

# MAPK/Erk-dependent phosphorylation of synapsin mediates formation of functional synapses and short-term homosynaptic plasticity

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## Summary

MAPK/Erk is a protein kinase activated by neurotrophic factors involved in synapse formation and plasticity, which acts at both the nuclear and cytoplasmic level. Synapsin proteins are synaptic-vesicle-associated proteins that are well known to be MAPK/Erk substrates at phylogenetically conserved sites. However, the physiological role of MAPK/Erk-dependent synapsin phosphorylation in regulating synaptic formation and function is poorly understood. Here, we examined whether synapsin acts as a physiological effector of MAPK/Erk in synaptogenesis and plasticity. To this aim, we developed an in vitro model of soma-to-soma paired *Helix* B2 neurons, that establish bidirectional excitatory synapses. We found that the formation and activity-dependent short-term plasticity of these synapses is dependent on the MAPK/Erk pathway. To address the role of synapsin in this pathway, we generated non-phosphorylatable and pseudo-phosphorylated *Helix* synapsin mutants at the MAPK/Erk sites. Overexpression experiments revealed that both mutants interfere with presynaptic differentiation, synapsin clustering, and severely impair post-tetanic potentiation, a form of short-term homosynaptic plasticity. Our findings show that MAPK/Erk-dependent synapsin phosphorylation has a dual role both in the establishment of functional synaptic connections and their short-term plasticity, indicating that some of the multiple extranuclear functions of MAPK/Erk in neurons can be mediated by the same multifunctional presynaptic target.

**Key words:** Synapsin, MAPK/Erk, Synaptogenesis, Post-tetanic potentiation, *Helix*, B2 neurons

## Introduction

Formation and plasticity of synapses are the result of complex molecular interactions that regulate assembly of pre- and post-synaptic specializations and modulate their functions. Synapsin proteins are a family of vesicle-associated phosphoproteins that are localized at presynaptic terminals (De Camilli et al., 1983; Huttner et al., 1983; Hirokawa et al., 1989; Kao et al., 1999) and promote neurite outgrowth and synaptogenesis, as well as regulating vesicle dynamics and neurotransmitter release (Greengard et al., 1993; Pieribone et al., 1995; Rosahl et al., 1995; Hilfiker et al., 1999; Chi et al., 2001). Synapsin is a substrate for several protein kinases, including PKA, CaMKs and MAPK/Erk, which strongly modulate its biochemical properties. However, the specific effect of MAPK/Erk-dependent synapsin phosphorylation in synaptogenesis has not been clarified, and its physiological role in neurotransmitter release and synaptic plasticity, mainly inferred from biochemical and imaging studies (Jovanovic et al., 1996; Jovanovic et al., 2000; Chin et al., 2002; Chi et al., 2003), has not been directly explored by electrophysiology.

The MAPK/Erk pathway has an important role in both synapse formation and plasticity. Evidence exists that this signalling cascade mediates the synaptogenic action of neurotrophic factors (Huang and Reichardt, 2001; Alonso et al., 2004; Hans et al., 2004), although its molecular targets during synaptogenesis are still unknown. Moreover, it has been proposed that the MAPK/Erk pathway

participates in long-term synaptic plasticity by regulating the activity of transcriptional factors upon nuclear translocation (Martin et al., 1997). Some studies show that MAPKs are also present and active in synaptic terminals, suggesting that this pathway might have several functions in distinct subcellular compartments during short- and long-term plasticity, acting through phosphorylation of synaptic targets, including synapsin (Sweatt, 2004; Boggio et al., 2007). Some studies have excluded the involvement of the MAPK/Erk pathway in short-term heterosynaptic plasticity (Martin et al., 1997; Purcell et al., 2003; Phares and Byrne, 2005). However, it has been observed that enhanced paired-pulse facilitation and long-term potentiation in mice expressing an activated form of H-Ras are abolished upon synapsin knockout, indicating that synapsin is an important target of the H-Ras-MAPK-Erk pathway in these forms of homosynaptic plasticity (Kushner et al., 2005).

In this work, we studied whether synapsin is a relevant MAPK/Erk target during synaptogenesis and short-term homosynaptic plasticity by directly manipulating the levels of synapsin and its mutant phosphoforms in presynaptic terminals. To this aim, we developed an in vitro model of synaptogenesis between B2 *Helix* neurons (Altrup and Speckmann, 1994) forming bidirectional excitatory synapses in presence of extrinsic trophic factors. Our experiments show that the formation and short-term plasticity of functional synapses are MAPK/Erk-dependent synapsin phosphorylation processes. In fact, using phosphorylation site

mutants of *Helix* synapsin that are either non-phosphorylatable, or mimic a persistent phosphorylation, we showed that presynaptic overexpression of non-phosphorylatable synapsin interferes with formation of active connections without affecting neurite outgrowth. Moreover, both phosphomutants virtually abolished post-tetanic potentiation (PTP) by exerting a dominant-negative effect over endogenous synapsin. Some of these results have been previously presented in abstract form (Giachello et al., 2008).

## Results

### Paired *Helix* B2 neurons form bidirectional soma-soma synapses in culture

In-vitro-reconstructed synaptic connections between identified neurons of *Helix*, *Aplysia* and other gastropods offer an advantageous model for studying synapse formation, as well as synaptic function. We previously observed that various types of *Helix* neurons can form uni- and bi-directional synapses when paired in culture in soma-to-soma configuration (Fiumara et al., 2005).

We found that in the presence of ganglia-derived trophic factors, paired B2 neurons from *Helix aspersa* buccal ganglia (Altrup and Speckmann, 1994; Fig. 1A) developed soma-to-soma chemical synapses (about 70% of cell pairs) in which excitatory postsynaptic potentials (EPSPs) were detected in a 1:1 relationship with presynaptic spikes. Interestingly, more than 70% of these chemical connections were bidirectional, whereas the remaining 30% were unidirectional (Fig. 1B,C). These synapses displayed distinct forms of activity-dependent synaptic enhancement. In particular, a presynaptic high-frequency stimulation of the presynaptic neuron (10 Hz for 10 seconds, Fig. 1D) induced a several-fold increase in the EPSP amplitude lasting for 3–4 minutes. This synaptic enhancement decayed exponentially with a time constant ( $\tau$ ) of about 1 minute, which is typical of post-tetanic potentiation (PTP) (Fisher et al., 1997).

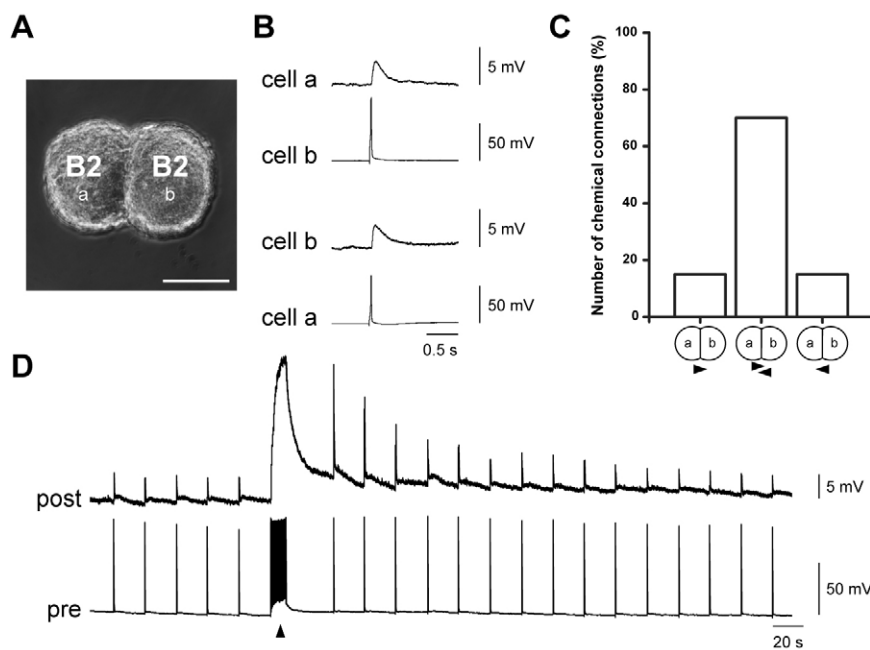
The bidirectionality of these connections allows us to apply a specific treatment either to both cells simultaneously or to only one of them by intracellular microinjection. Moreover, in comparison with other *Helix* synapses in vitro (Fiumara et al., 2005; Fiumara

et al., 2007), B2-B2 synapses showed faster EPSP kinetics, absence of low-frequency depression and a particularly robust PTP. Taken together, these features of B2-B2 synapses made them particularly suitable for this study.

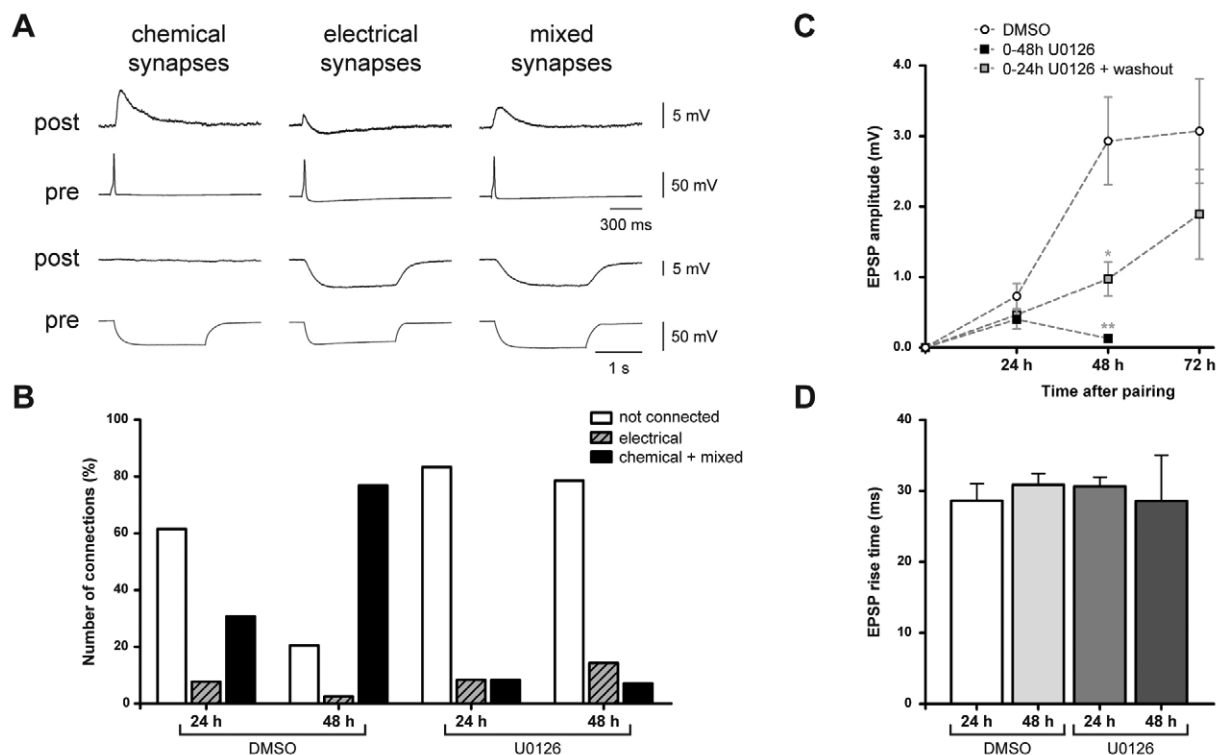
### MAPK/Erk activity interferes with the establishment of functional synapses between B2 neurons

We initially determined whether the MAPK/Erk pathway is involved in the formation of chemical connections between B2 neurons. To this aim, we studied the effect of pharmacological inhibition of the MAPK/Erk pathway on B2-B2 connections. Similarly to other *Helix* neuronal pairs in culture, B2-B2 chemical connections were associated in 25–30% of cases with an electrical component (Fig. 2A, mixed synapses). In rare instances, pure electrical synapses were detected. We compared the relative proportion of chemical, mixed and electrical connections between two groups of cells that were paired in the presence of the MEK inhibitor U0126 (50  $\mu$ M) or vehicle alone (DMSO), as a control. We found that U0126 treatment strongly affected the number of electrophysiologically detectable B2-B2 connections, compared with the DMSO control ( $P < 0.0001$ , Pearson's chi-square test; Fig. 2B). In the control group, the number of active synapses with a chemical component (chemical + mixed) significantly increased from 30.7% ( $n=13$ ) to 76.9% ( $n=39$ ), over 24 to 48 hours, respectively ( $P < 0.0001$ , Fisher's exact test). Conversely, no changes in the occurrence of chemical synapses were observed in U0126-treated synapses between the two time points (8.3% at 24 hours,  $n=12$ , vs 7.1% at 48 hours,  $n=28$ ;  $P=0.99$ , Fisher's exact test). Comparing the U0126 group with the DMSO one, we noticed a reduction in the number of functional chemical connections at 24 hours; an effect that became more evident 48 hours after the inhibitor application (24 hours: 8.3% in U0126 group,  $n=12$ , vs 30.7% in control group,  $n=13$ ;  $P=0.32$ , Fisher's exact test; 48 hours: 7.1% in U0126 group,  $n=28$ , vs 76.9% in control group,  $n=39$ ;  $P < 0.0001$ , Fisher's exact test).

Next, we examined the time course of EPSP amplitude changes in chemically interconnected B2-B2 pairs to establish whether the



**Fig. 1. *Helix* B2-B2 neuron pairs form unidirectional and bidirectional chemical synapses and exhibit short-term plasticity in vitro.** (A) Phase-contrast micrograph of a soma-to-soma pair of cultured *Helix* B2 neurons. Scale bar: 50  $\mu$ m. (B) Sample electrophysiological recordings of a bidirectional synapse between two B2 neurons (labeled 'a' and 'b' in panel A). (C) Occurrence of unidirectional or bidirectional synapses between chemically interconnected *Helix* B2-B2 neurons cultured in the presence of ganglia-derived trophic factor. The majority of B2-B2 pairs show bidirectional connections. (D) Representative induction and decay of PTP recorded from B2-B2 synapses in culture. The presynaptic neuron was electrically stimulated to fire an action potential five times at a basal frequency of 0.05 Hz and the corresponding EPSPs recorded in the postsynaptic cell kept hyperpolarized at  $-85$  mV to prevent firing. Next, a 10 Hz presynaptic stimulation was applied for 10 seconds (arrowhead). Thirty seconds after the tetanus, the presynaptic stimulation was brought back to the basal rate of 0.05 Hz.

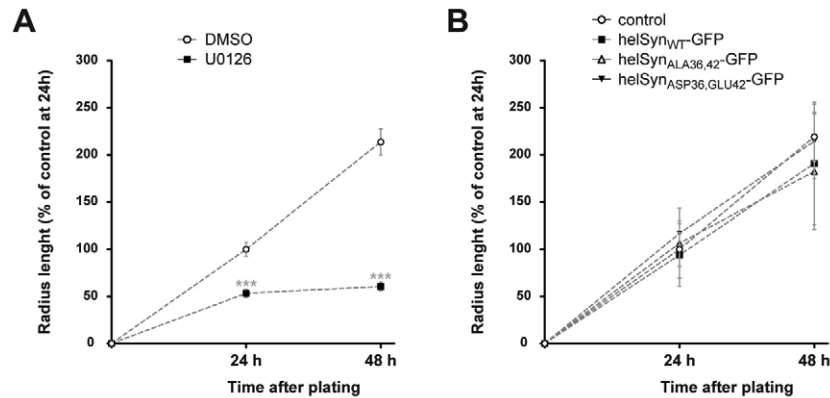


**Fig. 2. Activation of the MAPK/Erk cascade is involved in the formation of functional synapses between B2 neurons.** (A) Sample electrophysiological recordings illustrating the various types of synaptic transmission established by paired B2-B2 neurons. The presence and type of synaptic connections were evaluated by analyzing changes in the membrane potential of each cell during injection of either hyperpolarizing or depolarizing current (1 nA for 2 seconds) into the paired cell. (B) Time course of the occurrence of synapses with a chemical component (chemical + mixed) or electrical coupling between *Helix* B2-B2 neurons paired in the presence of the MEK inhibitor U0126 or vehicle (DMSO). (C) EPSP amplitudes recorded in chemically interconnected B2-B2 pairs at 24, 48 and 72 hours after pairing in the absence (DMSO) or presence of U0126 (0-48 hour U0126) reveal that chronic inhibition of MAPK/Erk virtually blocks the progressive increase in EPSP amplitude that accompanies synapse formation. Missing time-point is due to low signal-to-noise ratio. To estimate the recovery of functional transmission, B2-B2 pairs were tested after the washout of the inhibitor (0-24 hours U0126+washout). (D) Similar EPSP rise time values are measured in both DMSO- and U0126-treated B2-B2 pairs, indicating that pharmacological treatment did not affect the neurotransmitter release machinery. Values represent means  $\pm$  s.e.m. \*\* $P < 0.01$ ; \* $P < 0.05$ .

synaptic strength was affected by MAPK/Erk inhibition in a time-dependent manner. In the control group, there was a progressive increase in EPSP amplitudes with time after pairing (Fig. 2C). In B2-B2 neurons chronically exposed to U0126, a reduction in EPSP amplitudes was already apparent 24 hours after cell-cell pairing ( $0.39 \pm 0.13$  mV in 0-48 hours U0126-treated group,  $n=5$ , vs  $0.73 \pm 0.17$  mV in control group,  $n=12$ ;  $P > 0.05$ , Bonferroni's post-hoc test), and became more pronounced and significant 48 hours after pairing ( $0.12 \pm 0.01$  mV in 0-48 hours U0126-treated group,  $n=5$ , vs  $2.92 \pm 0.62$  mV in control group,  $n=12$ ;  $P < 0.01$ , Bonferroni's post-hoc test). A two-way ANOVA for repeated measures revealed a significant effect of the treatment ( $F_{(1,15)}=5.90$ ,  $P < 0.05$ ) and time ( $F_{(2,15)}=5.16$ ,  $P < 0.05$ ) and a significant treatment by time interaction ( $F_{(2,30)}=4.96$ ,  $P < 0.05$ ). To verify whether this effect on functional transmission was reversible, B2-B2 pairs incubated with U0126 for 24 hours were recorded after inhibitor washout (Fig. 2C, 0-24 hours U0126-treated + washout group). We observed a recovery of synaptic transmission with a time-course similar to that in the control group, but delayed by 24 hours (48 hours:  $0.97 \pm 0.24$  mV in treated group,  $n=7$ , vs  $2.92 \pm 0.62$  mV in control group,  $n=12$ ;  $P < 0.05$ , Bonferroni's post-hoc test; 72 hours:  $1.89 \pm 0.63$  mV in treated group,  $n=7$ , vs  $3.08 \pm 0.54$  mV in control group,  $n=12$ ;  $P > 0.05$ , Bonferroni's post-hoc test).

The effects of the extended U0126 treatment cannot be ascribed to cell damage, since several electrophysiological parameters including resting membrane potential (U0126:  $-53.00 \pm 2.47$  mV,  $n=8$ ; DMSO:  $-55.93 \pm 1.62$  mV,  $n=12$ ;  $P=0.314$ , Student's *t*-test), input resistance (U0126:  $53.65 \pm 3.10$  M $\Omega$ ,  $n=14$ ; DMSO:  $51.03 \pm 2.21$  M $\Omega$ ,  $n=24$ ;  $P=0.4867$ , Student's *t*-test) and spike threshold (U0126:  $-26.20 \pm 1.09$  mV,  $n=8$ ; DMSO:  $-26.06 \pm 1.82$  mV,  $n=12$ ;  $P=0.9525$ , Student's *t*-test) were not different after U0126 incubation compared with control conditions. Moreover, we also tested whether the neurotransmitter release kinetics of synapses formed in the presence of U0126 were somehow altered. We observed that EPSP rise time was comparable in the two different experimental groups, at both 24 and 48 hours (Fig. 2D;  $F_{(1,14)}=0.0016$ ,  $P=0.9684$ , two-way ANOVA for repeated measures).

In addition, we measured the neurite outgrowth of single B2 neurons plated on poly-L-lysine-pretreated dishes (Fig. 3A). We found that the presence of the inhibitor strongly reduced the extension of neurites at both 24 and 48 hours (treatment  $F_{(1,36)}=91.76$ ,  $P < 0.0001$ , time  $F_{(2,36)}=381.5$ ,  $P < 0.0001$  and treatment by time interaction  $F_{(2,72)}=124.4$ ,  $P < 0.0001$ ; two-way ANOVA for repeated measures), which was consistent with previous observations in other systems (Kim et al., 1997; Encinas et al., 1999; Dimitropoulou and Bixby, 2000; Kurihara et al., 2000).



**Fig. 3. Inhibition of the MAPK/Erk cascade affects neurite outgrowth through a synapsin phosphorylation-independent mechanism.** (A) Graphic representation of the neurite outgrowth of *Helix* B2 neurons plated on adhesive substrate in the presence of ganglia-derived trophic factors, upon application of either U0126 or DMSO as a control. Each time-point represents the percentage mean value of radius length of a circumferential line interconnecting the tips of the three longest neurites, normalized to the control mean value measured at 24 hours. The presence of the inhibitor strongly reduces the extension of neurite outgrowth at both 24 and 48 hours (24 hours:  $53.3 \pm 4.0\%$  in U0126 group,  $n=21$ , vs  $100.0 \pm 7.3\%$  in control group,  $n=17$ ;  $P < 0.001$ , Bonferroni's post-hoc test; 48 hours:  $60.6 \pm 4.6\%$  in U0126 group,  $n=21$ , vs  $213.7 \pm 13.8\%$  in control group,  $n=17$ ;  $P < 0.001$ , Bonferroni's post-hoc test). (B) Neurite growth profile of *Helix* B2 neurons overexpressing the different *Helix* synapsin (helSyn) mutants. No differences are observed among the experimental groups ( $n=6$  in each group) with respect to control (uninjected cells,  $n=20$ ). Values represent means  $\pm$  s.e.m. \*\*\* $P < 0.001$ .

Taken together, our data indicate that the MAPK/Erk pathway regulates both neurite outgrowth and the occurrence of functional chemical connections between B2 neurons.

#### Post-tetanic potentiation at B2-B2 synapses depends on MAPK/Erk activation

Next, we studied whether the MAPK/Erk pathway is also involved, in ways other than in synapse formation, in the functional plasticity of already established B2-B2 synapses. To this aim, we induced PTP, a form of short-term homosynaptic plasticity, before and after the bath application of the inhibitor U0126 (Fig. 4A,C) or vehicle alone (DMSO) as a control (Fig. 4B,C). In the same synapses, we observed that the presence of U0126 strongly reduced the expression of PTP at both 15 and 30 minutes after bath application. We also determined the reversibility of the U0126 effect by testing PTP 15 minutes after wash out of the inhibitor. We found that the U0126 effect was completely reversible and PTP was fully restored after washout. A two-way ANOVA for repeated measures revealed a significant effect of the treatment ( $n=7$ ;  $F_{(3,24)}=4.83$ ,  $P < 0.01$ ) and a significant treatment by time interaction ( $F_{(57,456)}=4.98$ ,  $P < 0.001$ ). In particular, the peak amplitude of PTP measured 30 seconds after tetanus was reduced to  $38 \pm 8.0\%$  of control amplitude at 15 minutes and to  $32.92 \pm 7.1\%$  at 30 minutes after the application ( $n=7$ ;  $F_{(3,18)}=12.79$ ,  $P=0.0001$ , one-way ANOVA for repeated measures; Fig. 4D). In the control group, we did not detect significant changes in PTP before or after addition of vehicle ( $n=5$ ; treatment  $F_{(1,16)}=0.54$ ,  $P=0.6614$  and treatment by time interaction  $F_{(57,304)}=0.30$ ,  $P=1.00$ , two-way ANOVA for repeated measures; Fig. 4B).

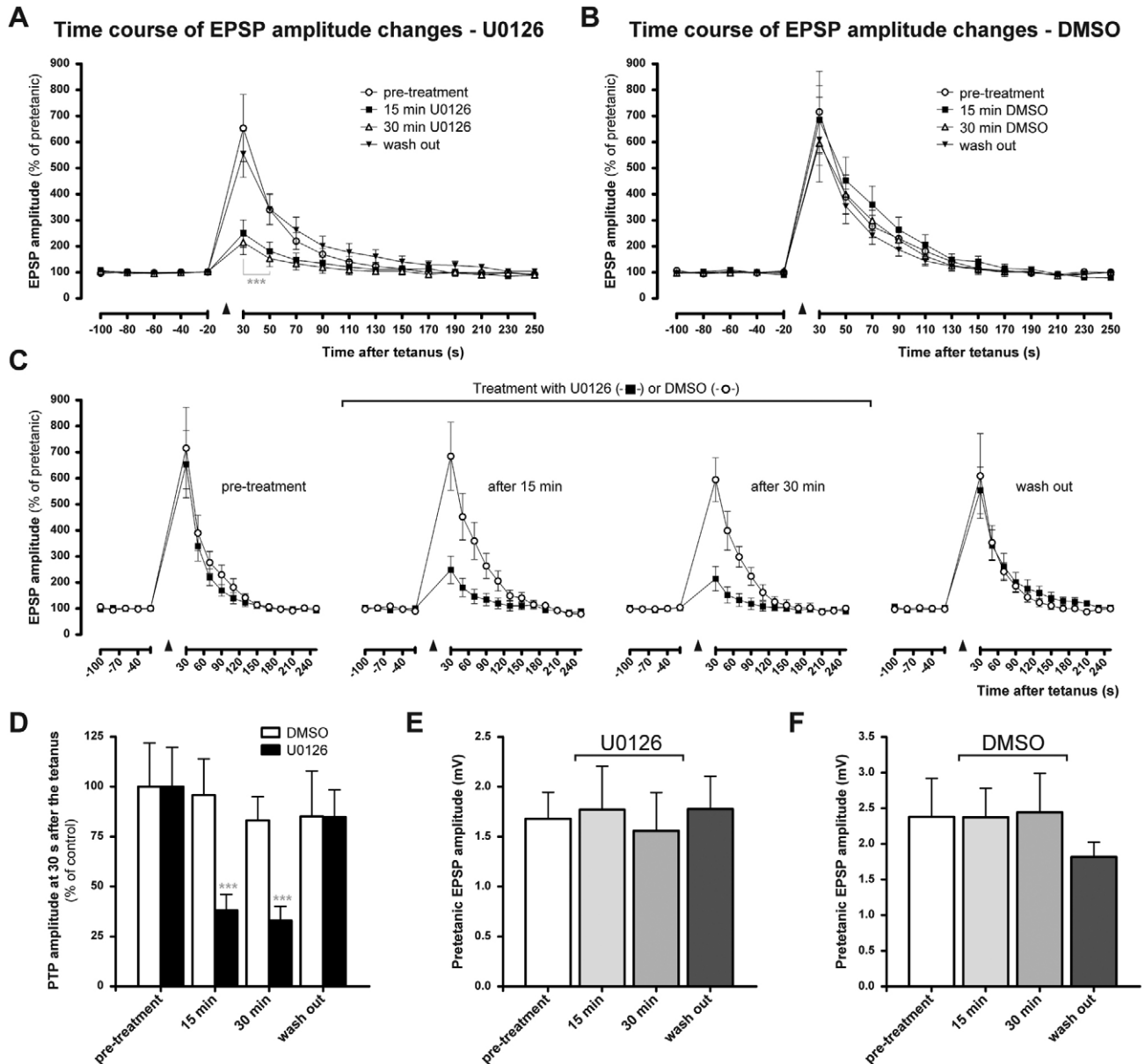
To rule out the possibility that the U0126 effect might be related to general changes in efficiency of synaptic transmission and not to a specific effect on short-term plasticity, we verified that the presence of U0126 or DMSO did not alter the basal EPSP amplitudes. To this aim, we compared the mean pre-tetanic basal EPSPs before and after application of U0126 and found no effects of the inhibitor or of vehicle alone on basal synaptic transmission (U0126:  $F_{(3,18)}=15.23$ ,  $P=0.774$ ,  $n=7$ ; DMSO:  $F_{(3,12)}=9.008$ ,

$P=0.3232$ ,  $n=5$ ; one-way ANOVA for repeated measures; Fig. 4E,F).

Taken together, these data indicate that MAPK/Erk pathway has an important role in expression of PTP at B2-B2 synapses. The fast and fully reversible effect of U0126 indicates that the role of MAPK/Erk activation in this form of plasticity is likely to be related to phosphorylation of synaptic substrates rather than to transcriptional effects.

#### *Helix* synapsin is a MAPK/Erk substrate at phylogenetically conserved phosphorylation sites

Synapsin proteins are major presynaptic components and are known targets for MAPK/Erk in vertebrates. To determine whether synapsin is a mediator of the MAPK/Erk actions in B2-B2 synaptic functionality and plasticity that we observed, we studied the primary sequence of *Helix* synapsin looking for potential MAPK/Erk consensus sites. MAPK/Erk is known to phosphorylate mammalian synapsin I (at sites 4/5 in domain B and site 6 in domain D) as well as *Aplysia* synapsin (Angers et al., 2002). Since molluscan synapsin lacks the D-domain, in search for conserved phosphorylation sites we focused our analysis on the N-terminal B-domain. A bioinformatic analysis with the GPS 2.1 software revealed three putative sites in this region (Ser36, Ser42 and Ser48). Whereas Ser36 and Ser42 had high probability scores, the Ser48 was only at the prediction threshold. Interestingly, Ser36 and Ser42 were highly conserved among the known invertebrate synapsin proteins (Fig. 5A). MAPK/Erk phosphorylation of the closely related *Aplysia* synapsin was previously reported, and the former two serine residues were identified as putative MAPK sites (Angers et al., 2002). Moreover, these two sites seemingly corresponded to sites 4 and 5 of mammalian synapsin. As illustrated in Fig. 5B, in a multiple alignment of mammalian synapsin I with the *Helix* and *Aplysia* orthologues, Ser36 aligned with mammalian site 4 (Ser62). Within this alignment, *Helix* Ser42 and the corresponding *Aplysia* site were shifted by one residue with respect to mammalian site 5. Based on this analysis, we concluded that *Helix* and *Aplysia* Ser36 and Ser42 might represent the MAPK/Erk phosphorylation sites in

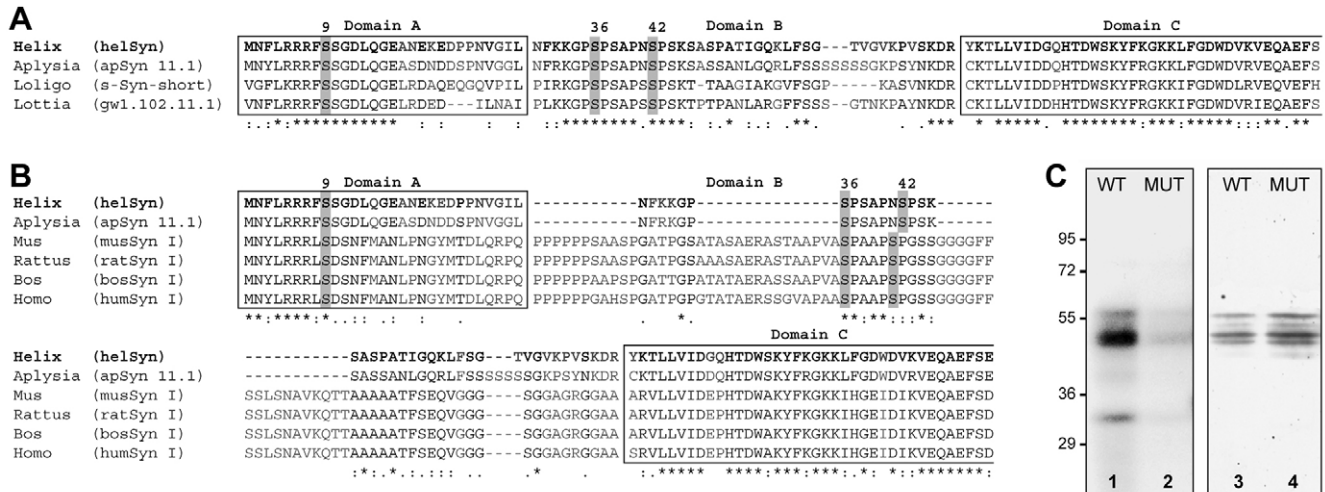


**Fig. 4. Bath application of MEK inhibitor U0126 impairs PTP expression in B2-B2 soma pairs.** (A-C) Time courses of EPSP amplitude changes in 50  $\mu$ M U0126-treated (A) and DMSO-treated (B) synapses. In U0126-treated synapses tested 15 or 30 minutes after inhibitor application, the expression of PTP is significantly impaired ( $P < 0.001$  vs control during the first minute after the tetanus, Bonferroni's post-hoc test), whereas no significant changes are observed in vehicle-treated synapses. After U0126 washout, PTP expression returns to pre-treatment level, indicating a fast reversibility of the effect. (D) Bar graph of the EPSP amplitudes measured at 30 seconds after tetanus. Values are normalized to the mean peak PTP amplitude measured before the treatment in each group. (E, F) Bar graphs of mean pre-tetanic EPSP amplitudes in U0126-treated (E) or DMSO-treated (F) synapses reveal no significant effects of the treatment. Values represent means  $\pm$  s.e.m. \*\*\* $P < 0.001$ .

the B-domain that are homologous to sites 4 and 5 of mammalian synapsin. To unambiguously confirm that *Aplysia* and *Helix* synapsin was indeed phosphorylated by MAPK/Erk at these two sites, we performed in vitro phosphorylation assays. We generated recombinant wild-type synapsin as well as mutant form in which both Ser36 and Ser42 were replaced with alanine to prevent phosphorylation upon incubation with activated MAPK/Erk2 in presence of radioactively labeled ATP. Indeed, replacement of Ser36 and Ser42 with alanine dramatically decreased synapsin phosphorylation by MAPK/Erk, indicating that Ser36 and Ser42 are the major MAPK/Erk phosphorylation sites (Fig. 5C).

#### Phosphorylation of *Helix* synapsin B-domain modulates synapsin clustering

To assess the functional role of synapsin MAPK/Erk phosphorylation sites, in addition to the non-phosphorylatable mutant, we generated a pseudo-phosphorylated mutant in which Ser36 and Ser42 were substituted with aspartate and glutamate, respectively, to mimic the negative charges present on phosphorylated serines. As a first step in functional characterization of synapsin mutants, we compared their subcellular distribution after overexpression in B2 neurons as GFP-fusion proteins. To this end, B2 neuron somata were microinjected with mRNAs encoding either



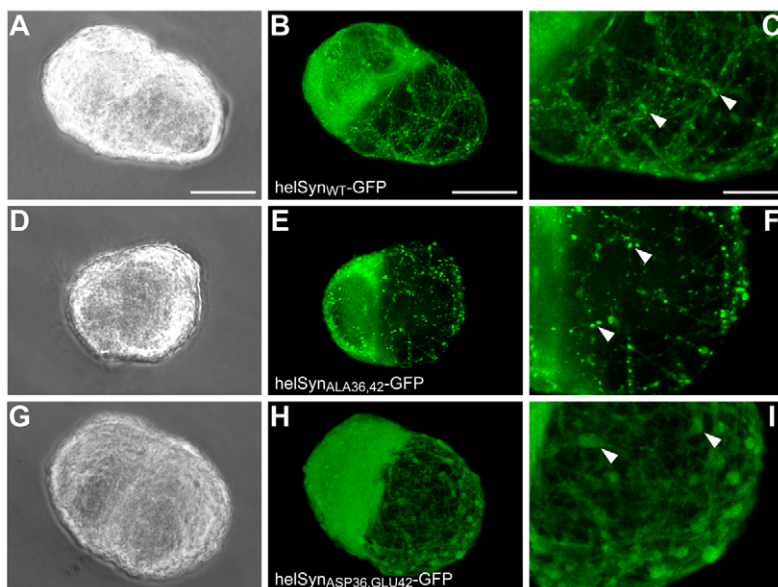
**Fig. 5. Site-directed mutagenesis of *Helix* synapsin B-domain.** (A) Multiple sequence alignment of *Helix* synapsin and three other related gastropod synapsin proteins. (B) Partial multiple sequence alignment between mammalian synapsin I and invertebrate synapsin. In spite of the lower sequence homology of the B-domain compared with domains A and C, the mammalian phosphorylation sites for MAPK/Erk (sites 4/5) are well conserved in invertebrate synapsin (Ser36 and Ser42 are highlighted). Perfectly conserved positions are labeled with asterisks, high- and low-match amino acid residues are indicated with colons and dots, respectively. (C) In vitro phosphorylation assay of recombinant wild-type and non-phosphorylatable synapsin with activated MAPK/Erk2. The blot autoradiogram (lanes 1 and 2) reveals that only the band corresponding to wild-type synapsin (lane 1) is intensely labeled by  $^{32}\text{P}$ , whereas equal amounts of synapsin mutated in the putative MAPK/Erk consensus sites (lane 2) shows a negligible phosphate incorporation, indicating that Ser36 and Ser42 are MAPK/Erk phosphorylation sites homologous to mammalian sites 4/5. The immunoblot with anti-Syn antibodies reveals that both wild-type (lane 3) and mutant (lane 4) synapsin were loaded in similar amounts.

wild-type Syn-GFP (Syn<sub>WT</sub>-GFP), the non-phosphorylatable mutant Syn<sub>Ala36,Ala42</sub>-GFP, or the pseudo-phosphorylated mutant Syn<sub>Asp36,Glu42</sub>-GFP. The injected cells were then paired with other B2 somata. After 48 hours, the B2-B2 soma-to-soma pairs were electrophysiologically tested for the presence of chemical synaptic connections, and imaged with confocal microscopy (Fig. 6A-I).

The distribution pattern of Syn<sub>WT</sub>-GFP was comparable with that observed after presynaptic overexpression of the same protein at C1-B2 soma-to-soma synapses described previously (Fiumara et al., 2007). In particular, Syn<sub>WT</sub>-GFP was more intensely clustered along the presynaptic neurites projecting onto the postsynaptic cell

(Fig. 6A-C). These sites of clustering represent the putative synaptic sites at soma-to-soma synapses (Fiumara et al., 2005; Fiumara et al., 2007; Naruo et al., 2005). A more punctate distribution was observed for the non-phosphorylatable mutant Syn<sub>Ala36,Ala42</sub>-GFP (Fig. 6D-F), which was much more intensely clustered in the same areas as Syn<sub>WT</sub>-GFP. We detected a similar pattern of distribution of the endogenous synapsin in uninjected B2 neurons incubated with the U0126 inhibitor (supplementary material Fig. S1).

Conversely, the pseudo-phosphorylated mutant Syn<sub>Asp36,Glu42</sub>-GFP exhibited a diffuse and uniform pattern of expression along neurites, with negligible signs of clustering (Fig. 6G-I). To quantify



**Fig. 6. Presynaptic overexpression of wild-type and mutant forms of Syn-GFP at B2-B2 synapses.** Representative phase-contrast (left panels) and confocal fluorescence (middle and right panels) images of *Helix* B2-B2 pairs captured 48 hours after mRNA injection in the presynaptic cell. (A,B) Overexpressed *Helix* Syn<sub>WT</sub>-GFP is concentrated in varicose structures along neurites projecting from the pre- to postsynaptic cell. At higher magnification (C), arrowheads indicate the areas in which synapsin is more concentrated. (D,E) The overexpression of non-phosphorylatable mutant displays a more clustered distribution pattern in presynaptic compartment and along neurites. At higher magnification (F), arrowheads indicate clusters of non-phosphorylatable synapsin mutant. (G,H) *Helix* Syn<sub>Asp36,Glu42</sub>-GFP exhibits a diffuse distribution pattern with no apparent differences in clustering among soma and neurites. (I) Detail of varicosities (arrowheads) and neurites grown on postsynaptic cell showing a relatively homogeneous fluorescence. Scale bars: 50  $\mu\text{m}$  (left and middle panels); 20  $\mu\text{m}$  (right panels).

the fluorescence signal,  $50 \times 50 \mu\text{m}$  areas were selected in five cell pairs for each experimental group, and computationally converted into a matrix of pixel values (supplementary material Fig. S2A). Statistical analysis revealed significant changes in pixel value variance, indicating a phosphorylation-dependent subcellular distribution of synapsin. In fact, fluorescence intensities were characterized by an even distribution for the pseudo-phosphorylated form and a markedly uneven distribution for the non-phosphorylatable form of Syn-GFP ( $F_{(2,12)}=23.77$ ,  $P<0.0002$ ,  $n=5$  in each group, one-way ANOVA; supplementary material Fig. S2B).

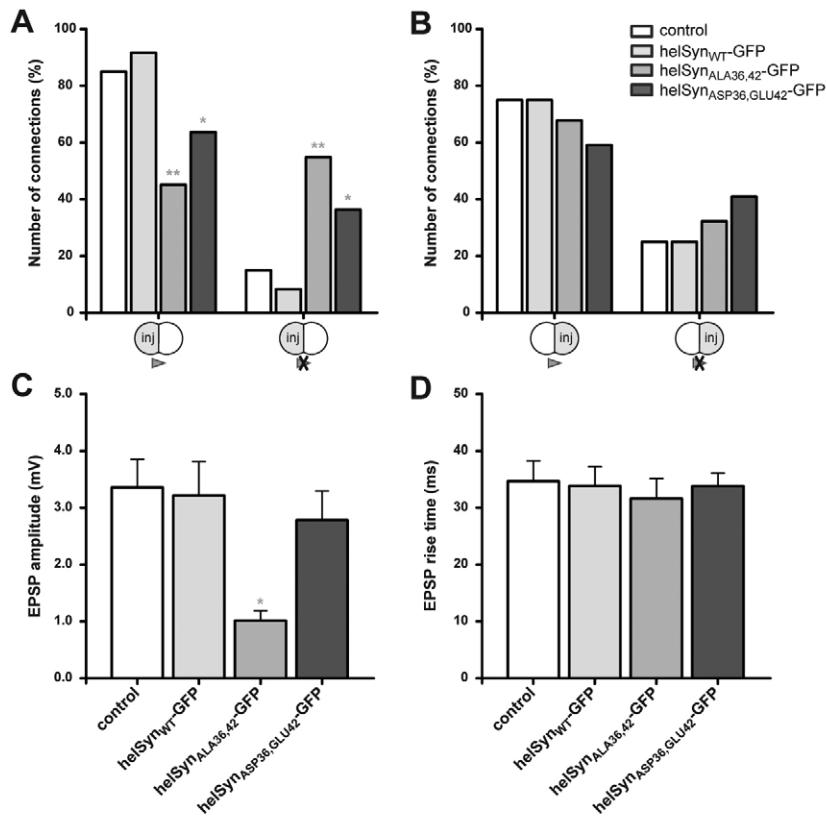
To better evaluate the degree of clustering in discrete structures of synapsin-GFP phosphoforms, we isolated areas encompassing a single cluster and drew a plot of the corresponding intensity values (supplementary material Fig. S2C). From nonlinear fitting of the fluorescence intensity profiles, we measured the half-intensity width parameter (i.e. the distance between the peak of fluorescence and half-intensity point). The subcellular distribution of non-phosphorylatable synapsin greatly differed from the wild-type form, and was highly concentrated at putative presynaptic spots (Fiumara et al., 2005; Fiumara et al., 2007), suggesting a reduced degree of dissociation from the presynaptic vesicle clusters (supplementary material Fig. S2D). However, the pseudo-phosphorylated mutant showed a broader shape curve that indicates a more diffuse distribution of the fluorescence intensity, suggesting a reduced binding capacity to synaptic vesicles. These data indicate that MAPK/Erk-dependent phosphorylation modulates the extent of clustering of *Helix* synapsin at presynaptic sites ( $F_{(3,36)}=28.68$ ,  $P<0.0001$ , one-way ANOVA) and are consistent with previous studies that show significant changes in clustering and dispersion of mammalian synapsin after mutagenesis of MAPK/Erk phosphorylation sites (Chi et al., 2003).

### MAPK/Erk-dependent synapsin phosphorylation regulates functional synaptic connectivity at presynaptic level

To elucidate the potential role of synapsin as a downstream effector of MAPK/Erk during synapse formation, we compared the effect of wild-type synapsin and of its phosphorylation site mutants on the formation of functional synaptic contacts.

To this aim, we took advantage of the bidirectional B2-B2 synaptogenesis model to compare the relative proportion of cell pairs in which the overexpressing neuron was presynaptic with the untreated counterpart and vice versa (Fig. 7A-B). As a control, we used cell pairs in which one cell was injected with saline buffer (mock treated). We found that neurons overexpressing Syn<sub>WT</sub>-GFP were able to establish a connection as presynaptic cells in 91.6% ( $n=24$ ) of the cases, similarly to the control group (85.0%,  $n=20$ ,  $P=0.64$ , Fisher's exact test, Fig. 7A). Conversely, overexpression of the non-phosphorylatable mutant Syn<sub>Ala36,Ala42</sub>-GFP strongly reduced the presynaptic differentiation of injected neuron (45.2% of cases;  $n=31$ ,  $P<0.01$ , Fisher's exact test). A similar trend was observed for the pseudo-phosphorylated mutant Syn<sub>Asp36,Glu42</sub>-GFP (63.6% of cases;  $n=66$ ,  $P<0.05$ , Fisher's exact test). In both Syn<sub>Ala36,Ala42</sub>-GFP and Syn<sub>Asp36,Glu42</sub>-GFP groups, the impairment was paralleled by a clear-cut increase in the proportion of pairs in which there was not a connection from the injected cell to the other cell. To verify that microinjection treatment had no consequences in the cell postsynaptic to the injected one, we also analyzed the presynaptic differentiation of the paired untreated neuron. No alterations were found among the experimental groups ( $P=0.39$ , Pearson's chi-square test, Fig. 7B), indicating that the effects of synapsin phosphomutants were essentially presynaptic.

In addition, we measured EPSP amplitudes in B2-B2 soma pairs overexpressing the different forms of synapsin in presynaptic



**Fig. 7. Overexpression of MAPK/Erk non-phosphorylatable mutant of *Helix* synapsin affects the occurrence of active connections and synaptic strength at B2-B2 neuronal pairs.** Presynaptic role preference, in chemically connected B2-B2 pairs, of the somata overexpressing the different forms of *Helix* synapsin (A) compared with untreated paired somata (B). As a control, we used B2-B2 pairs in which one cell was injected with saline buffer. The overexpression of both synapsin phosphoforms influenced the presynaptic differentiation of treated neuron without affecting the presynaptic role preference of the untreated partner cell. (C) Bar graph of mean basal EPSP amplitudes recorded from B2-B2 neurons overexpressing the various mutant forms of *Helix* synapsin. The mean EPSP amplitude is significantly reduced at synapses overexpressing presynaptically Syn<sub>Ala36,Ala42</sub>-GFP with respect to the control group. (D) Rise times of EPSPs measured between 10% and 90% of the peak amplitude in each experimental group ( $n=10$ ). No significant difference was found between experimental groups. Values represent means  $\pm$  s.e.m. \*\* $P<0.01$ ; \* $P<0.05$ .

compartment (Fig. 7C). We found that the mean EPSP amplitude was not affected by overexpression of Syn<sub>WT</sub>-GFP ( $3.22 \pm 0.60$  mV,  $n=15$ ), and was only slightly reduced by the pseudo-phosphorylated form of synapsin ( $2.79 \pm 0.51$  mV,  $n=14$ ), compared with control cells ( $3.36 \pm 0.50$  mV,  $n=33$ ). Conversely, the overexpression of non-phosphorylatable mutant Syn<sub>Ala36,Ala42</sub>-GFP significantly reduced basal EPSP amplitude to about 30% of control values ( $1.01 \pm 0.18$  mV,  $n=11$ ,  $F_{(3,69)}=2.821$ ,  $P<0.05$ , one-way ANOVA followed by Bonferroni's post-hoc test).

To rule out a direct interference of *Helix* synapsin mutants with the neurotransmitter release machinery, we measured the EPSP rise time in the four groups of injected neurons ( $34.68 \pm 3.6$  ms in control group,  $33.84 \pm 3.4$  ms in Syn<sub>WT</sub>-GFP group,  $31.63 \pm 3.5$  ms in Syn<sub>Ala36,Ala42</sub>-GFP group and  $33.81 \pm 2.2$  ms in Syn<sub>Asp36,Glu42</sub>-GFP group; Fig. 7D) and they were not statistically different ( $F_{(3,36)}=0.16$ ,  $P=0.92$ , one-way ANOVA).

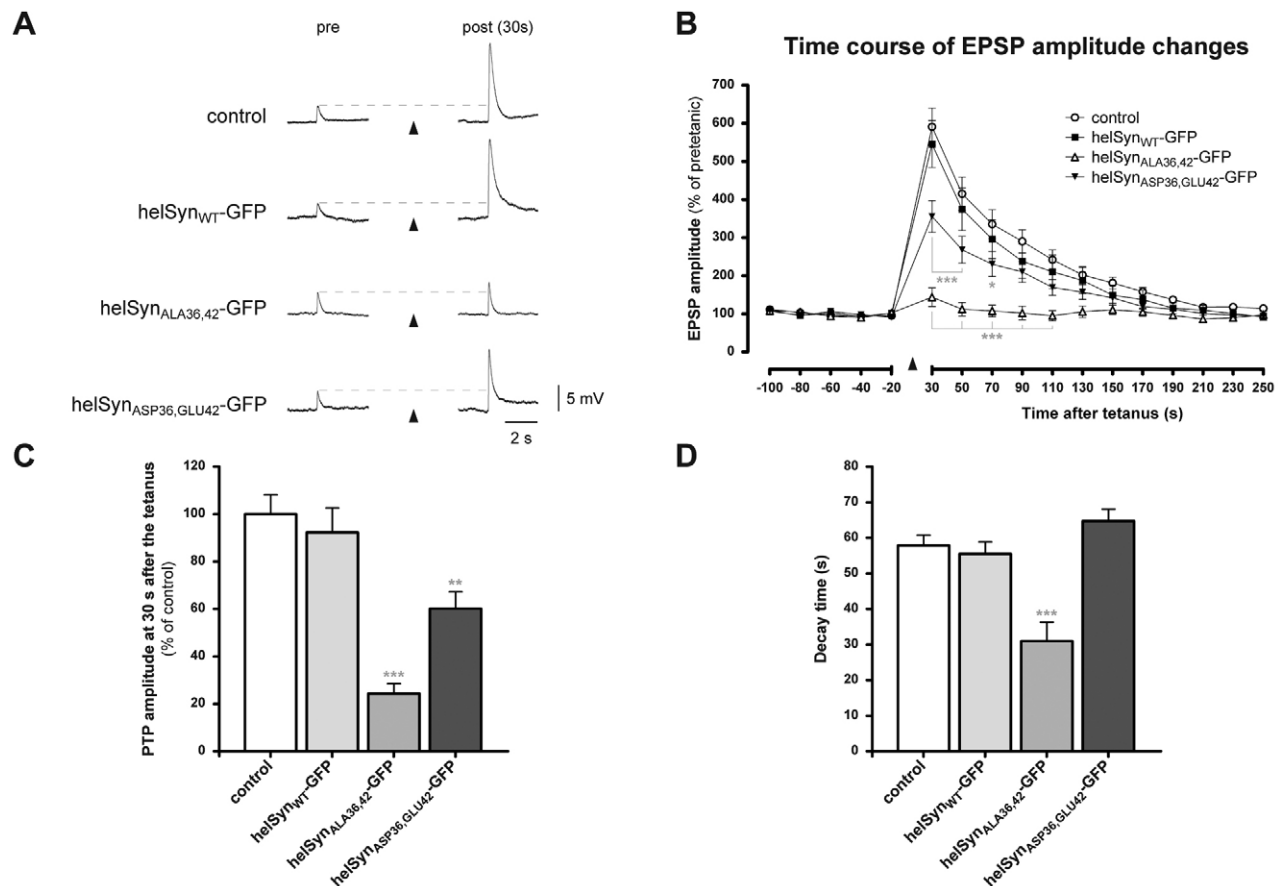
Since we observed that the MAPK/Erk pathway affects neurite outgrowth, we tested the hypothesis that this action might be mediated by synapsin phosphorylation. To this aim, we measured neurite outgrowth in single B2 overexpressing neurons. Under these experimental conditions, we did not observe any alterations with

respect to uninjected cells (treatment  $F_{(3,34)}=0.09$ ,  $P=0.96$  and treatment by time interaction  $F_{(6,68)}=0.26$ ,  $P=0.95$ , two-way ANOVA for repeated measures; Fig. 3B), thus ruling out the possibility that the effects of the mutants on the formation of functional synaptic contacts that we observed are related to differences in neurite growth.

Taken together, these findings indicate that MAPK/Erk-dependent phosphorylation of *Helix* synapsin is important for the establishment of functional synaptic connections through presynaptic mechanisms that are independent of neurite outgrowth.

### Presynaptic MAPK/Erk-dependent synapsin phosphorylation mediates PTP at B2-B2 synapses

To evaluate the role of synapsin MAPK/Erk-dependent phosphorylation in short-term plasticity, we recorded the induction and decay of PTP at B2-B2 synapses in which the presynaptic cell overexpressed either wild-type or mutant synapsin (Fig. 8A-D). In each synaptic pair, we used as an internal control the B2-B2 synapse in which the uninjected neuron was the presynaptic cell. The amplitude of PTP is known to be related to the basal strength of synaptic connections (Fiumara et al., 2005). Therefore,



**Fig. 8. Presynaptic overexpression of MAPK/Erk phosphorylation mutants of *Helix* synapsin impairs PTP.** (A) Sample electrophysiological recordings of EPSPs before and 30 seconds after the tetanic stimulation at B2-B2 synapses overexpressing presynaptically either wild-type *Helix* synapsin or its phosphorylation site mutants. (B) Time course of EPSP amplitude changes in the four experimental groups. (C) Mean amplitudes of the peak PTP measured 30 seconds after tetanus. EPSP amplitudes recorded 30 seconds after tetanization in control neurons ( $n=21$ ) and in neurons overexpressing Syn<sub>WT</sub>-GFP ( $n=12$ ) are similar. In the Syn<sub>Ala36,Ala42</sub>-GFP group ( $n=10$ ), the peak PTP was reduced to  $24.4 \pm 4.1\%$  of the control. A less-pronounced decrease ( $60.2 \pm 7.0\%$ ;  $n=13$ ) was also observed in neurons overexpressing Syn<sub>Asp36,Glu42</sub>-GFP. Values were normalized to the mean peak PTP amplitude measured in the control group. One-way ANOVA analysis confirms the highly significant effect of treatment ( $F_{(3,52)}=15.10$ ;  $P<0.0001$ ). (D) Bar graph of the decay time constants of PTP measured in B2-B2 synapses showing that only overexpression of Syn<sub>Ala36,Ala42</sub>-GFP significantly reduces  $\tau$ . Values represent means  $\pm$  s.e.m. \*\*\* $P<0.001$ , \*\* $P<0.01$ , \* $P<0.05$ .



for these experiments we selected synapses of comparable strength in each group (basal EPSP 0.5–5 mV), and we verified that the mean amplitudes of the last five pre-tetanic EPSPs were homogenous among the experimental groups ( $F_{(3,52)}=1.954$ ,  $P=0.13$ , one-way ANOVA). A two-way ANOVA for repeated measures revealed overall a significant effect of treatment, i.e. the overexpression of synapsin phosphoforms ( $F_{(3,52)}=8.04$ ,  $P<0.001$ ), and a significant treatment by time interaction ( $F_{(48,832)}=8.14$ ,  $P<0.0001$ ; Fig. 8B). In particular, we found that PTP was not affected by overexpression of Syn<sub>WT</sub>-GFP (peak PTP amplitude at 30 seconds after tetanus: 545.7±61.4% in Syn<sub>WT</sub>-GFP group,  $n=12$ , vs 591.2±48.5%,  $n=21$ , in control synapses). This result is consistent with our previous observations at *Helix* C1-B2 synapses (Fiumara et al., 2007). Conversely, overexpression of the non-phosphorylatable mutant Syn<sub>Ala36,Ala42</sub>-GFP determined a pronounced impairment of PTP. In fact, the peak PTP amplitude (144.0±24.4%,  $n=10$ ) was decreased to 24.4±4.1% of the control value (Fig. 8C) and the amplitude of post-tetanic EPSPs remained significantly reduced for 2 minutes after the tetanus with respect to control ( $P<0.001$ , Bonferroni's post-hoc test). In synapses overexpressing the pseudo-phosphorylated Syn<sub>Asp36,Glu42</sub>-GFP, we observed a less-dramatic reduction in PTP amplitude. The peak measured at 30 seconds was 355.8±41.5% ( $n=13$ ), corresponding to 60.2±7.0% of control amplitude ( $P<0.01$ , Bonferroni's post-hoc test).

We also analyzed the time course of PTP decay fitted according to a mono-exponential function to estimate the decay constant ( $\tau$ ) in control and treated synapses. We found a  $\tau$  value of 57.8±3.0 seconds in the control group, 55.5±3.3 seconds in the Syn<sub>WT</sub>-GFP group, 30.9±5.3 seconds in the Syn<sub>Ala36,Ala42</sub>-GFP group and 64.7±3.3 seconds in the Syn<sub>Asp36,Glu42</sub>-GFP group (Fig. 8D). On the whole, only the non-phosphorylatable mutant form gave a significant reduction of PTP decay time ( $F_{(3,36)}=14.62$ ,  $P<0.0001$ , one-way ANOVA), whereas overexpression of the pseudo-phosphorylated form of synapsin affected the PTP amplitude but not its decay. Taken together, these results show that MAPK/Erk-dependent synapsin phosphorylation, besides its role in the generation of functional B2-B2 synapses, is also an important mediator of their short-term plasticity.

## Discussion

In this study, we found that MAPK/Erk-dependent phosphorylation of synapsin has a dual role in regulating both functional synapse formation and activity-dependent short-term plasticity. These findings extend the established role of MAPK/Erk beyond the regulation of nuclear transcription to the modulation of synaptic formation and function through a synapsin-dependent mechanism.

A well-characterized role of MAPK/Erk in neurons is the regulation of gene expression upon nuclear translocation (Martin et al., 1997). However, growing experimental evidence suggests that this pathway also exerts other fast actions mediated by phosphorylation of synaptic protein substrates (Sweatt, 2004; Boggio et al., 2007). Bioinformatic analysis of *Helix*, *Aplysia*, and other gastropod synapsin proteins that display a quite well-conserved primary sequence, revealed the presence of two putative MAPK/Erk phosphorylation sites in the B-domain, consistent with the localization of mammalian synapsin phosphorylation sites 4 and 5. The same sites were predicted to be MAPK/Erk consensus sites in *Aplysia* synapsin (Angers et al., 2002). Interestingly, in an alignment of four molluscan synapsin proteins, the highest degree of amino acid identity in B-domain is observed around these residues,

suggesting the high functional importance of this region. Moreover, the two molluscan MAPK/Erk sites align quite well with mammalian sites, despite the low degree of sequence conservation between vertebrate and invertebrate synapsin proteins in this region. An in vitro phosphorylation assay validated this prediction, confirming that molluscan synapsin is a MAPK/Erk substrate and this phosphorylation is strongly reduced by Ser36 to Ala and Ser42 to Ala mutations. In addition to the highly conserved PKA-CaMKI/IV phosphorylation site in the A-domain described in our previous study (Fiumara et al., 2004), the present findings show that molluscan synapsin also possesses MAPK/Erk functional phosphorylation sites.

## The MAPK/Erk-synapsin pathway as a mediator of functional synapse formation

MAPK/Erk is one of the major intracellular effectors of neurotrophic factors and has been implicated in both synaptogenesis and plasticity (Martinez et al., 2008; Vicario-Abejón et al., 1998; Vicario-Abejón et al., 2002; Collin et al., 2001; Tyler and Pozzo-Miller, 2001; Alonso et al., 2004). We have previously found that ganglia-derived trophic factors have an essential role in formation of excitatory synapses between *Helix* neurons through a *trk*-dependent mechanism (Fiumara et al., 2005), although the existence of canonical neurotrophin-*trk* signalling in invertebrates is debated (McKay et al., 1999; Chao, 2000; Jaaro et al., 2001). However, the recent identification of neurotrophin and *trk* receptor orthologues in *Aplysia* (Kassabov et al., 2007), indicates a phylogenetic conservation of neurotrophin signalling from gastropods to vertebrates. Here, we found that MAPK/Erk inhibition affects neurite outgrowth, as well as the formation of functional connections induced by ganglia-derived trophic factors (Fiumara et al., 2005), suggesting that this kinase is a mediator of neurotrophic factor actions in *Helix* nervous system. These findings indicate a phylogenetically conserved role, from molluscs to mammals, of the neurotrophin-MAPK/Erk pathway in regulating neuronal growth and synaptic connectivity.

Pharmacological inhibition of MAPK/Erk with U0126 caused a reduction in the occurrence of functional chemical synapses. Although in principle this effect might also be partially attributed to aspecific U0126 actions on synaptic transmission per se and/or on neuritic growth – rather than on synaptogenesis – several lines of evidence point to a direct involvement of the MAPK/Erk-synapsin pathway in the formation of functional B2-B2 connections. In fact, we did not observe changes in basal transmission after 30 minutes of U0126 application on preformed B2-B2 synapses, despite the almost complete impairment of PTP that was rapidly and completely reverted after inhibitor washout. This indicates that U0126 does not affect basal synaptic transmission in such a manner to prevent the detection of already established synaptic connections. This conclusion is consistent with the results of experiments performed on *Aplysia* synapses under similar conditions (Khoutorsky and Spira, 2009). Moreover, we found that washout of U0126 did not lead to a fast recovery of synaptic strength in B2-B2 neurons chronically treated with inhibitor for 24 hours starting at the time of cell-cell pairing. In this case, once the inhibitor was washed out, we observed a very slow increase in synaptic strength that reached control levels over a time span of 2 days after washout of the inhibitor, with kinetics similar to those of the control group. This slow recovery indicates the formation of new synaptic contacts after washout rather than a re-establishment of synaptic transmission after a transient inhibition. In addition, the measurement of other

electrophysiological parameters – such as EPSP rise time – also rules out the possibility that our experimental manipulations would disrupt the function of neurotransmitter release machinery to the extent of preventing the electrophysiological detection of functional synaptic contacts.

We found that the presence of the MEK inhibitor U0126 strongly reduces the neurite growth of B2 neurons. This might indicate that MAPK/Erk inhibition could affect synapse formation both through this growth impairment (leading to a reduction of the cell-cell surface contact) and through a direct effect on the assembly of synaptic structures. However, we also demonstrated that presynaptic overexpression of MAPK/Erk phosphorylation mutants of synapsin regulates the occurrence and the strength of synaptic connections through a dominant-negative effect over endogenous synapsin without affecting neurite outgrowth. This indicates that MAPK-dependent synapsin phosphorylation regulates the occurrence of chemical synapses through a growth-independent mechanism. The fact that both non-phosphorylatable and pseudo-phosphorylated synapsin mutants had similar effects suggests that cycles of MAPK/Erk phosphorylation are required for proper activity of synapsin during synapse formation. Repeated episodes of phosphorylation and dephosphorylation might regulate cytoskeletal assembly and vesicle clustering at synaptic terminals, consistent with the established role of MAPK/Erk phosphorylation in modulating the synapsin affinity for actin (Jovanovic et al., 1996).

#### The MAPK/Erk-synapsin pathway as a mediator of short-term homosynaptic plasticity

MAPK/Erk has a fundamental role in many forms of long-term synaptic plasticity in vertebrates (Kawasaki et al., 1999; Roberson et al., 1999; Huang et al., 2000; Zhang et al., 2003) and invertebrates (Bailey et al., 1997; Martin et al., 1997; Sharma et al., 2003), by regulating gene expression upon nuclear translocation. In addition, this kinase has also been implicated in the short-term regulation of neurotransmitter release and synaptic function through the phosphorylation of cytoplasmic substrates. For instance, MAPK/Erk activation in presynaptic terminals regulates synaptic vesicle dynamics upon AMPA receptor activation (Schenk et al., 2005) or BDNF-induced neurotransmitter release from mammalian synaptosomes (Jovanovic et al., 2000). We previously found that target-dependent modulation of neurotransmitter release from *Helix* presynaptic terminals crucially depends on MAPK/Erk activation (Ghirardi et al., 2004). Although MAPK/Erk activity does not appear to be required for short-term heterosynaptic facilitation induced by serotonin in *Aplysia* sensorimotor synapses (Martin et al., 1997; Purcell et al., 2003; Phares and Byrne, 2005), other studies in invertebrates show that modulation of short- and long-term synaptic plasticity paradigms is mediated by MAPK/Erk (Tancredi et al., 2000; Chin et al., 2002; Roberto et al., 2003; Giachello et al., 2008; Khoutorsky and Spira, 2009). An involvement of MAPK/Erk in short-term plasticity is also supported by studies in transgenic mice that express a constitutively active form of H-Ras, which exhibit an enhancement of paired-pulse facilitation and long-term potentiation that is dependent on MAPK/Erk activation (Kushner et al., 2005). Our findings are consistent with this view, showing directly that PTP, a specific form of short-term homosynaptic enhancement, crucially depends on MAPK/Erk activation, which might occur upon intracellular calcium build-up during the tetanus (Impey et al., 1999; Agell et al., 2002) or via cross-talk with other calcium-dependent pathways (Soderling, 1999; Ferguson and Storm, 2004; Shaul and Seger, 2007; Gerits et al., 2008). Another possibility

is that the high-frequency presynaptic activity triggers pre- and/or post-synaptic release of neurotrophins, which, after binding to pre- and/or post-synaptic *trk* receptors, ultimately leads to MAPK/Erk activation (Lu et al., 2002).

PTP is a relatively long-lasting form of short-term homosynaptic plasticity that is thought to rely on several concurring mechanisms modulating several steps of synaptic vesicle cycle (Zucker and Regehr, 2002; Habets and Borst, 2005; Felmy and von Gersdorff, 2006). The supply of synaptic vesicles from a reserve to a readily releasable pool is one of the key steps that sustain the enhanced neurotransmitter release during PTP (Kuromi and Kidokoro, 2003; Kidokoro et al., 2004). Synapsin proteins are strongly implicated in maintenance of presynaptic vesicular pools and in the regulation of vesicle mobility among them during short-term plasticity (Humeau et al., 2001; Cousin et al., 2003; Giovedi et al., 2004). Our findings suggest that MAPK/Erk-dependent synapsin phosphorylation is a key regulator of this process. This conclusion is supported primarily by the fact that presynaptic overexpression of non-phosphorylatable mutant impairs PTP, indicating that MAPK/Erk-dependent synapsin phosphorylation has a permissive role for mobilization of synaptic vesicles (Prekeris and Terrian, 1997). Accordingly, we observed a higher degree of clustering of overexpressed GFP-tagged synapsin mutant compared with the wild-type protein, suggesting a stronger association with vesicles, similarly to what we reported for the PKA-CaMKI/IV site mutant (Fiumara et al., 2007). Both observations are consistent with previous morphological studies showing that serotonin-induced dispersion of synapsin clusters in *Aplysia* neurons depends on both PKA and MAPK/Erk activity (Angers et al., 2002), and that PKA and MAPK/Erk phosphorylation regulates the mobility of synapsin as well as the trafficking of synaptic vesicles in nerve terminals upon stimulation (Chi et al., 2003). The lack of any obvious effects of overexpression of synapsin mutants on EPSP kinetics suggests that this mechanism does not interfere with the function of this protein at post-docking stages of synaptic vesicle cycle (Hilfiker et al., 1998; Hilfiker et al., 2005; Humeau et al., 2001), as also shown for the PKA-CaMKI/IV site in the A-domain (Fiumara et al., 2007).

The present findings, together with our previous study (Fiumara et al., 2007), indicate that PTP is associated with the phosphorylation of several synapsin sites by distinct kinases that are concurrently activated by tetanic stimulation. The functional meaning of these multiple regulatory events during plasticity might be to fine-tune trafficking and availability of synaptic vesicles for release to the type, intensity, and duration of presynaptic activation. Various kinase pathways can be differentially recruited and with specific time courses, depending on the frequency, the number of presynaptic spikes and the presence of heterosynaptic modulators. Interestingly, MAPK/Erk-dependent synapsin mobilization upon presynaptic stimulation is frequency dependent (Chi et al., 2003). Therefore, the type and sequence of synapsin phosphorylation reactions might specify a change in the functional properties of these proteins that is appropriate for each type of presynaptic activity pattern.

#### Materials and Methods

##### Materials

All reagents and materials were of analytical grade and purchased from Sigma (Milan, Italy).

##### Cell culture and electrophysiology

Juvenile *Helix aspersa* and *Helix pomatia* land snails were purchased from local breeders. Cell cultures were performed as previously described (Ghirardi et al., 1996). Briefly, buccal B2 neurons were isolated and grown under non-adhesive conditions. Floating somata were then paired to form B2-B2 soma-to-soma synapses (Fiumara

et al., 2005). In growth experiments, single B2 somata were plated on poly-L-lysine pretreated dishes and analyzed at 24 and 48 hours as previously described (Milanese et al., 2008). Conventional intracellular recordings of synaptic activity were performed as previously reported (Fiumara et al., 2005; Fiumara et al., 2007).

#### Pharmacological treatment

U0126 (Sigma, Milan, Italy), a selective inhibitor of MEK, was used to block the MAPK/Erk cascade pathway. In synaptogenesis experiments, prior to pairing, isolated B2 neurons were incubated for 2 hours either in 50  $\mu$ M U0126 or in 0.25% DMSO. Cells were subsequently paired in ganglia-conditioned medium (Fiumara et al., 2005) in the presence of either U0126 or DMSO. Electrophysiological recordings were performed at 24, 48 and 72 hours after cell-cell pairing.

#### Bioinformatics

Prediction of kinase-specific phosphorylation sites in the amino acid sequence of *Helix* synapsin (GenBank AY533823) was performed using the Group-based Phosphorylation Scoring method, GPS 2.1 (<http://gps.biocuckoo.org/online.php>) (Xue et al., 2008). A high threshold setting and a cut-off value of 3.757 were chosen to minimize the false-positive rate. Phylogenetic analysis of putative phosphorylation sites in the synapsin B-domain was carried out with the multiple alignment software AlignX, part of Vector NTI Advance 9 suite (Invitrogen, Carlsbad, CA), based on the ClustalW algorithm.

#### Site-directed mutagenesis of GFP-tagged *Helix* synapsin

*Helix* synapsin (Syn<sub>WT</sub>) sequence subcloned into the expression vector pCS2-mt-GFP (kindly provided by Mike Klymkowsky, University of Colorado, Boulder, CO) was used as a template for site-directed mutagenesis by QuikChange Site-Directed Mutagenesis Kit (Stratagene, Milan, Italy) following the manufacturer's procedure with two antiparallel primers carrying codons for the required substitutions. To remove consensus phosphorylation sites, Ser36 and Ser42 were replaced with alanine residues generating a non-phosphorylatable mutant (Syn<sub>Ala36,Ala42</sub>-GFP). Primers used were: forward, TAAGAAGGGGCCGCTCCAGTGCCTAACGCACCATCCAAGA; reverse, TCTTGGATGGTGCCTAGGGGCACTGGGAGCCGGCCCTTCTTA. Moreover, substitutions of negatively charged aspartate and glutamate residues at positions 36 and 42 were exploited to mimic the effect of a constitutive phosphorylation (Syn<sub>Asp36,Glu42</sub>-GFP). Primers used were: forward, TAAGAAGGGGCCGCTCCAGTGCCTAACGCACCATCCAAGA; reverse, TCTTGGATGGTGCCTAGGGGCACTGGGAGCCGGCCCTTCTTA. The presence of mutations was verified by sequence analysis.

#### Expression and production of synapsin recombinant proteins

The wild-type and non-phosphorylatable synapsin mutant were subcloned into pGEX-4T vector and transformed in BL21 cells. Large-scale cultures of Luria broth containing ampicillin (100  $\mu$ g/ml) were inoculated with small overnight cultures, grown at 37°C to log phase and induced with isopropyl  $\beta$ -D-thiogalactopyranoside (100  $\mu$ M) for 3–5 hours. GST-synapsin fusion proteins were extracted from bacterial lysates, purified to homogeneity by affinity chromatography on glutathione-Sepharose and dialyzed against 25 mM Tris-HCl, 50 mM NaCl, pH 7.4. The purified synapsin-GST fusion proteins were then subjected to cleavage with thrombin (1 U/mg fusion protein) for 3 hours at 22°C and repurified by a further passage through the glutathione-Sepharose column following the manufacturer's procedure.

#### Phosphorylation of wild-type and MAPK/Erk non-phosphorylatable synapsin mutant

For the analysis of synapsin phosphorylation by activated MAPK/Erk2, equal amounts of either wild-type and non-phosphorylatable mutant (Ser36Ala and Ser42Ala) were incubated in a buffer containing 20 mM Tris-HCl, pH 7.4, 20 mM MgCl<sub>2</sub>, 2 mM MnCl<sub>2</sub>, 0.5 mM dithiothreitol, 0.5 mM Na<sub>2</sub>VO<sub>4</sub>, 1.5  $\mu$ g/ml purified active MAPK/Erk2. The incubation were started by the addition of 50  $\mu$ M [ $\gamma$ -<sup>32</sup>P]ATP (2  $\mu$ Ci/sample), carried out at 30°C for 30 minutes, and stopped by the addition of concentrated sample buffer (Laemmli, 1970) and boiled for 2 minutes. Phosphorylated samples were resolved by SDS-PAGE on 10% polyacrylamide gels. Gels were then fixed, stained with Coomassie brilliant blue and exposed to Kodak X-Omat films. Parallel samples were assessed by immunoblotting with an antibody (G143) recognizing the highly conserved domain A of synapsin.

#### In vitro transcription and intracellular injection of mRNAs

In vitro transcription was performed according to the protocol of Sahly and colleagues (Sahly et al., 2003) with minor modifications, using the RiboMAX Large Scale RNA Production System SP6 (Promega, Milan, Italy). The quality of the synthesized mRNA was checked by agarose gel electrophoresis and spectrophotometrically quantified. Generally, the final concentration of purified mRNAs ranged from 1 to 3  $\mu$ g/ $\mu$ l and it was intracellularly injected in B2 somata as previously reported (Fiumara et al., 2007).

#### Cell imaging and fluorescence analysis

Cell images were captured with a Monochrome Evolution QE camera (Mediacybernetics, Bethesda, MD) using an Eclipse TE200 inverted microscope (Nikon Instruments, Tokyo, Japan) equipped with phase contrast and epifluorescence

optics. Confocal images were acquired with a Fluoview 300 confocal laser-scanning microscope (Olympus, Hamburg, Germany). Quantitative analysis of fluorescence distribution was performed on selected areas (50 $\times$ 50  $\mu$ m, with an almost complete coverage of the post-synaptic cell) digitally converted into matrices of pixel values and plotted using the Bitmap Analysis and the Surface Plot tool of Image Pro Plus version 6.3 (Mediacybernetics, Bethesda, MD). To analyze the dispersion of GFP-tagged proteins, the Line Profile tool of Image Pro Plus was applied to areas of 20 $\times$ 20 pixels (25  $\mu$ m<sup>2</sup>) encompassing a single cluster, obtaining a plot of the pixel intensity values that was fitted by a bell-shaped curve. All figures were assembled using Photoshop CS2 version 9.0 software (Adobe Systems, San Jose, CA).

#### Statistical analysis

Data were expressed as means  $\pm$  s.e.m. Statistical analysis was performed using GraphPad Prism version 5 (GraphPad Software, San Diego, CA). Statistical significance between group means was assessed using Student's *t*-test or ANOVA analysis (one or two-way and with or without repeated measures where appropriate) followed by the Bonferroni post-hoc test. Contingency tests, such as Pearson's chi square test or Fisher's exact test, were performed as indicated. Significance levels were set at *P*<0.05.

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Supplementary material available online at

<http://jcs.biologists.org/cgi/content/full/123/6/881/DC1>

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