Effects of phosphorylation and neuronal activity on the control of synapse formation by synapsin I

Laura E. Perlini¹,²,*, Francesca Botti¹,*, Eugenio F. Fornasiero¹, Maila Giannandrea¹, Dario Bonanomi¹,², Mario Amendola¹,³, Luigi Naldini¹,³, Fabio Benfenati²,⁴ and Flavia Valtorta¹,§

¹San Raffaele Scientific Institute and Vita-Salute University, Via Olgettina 58, 20132 Milano, Italy
²Department of Neuroscience and Brain Technologies, The Italian Institute of Technology, Via Morego 30, 16163 Genova, Italy
³TIGET, Telethon Institute for Genetics and Medicine, Via Olgettina 58, 20132 Milano, Italy
⁴Department of Experimental Medicine, Section of Physiology, University of Genoa and National Institute of Neuroscience, Viale Benedetto XV, 3, 16132 Genova, Italy

*These authors contributed equally to this work
§Author for correspondence (valtorta.flavia@hsr.it)

Summary

Synapsins are synaptic vesicle (SV)-associated proteins that regulate synaptic transmission and neuronal differentiation. At early stages, Syn I and II phosphorylation at Ser9 by cAMP-dependent protein kinase (PKA) and Ca²⁺/calmodulin-dependent protein kinase I/IV modulates axon elongation and SV-precursor dynamics. We evaluated the requirement of Syn I for synapse formation by siRNA-mediated knockdown as well as by overexpression of either its wild-type (WT) form or its phosphorylation mutants. Syn I knockdown at 14 days in vitro caused a decrease in the number of synapses, accompanied by a reduction of SV recycling. Although overexpression of WT Syn I was ineffective, overexpression of its phosphorylation mutants resulted in a complex temporal regulation of synapse density. At early stages of synaptogenesis, phosphomimetic Syn I S9E significantly increased the number of synapses. Conversely, dephosphomimetic Syn I S9A decreased synapse number at more advanced stages. Overexpression of either WT Syn I or its phosphomimetic S9E mutant rescued the decrease in synapse number caused by chronic treatment with tetrodotoxin at early stages, suggesting that Syn I participates in an alternative PKA-dependent mechanism that can compensate for the impairment of the activity-dependent synaptogenic pathway. Altogether these results indicate that Syn I is an important regulator of synapse formation, which adjusts synapse number in response to extracellular signals.

Key words: Synaptic vesicle, cAMP-dependent protein kinase (PKA), Tetrodotoxin (TTX), Hippocampal neuron, Lentiviral vector

Introduction

The synapsins are evolutionarily conserved neuron-specific phosphoproteins (Kao et al., 1999) that localise at the presynaptic level, where they interact with synaptic vesicle (SV) proteins, phospholipids and actin, regulating SV homeostasis (Cesca et al., 2010). In mammals synapsins are encoded by three genes: Syn1, Syn2 and Syn3. Alternative splicing of these genes gives rise to several protein isoforms that share common domains (De Camilli et al., 1990; Kao et al., 1998; Sudhof et al., 1989). Synapsin I (Syn I; also known as synapsin-1), the best-characterized member of the family, has been shown to control the availability of SVs for exocytosis by linking them to each other and to the actin cytoskeleton of the nerve terminal, thereby regulating SV functional pools and tuning neurotransmitter release (Benfenati et al., 1992; Ceccaldi et al., 1995; Valtorta et al., 1992).

Although Syn I has been studied for more than 30 years, some aspects of its function are still not fully understood. The most controversial role of Syn I concerns its involvement in neuritogenesis and synaptogenesis (Fornasiero et al., 2010). A large body of literature indicates that synapsins exert several roles in neuronal development. Firstly, the expression levels of synapsins correlate with the structural and functional maturation of neurites and synaptic contacts (Chin et al., 1995; Ferreira et al., 1994; Lu et al., 1992; Valtorta et al., 1995). Secondly, overexpression of Syn I or Syn II in Xenopus motor neurons increases quantal secretion properties of newly formed synapses, which is paralleled by an increased SV density, SV clustering and development of synaptic specializations (Lu et al., 1992; Schaeffer et al., 1994; Valtorta et al., 1995). Syn I, II, III, I/II or I/II/III knockout (KO) mice develop normally and display an overall normal brain cytoarchitecture, neuronal morphology and synapse number (Gittler et al., 2004a; Rosahl et al., 1995). Primary cultures of neurons derived from Syn I, II or I/II KO mice have a delay in synapse formation, although no differences in synapse density were observed in mature cultures (Chin et al., 1995; Rosahl et al., 1995; Ferreira et al., 1998).

Synapsins integrate intracellular signals, as prominent substrates of protein kinases. Among these, cAMP-dependent protein kinase (PKA) and Ca²⁺/calmodulin-dependent protein kinase I/IV (CaMK I/IV), phosphorylate all Syn isoforms at a single site (site 1, Ser9 in Syn I), which is highly conserved across vertebrate and invertebrate species (Kao et al., 1999; Candiani et al., 2010). Phosphorylation of site 1 is involved in precocious stages of neuron development, as seen in Xenopus spinal neurons, where phosphorylation of Syn II at this site stimulates neurite outgrowth both in vitro and in vivo (Kao et al., 2002). In rat hippocampal neurons phosphorylation of the same
site in Syn I regulates the localisation of SVs and their rate of recycling in axonal growth cones and mature SVs (Bonanomi et al., 2005). It is unclear if the latter effect modulates the putative synaptogenic activity of Syn I; however it is known that the regulation of exo-endocytotic cycles of SV precursors at the nascent synapse is important for synapse establishment (Matteoli et al., 1992). Taken together, these observations suggest that synapsins might regulate synaptogenesis in a phosphorylation-dependent manner, although the exact role and regulation of Syn I in formation of synaptic contacts has never been carefully investigated.

In this study, we investigated the effects of changes in the levels of expression of Syn I and in its state of phosphorylation at site I in the regulation of synaptogenesis. We show that physiological levels of expression are necessary for synapse formation, and that the ratio between phosphorylated and dephosphorylated Syn I appears to be more important for synapse formation than the absolute levels of the protein. Moreover, we report that Syn I phosphorylation rescues the decrease in synapse number caused by chronic treatment with tetrodotoxin at early stages of development, thus compensating for the defective synapse formation caused by blockade of electrical activity.

Results
The knockdown of Syn1 by siRNA decreases the expression of an array of synaptic proteins involved in synaptic transmission

To investigate the function of Syn I during synapse formation in hippocampal cultures we used RNA interference (RNAi) technology. We identified a Syn I target-specific small interfering RNA (siRNA) sequence and tested its efficacy in HeLa cells by co-transfecting Syn Ia and IIa expression plasmids with the duplex. To minimize the possibility of nonspecific off-target effects, siRNAs were applied at low concentration (20 nM). As a control, we used luciferase (luc) siRNA. Syn1 siRNA specifically and efficiently downregulated Syn Ia without affecting Syn IIA expression (supplementary material Fig. S1A). The same siRNA was able to downregulate the endogenously expressed protein in primary hippocampal neurons (supplementary material Fig. S1B). To evaluate Syn1 siRNA efficacy at single synaptic boutons, we co-transfected Syn1 siRNA and a plasmid encoding synaptophysin (Syp)–YFP and measured Syn I fluorescence intensity at single YFP-positive synapses. Syn I levels were decreased in at least 63% of Syp–YFP-positive synapses compared with the control and 58% compared with luc siRNA synapses (supplementary material Fig. S1C). Moreover, we realized that the transfection of neurons with Syn1 siRNA induced a decrease in Syn I level also in Syp–YFP-negative synapses. In detail, Syn I level was decreased in 38% of total synapses compared with the control and 36% compared with the luc siRNA (data not shown). Owing to this widespread effect on single synapses in our cultures, we decided to evaluate the effect of Syn1 siRNA on the total number of synapses, even though this procedure underestimates the effects of siRNAs.

We then analyzed the effect of acute Syn1 knockdown on synaptic protein expression in primary hippocampal neurons. Immunoblot analysis revealed that treatment of 14 days in vitro (DIV) hippocampal neurons for 7 days with Syn1 siRNA effectively decreased the levels of Syn Ia/Ib as well as the levels of several synaptic proteins, including other SV proteins such as Syn IIa/Ib, synaptotagmin I (Syt I), Syp I, vesicular glutamate transporter-1 (VGLUT-1), vesicular γ-aminobutyric acid (GABA) transporter (VGAT) and Rab3a, the endosomal protein Rab5, the t-SNARE protein syntaxin 1 (Syx 1) and the postsynaptic density protein PSD 95 (Fig. 1A,B). Noticeably, for all synaptic proteins the extent of the decrease was similar to that of Syn I. The reduction in the levels of both SV proteins and presynaptic and postsynaptic proteins suggests a decrease in synapse formation and/or stability upon Syn1 knockdown.

Fig. 1. siRNA-mediated knockdown of Syn1 in hippocampal neurons decreases several components of synapses. Representative western blot (A) and quantitative analysis (B) of the expression levels of synaptic proteins in total extracts of primary hippocampal neurons (21 DIV) that had been transfected at 14 DIV with Syn1 siRNA. Data were normalized to the values of samples transfected with luc siRNA (dashed line) and analyzed using two-way ANOVA followed by Tukey’s post-hoc test. *P<0.01 vs the levels of the same protein in luc-siRNA-treated samples; **P<0.01 vs Syn I levels in Syn1-siRNA-treated samples (n=3).
The knockdown of Syn1 by siRNA induces a parallel decrease of glutamatergic and GABAergic synapses

The simultaneous presence of an SV population and a presynaptic scaffold that stabilizes the presynaptic terminal is an important feature that defines a synapse. We used double staining for the synapse scaffold (bassoon; Bsn) and SV clusters (either Syt I, VGLUT1 or VGAT) to identify bona fide synapses in developing neurons. Because differential alterations at glutamatergic and GABAergic synapses have been described in Syn1 null mice (Gitler et al., 2004a), we separately analyzed excitatory and inhibitory synapse formation in order to identify any differential effect of Syn1 knockdown or overexpression. To this aim, we used the colocalisation between VGLUT1 and Bsn to define glutamatergic synapses and the colocalisation between VGAT and Bsn to define GABAergic synapses.

To investigate whether Syn1 regulates the number of synaptic contacts, we transfected Syn1 siRNA in hippocampal neurons at 14 DIV, when neurotogenesis is virtually complete and synaptogenesis is the predominant ongoing neurodevelopmental process, and, after 7 days, we counted the number of VGLUT1- and VGAT-positive puncta present in the first 30 μm of dendrites proximal to the cell body (see Materials and Methods; Fig. 2A). We found that acute knockdown of Syn1 caused a significant decrease in the number of both glutamatergic (−28.1 ± 3.2% vs non-transfected neurons; −26.6 ± 2.8% vs neurons transfected with luc siRNA) and GABAergic synapses (−17.9 ± 2.4% vs non-transfected neurons; −18.6 ± 2.8% vs neurons transfected with luc siRNA; Fig. 2B).

To test whether SV exo-endocytosis at single synapses was also impaired after acute Syn1 knockdown, we monitored SV recycling using the fluorescent styryl dye FM 4-64 (Betz et al., 1996). The entire pool of recycling SVs was loaded using high recycling using the fluorescent styryl dye FM 4-64 (Betz et al., also impaired after acute

The overexpressed phosphorylation site mutants of Syn1 are correctly targeted to presynaptic sites in primary hippocampal neurons

Because knockdown of Syn1 decreases the formation of synapses, we sought to determine whether synapse number is dependent on the expression levels of Syn1. To this aim, we overexpressed the enhanced cyan fluorescent protein (ECFP)-tagged WT protein and its site I phosphorylation mutants in rat primary hippocampal neurons. As a control of infection we used ECFP–VAMP2, i.e. the fluorescent chimera of an SV protein that was previously shown not to have detectable effects on synaptogenesis. Hippocampal neurons were transduced at 3 DIV and fixed at 13 DIV, allowing transgene overexpression during the most intense period of synapse formation. To exclude a bias due to a heterogeneous protein expression, cultures were infected with high efficiency (supplementary material Fig. S2), and the presence of the tagged protein was verified at all analyzed synapses. VAMP2, WT Syn 1 and Syn I S9A localised at the presynaptic site in mature hippocampal neurons, as previously described (Bonanomi et al., 2005; Pennuto et al., 2002). The pseudophosphorylated mutant Syn I S9E was also efficiently expressed and localised in the presynaptic compartment similarly to the WT and S9A isoforms, as confirmed by its colocalisation with the presynaptic markers Syt I and Bsn (Fig. 4A). All the exogenously expressed Syn I isoforms were expressed in comparable amounts and exhibited a similar nerve terminal targeting (Fig. 4B,C). Thus, the localisation of Syn 1 was not affected by mutagenesis of phosphorylation site 1, confirming that phosphorylation of Syn 1 at this site does not affect nerve terminal targeting (Gitler et al., 2004b), and allowing direct comparisons between the effects of the phosphorylation mutants on synapse formation.

Fig. 2. Acute Syn1 knockdown decreases the total number of glutamatergic and GABAergic synapses in mature hippocampal neurons. (A) Fluorescence images of hippocampal neurons transfected with either luc-siRNA, Syn1 siRNA or left untreated (Ctrl) at 14 DIV and analyzed at 21 DIV. Glutamatergic and GABAergic synaptic boutons were identified by double immunostaining with Bsn (red in the merge panels), and either VGLUT-1 or VGAT (green in the merge panels). The colocalisation panels (Col) highlight the puncta double positive for Bsn and either VGLUT-1 or VGAT that were identified as glutamatergic and GABAergic synaptic boutons. Despite a broad distribution of the fluorescence intensity values due to variability among individual synapses, we found that treatment of neurons with Syn1 siRNA significantly reduced depolarization-induced FM 4-64 uptake by 20.2 ± 1.8% (Fig. 3B,C) suggesting a decrease in the number of active synapses.
Overexpression of WT Syn I does not further increase the number of synapses in mature hippocampal neurons

Statistical analysis of the number of infected presynaptic nerve terminals present in the first 30 μm of dendrites proximal to the cell body revealed that overexpression of WT Syn I did not affect the total number of synapses compared with uninfected neurons and control VAMP2-infected neurons (Fig. 5A,B). This indicates that, although a decrease in Syn I impairs synaptogenesis, further
increases in Syn I expression above physiological levels are ineffective, suggesting that physiological levels of Syn I are sufficient to fully exert its synaptogenic potential.

**Syn I phosphorylation at site 1 regulates the formation of both glutamatergic and GABAergic synapses at various developmental stages**

If Syn I controls synapse formation, then its state of phosphorylation might influence its activity. To test this, in the same set of experiments we evaluated the synaptogenic potential of site 1 phosphorylation mutants. Indeed, overexpression of the dephosphomimetic Syn I S9A mutant caused a significant decrease (−17.7 ± 3.7%) in the total number of synapses at 13 DIV (Fig. 5A,B), whereas the phosphomimetic S9E mutant had no effect. These results suggest that phosphorylation of Syn I at site 1 plays an important role in the control of synapse formation. To investigate in detail the time course of this control during neuronal development, neurons infected at 3 DIV were followed and analyzed at 7, 10 and 13 DIV by counting separately glutamatergic and GABAergic synaptic boutons, as described above (Fig. 6A). The time courses show that the effects of Syn I mutants were very similar in both glutamatergic and GABAergic synapse populations (Fig. 6B). At the beginning of synaptogenesis (7 DIV), only the pseudophosphorylated mutant caused a significant increase in synapse number with respect to uninfected or VAMP2-infected neurons (+20.8 ± 5.4% in glutamatergic synapses and +13.5 ± 3.4% in GABAergic synapses), whereas overexpression of either WT Syn I or Syn I S9A was ineffective (Fig. 6B). By contrast, at later times (10 and 13 DIV), Syn I S9A was seen to be exerting its inhibitory influence and caused a significant decrease in the number of both glutamatergic and GABAergic synapses with respect to uninfected or VAMP2-infected neurons (−14.5 ± 2.0% in glutamatergic and −15.5 ± 5.1% in GABAergic synapses at 10 DIV; −24.6 ± 5.1% in glutamatergic and −12.6 ± 1.3% in GABAergic synapses at 13 DIV). At these developmental times, overexpression of either WT Syn I or Syn I S9E was ineffective (Fig. 6B).

**Overexpression of WT or pseudophosphorylated Syn I rescues the early impairment of synaptogenesis caused by blockade of synaptic activity**

Because Syn I has a role in the transduction of extracellular signals into regulation of neurotransmitter release, we next examined whether the effects of Syn I in the regulation of synaptogenesis require synaptic activity. We chronically treated rat hippocampal neuronal cultures with tetrodotoxin (TTX) to prevent action potential-evoked synaptic activity, starting from 4 DIV. Because the effects of Syn I mutants on the glutamatergic and GABAergic synapse populations had very similar trends, we analyzed these effects on the whole synapse population using the colocalisation between Syt I (a common marker of excitatory and inhibitory synapses) and Bsn to identify synaptic contacts, as described above (Fig. 7A,C).

Chronic exposure to TTX led to an almost complete blockade of synaptic activity (supplementary material Fig. S3) and significantly impaired synapse formation during the early stages of synaptogenesis (−18.7 ± 5.7% and −21.4 ± 2.6% at 7 and 10 DIV, respectively). However, this inhibition was spontaneously overcome at later stages of development (13 DIV), indicating that the influence of synaptic activity on synaptogenesis at early stages is spontaneously rescued at later time points (Fig. 7A,B). To test whether the delay in synaptogenesis after TTX block involves Syn I, we overexpressed Syn I and its phosphorylation mutants in TTX-treated cells. Interestingly, at 7 DIV, the TTX-induced impairment in synapse formation was rescued by overexpression of either WT Syn I or Syn I S9E, whereas it was unaffected by overexpression of either VAMP2 (−19.1 ± 2.6) or Syn I S9A (−22.6 ± 8.3%; Fig. 7C,D). Similar effects were observed at 10 DIV, although the TTX-induced decrease in synapse number was fully and significantly rescued only by overexpression of Syn I S9E, but not by overexpression of VAMP2 (−24.28 ± 3.8%), WT Syn I (−10.7 ± 3.8%) or Syn I S9A (−20.7 ± 3.9%; Fig. 7C,D). At 13 DIV, when the inhibitory effect of activity blockade spontaneously subsided, the overexpressed proteins had no detectable effects, except for the...
Syn I S9A mutant, which still had the ability to inhibit synapse formation (18.6 ± 0.7%; Fig. 7C,D), similar to what was observed in untreated neurons at the same developmental stage (see above).

**Discussion**

A large body of experimental data suggests that synapsins modulate neuronal development. Syn I loaded into early blastomeres of *Xenopus* embryos increased both the functional and morphological maturation of developing synaptic contacts (Lu et al., 1992; Valtorta et al., 1995). Consistently, ectopic expression of synapsins in non-neuronal cells dramatically promoted the extension of neurite-like processes and the formation of synaptic-like varicosities (Han et al., 1991).

Constitutive Syn I knockout neurons had delayed neurite outgrowth and synapse formation in vitro, although development had almost caught up at mature stages (Chin et al., 1995; Ferreira et al., 1998; Rosahl et al., 1995). However, hippocampal mossy fibre terminals and cerebellar granule cell terminals from Syn1−/− mice showed presynaptic defects that could be linked to impaired synapse formation (Takei et al., 1995).

To better understand the role of synapsins in synaptogenesis, we characterized the effects of acute knockdown and overexpression of Syn1 and its site 1 phosphorylation mutants in rat hippocampal neurons in culture. Cultured embryonic hippocampal neurons represent an excellent in vitro model to study synapse formation (Dotti et al., 1988). The whole processes
of differentiation and synaptogenesis have been extensively studied and well characterized in this system: neurons start to display synapses at around 5 DIV (Fletcher et al., 1994; Bonanomi et al., 2008) and synaptogenesis proceeds at least until 16–19 DIV, when dendritic protrusions are stabilized and become spines (Ziv and Smith, 1996). However, a study of the effects of acutely decreased Syn I expression on synapse formation without affecting the early stages of neurite outgrowth has never been performed. In this paper, we investigated the effects of an acute knockdown of Syn I by siRNA technology and of the overexpression of distinct Syn I conformers. In particular, we overexpressed WT Syn I or its phosphomimetic and dephosphomimetic mutants of Ser9 (site 1), the major Syn phosphorylation site that has been shown to be directly involved in the early stages of neuronal development (Bonanomi et al., 2005; Kao et al., 2002).

Specific knockdown of Syn I by siRNA performed at the time of synaptogenesis was associated with a decrease in a large array of synaptic proteins, including proteins of SVs, as well as proteins of the presynaptic and postsynaptic membranes. These effects are only partially consistent with those observed in Syn I KO mice in which the main impairment was on the levels of SV

Fig. 7. Chronic treatment with TTX and overexpression of Syn I phosphorylation mutants have complex effects in the various stages of synaptogenesis. (A) Representative fluorescence images of hippocampal neurons at 10 DIV chronically treated with TTX starting at 4 DIV. Syt I is shown in green and Bsn is shown in red. For further details, see legend to Fig. 2. Scale bar: 10 μm. (B) The number of infected synapses counted on a 30 μm dendrite length (means ± s.e.m.) is shown as a function of the developmental time (7, 10 and 13 DIV) in TTX-treated (red trace) and untreated (blue trace) neurons. Kruskal–Wallis and Tukey’s post-hoc tests were applied on each time point. *P<0.05; *P<0.05 TTX treated vs untreated. (C) Representative fluorescence images of hippocampal neurons at 10 DIV that had been infected at 3 DIV with the lentiviral constructs encoding for CFP chimeras of VAMP2, WT Syn I, Syn I S9A or Syn I S9E and chronically treated with TTX. The overexpressed protein is shown in blue, Syt I is shown in green and Bsn is shown in red. For further details, see legend to Fig. 2. (D) Number of infected synapses counted on a 30 μm dendrite length (means ± s.e.m. of three independent experiments). The blue dashed line represents the mean synapse number for uninfected and untreated neurons, and the red dashed line represents the mean synapse number for uninfected TTX-treated neurons (values taken from B). WT Syn I and Syn I S9E, but not Syn I S9A, rescued the effect of TTX treatment, whereas Syn I S9A did not affect the TTX effect at early stages of development and decreased synapse number at later stages when the TTX effect was overcome. Statistical analysis was performed using the Kruskal–Wallis and post-hoc Tukey’s tests applied to the whole data sample. **P<0.01 vs untreated/uninfected; *P<0.05; *P<0.01 vs TTX-treated uninfected; n=40 dendrites (total length >3600 μm)/experimental condition for each of three independent experiments.
proteins. A specific decrease in the levels of plasma membrane and scaffolding proteins suggests a decreased number of synaptic contacts, in addition to a decrease in the number of SVs per synapse. This was demonstrated by a parallel decrease in both glutamatergic and GABAergic synapses and by a decreased uptake of the styryl dye FM 4-64, which indicates that the decrease involved actively recycling synapses.

When Syn I was overexpressed by viral infection at early stages of neuronal development, all Syn I conformers correctly localised at presynaptic sites, and targeting was not affected by mutagenesis of phosphorylation site 1, consistent with previous observations (Gitler et al., 2004b; Menegon et al., 2006). This allowed us to investigate the dependence of synapse formation on the expression levels and phosphorylation state of Syn I. Overexpression of WT Syn I did not affect glutamatergic or GABAergic synapses between 7 and 13 DIV, suggesting that under normal conditions the absolute amount of Syn I above physiological levels is not a major determinant for synapse development. By contrast, the state of phosphorylation of Syn I on site 1 was important for synapse development: the overexpression of Syn I S9E accelerated synapse formation as observed at 7 DIV whereas, at later stages, the presence of Syn I S9A impaired a further increase in the number of synapses. This effect was more pronounced in glutamatergic synapses, but was also present in GABAergic synapses.

It is known that phosphorylation of Syn I on site 1 regulates Syn I–SVs and Syn I–actin interactions (Benfenati et al., 1992; Chiaregatti et al., 1996; Fesce et al., 1992; Hosaka et al., 1999; Stefani et al., 1997; Valtorta et al., 1992). Thus, the pseudophosphorylated mutant should scarcely interact with actin and SVs, similarly to the PKA-phosphorylated WT Syn I. By contrast, the non-phosphorylatable mutant should strongly bind to both actin and SVs, similar to dephosphorylated WT Syn I. Under overexpression conditions, the mutants, present in higher concentration, should compete with the endogenous protein. Previous data demonstrated that, when overexpressed in a WT background, the Syn I S9A mutant was not able to diffuse into the axonal shaft after PKA activation, in contrast to WT Syn I (Menegon et al., 2006). Furthermore, neurons expressing Syn I S9A took up less FM 4-64 and exhibited a higher synaptic depression than WT-Syn I-expressing neurons expressing Syn I S9A overexpression in untreated neurons. Overexpression of the pseudophosphorylated mutant, but not of WT Syn I or its non-phosphorylatable mutant, rescued this effect, implying that, at this stage of development, Syn I is probably part of an alternative synaptogenic pathway that is PKA, but not activity, dependent, suggesting that the two parallel (activity- and PKA-dependent) pathways act additively and that activation of PKA is not exclusively coupled to electrical activity at this stage. This is also consistent with the lack of effect of Syn I S9E in increasing synapse number above control levels under the chronic effect of TTX. At 10 DIV the TTX chronic treatment decreased the number of synapses to a similar level to that observed for Syn I S9A overexpression in untreated neurons. Overexpression of the pseudophosphorylated mutant, but not of WT Syn I or its non-phosphorylatable mutant, rescued this effect, implying that, at this developmental stage, the effect of Syn I on synapse development is related to neuronal activity and that PKA activation has become activity dependent. At 13 DIV, a stage in which the TTX chronic treatment had no effect on synapse number, the Syn I S9A mutant retained its capacity to impair synaptogenesis, indicating that at later stages of synaptogenesis Syn I acts through mechanisms that are not linked to neuronal activity.

In conclusion, the evidence that synapsins exhibit actin-binding and SV-clustering activities in vitro (Bahler and Greengard, 1987; Valtorta et al., 1992) led to the hypothesis that these activities underlie neurodevelopmental processes. However, results obtained from the constitutive Syn knockout indicate that synapsins are more modulators rather than determinants of synapse formation. An obvious mechanism for regulation of synaptogenesis could be the switch between a positive and negative form of the protein, which could occur through post-translational modifications such as phosphorylation. A similar mechanism has been demonstrated for Syn II during
morphological development of neurons because the dephosphomimetic mutant inhibits, whereas its phosphomimetic mutant increases neurite outgrowth (Kao et al., 2002). Our data support the idea that in addition to a basal amount of Syn being required for synapse formation, the pattern of active Syn conformers could be a key factor in the regulation of synaptogenesis. However, because Syn exhibits complex molecular interactions (Cesca et al., 2010) and not all its functional properties are phosphorylation dependent (see Bonanomi et al., 2005), phosphorylation might have a positive or a negative role depending on the interactions involved. Thus, dephosphorylation mutant and phosphomimetic forms of the protein do not necessarily exhibit opposite effects under all circumstances.

Electrical activity activates neurotransmitter release and several signal transduction pathways, including those triggered by cAMP and Ca²⁺. In particular, a large body of experimental data suggests that PKA activity and neurotransmitter-induced signalling are correlated and strongly involved in synaptogenesis. In fact, the repeated activation of PKA by either a cAMP analogue or forskolin in primary rat hippocampal neurons (Yamamoto et al., 2005) or by glutamate in hippocampal slices (Tominaga-Yoshino et al., 2008) leads to a slowly developing long-lasting synaptogenesis. However, the expression of dominant-negative PKA in zebrafish olfactory sensory neurons (Tominaga-Yoshino et al., 2008) leads to a slowly developing long-lasting synaptogenesis. These results further confirm the central role in neural PKA plays a key role in the regulation of neurite outgrowth (Kao et al., 2002). Our data confirm the central role in neural PKA plays a key role in the regulation of neurite outgrowth (Kao et al., 2002).

Materials and Methods

Materials

The mouse monoclonal antibody anti-Syn I/II (clone 19.11) and the polyclonal antibody against Syn were made in-house. The rabbit antisera VGLUT1, VGAT, Rab5 and the monoclonal antibodies against syntaxin 1, PSD95, Rab3a and the Syn I laminal epitope were purchased from Synaptic Systems (Goettingen, Germany). The monoclonal antibody against the bassoon (Bsn) epitope was from Stressgen Bioreagents (Victoria, BC, USA). The monoclonal antibody against tubulin III (Tubulin III) was from Covance (Dedham, MA, USA) and the polyclonal antibody against GFP was from Invitrogen (Carlsbad, CA, USA). Tetramethyl Rhodamine iso-thiocyanate (TRITC)- and fluorescein isothiocyanate (FITC)-conjugated secondary antibodies were from Jackson ImmunoResearch. Hoechst 33342 was from Sigma-Aldrich (St. Louis, MO, USA). FM 4-64 was from Invitrogen and FM 1-43 was from Molecular Probes (Eugene, OR, USA). The S9E mutation was produced through PCR mutagenesis using the QuickChange Mutagenesis kit (Stratagene-Medical, Milano, Italy) at the University of Milano-Bicocca.

Materials and Methods

Materials

The mouse monoclonal antibody anti-Syn I/II (clone 19.11) and the polyclonal antibody against Syn were made in-house. The rabbit antisera VGLUT1, VGAT, Rab5 and the monoclonal antibodies against syntaxin 1, PSD95, Rab3a and the Syn I laminal epitope were purchased from Synaptic Systems (Goettingen, Germany). The monoclonal antibody against the bassoon (Bsn) epitope was from Stressgen Bioreagents (Victoria, BC, USA). The monoclonal antibody against tubulin III (Tubulin III) was from Covance (Dedham, MA, USA) and the polyclonal antibody against GFP was from Invitrogen (Carlsbad, CA, USA). Tetramethyl Rhodamine iso-thiocyanate (TRITC)- and fluorescein isothiocyanate (FITC)-conjugated secondary antibodies were from Jackson ImmunoResearch. Hoechst 33342 was from Sigma-Aldrich (St. Louis, MO, USA). FM 4-64 was from Invitrogen and FM 1-43 was from Molecular Probes (Eugene, OR, USA). The S9E mutation was produced through PCR mutagenesis using the QuickChange Mutagenesis kit (Stratagene-Medical, Milano, Italy) at the University of Milano-Bicocca.

Materials

The mouse monoclonal antibody anti-Syn I/II (clone 19.11) and the polyclonal antibody against Syn were made in-house. The rabbit antisera VGLUT1, VGAT, Rab5 and the monoclonal antibodies against syntaxin 1, PSD95, Rab3a and the Syn I laminal epitope were purchased from Synaptic Systems (Goettingen, Germany). The monoclonal antibody against the bassoon (Bsn) epitope was from Stressgen Bioreagents (Victoria, BC, USA). The monoclonal antibody against tubulin III (Tubulin III) was from Covance (Dedham, MA, USA) and the polyclonal antibody against GFP was from Invitrogen (Carlsbad, CA, USA). Tetramethyl Rhodamine iso-thiocyanate (TRITC)- and fluorescein isothiocyanate (FITC)-conjugated secondary antibodies were from Jackson ImmunoResearch. Hoechst 33342 was from Sigma-Aldrich (St. Louis, MO, USA). FM 4-64 was from Invitrogen and FM 1-43 was from Molecular Probes (Eugene, OR, USA). The S9E mutation was produced through PCR mutagenesis using the QuickChange Mutagenesis kit (Stratagene-Medical, Milano, Italy) at the University of Milano-Bicocca.

Materials

The mouse monoclonal antibody anti-Syn I/II (clone 19.11) and the polyclonal antibody against Syn were made in-house. The rabbit antisera VGLUT1, VGAT, Rab5 and the monoclonal antibodies against syntaxin 1, PSD95, Rab3a and the Syn I laminal epitope were purchased from Synaptic Systems (Goettingen, Germany). The monoclonal antibody against the bassoon (Bsn) epitope was from Stressgen Bioreagents (Victoria, BC, USA). The monoclonal antibody against tubulin III (Tubulin III) was from Covance (Dedham, MA, USA) and the polyclonal antibody against GFP was from Invitrogen (Carlsbad, CA, USA). Tetramethyl Rhodamine iso-thiocyanate (TRITC)- and fluorescein isothiocyanate (FITC)-conjugated secondary antibodies were from Jackson ImmunoResearch. Hoechst 33342 was from Sigma-Aldrich (St. Louis, MO, USA). FM 4-64 was from Invitrogen and FM 1-43 was from Molecular Probes (Eugene, OR, USA). The S9E mutation was produced through PCR mutagenesis using the QuickChange Mutagenesis kit (Stratagene-Medical, Milano, Italy) at the University of Milano-Bicocca.

Materials

The mouse monoclonal antibody anti-Syn I/II (clone 19.11) and the polyclonal antibody against Syn were made in-house. The rabbit antisera VGLUT1, VGAT, Rab5 and the monoclonal antibodies against syntaxin 1, PSD95, Rab3a and the Syn I laminal epitope were purchased from Synaptic Systems (Goettingen, Germany). The monoclonal antibody against the bassoon (Bsn) epitope was from Stressgen Bioreagents (Victoria, BC, USA). The monoclonal antibody against tubulin III (Tubulin III) was from Covance (Dedham, MA, USA) and the polyclonal antibody against GFP was from Invitrogen (Carlsbad, CA, USA). Tetramethyl Rhodamine iso-thiocyanate (TRITC)- and fluorescein isothiocyanate (FITC)-conjugated secondary antibodies were from Jackson ImmunoResearch. Hoechst 33342 was from Sigma-Aldrich (St. Louis, MO, USA). FM 4-64 was from Invitrogen and FM 1-43 was from Molecular Probes (Eugene, OR, USA). The S9E mutation was produced through PCR mutagenesis using the QuickChange Mutagenesis kit (Stratagene-Medical, Milano, Italy) at the University of Milano-Bicocca.

Materials

The mouse monoclonal antibody anti-Syn I/II (clone 19.11) and the polyclonal antibody against Syn were made in-house. The rabbit antisera VGLUT1, VGAT, Rab5 and the monoclonal antibodies against syntaxin 1, PSD95, Rab3a and the Syn I laminal epitope were purchased from Synaptic Systems (Goettingen, Germany). The monoclonal antibody against the bassoon (Bsn) epitope was from Stressgen Bioreagents (Victoria, BC, USA). The monoclonal antibody against tubulin III (Tubulin III) was from Covance (Dedham, MA, USA) and the polyclonal antibody against GFP was from Invitrogen (Carlsbad, CA, USA). Tetramethyl Rhodamine iso-thiocyanate (TRITC)- and fluorescein isothiocyanate (FITC)-conjugated secondary antibodies were from Jackson ImmunoResearch. Hoechst 33342 was from Sigma-Aldrich (St. Louis, MO, USA). FM 4-64 was from Invitrogen and FM 1-43 was from Molecular Probes (Eugene, OR, USA). The S9E mutation was produced through PCR mutagenesis using the QuickChange Mutagenesis kit (Stratagene-Medical, Milano, Italy) at the University of Milano-Bicocca.

Materials

The mouse monoclonal antibody anti-Syn I/II (clone 19.11) and the polyclonal antibody against Syn were made in-house. The rabbit antisera VGLUT1, VGAT, Rab5 and the monoclonal antibodies against syntaxin 1, PSD95, Rab3a and the Syn I laminal epitope were purchased from Synaptic Systems (Goettingen, Germany). The monoclonal antibody against the bassoon (Bsn) epitope was from Stressgen Bioreagents (Victoria, BC, USA). The monoclonal antibody against tubulin III (Tubulin III) was from Covance (Dedham, MA, USA) and the polyclonal antibody against GFP was from Invitrogen (Carlsbad, CA, USA). Tetramethyl Rhodamine iso-thiocyanate (TRITC)- and fluorescein isothiocyanate (FITC)-conjugated secondary antibodies were from Jackson ImmunoResearch. Hoechst 33342 was from Sigma-Aldrich (St. Louis, MO, USA). FM 4-64 was from Invitrogen and FM 1-43 was from Molecular Probes (Eugene, OR, USA). The S9E mutation was produced through PCR mutagenesis using the QuickChange Mutagenesis kit (Stratagene-Medical, Milano, Italy) at the University of Milano-Bicocca.

Materials

The mouse monoclonal antibody anti-Syn I/II (clone 19.11) and the polyclonal antibody against Syn were made in-house. The rabbit antisera VGLUT1, VGAT, Rab5 and the monoclonal antibodies against syntaxin 1, PSD95, Rab3a and the Syn I laminal epitope were purchased from Synaptic Systems (Goettingen, Germany). The monoclonal antibody against the bassoon (Bsn) epitope was from Stressgen Bioreagents (Victoria, BC, USA). The monoclonal antibody against tubulin III (Tubulin III) was from Covance (Dedham, MA, USA) and the polyclonal antibody against GFP was from Invitrogen (Carlsbad, CA, USA). Tetramethyl Rhodamine iso-thiocyanate (TRITC)- and fluorescein isothiocyanate (FITC)-conjugated secondary antibodies were from Jackson ImmunoResearch. Hoechst 33342 was from Sigma-Aldrich (St. Louis, MO, USA). FM 4-64 was from Invitrogen and FM 1-43 was from Molecular Probes (Eugene, OR, USA). The S9E mutation was produced through PCR mutagenesis using the QuickChange Mutagenesis kit (Stratagene-Medical, Milano, Italy) at the University of Milano-Bicocca.
Trypsin in HDSS at 37°C, the whole hippocampi were washed three times with HBSS to remove trypsin and then mechanically dissociated. Neurons stained with vital dye (Trypan Blue; Sigma-Aldrich) were counted using a Burker chamber. Neurons were plated on poly-L-lysine (Sigma-Aldrich; 0.1 mg/ml)-treated glass coverslips in order to obtain 100,000 cells/coverslip (low density culture). Cells were plating in medium (MEM supplemented with 10% horse serum, 3.3 mM glucose and 2 mM glutamine) and incubated for 4 hours at 37°C in a 5% CO2 humidified atmosphere to allow adhesion to the substrate. Given that the density of neurons in culture influences synapse number (Fletcher et al., 1994), we always plated the same number of neurons (100,000 neurons on 24 mm coverslip). After plating, coverslips were transferred into a cell-culture dish covered with a glia monolayer prepared as described above containing hippocampal medium conditioned for at least 24 hours. Coverslips were turned upside down, with neurons facing down, toward the glia.

**Transfection and transduction procedures**

**HeLa cell transfection**

The day before transfection, cells were trypsinised, suspended in fresh medium without antibiotics and plated in 35-mm Petri dishes. Transfection was carried out with Lipofectamine 2000 (Invitrogen) according to manufacturer’s instructions using 2 μg ECFP/Synl or ECFP/SynII with 20 nM siRNA per 35-mm dish. Cells were lysed 48 hours after transfection.

**Neuron transfection**

Transfections were performed with Lipofectamine 2000 (Invitrogen) according to manufacturer’s instructions using 20 nM siRNA oligonucleotides. Neurons at 14 DIV were placed in a clean dish and incubated for 1 hour with hippocampal medium containing Lipofectamine and siRNA, then returned to the original dishes and analyzed at 21 DIV.

**Neuron transduction**

Infection was carried out on 3 DIV neurons as previously described (Bonanomi et al., 2005). Coverslips were placed in clean dishes containing glia-conditioned hippocampal medium in the presence of viral supernatant for 12–15 hours at 37°C in a 5% CO2 humidified atmosphere. After transduction, neurons were returned to the original dishes and maintained in culture in glia-conditioned medium until processing.

**Pharmacological treatment**

TTX was resuspended in 10 mM sodium acetate, pH 4.5, and used at a final concentration of 1 μM. The medium was changed every 3 days and fresh drug was added.

**Cell labelling protocols**

Standard immunofluorescence experiments were carried out as previously described (Pennuto et al., 2003). Briefly, cells were rinsed once with Krebs-Ringer’s solution (KRB)-EGTA (130 mM NaCl, 5 mM KCl, 1.2 mM KH₂PO₄, 12 mM MgSO₄, 2 mM MgCl₂, 2 mM EGTA, 25 mM HEPES, 6 mM glucose, pH 7.4), fixed for 20 minutes with 4% parafformaldehyde, 4% sucrose in 120 mM sodium phosphate buffer, pH 7.4, and then incubated overnight at 4°C in a humidified chamber with the primary monoclonal antibody appropriately diluted in goat serum dilution buffer (GSDB; 15% goat serum, 450 mM NaCl, 0.3% Triton X-100, 20 mM sodium phosphate buffer, pH 7.4). Incubation with the polyclonal primary antibodies was carried out for 2 hours at room temperature. Cells were washed twice within 20 minutes with HS and then incubated with the appropriate secondary antibodies for 90 minutes at room temperature. After three washes with HS for 30 minutes each and one 10 minute wash with PBS, coverslips were mounted with 70% glycerol in PBS supplemented with phenylenediamine (1 mg/ml) as an anti-bleaching agent. All the reagents used were from Sigma-Aldrich.

**Image acquisition, processing and statistical analysis**

Image processing was viewed with an Axiosvert 135 inverted microscope (Zeiss, Oberkochen, Germany) equipped with epifluorescence optics. Images were recorded with a C4742-98 ORCA II cooled charge-coupled device camera (Hamamatsu Photonics, Hamamatsu City, Japan) and processed using Image Pro Plus 4.5 (Media Cybernetics, Silver Spring, MD) and Adobe Photoshop 6.0 (Adobe System, San Jose, CA). To obtain RGB images we acquired a single grey image for every colour channel, then the three grey images were merged and processed using Adobe Photoshop CS (Adobe Systems). To better visualize colocalised puncta, after excluding the cell body, we adjusted the fluorescence level by applying the auto-level function, and then inverted the colours. The nerve terminal targeting of the exogenously expressed synapsins was determined by calculating the ‘targeting factor’, i.e. the ratio between the total number of Syn-I- positive puncta and the number of Syt-I- Syn-I-double-positive puncta. In the literature, synapses in are often defined simply as discrete puncta of single presynaptic proteins (Chin et al., 1995; Ferreira et al., 1998; Pagonami et al., 2010). However, to avoid counting structures other than synapses (e.g. transport packets), we evaluated the simultaneous presence of a scaffolding component (Bsn) with SV proteins. Bsn and SVs are transported by two distinct families of transport vesicles that probably utilize different molecular motors and/or adaptors (Zhai et al., 2001; McAllister, 2007). To test the validity of our method, we counted the single positive Syt, Bas and overexpressed protein puncta of a sample experiment (supplementary material Fig. S4). Indeed, no significant difference between the number of puncta labelled for either Syt or overexpressed protein (either VAMP2, WT or mutant synapsin) was detected. By contrast, there were significantly more Bsn-positive puncta under all experimental conditions, suggesting that structures other than synapses were labelled.

In inverted images we counted the number of blue (double colocalisation) or black (triple colocalisation) puncta present within a 30 μm dendrite tract starting from the cell body. Measuring a longer tract of dendrite was troublesome, because often dendrites do not follow linear routes. During the analysis we considered only puncta characterised by a signal to noise (S/N) ratio greater than 3 and an area in the range of 0.3–0.8 μm². We measured at least 20 neurons for each sample and selected at least two dendrites for each neuron.

To analyze FM-64 uptake at axonal boutons, we analyzed three separate coverslips of cultured neurons and acquired 10 images per condition in each experiment. Using the Gran Filter Plugin of NIH ImageJ software, we created a mask to select synapses with an area of 0.3–0.8 μm² and we measured FM-64 fluorescence intensity in each terminal. Statistical analysis was performed using the Kruskal–Wallis one-way analysis of variance on ranks followed by Tukey’s post-hoc test.

**Miscellaneous procedures**

**Protein concentrations**

Protein concentrations were determined by the Bradford or BCA assays (Thermo Scientific, Rockford, IL, USA). SDS-PAGE was performed according to Laemmli (Laemmli, 1970). Immunoblotting was performed using peroxidase-conjugated secondary antibodies coupled with the ECL chemiluminescence detection system. Statistical analysis of data obtained from western blot quantification was performed by two-way analysis of variance or Kruskal–Wallis test, followed by the Tukey’s post-hoc test.

**Acknowledgements**

We thank Elena Monzani for technical support.

**Funding**

This study was supported by research grants from the Italian Ministry of University and Research [PRIN grant number 2008T4ZCNL to F.B. and F.V.]; the Compagnia di San Paolo, Torino [grant number 2006-1964 to F.B. and 2008-2207 to F.V.]; and Telethon-Italy [grant number GGP09134 to F.B. and F.V.].

Supplementary material available online at


**References**


Synapsin I controls synaptogenesis


