins supports the notion that the basic mechanisms by which the synapsins regulate synaptic transmission have been evolutionarily conserved.

## 3. LOCALIZATION AND DIFFERENTIAL EXPRESSION OF SYNAPSINS

*In vivo*, synapsins have been localized to the surface of synaptic vesicles by immunogold or immunoferritin labelling and electron microscopy in a variety of preparations (De Camilli *et al.* 1983; Valtorta *et al.* 1988; Hirokawa *et al.* 1989; Mandell *et al.* 1992; Torri-Tarelli *et al.* 1992; Pieribone *et al.* 1995). In all cases, synapsins were found associated both with vesicles away from the plasma membrane and, to a lesser extent, with docked synaptic vesicles (figure 2). In addition, quick-freeze deep-etch electron microscopy was employed to study the localization of synapsins with respect to both synaptic vesicles and cytoskeletal elements (Landis *et al.* 1988; Hirokawa *et al.* 1989; Gotow *et al.* 1991; Takei *et al.* 1995). Active zones were found to contain a few actin filaments and microtubules. A meshwork of short, linking strands was observed to connect vesicles to those cytoskeletal structures and especially to each other (Landis *et al.* 1988; Hirokawa *et al.* 1989). In addition, the linking strands also bound to larger filaments, thought to be brain spectrin, which directly extended from the presynaptic membrane (Landis *et al.* 1988; Hirokawa *et al.* 1989). Rotary shadowing of quick-frozen, deep-etched samples of purified bovine brain synapsin I suggested that the molecule had an overall shape similar to those linking strands, which were thus proposed to represent the vesicle protein synapsin I (Landis *et al.* 1988; Hirokawa *et al.* 1989). Physicochemical studies are consistent with these morphological studies, suggesting a lollipop shape for the synapsin I molecule (Ueda & Greengard 1977; Benfenati *et al.* 1990). However, similar structures have been seen in ribbon synapses which lack synapsins (Usukura & Yamada 1987), and independent studies using purified, non-frozen samples of bovine brain synapsin I suggest that the protein has an overall shape distinct from these linking strands (J. Heuser, F. Valtorta and P. Greengard, unpublished observations). Thus, the precise molecular structure and cytoskeletal association of synapsins *in vivo* is currently unresolved.

Like most other synaptic proteins (Südhof 1995), the various gene products of the synapsin family are differentially expressed in subsets of neurons (Südhof *et al.* 1989; Mandell *et al.* 1990, 1992; Stone *et al.* 1994). Double labelling experiments with antibodies to choline acetyltransferase or glutamic acid decarboxylase in certain brain regions suggested that a high level of expression of synapsin II relative to synapsin I may be associated with excitatory synapses, while a high relative level of synapsin I expression may be associated with inhibitory synapses (Mandell *et al.* 1992). However, the functional relevance of this differential expression pattern is largely unknown. In addition, the different synapsin isoforms generated by alternative splicing have also been

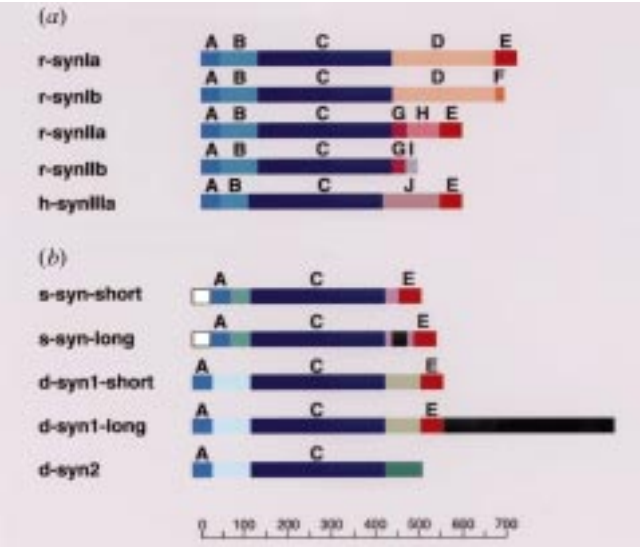


Figure 1. Domain model of the synapsin family. (*a*) Domain model of vertebrate synapsins, including rat synapsins Ia (r-synIa) and Ib (r-synIb), rat synapsins IIa (r-synIIa) and IIb (r-synIIb), and human synapsin IIIa (h-synIIIa). (*b*) Domain model of invertebrate synapsins, including the two alternatively spliced squid synapsin isoforms (s-syn-short and s-syn-long), as well as *Drosophila* synapsin1-short (d-syn1-short) and synapsin1-long (d-syn1-long), which is generated by a stop-codon read-through, and the alternatively spliced isoform synapsin2 (d-syn2). Scale bar, amino acid residues.

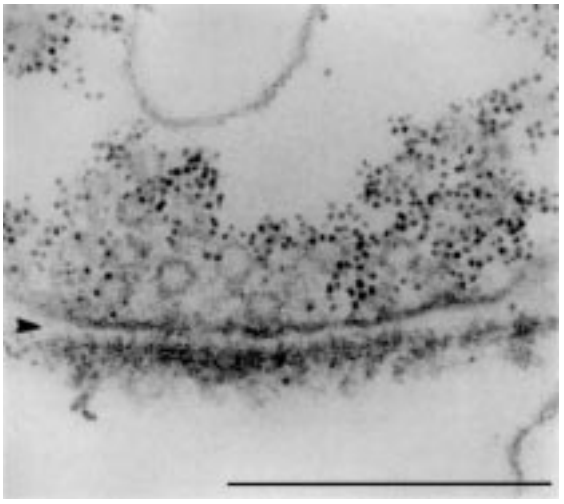

Figure 2. Subcellular localization of synapsins. Electron micrograph showing the distribution of synapsin I immunoreactivity in isolated rat brain synaptosomes. The dark ferritin particles are concentrated around synaptic vesicles both away from the plasma membrane, as well as docked at the plasma membrane opposite the postsynaptic density (arrowhead indicates synaptic cleft). Scale bar, 0.5 µm. Reproduced from *J. Cell Biol.* (1983) **96**, 1355–1373 by copyright permission of the Rockefeller University Press.

reported to be differentially expressed among neurons (Südhof *et al.* 1989). These data suggest that the a-type and b-type synapsin isoforms might be functionally distinct and that alternative splicing mechanisms in certain brain regions might be somehow regulated to generate the preferential expression of the a-type or b-type isoforms.

## 4. SYNAPSIN INTERACTIONS WITH LIPIDS AND PROTEINS

In an attempt to gain insight into the potential role(s) of synapsins in neurotransmitter release, the ability of synapsins to interact with protein and lipid components *in vitro* has been investigated in a multitude of studies. Synapsin I is present in the presynaptic nerve terminal at an average concentration of about 10–20 µM, and it has been estimated that 10–30 synapsin I molecules are present on a single synaptic vesicle (Schiebler *et al.* 1986; De Camilli *et al.* 1990; Ho *et al.* 1991), suggesting that they cover a large portion of the synaptic vesicle surface. The binding of synapsin I to synaptic vesicles consists of multiple interactions of distinct sites of synapsin I with phospholipid as well as protein components of the vesicles (Benfenati *et al.* 1989*a*,*b*). Synapsin I is the most surface-active protein known to date (Ho *et al.* 1991). It can stabilize phospholipids in the bilayer arrangement, which might contribute to the maintenance of vesicle integrity and size uniformity, as well as to the prevention of random fusion events (Benfenati *et al.* 1993). The binding of synapsin I to synthetic phospholipid vesicles is saturable, reversible and exhibits an absolute requirement for acidic phospholipids (Benfenati *et al.* 1989*b*). This interaction consists of two sequential steps: an initial electrostatic interaction with the polar head groups on the surface of the membrane, followed by a conformational change which leads to the penetration of specific regions within domain C of the synapsin molecule into the hydrophobic core of the lipid membrane bilayer (Benfenati *et al.* 1989*b*; J. Cheetham and P. Greengard, unpublished observations).

While synapsin–lipid interactions cannot account for the specific localization of synapsins, they help explain the high affinity ($K_d$=10 nM) with which synapsins interact with synaptic vesicles (Benfenati *et al.* 1989*b*). The exclusive association of synapsins with small synaptic vesicles suggests the existence of one or more binding partners on the vesicle membrane that specifically target synapsins to this location. To date, two protein kinases have been identified as synaptic vesicle-associated binding partners for synapsins. A vesicle-associated form of CaM kinase II was shown to interact with synapsin I (Benfenati *et al.* 1992*a*), and a vesicle-associated form of c-src was reported to interact with synapsins I and II (Onofri *et al.* 1997; Foster-Barber & Bishop 1998). Since both kinases are also present in the cytoplasm within the synaptic terminal, neither interaction can account for the specific and exclusive localization of synapsins, and additional synapsin binding partners are likely to be found on synaptic vesicles.

Synapsins also interact with various components of the cytomatrix. The interactions of synapsins with actin have been most extensively studied. Synapsin I was found to bind to actin filaments, although the affinity of this interaction is about 100-fold lower ($K_d$=1–2 µM) than the affinity of synapsin I for synaptic vesicles (Bähler & Greengard 1987; Petrucci & Morrow 1987; Bähler *et al.* 1989; Benfenati *et al.* 1992*b*). Structure–function analyses showed that a distinct region within domain C is

responsible for the binding of synapsin I to actin filaments (Bähler *et al.* 1989). Synapsin I also interacts with microtubules ($K_d$=5 μM) as well as neurofilaments and brain spectrin (fodrin) ($K_d$=9 nM) (Baines & Bennett 1985, 1986; Goldenring *et al.* 1986; Iga *et al.* 1997). Nerve terminals contain actin filaments and microtubules, as well as structures extending from the presynaptic active zone corresponding in size to brain spectrin (Landis *et al.* 1988; Hirokawa *et al.* 1989), suggesting that these interactions might occur *in vivo*.

## 5. ARE SYNAPSINS ENZYMES?

The recent elucidation of the synapsin I domain C crystal structure suggests the possibility that synapsins might perform enzymatic roles (Esser *et al.* 1998). Surprisingly, more than 80% of the Cα atoms of synapsin I domain C are superimposable with those from a group of ATP-using enzymes (Esser *et al.* 1998). Recombinant domain C regions from synapsins I, II and III are able to bind ATP *in vitro* with high affinity ($K_d$=150–450 nM). Interestingly, while all three synapsins bind ATP with similar affinities, their binding is differentially regulated: ATP binding to synapsin I is $Ca^{2+}$-activated, ATP binding to synapsin II is $Ca^{2+}$-independent, and ATP binding to synapsin III is $Ca^{2+}$-inhibited (Hosaka & Südhof 1998*a*,*b*).

All residues involved in ATP binding are conserved in vertebrate and squid synapsins, but not in *Drosophila* synapsins, and the functional relevance of ATP binding of synapsins is currently unclear. The purified proteins do not hydrolyse ATP at a detectable rate, suggesting that synapsins are not constitutively active ATPases. However, ATP hydrolysis is likely to depend on the proper localization and substrate binding of synapsin. In addition, the crystal structure indicates that domain C forms a stable dimer, and enzymatic activity might depend on the correct multimeric state of the protein. One report has suggested that synapsin I isolated from rat brain, presumably in its dimeric state, might be a diacylglycerol kinase (Kahn & Besterman 1991). These results could not be confirmed in an independent study (Hosaka & Südhof 1998*a*), and the suggestion that synapsins might be enzymes which use ATP to catalyse some unidentified reaction remains an interesting, but currently unresolved hypothesis.

## 6. REGULATION OF SYNAPSIN FUNCTION BY PHOSPHORYLATION

Synapsins were initially identified as major brain-specific substrates for multiple protein kinases both *in vitro* and *in vivo* (De Camilli *et al.* 1990). In some cases, their phosphorylation state is associated with profound conformational changes as well as changes in their biochemical properties, suggesting that this post-translational modification serves as an important, evolutionarily conserved functional regulatory switch.

All synapsin isoforms currently identified contain a consensus phosphorylation site within domain A for both protein kinase A and CaM kinase I (site 1). In vertebrates, site 1 of synapsins I and II is phosphorylated both *in vitro* (Czernik *et al.* 1987) and *in vivo* in response to increased levels of second messengers (Huttner & Greengard 1979). Phosphorylation at site 1 leads to subtle changes in the overall conformation of the holoprotein (Benfenati *et al.* 1990), moderately decreases the affinity with which synapsins bind to actin (Bähler & Greengard 1987), but does not significantly change the binding of synapsins to lipids or synaptic vesicles (Schiebler *et al.* 1986; Benfenati *et al.* 1989*b*). The functional relevance of phosphorylation at this site is currently unknown.

All synapsin isoforms currently identified contain multiple consensus sites for proline-directed protein kinases, including mitogen-activated protein kinase (MAP kinase) and cyclin-dependent kinase (cdk5). MAP kinase phosphorylates vertebrate synapsin I *in vitro* at three distinct sites (termed sites 4, 5 and 6); sites 4 and 5 are located within domain B, while site 6 is located within domain D and also subject to phosphorylation by cdk5 (Jovanovic *et al.* 1996; Matsubara *et al.* 1996). Similar to site 1 phosphorylation, phosphorylation at sites 4–6 results in a moderate decrease in the ability of synapsins to interact with actin, but has no effect on the binding of synapsins to synaptic vesicles (Jovanovic *et al.* 1996). While these sites are phosphorylated *in vivo* (Romano *et al.* 1987*a*,*b*; Jovanovic *et al.* 1996), the functional consequence of this post-translational modification remains to be elucidated.

Finally, synapsins have been reported to be differential targets for phosphorylation by CaM kinase II. Synapsin I, but not synapsin II, is phosphorylated by CaM kinase II *in vitro* at two sites within domain D (termed sites 2 and 3) (Czernik *et al.* 1987). Similar consensus sites for CaM kinase II phosphorylation are present within domain J of synapsin III, as well as within selected variants of squid and *Drosophila* synapsins, although their accessibility for phosphorylation remains to be determined. Interestingly, phosphorylation at sites 2 and 3 results in profound changes in the biochemical properties of synapsin I. It results in a major conformational change of the molecule (Benfenati *et al.* 1990), as well as in a drastic decrease in the ability of synapsin I to interact with actin (Bähler & Greengard 1987; Petrucci & Morrow 1987; Valtorta *et al.* 1992) and in a decreased affinity for synaptic vesicles (Schiebler *et al.* 1986).

*In vivo*, phosphorylation of synapsin I at sites 2 and 3 is low under basal conditions and increases upon prolonged physiological or pharmacological activation of $Ca^{2+}$-dependent protein kinases. Several *in vivo* studies support the biochemical evidence that phosphorylation at sites 2 and 3 results in a decrease in the affinity of synapsin I for synaptic vesicles and the cytoskeleton. For example, an increase in the amount of synapsin I present in the soluble fraction is observed upon continuous $K^+$-evoked depolarization of synaptic nerve endings (synaptosomes), which consists mostly of phosphosynapsin I (at sites 1, 2 and 3) (Sihra *et al.* 1989). After prolonged electrical stimulation of the frog neuromuscular junction (30 min, 20 Hz), immunogold electron microscopy revealed a 30% decrease in the amount of synapsin I associated with synaptic vesicles compared to control, non-stimulated conditions (Torri Tarelli *et al.* 1992). The extent of dissociation of synapsin I from the vesicle membrane was dependent on the rate of stimulation, with no decrease in the amount of bound synapsin

I observed at low rates of secretion (Torri Tarelli *et al.* 1990).

Purified bovine synapsin I molecules were introduced into several presynaptic preparations to assess the functional role of synapsin phosphorylation (Llinás *et al.* 1985, 1991; Lin *et al.* 1990; Hackett *et al.* 1990; Nichols *et al.* 1992). Dephospho-synapsin I, or synapsin I phosphorylated at site 1, inhibited neurotransmitter release, while synapsin I phosphorylated at sites 2 and 3 by CaM kinase II was without effect (Llinás *et al.* 1985, 1991; Nichols *et al.* 1992). These observations, together with biochemical evidence that synapsins were able to bind to synaptic vesicles and to cytoskeletal elements, led to the development of a testable model for the role of synapsins in synaptic function. Synapsins were proposed to reversibly cross-link synaptic vesicles to each other and possibly also to an actin-containing cytoskeletal meshwork, thereby maintaining a reserve pool of vesicles away from the plasma membrane. Assuming a dynamic equilibrium between the reserve and the releasable pool of vesicles, the size of the synapsin-dependent reserve pool would then determine the availability of vesicles for exocytosis. In addition, it was proposed that synapsin I could regulate the amount of neurotransmitter release through its ability to undergo cycles of phosphorylation and dephosphorylation at sites 2 and 3.

## 7. SYNAPSINS REGULATE THE RESERVE POOL OF SYNAPTIC VESICLES

Two additional types of experiments have been performed to study the function(s) of synapsins in neurotransmitter release *in vivo*: (i) transient perturbation of synapsin levels by the introduction of either antibodies to synapsins, or peptides derived from synapsin sequences, into living presynaptic nerve terminals, or (ii) permanent disruption of synapsin function by deletion of synapsin gene(s) in mice. All experimental data support the notion that synapsins regulate the availability of vesicles for exocytosis, and some data also indicate that synapsins play additional role(s) in the regulation of neurotransmitter release.

It was proposed that exogenous synapsin I inhibited synaptic transmission by recruiting vesicles from the releasable pool back into the reserve pool, without interfering with the release process itself (Llinás *et al.* 1985). Based on this model, disruption of synapsin function might be expected to increase neurotransmitter release, by increasing the number of vesicles available for release. However, if these freed vesicles are no longer held in place, but instead diffuse away from the vesicle cluster into the large volume of the presynaptic axon, an inhibition of neurotransmitter release might be observed. To distinguish between these two possibilities, ultrastructural studies were performed at the reticulospinal axon of lamprey (Pieribone *et al.* 1995). Injection of an antibody directed against the a-type synapsins resulted in the selective loss of vesicles located away from the plasma membrane (100–500 nm), while the number of vesicles in the region close to the plasma membrane (0–100 nm) was unchanged (Pieribone *et al.* 1995) (figure 3*a*,*b*). These findings suggest that synapsins contribute to the stability of a major portion of the synaptic vesicle cluster, but not to the stability of the subpopulation of vesicles adjacent to the plasma membrane. Action potential-evoked release at low frequencies was unchanged in terminals injected with antibody, suggesting that vesicular release could be fully supported under these conditions by the pool of vesicles immediately adjacent to the active zone. However, in antibody-injected terminals, release was drastically decreased upon high-frequency stimulation which elicits synaptic depression, suggesting that the large, synapsin-dependent cluster of vesicles away from the membrane is required to sustain release of neurotransmitter in response to high levels of neuronal activity. While the function of synapsin bound to vesicles immediately adjacent to the plasma membrane could not be elucidated, these studies strongly support the proposed model for synapsin function in maintaining a reserve pool of vesicles.

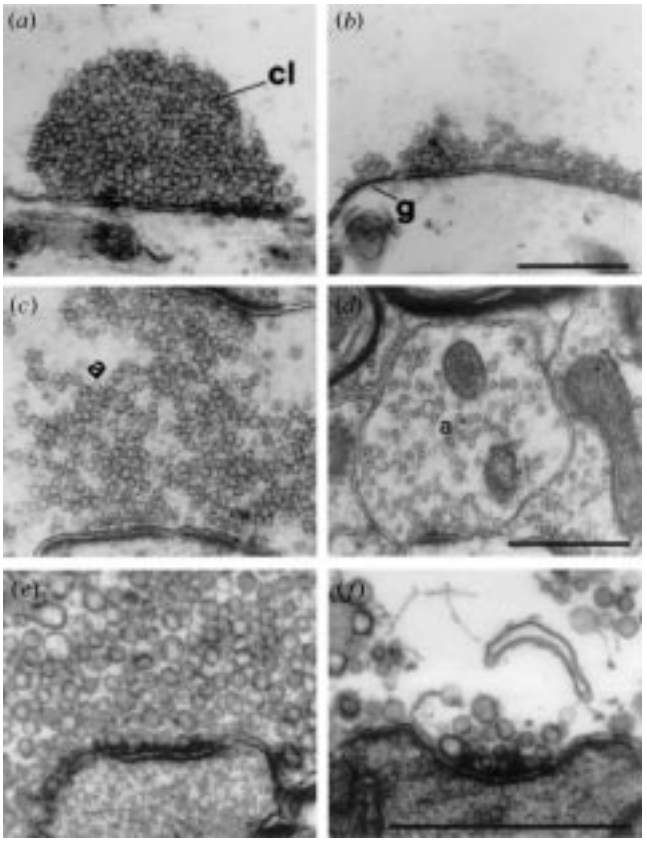


Figure 3. Ultrastructural changes in various preparations upon disruption of synapsin function. (*a*,*b*) Electron micrographs of synapses from (*a*) control IgG antibody-injected, or (*b*) synapsin antibody-injected lamprey reticulospinal axons. Reprinted with permission from *Nature* (Pieribone *et al.* 1995). Copyright (1995) Macmillan Magazines Limited. (*c*,*d*) Electron micrographs of synapses from (*c*) wildtype or (*d*) synapsin I-deficient mice. Reproduced from Li *et al.* (1995). Copyright National Academy of Sciences, USA. (*e*,*f*) Electron micrographs of active zones from (*e*) control peptide-injected or (*f*) synapsin domain E peptide-injected squid giant synapses. Reproduced from Hilfiker *et al.* (1998). Copyright permission of *Nature Neuroscience*. a, Axoplasmic matrix; cl, synaptic vesicle cluster; g, gap junction. Scale bars, 0.5 μm.

The role of synapsins in neurotransmitter release has also been investigated by using mice deficient in either synapsin I, synapsin II or synapsins I/II. Generally, the interpretation of data obtained from these types of studies

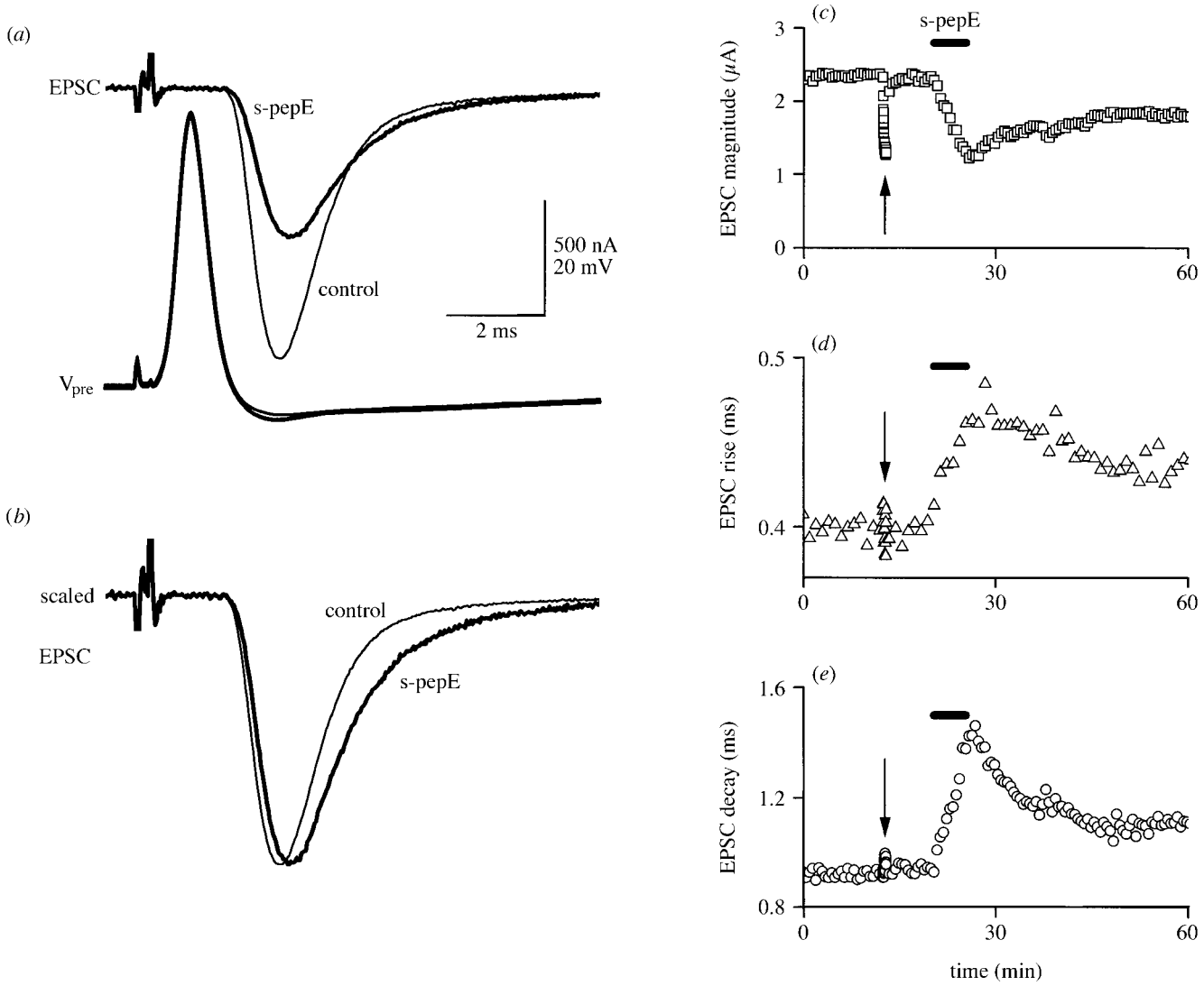


Figure 4. Synapsins regulate the kinetics of neurotransmitter release. (*a*) Presynaptic potentials (Vpre) and postsynaptic currents (EPSC) before (control) and after (s-pepE) injection of the domain E peptide into the squid giant synapse. (*b*) Postsynaptic currents from (*a*) scaled to identical peak amplitudes illustrates the slowing of the rise and decay of the EPSC. (*c*,*d*,*e*) Time-course of the slowing of the release kinetics on domain E peptide injection. (*c*) EPSC amplitude; (*d*) 20–80% rise time-constant; (*e*) single-exponential decay time-constant. The arrow indicates a train of presynaptic stimuli (30 s, 1 Hz) which depressed EPSC amplitude without affecting the kinetics of release. Reproduced from Hilfiker *et al.* (1998) by copyright permission of *Nature Neuroscience*.

is complicated. For example, the chronic deficiency of a protein of interest often leads to compensatory increases or decreases in other proteins, and a variety of proteins are up- or down-regulated in mice lacking synapsin I, II or I/II (Rosahl *et al.* 1995). In addition, the phenotypic changes resulting from a specific gene deletion are often attenuated or alleviated by the presence of other, homologous genes with redundant functions, and the recent identification of the existence of a third synapsin gene suggests that the degree of severity in the phenotypic changes of the synapsin knock-outs might be attenuated by the expression of synapsin III isoforms (Kao *et al.* 1998; Hosaka & Südhof 1998*b*). Nevertheless, studies of this type have yielded important information as to the role of synapsins in neurotransmitter release.

Synapsin-deficient mice have been generated independently by three different laboratories, and two studies have reported a selective decrease of vesicles away from the plasma membrane in mice lacking synapsin I (Li *et al.* 1995; Takei *et al.* 1995) (figure 3*c*,*d*), as well as in mice lacking synapsin II or I/II (A. M. Magarinos, B. McEwen and P. Greengard, unpublished observations). In a third study, an overall decrease in the number of synaptic vesicles was observed in terminals deficient in synapsins I/II; it was stated that there was no selective depletion of vesicles away from the membrane (Rosahl *et al.* 1995). While the discrepancy between the latter study and the others is currently unresolved, the observed increase in synaptic depression in the synapsin I/II-deficient mice (Rosahl *et al.* 1995) is consistent with the proposal that synapsins regulate the size of a reserve pool of vesicles.

Recently, the squid giant synapse has again been used to investigate the role of synapsins in neurotransmitter release. Disrupting synapsin function by injecting a peptide derived from domain E of squid synapsins selectively and drastically decreased the number of vesicles away from the plasma membrane, but had no effect on the number of morphologically docked vesicles

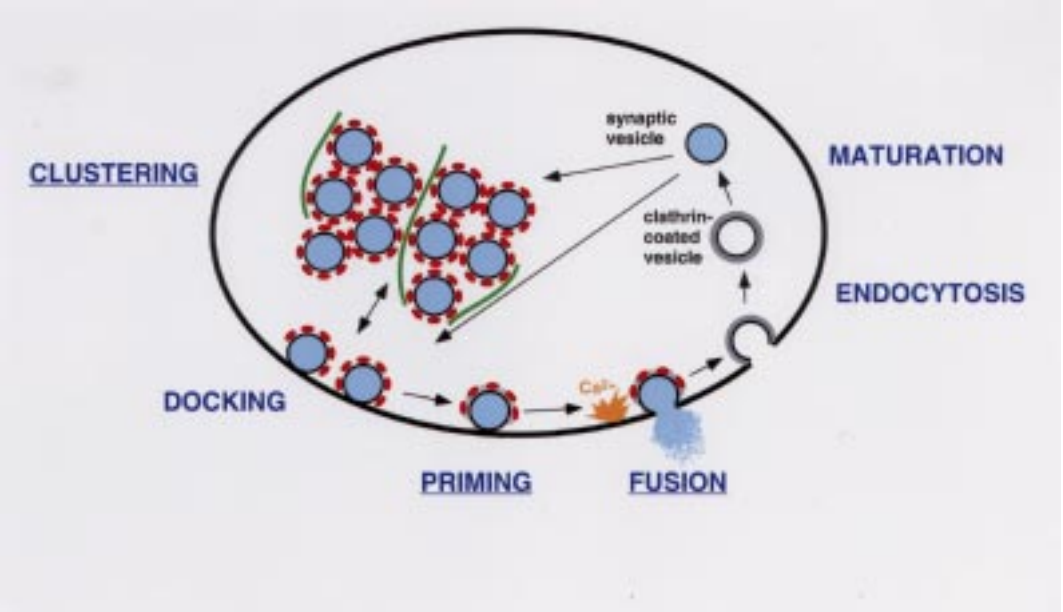


Figure 5. A model for synapsin function. Synapsins (red) are proposed to perform several distinct functions within the synaptic vesicle life cycle (underlined). They tether synaptic vesicles (blue) to each other and possibly also to a cytoskeletal meshwork in the nerve terminal (green wavy lines), thereby maintaining a reserve pool of vesicles away from the active zone. In addition, they are suggested to act downstream from vesicle docking, in either priming or fusion reactions.

(Hilfiker *et al.* 1998) (figure 3*e*,*f* ). In addition, peptide injection led to enhanced synaptic depression, again supporting the notion that synapsins maintain a vesicle cluster away from the membrane which is needed during times of high synaptic activity.

## 8. SYNAPSINS AND SHORT-TERM SYNAPTIC PLASTICITY

Studies with synapsin-deficient mice suggest specific roles for synapsins in certain forms of synaptic plasticity. At most synapses, when two stimuli are given within a short time interval, the second response is larger than the first response. This form of plasticity, termed paired-pulse facilitation (PPF), is due to an increased release of neurotransmitter and is very short-lived, occurring when interstimulus intervals are in the millisecond range and decaying with an equally rapid time-course (Zucker 1989). In contrast, post-tetanic potentiation (PTP) can be elicited by trains of presynaptic stimuli, and the increase in postsynaptic responses can last for many seconds (Zucker 1989).

Synapsin I-deficient mice were reported to exhibit an increase in PPF (Rosahl *et al.* 1993), while mice deficient in synapsin II showed no changes in PPF (Rosahl *et al.* 1995). These data suggested that synapsin I, but not synapsin II, may function to selectively limit the increase in neurotransmitter release elicited after an initial stimulus. However, mice deficient in synapsins I/II showed normal PPF (Rosahl *et al.* 1995), and disrupting the function of both squid synapsin isoforms revealed no changes in PPF (Hilfiker *et al.* 1998). Thus, the relevance, if any, of the observed changes in PPF in the synapsin I-deficient mice is currently unclear.

Mice lacking synapsin II or synapsins I/II were found to exhibit a decrease in PTP (Rosahl *et al.* 1995). It is currently unresolved whether the mobilization of vesicles from a reserve pool to a releasable pool or the priming of docked vesicles contribute to certain forms of short-term plasticity such as PTP (Zucker 1989). In this respect, it is interesting to note that synapsin I-deficient mice showed neither a change in synaptic depression nor PTP, but the values of those parameters were altered more drastically in the synapsin I/II-deficient mice as compared to the synapsin II-deficient mice. This observation correlates with the more drastic depletion and 'unclustering' of vesicles observed in the synapsin II and synapsin I/II-deficient mice (A. M. Magarinos, B. McEwen and P. Greengard, unpublished observations) compared to the synapsin I-deficient mice, suggesting that the size of a reserve pool might influence both synaptic depression and PTP.

In summary, while far from being understood, these studies suggest that individual synapsins might perform distinct roles in modulating different forms of short-term synaptic plasticity.

## 9. SYNAPSINS REGULATE THE KINETICS OF NEUROTRANSMITTER RELEASE

Several observations suggest an additional role for synapsins in modulating the release of individual vesicles from an immediately releasable pool. Studies of neurotransmitter release using FM1-43 in primary cultures derived from synapsin I-deficient mice showed a decrease in the size of both the total pool of vesicles and the immediately releasable pool of vesicles, suggesting that these two pools are in a dynamic equilibrium with each other (Ryan *et al.* 1996). However, while release probability was decreased, the number of docked vesicles in the synapsin I-deficient mice was unchanged (Li *et al.* 1995).

At the squid giant synapse, presynaptic injection of a peptide corresponding to a region of domain E of both

squid synapsins could completely inhibit neurotransmitter release, although the number of docked vesicles was unchanged under these conditions (Hilfiker *et al.* 1998). In this preparation, the number of docked vesicles is not a static morphological feature, but can be either up- or down-regulated by altering the function of a number of other synaptic proteins (Bommert *et al.* 1993; Hess *et al.* 1993; Hunt *et al.* 1994; DeBello *et al.* 1995; Schweizer *et al.* 1998; Burns *et al.* 1998; Dresbach *et al.* 1998). These data supported the possibility that synapsins might perform an additional function in regulating the availability of docked vesicles to fuse upon stimulation.

Direct experimental evidence in support of a post-docking role of synapsins was obtained by measuring excitatory postsynaptic currents (EPSCs). Injection of the domain E peptide was found to reduce the amount of transmitter release, but also slowed the onset and decay of the EPSC (Hilfiker *et al.* 1998) (figure 4*a–e*). Slowing of the release kinetics is a highly unusual event and is not simply a consequence of reduced transmitter release. Most manipulations that decrease neurotransmitter release, such as injection of peptides derived from a variety of presynaptic proteins, including α-SNAP, synaptotagmin, synaptobrevin–VAMP, syntaxin, SNAP-25, secl or rabphilin, reduction of external $Ca^{2+}$ concentrations or synaptic depression all inhibit release, but do not slow the time-course of release (Bommert *et al.* 1993; Hess *et al.* 1993; Hunt *et al.* 1994; DeBello *et al.* 1995; O'Connor *et al.* 1997; Burns *et al.* 1998; Dresbach *et al.* 1998). Most importantly, this kinetic effect also cannot be attributed to the overall reduction in vesicle pool size, because other treatments that reduce the number of synaptic vesicles, such as injection of GTPγS or of peptides derived from α-SNAP and rabphilin, do not modify release kinetics (Hess *et al.* 1993; DeBello *et al.* 1995; Burns *et al.* 1998). Thus, it seems likely that the domain E peptide directly slows the kinetics of synaptic vesicle fusion events.

The presynaptic mechanisms responsible for the shape or waveform of the macroscopic postsynaptic EPSC are largely unknown. The macroscopic EPSC is thought to be a composite of individual, evoked miniature EPSCs which are released with a certain probability within a given amount of time (Diamond & Jahr 1995). The kinetic changes produced by the domain E peptide could result from slowing of the reaction that mediates the fusion of individual synaptic vesicles or from an effect resulting in the desynchronization of these individual fusion events. Thus, synapsins could be directly or indirectly involved in the actual fusion reaction, or in some priming reaction which precedes fusion. Interestingly, the only other molecular perturbations known to slow both the onset and decay of the EPSC at the squid giant synapse involve certain peptides derived from NSF (Schweizer *et al.* 1998). These peptides inhibit the ATPase activity of NSF, which has previously been suggested to promote conformational changes that activate SNARE proteins and provide the energy required to drive membrane fusion upon $Ca^{2+}$ entry (Söllner *et al.* 1993*a*,*b*; Hanson *et al.* 1995). Recently, ATP hydrolysis by NSF has been suggested to promote the disassembly of used SNARE complexes, allowing subsequent rounds of vesicle docking, priming and fusion to occur (Søgaard *et al.* 1994; Nichols *et al.* 1997; Weber *et al.* 1998). In either case, the slower kinetics produced by NSF peptides are thought to result from the fusion of individual, incompletely primed vesicles (Schweizer *et al.* 1998). Interestingly, both NSF and synapsins bind ATP, suggesting that ATP-dependent steps might regulate the timing of neurotransmitter release.

The physiological role of synapsin domain E is currently unknown. It seems possible that the peptide injection experiments caused a disruption of a domain E-mediated interaction of synapsins with a binding partner of unknown identity. Such a domain E-mediated interaction might be responsible for the proper targeting and/or binding of synapsins to synaptic vesicles. Synapsins might be important for the proper priming and/or fusion of vesicles by recruiting additional factors to the vesicle which are needed in further downstream steps, or they might act as spacer molecules between docked vesicles, allowing other interactions between vesicle and plasma membrane proteins to occur (Weber *et al.* 1998). Finally, synapsins might directly catalyse an ATP-dependent reaction downstream from vesicle docking. Additional experiments, such as the identification of the binding partner(s) for synapsin domain E or the characterization of the potential enzymatic activity of synapsins, will help to distinguish among these possibilities.

## 10. CONCLUSIONS

The current data suggest that synapsins are involved in two distinct reactions that are crucial for exocytosis in presynaptic terminals: (i) synapsins and, more specifically, domain E-mediated interactions are necessary for the maintenance of a pool of vesicles (Pieribone *et al.* 1995; Hilfiker *et al.* 1998); and (ii) domain E-mediated interactions regulate some event(s) which determine the time-course of neurotransmitter release (Hilfiker *et al.* 1998) (figure 5).

A synapsin-dependent reserve pool of vesicles allows exocytosis to occur at rates which can transiently exceed the rate of endocytosis, enabling nerve terminals to respond to a wide variety of synaptic activities (Brodin *et al.* 1997). A synapsin-mediated clustering of synaptic vesicles was also suggested to enhance the stability of vesicles and prevent their degradation (Rosahl *et al.* 1995). Thus, the synapsin-dependent reserve pool of vesicles might serve two distinct functions: it might provide a constant source of vesicles to the releasable pool and, in addition, it might sequester vesicles from some type of metabolic pathway that would otherwise target synapsin-depleted vesicles for degradation.

Synapsins also regulate the kinetics of neurotransmitter release, through a mechanism which is currently unknown. It is possible that this action is a result of a synapsin-mediated mobilization of vesicles into the releasable pool. However, the results are most readily explained by postulating that synapsins are more directly involved in the priming or fusion of vesicles, a notion which is consistent with studies showing the presence of synapsins on docked vesicles, albeit at reduced levels (Valtorta *et al.* 1988; Hirokawa *et al.* 1989; Torri Tarelli *et al.* 1990; Pieribone *et al.* 1995).

Interestingly, domain E is selectively present in the vertebrate a-type synapsin isoforms, but not the b-type isoforms, suggesting that alternative splicing generates

synapsin isoforms that have distinct roles in regulating the synaptic vesicle life cycle. The differential functional roles of the b-type versus the a-type synapsin isoforms is currently unknown. The primary sequences of the COOH-terminal domains of the b-type isoforms are not conserved across species, suggesting that these domains might not be functionally relevant. Future studies will be needed to fully understand the role of the different synapsin isoforms in synaptic vesicle trafficking, synaptic plasticity and the kinetics of neurotransmitter release.

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