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Phosphorylation of synapsin domain A is required for post-tetanic potentiation

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Summary

Post-tetanic potentiation (PTP) is a form of homosynaptic plasticity important for information processing and short-term memory in the nervous system. The synapsins, a family of synaptic vesicle (SV)-associated phosphoproteins, have been implicated in PTP. Although several synapsin functions are known to be regulated by phosphorylation by multiple protein kinases, the role of individual phosphorylation sites in synaptic plasticity is poorly understood. All the synapsins share a phosphorylation site in the N-terminal domain A (site 1) that regulates neurite elongation and SV mobilization. Here, we have examined the role of phosphorylation of synapsin domain A in PTP and other forms of short-term synaptic enhancement (STE) at synapses between cultured *Helix pomatia* neurons. To this aim, we cloned *H. pomatia* synapsin (helSyn) and overexpressed GFP-tagged wild-type helSyn or site-1-mutant helSyn mutated in the presynaptic compartment of C1-B2 synapses. We found that PTP at these synapses depends both on Ca^{2+}/calmodulin-dependent and cAMP-dependent protein kinases, and that overexpression of the non-phosphorylatable helSyn mutant, but not wild-type helSyn, specifically impairs PTP, while not altering facilitation and augmentation. Our findings show that phosphorylation of site 1 has a prominent role in the expression of PTP, thus defining a novel role for phosphorylation of synapsin domain A in short-term homosynaptic plasticity.

Keywords

Helix pomatia synapsin; Short-term homosynaptic plasticity; Synaptic vesicle proteins; Protein phosphorylation; mRNA microinjection; Cell culture

Introduction

Post-tetanic potentiation (PTP) is a widespread form of homosynaptic plasticity that, together with other forms of short-term synaptic enhancement (STE), is considered as one of

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the neural substrates of short-term memory and synaptic computation (Fisher et al., 1997; Zucker and Regehr, 2002; Abbott and Regehr, 2004). Even though PTP – and other forms of STE – are generally thought to depend on the activity-dependent increase of Ca^{2+} concentration in presynaptic terminals, the downstream molecular effectors of Ca^{2+} elevation in these plastic phenomena have been only partially defined (Zucker and Regehr, 2002; Brager et al., 2003).

Synapsins are a phylogenetically conserved family of synaptic vesicle (SV)-associated proteins that, among other functions, have been implicated in the regulation of STE, and other types of short-term plasticity in various vertebrate and invertebrate systems (Rosahl et al., 1993; Rosahl et al., 1995; Humeau et al., 2001; Sun et al., 2006; Kielland et al., 2006). The biological activity of these proteins is largely regulated by phosphorylation at distinct sites by multiple protein kinases, including protein kinase A (PKA) and Ca^{2+}/calmodulin-dependent kinases (CaMK) I, II and IV (Hilfiker et al., 1999). All known synapsin isoforms contain a short N-terminal domain (domain A) with a highly conserved single phosphorylation site (site 1) for both PKA, and CaMKI and CaMKIV. The phosphorylation of this site has been shown to reversibly regulate the interactions of these proteins with SVs (Hosaka et al., 1999), and to regulate neurite outgrowth (Kao et al., 2002) and SV dynamics in developing vertebrate neurons (Bonanomi et al., 2005), as well as synapsin distribution (Angers et al., 2002) and neurotransmitter release in invertebrate neurons (Fiumara et al., 2004). However, the possible role of this phosphorylation site in STE has not yet been investigated.

To study the role of phosphorylation of synapsin domain A in PTP and other forms of STE, we took advantage of in-vitro-reconstructed giant synapses of identified *Helix pomatia* neurons, which are capable of expressing multiple forms of STE that have been characterized previously (Fiumara et al., 2005). To this aim, we have cloned the synapsin orthologue in *H. pomatia* (helSyn) and compared the effect of the selective presynaptic overexpression of either wild-type or site-1-mutant helSyn on the short-term plasticity of synapses between *H. pomatia* C1 and B2 neurons. We have found that PTP at these synapses depends on the activation of CaMKs and PKA, and that the presynaptic overexpression of a non-phosphorylatable synapsin mutant acts in a dominant-negative manner by severely impairing PTP, but not facilitation or augmentation. Although both the wild-type and mutant helSyn forms were targeted to the synaptic compartments, the mutant form was densely packed at both synaptic and extrasynaptic sites. Together, these observations indicate that synapsin site-1-phosphorylation-dependent changes in SV trafficking and neurotransmitter release are involved in the expression of PTP.

Results

Cloning of *H. pomatia* synapsin and generation of a domain A phosphorylation site mutant

As a first step in our investigation, we cloned the *H. pomatia* synapsin orthologue by reverse transcriptase (RT)- and rapid amplification of cDNA ends (RACE)-PCR, using primers corresponding to conserved sequences of synapsins in related species. We identified a 1.9-kb sequence (GenBank accession number AY533823) showing close homology with *A. californica* synapsin (apSyn) (Angers et al., 2002) (Fig. 1A). This putative helSyn showed a general domain arrangement similar to that of other known vertebrate and invertebrate synapsins, in that helSyn contains the A, C and E domains (Fig. 1A). In particular, the N-terminal A domain shows a high degree of sequence similarity with apSyn (76.7%; Fig. 1B) and vertebrate synapsins (22.58% similarity to rat synapsin I).

Potential consensus sequences for several kinases are present along the helSyn sequence, including the highly conserved PKA and CaMKI/IV phosphorylation site in the A domain

(site 1, corresponding to Ser9, Fig. 1C). We have previously shown that this is a major phosphorylation site in apSyn and that its phosphorylation is involved in the modulation of neurotransmitter release (Fiumara et al., 2004;Bonanomi et al., 2005;Menegon et al., 2006). To study the role of this phosphorylation site in short-term homosynaptic plasticity, we generated a helSyn mutant in which Ser9 was replaced with alanine (helSynALA9, Fig. 1C). This mutation has been previously shown to effectively prevent domain A phosphorylation in both vertebrate (Hosaka et al., 1999) and invertebrate (Fiumara et al., 2004) synapsins.

Post-tetanic potentiation at C1-B2 synapses involves the activation of CaMKs and PKA

In vitro-reconstructed C1-B2 synapses show different forms of STE, including PTP (Fiumara et al., 2005). PTP has been shown to depend on different kinase pathways in different types of synapses (e.g. Wang and Maler, 1998; Alle et al., 2001; Brager et al., 2003). As a preliminary step in studying the role of synapsin site 1 phosphorylation in PTP, we tested whether the protein kinases that can phosphorylate this site (PKA and CaMKI/IV) have some role in PTP at C1-B2 synapses.

To this aim, we tested the effect inhibitors of these protein kinases have on PTP at C1-B2 synapses (Fig. 2A). PTP was tested in the same C1-B2 synapses before (time 0) and 30 minutes after the application of inhibitors. This testing procedure itself – without kinase inhibitors – did not cause EPSP or PTP rundown in control synapses (Fig. 2B). In controls, the peak amplitude of PTP measured at 30 minutes (372.66±57.64%) was 106.86±22.59% of the PTP peak amplitude measured at time 0 (355.16±68.63%) (Fig. 2F). Similarly, the pre-tetanic EPSP amplitude was not different at time 0 and 30 minutes after (2.77±0.64 mV vs 2.65±0.59 mV, respectively, $P>0.05$, paired Student's *t*-test; Fig. 2G).

We observed that KN-93, an inhibitor of CaMKs (Corcoran and Means, 2001), had a dramatic effect on PTP (Fig. 2C). The peak amplitude of PTP measured at 30 minutes (144.57±7.25%) was 18.25±2.97% of the PTP peak amplitude measured at time 0 (344.14±34.98%) (Fig. 2F). Conversely, KN-93 did not seem to alter the basal synaptic transmission because the pre-tetanic EPSP amplitude was not different before and 30 minutes after the application of the inhibitor (3.64±0.63 mV vs 3.57±0.79 mV, respectively, $P>0.05$, paired Student's *t*-test; Fig. 2G). The presynaptic nature of this effect was confirmed by the observation that the effect of KN-93 on PTP was mimicked by the presynaptic injection of the MLCK peptide, an inhibitor of CaMKs, but not by the injection of the inactive MLCK control peptide (Fig. 2D). The peak amplitude of PTP measured in a group of synapses in which the C1 neuron was injected with the MLCK peptide (148.14±14.47%) was 17.18±5.65% of the PTP peak amplitude measured in control synapses injected with the control peptide (356.85±57.39%) (Fig. 2F). The MLCK peptide injection did not significantly alter the basal synaptic transmission as the pre-tetanic EPSP amplitude was not different in the two experimental groups (5.92±1.37 mV vs 3.92±0.70 mV, respectively, $P>0.05$, unpaired Student's *t*-test; Fig. 2G).

Rp-cAMPS, an inhibitor of PKA, had a less pronounced but still significant effect on PTP (Fig. 2E). The peak amplitude of PTP measured at 30 minutes (212.66±20.13%) was 45.24±8.08% of the PTP peak amplitude measured at time 0 (349±30.46%) (Fig. 2F). As for KN-93, Rp-cAMPS did not seem to alter the basal synaptic transmission because the pre-tetanic EPSP amplitude was not different before and 30 minutes after the application of the inhibitor (3.35±0.66 mV vs 3.28±0.55 mV, respectively, $P>0.05$, paired Student's *t*-test; Fig. 2G). These results show that both protein kinases that are known to phosphorylate synapsin domain A are involved in mediating PTP at C1-B2 synapses.

Selective presynaptic overexpression of GFP-tagged wild-type and mutant synapsin

To directly assess the role of synapsin site 1 phosphorylation in synaptic plasticity, we overexpressed GFP-tagged wild-type or mutant helSyn in the presynaptic compartment of in vitro reconstructed soma-to-soma giant synapses between *H. pomatia* C1 and B2 neurons (Fig. 3A) (Fiumara et al., 2005). To this aim, C1 neurons were intracellularly injected with in-vitro-synthesized mRNA encoding either helSyn-GFP or helSynALA9-GFP. As a control, some cells were injected with mRNA encoding for GFP alone, whereas some other control cells were not injected at all.

In comparing the subcellular distribution pattern of the overexpressed proteins, we observed that GFP alone was diffusely and homogeneously distributed throughout the whole cytoplasm of the overexpressing neurons, without any apparent concentration in specific compartments (Fig. 3A-D). By contrast, helSyn-GFP fluorescence was more concentrated in punctate structures localized in the contact area between the pre- and post-synaptic cells and along presynaptic neurites projecting onto the postsynaptic cell (Fig. 3E,F). These areas of the soma-to-soma pairs contained the majority of the SV clusters and synaptic structures, as observed using electron microscopy (EM; Fig. 3I-M) (see also Fiumara et al., 2005). The non-phosphorylatable mutant helSynALA9-GFP was even more strongly concentrated in multiple spots in the same areas (Fig. 3G,H). Interestingly, these puncta were also diffusely present under the surface of the non-synaptic somatic membrane, a compartment that has been previously shown by EM observations to contain synaptic vesicles and to be capable of non-synaptic neurotransmitter release (Fiumara et al., 2004).

Post-tetanic potentiation at C1-B2 synapses requires phosphorylation of synapsin domain A

To determine the relevance of phosphorylation of synapsin domain A for PTP, we compared the effect of presynaptic tetanic stimulation in control C1-B2 synapses with that in C1-B2 synapses overexpressing in the presynaptic compartment either GFP alone, helSyn-GFP or the non-phosphorylatable mutant helSynALA9-GFP. PTP induction and decay were recorded in these four experimental groups, as shown in Fig. 4A,B. A statistical comparison of these four groups using two-way ANOVA for repeated measures revealed a significant effect of the treatment, i.e. the overexpression of the different proteins ($F_{(3,54)}$=3.26; P<0.03) and a significant treatment × time interaction ($F_{(15,270)}$=2.71; P<0.001) for 2 minutes after tetanus (Fig. 4B).

In analyzing specific effects, we found that GFP overexpression by itself did not interfere with PTP expression. The peak amplitude of PTP in synapses overexpressing GFP alone and in control synapses were not statistically different (Fig. 4B,C; PTP amplitude at 30 seconds was 249.33±22.63%, n=13, in the control group and 265.46±33.01%, n=6, in the GFP group, P=0.47, Newman-Keuls post hoc test). PTP decay was also almost parallel in the two groups. Similarly, the overexpression of helSyn-GFP did not seem to interfere with PTP induction and decay. The peak PTP amplitude at 30 seconds was 249.33±22.63% (n=13) in the control group and 234.45±36.97% in the helSyn-GFP group (n=17; P=0.50, Newman-Keuls test; Fig. 4B,C).

Conversely, helSynALA9 overexpression had a remarkable effect of PTP expression at C1-B2 synapses. In the helSynALA9-GFP group, we observed that PTP was almost abolished, as the amplitude of EPSPs measured before and 30 seconds after tetanus in the same helSynALA9-GFP group were not significantly different (P=0.90; Newman-Keuls test) and the peak post-tetanic increase in the amplitude of the EPSP at 30 seconds after tetanus (+36.72±14% n=22; Fig. 4B,C) was strongly reduced to less than 30% of that of the control

group (+149.33±22.63%, *n*=13, *P*<0.001, Newman-Keuls test) and the helSyn-GFP group (+134.45±36.97%, *n*=17; *P*<0.001, Newman-Keuls test).

Since PTP amplitude at C1-B2 synapses is strongly related to the basal strength of the synaptic connections (Fiumara et al., 2005), we compared the pre-tetanic EPSP amplitude between the different experimental groups in order to rule out the possibility that the reduction in PTP amplitude observed in the helSynALA9-GFP group might be due to an increase in basal strength of these synapses with respect to controls. As a matter of fact, we found that the pre-tetanic EPSP amplitude was not different between the experimental groups (3.14±0.71 mV in the control group, *n*=13, vs 2.63±0.53 mV in the GFP group, *n*=6, vs 2.67±0.55 mV in the helSyn-GFP group, *n*=17, vs 2.51±0.49 mV in the helSynALA9-GFP group, *n*=22; $F_{(3,54)}$=0.21, *P*=0.89, one-way ANOVA; Fig. 4D). The overexpression of helSyn and helSynALA9 did not seem to interfere with EPSP kinetics. For instance, the EPSP rise time in the different groups was not significantly different (166.6±24.08 ms in the control group, *n*=13, vs 160.90±41.89 ms in the GFP group, *n*=6, vs 142.50±17.07 mV in the helSyn-GFP group, *n*=17, vs 136.2±7.88 mV in the helSynALA9-GFP group, *n*=22; $F_{(3,54)}$=0.65, *P*=0.58, one-way ANOVA; Fig. 4E). These results show that overexpression of the non-phosphorylatable helSynALA9 mutant in the presynaptic compartment has a dominant-negative effect on the expression of PTP at these synapses. This strongly indicates that the phosphorylation of synapsin domain A is required for C1-B2 synapses to display PTP.

Phosphorylation of synapsin domain A is not required for frequency facilitation and augmentation at C1-B2 synapses

At the same time, we also investigated whether helSynALA9 overexpression also interferes with other more short-lived forms of STE expressed by C1-B2 synapses, namely facilitation and augmentation (Fiumara et al., 2005). We found that both forms of plasticity were not significantly altered in the helSynALA9-GFP group with respect to the helSyn-GFP group or to the control group (Fig. 5). To analyze the effect of synapsin overexpression within the time domain of facilitation, given the temporal kinetics of the EPSPs at *H. pomatia* C1-B2 synapses (Cottrell and Macon, 1974; Fiumara et al., 2005), we used a previously defined protocol that allows to resolve single EPSPs evoked by trains of action potentials (Fig. 5A) (Fiumara et al., 2005). The facilitation index was 268.93±51.52% (*n*=9) in the control group vs 315.02±83.08% (*n*=12) in the helSyn-GFP group vs 316.68±52.53% (*n*=17) in the helSynALA9-GFP group, $F_{(2,32)}$=0.24; *P*=0.78, one-way ANOVA; Fig. 5B). As for the PTP experiments, the basal EPSP amplitude was not different between the groups (1.62±0.23 mV in the control group vs 2.61±0.82 mV in the helSyn-GFP group vs 1.59±0.27 mV in the helSynALA9-GFP group, $F_{(2,32)}$=0.67; *P*=0.51, one-way ANOVA).

The time course of augmentation was also very similar irrespective of the treatment ($F_{(2,34)}$=0.38, *P*=0.68), as well as its peak value measured at 2 seconds after short trains of five action potentials (262.74±57.37%, *n*=7, in the control group vs 256.30±43.12%, *n*=13, in the helSyn-GFP group vs 291.59±42.36%, *n*=17, in the helSynALA9-GFP group (Fig. 5C,D). The basal EPSP amplitude was also not different between the groups (3.11±1.00 mV, *n*=7, in the control group vs 1.95±0.47 mV, *n*=13, in the helSyn-GFP group vs 1.40±0.19 mV, *n*=17, in the helSynALA9-GFP group, $F_{(2,34)}$=2.81, *P*=0.74, one-way ANOVA), showing that the observed results were not biased by EPSP amplitude-related differences in the degree of synaptic facilitation and augmentation (Fiumara et al., 2005).

Discussion

Synapsins account for more than 6% of SV proteins (Huttner et al., 1983) and intervene in diverse aspects of synaptic development and function (Hilfiker et al., 1999; Ferreira and

Rapoport, 2002). These proteins are substrates for several protein kinases phosphorylating them on distinct sites (De Camilli et al., 1990; Hilfiker et al., 1999). In this study, we have assessed the role of the phosporylation of synapsin domain A in PTP and other forms of STE at invertebrate synapses. We showed that the phosphorylation of synapsin site 1 is required for the expression of potentiation after presynaptic tetanic stimulation, whereas it is not necessary for facilitation and augmentation.

Synapsins have been identified in several species, ranging from simple invertebrates to humans, showing a relatively high degree of phylogenetical conservation (Kao et al., 1999). We have cloned the *H. pomatia* (Gastropoda, Pulmonata) synapsin orthologue, that shows a close homology with the *A. californica* (Gastropoda, Opistobranchia) synapsin isoform 11.1 (Angers et al., 2002). As the *A. californica* orthologue, helSyn contains the N-terminal domain A, the domain C, and the C-terminal domain E. Both the A and C domains show a high degree of homology with vertebrate synapsins. Interestingly, these molluscan synapsins are structurally similar to mammalian synapsin II in that they lack the D domain, which is instead present in synapsin I (Greengard et al., 1993). The D domain of synapsin I contains two phosphorylation sites for CaMKII (sites 2 and 3). These sites have been previously proposed as CaMKII targets for phosphorylation during PTP (Greengard et al., 1993; Zucker and Regehr, 2002). However, PTP is not altered in synapsin I knockout mice, whereas it is impaired in synapsin II and synapsin-I–synapsin-II knockouts (Rosahl et al., 1995; Kielland et al., 2006), and CaMKII activity was found not to be required for PTP in several synapses (Malinow et al., 1988; Kamiya and Zucker, 1994). Moreover, synapsin II has a relevant role in regulating SV mobilization in synaptic terminals (Chi et al., 2003). Taken together, these observations strongly suggest that the various synapsins isoforms, notwithstanding a certain degree of redundancy, have specific roles in distinct paradigms of short-term synaptic plasticity. In particular, synapsin II, rather than synapsin I, seems to be involved predominantly in PTP regulation. In contrast to vertebrates, in which synapsins constitute a multigene family of proteins (Kao et al., 1999), invertebrate synapsins are encoded by a single gene. Moreover, we have previously shown that *A. californica* synapsin, which – similarly to mammalian synapsin II – lacks the D domain, is a poor substrate for CaMKII, whereas it is an excellent substrate for phosphorylation at the A domain site (Fiumara et al., 2004). Observation that the domain A might be involved in regulating synapsin-SV interactions and neurotransmitter release in a phosphorylation-dependent manner (Hosaka et al., 1999; Fiumara et al., 2004; Hilfiker et al., 2005), led us to test the hypothesis that the phosphorylation of domain A rather than domain D is involved in PTP.

We have found that PTP in the C1-B2 synapse is dependent on CaMKs, because the CaMK inhibitor KN-93 almost abolished PTP. This effect was presynaptic, because it was mimicked by the presynaptic injection of calmodulin-inhibiting peptide. Similar observations have been reported for *A. californica* sensorimotor synapse (Jin and Hawkins, 2003). In addition, we also found that PKA activation is required for PTP at C1-B2 synapses, even though in a less dramatic manner, because PTP was also reduced by the selective PKA inibitor Rp-cAMPS. PKA might be activated in parallel with CaMKs as a consequence of the Ca^{2+}/calmodulin-mediated activation of adenylyl cyclase during the tetanus (Ferguson and Storm, 2004; Menegon et al., 2006). PKA has been previously shown to be a powerful regulator of neurotransmitter release from the *H. pomatia* C1 neuron under low-release conditions (Ghirardi et al., 2004). Altogether, these results show that CaMKI/IV and PKA are potentially involved in PTP at C1-B2 synapses, providing supportive evidence for the possible role of synapsin domain A phosphorylation in PTP at these connections. However, the data do not rule out the possibility that CaM-dependent kinases other than CaMKI and CaMKIV (e.g. MLCK) (Verstreken et al., 2005) are involved in PTP at C1-B2 synapses and, that CaMKI and PKA can phosphorylate many substrates other than synapsins involved as well in PTP. In the experiments with CaMKs inhibitors there was a reduction of

about 10-20% of the EPSP that was apparent in the later phases after tetanus, as compared with pre-tetanic values. This effect was not totally unexpected, because KN-93 and the MLCK peptide inhibit several Ca^{2+}/calmodulin-dependent kinases, some of which are involved in vesicular trafficking. Therefore, their combined inhibition might possibly cause a relative post-tetanic depletion of synaptic vesicles that may explain both the PTP reduction in the earlier phase and the subsequent slight depression observed in the later phase.

The overexpression of GFP-tagged wild-type and mutant synapsin allowed us to compare the subcellular distribution of the protein as a function of its phosphorylation state. We found that both the wild-type and non-phosphorylatable mutant were more concentrated in areas in which EM shows the presence of synaptic structures, i.e. the contact area between the two cell bodies and the neurites projecting from the presynaptic onto the postsynaptic cell (Fiumara et al., 2005; Naruo et al., 2005). These findings are consistent with the previous observation (Gitler et al., 2004) that even the deletion of synapsin domain A does not substantially alter the synaptic targeting of mammalian synapsins. We also observed a more intense clustering of helSynALA9-GFP with respect to the wild-type protein. Phosphorylation of site 1 has been shown to regulate the interaction of synapsins with SVs (Hosaka et al., 1999), their distribution (Angers et al., 2002) and mobility in growth cones (Bonanomi et al., 2005) and synaptic terminals (Chi et al., 2001). The higher degree of clustering of helSynALA9 might be owing to a stronger association of this mutant with SVs and/or to a lower rate of its dispersion and reclustering cycles even under basal conditions (Hosaka et al., 1999; Chi et al., 2001; Bonanomi et al., 2005). Further investigations with immunogold-EM, using antibodies against GFP to identify the overexpressed proteins, will be necessary to directly define these phenomena at the ultrastructural level.

At the electrophysiological level, we observed that helSyn overexpression did not alter basal synaptic transmission or PTP, consistent with previous observations in crayfish synapses (Dearborn, et al., 1998), whereas helSynALA9 overexpression specifically impaired PTP by acting in a dominant-negative fashion over the endogenous synapsin. This dominant-negative effect was prevalent in the earlier phase of PTP, as compared with the later phase starting ~1 minute after the tetanus. This phenomenon might be related to a delayed SV mobilization, which would allow the synapse to sustain an increased neurotransmitter release only in later phases after the tetanus, as compared with control synapses that can readily increase transmission in the earlier post-tetanic phase owing to faster SV trafficking. It is also noticeable that an increased level of the wild-type protein did not alter the basal strength of the mature and fully functional connections of synaptically paired C1 neurons, whereas it was capable of increasing the non-synaptic neurotransmitter release capability of the same neurons cultured under low-release conditions in the presence of a non-physiological target (Fiumara et al., 2001; Fiumara et al., 2004)

helSynALA9 did not interfere with the basal synaptic transmission and other forms of STE, namely facilitation and augmentation, although we cannot rule out the possibility that a higher level of overexpression might be necessary to reveal additional effects on facilitation and/or augmentation. The specificity – under our experimental conditions – of the effect on PTP of the site 1 mutant supports the view that, even though they are all initiated by an activity-dependent build-up of Ca^{2+} in presynaptic terminals, the different forms of STE depend on rather different molecular mechanisms downstream (Zucker and Regehr, 2002). Synapsins have been shown previously to be involved in PTP in vertebrate and invertebrate synapses (Rosahl et al., 1995; Humeau et al., 2001; Kielland et al., 2006). Our findings show for the first time that the role of synapsins in PTP relies essentially on the phosphorylation of their N-terminal domain A.

A number of possible mechanisms responsible for PTP have been proposed, mainly based on the activity-dependent regulatory changes in the different steps controlling SV dynamics and neurotransmitter release (Zucker and Regehr, 2002; Kidokoro et al., 2004; Felmy and von Gersdorff, 2006). Synapsins are thought to be involved in the maintenance of the reserve pool of SVs by means of their interaction with these organelles and the cytoskeleton (Pieribone et al., 1995; Rosahl et al., 1995), and to regulate the mobilization of vesicles from the reserve pool to the releasable pool under conditions of increased presynaptic activity (Jovanovic et al., 2001; Chi et al., 2003). A post-docking role of synapsins has also been shown (Hilfiker et al., 1998; Hilfiker et al., 2005; Humeau et al., 2001; Tao-Cheng, 2006). Synapsin dissociation from SVs and its modulatory role on neurotransmitter relase have been shown to depend to a large extent on domain A phosphorylation (Hosaka et al., 1999; Angers et al., 2002; Fiumara et al., 2004; Hilfiker et al., 2005).

Together with these observations, our results strongly support the view that the activity-dependent phosphorylation of synapsin domain A by CaMKI and CaMIV, and/or PKA is an important step in the expression of PTP. This phosphorylation could be required, possibly as a permissive step (Prekeris and Terrian, 1997), for the mobilization of SVs from the reserve pool, which is likely to be necessary to sustain PTP. Interestingly, SV mobilization from the reserve pool during PTP in *Drosophila* is strongly dependent on PKA activation (Kuromi and Kidokoro, 2002; Kidokoro et al., 2003; Kidokoro et al., 2004), even though the molecular mediators of this phenomenon have not yet been identified. Our observations strongly indicate the possibility that the synapsins are one of these mediators. Phosphorylation of synapsin domain A might also modulate PTP by altering the presynaptic release probability. PTP at the calyx of Held synapse has been shown to depend on an increase in the readily releasable pool of SVs and in the presynaptic release probability (Habets and Borst, 2005). In the same synapse, the synapsins have been implicated in the modulation of the release probability, at least during synaptic depression, an effect that may be mediated by the activation of CaMKs (Sun et al., 2006). However, microinjection of domain A peptide into the squid giant synapse had no effect on vesicle pool size, synaptic depression, or transmitter release kinetics, indicating that this domain may be predominantly involved in regulating SV trafficking at pre-docking stages (Hilfiker et al., 2005).

The role of phosphorylation of the synapsin site 1 in neuronal development and function has been extensively investigated in recent years. Phosphorylation of this site has been shown to regulate neurite extension, vesicle dynamics and neurotransmitter release (Kao et al., 2001; Kao et al., 2005; Chi et al., 2001; Chi et al., 2003; Fiumara et al., 2004; Hilfiker et al., 2005; Bonanomi et al., 2005). The present study defines the role of synapsin domain A phosphorylation in short-term activity-dependent synaptic plasticity (STE). An intriguing matter for future investigations is the analysis of the potential role of this synapsin domain as a regulator of the functional and structural aspects of long-term synaptic plasticity.

Materials and Methods

Materials

All chemicals and reagents used in this study were purchased from Sigma (Milano, Italy), unless stated otherwise.

Animals

Juvenile *Helix pomatia* snails were provided by local breeders and maintained in the laboratory as previously reported (Fiumara et al., 2005).

Cloning and site-directed mutagenesis of *H. pomatia* synapsin

Highly conserved regions of the synapsin gene were identified by comparing sequences from *Aplysia californica, Loligo pealei* and *Rattus norvegicus* synapsins, and two pairs of primers (S1-S2, S3-S4) were designed: S1 forward, 5′-AAGCCAGACTTTGTGTTGATACG-3′; S2 reverse, 5′-ATTTCCTTGTGGTTCGGGTAG-3′; S3 forward, 5′-TCAGGAAACTGGAAGGCCAA-3′; S4 reverse, 5′-ACATCCAGTCCAAACAG-3′. These oligonucleotides were used in reverse transcription-polymerase chain reaction (RT-PCR) on total RNA extracted from the *H. pomatia* nervous system that gave two cDNA products, S1-S2 and S3-S4, of 111 bp and 235 bp respectively. A further RT-PCR cycle using the S1-S4 primers, generated a product of 535 bp. Rapid amplification of cDNA ends (RACE)-PCR (Generacer Kit, Invitrogen, Milan, Italy) in both 5′ and 3′ directions of the S1-S4 clone gave two overlapping sequences that were cloned for nucleotide sequencing in the PCR4-Topo vector (Invitrogen) using standard recombinant DNA techniques (Sambrook et al., 1989). The full-length cDNA of 1895 bp was then cloned in the pGEM-T vector (Promega, Milan, Italy), after a fusion reaction of the overlapping 5′- and 3′-end fragments. Protein and DNA databases were searched for sequence similarities using the BLAST algorithm at the NCBI site (www.ncbi.nlm.nih.gov/blast). This *H. pomatia* synapsin (helSyn) sequence was deposited in the GenBank database under accession number AY533823.

In order to generate mutations in the helSyn region encompassing the putative PKA/CaMKI/IV phosphorylation site (Ser9) in the domain A, the QuickChange site-directed mutagenesis kit (Stratagene, Milan, Italy) was used following the manufacturer's procedure. The cDNA containing the Ser to Ala (non-phosphorylatable mutant, helSynALA9) was amplified by PCR using the following primers: Ser to Ala forward, 5′-TTTCCTCCGACGTCGATTTGCATCCGGGGAC-3′; Ser to Ala reverse, 5′-GTCCCCGGATGCAAATCGACGTCGGAGGAAA-3′; and the amplified products were subcloned into pGEX-4T1.

In vitro transcription of mRNAs

The helSyn and helSynALA9 sequences were subcloned in frame with the green fluorescent protein (GFP) sequence (Chalfie et al., 1994) in the pCS2-mt-GFP vector (kindly provided by M. Klymkowsky, University of Colorado, Boulder, CO), after cutting out the Myc-tags, using standard recombinant DNA techniques. The in vitro transcription of helSyn-GFP, helSynALA9-GFP and GFP mRNAs was performed according to the protocol of Sahly et al. (Sahly et al., 2003) with minor modifications, using the RiboMAX Large Scale RNA Production System–SP6 (Promega). The concentration of mRNA used in our intracellular injection experiments was usually 1-3 μg/μl in 200 mM KCl.

Cell culture

Soma-to-soma serotonergic synapses between C1 and B2 neurons of *H. pomatia* in culture were obtained as previously described (Fiumara et al., 2005). In those experiments in which intracellular injections were required, cells were paired in untreated Falcon #3001 dishes and allowed to adhere to the plastic substrate. The range of variation of EPSP amplitudes at C1-B2 synapses in culture is very broad and, when comparing different batches of cultures, basal EPSP at these synapses can vary from an average of 0.1 mV to 25-30 mV. This variability in the basal strength of these synapses is determined by several factors, including the batch of conditioned culture medium that is used to promote synaptogenesis (Fiumara et al., 2005). To minimize the effect of these potential sources of intrinsic variability, the different experimental treatments were applied in parallel to cells from the same culture batches.

Intracellular injections of mRNAs

Before injection, the acqueous mRNA solution (1-3 μg/μl) was added with 10% (v/v) of 2 M KCl and loaded (0.5-1 μl) in the tip of a conventional beveled glass microelectrode, as those used for intracellular recording. Some hours after C1-B2 pairing, the C1 neurons were impaled with the mRNA-containing electrode and loaded with mRNA using short pressure pulses (10-20 pulses of 0.3-0.5 seconds; 2-20 psi) delivered through a pneumatic picopump (PV820, WPI, Sarasota, FL) connected to the electrode holder. The injection procedure was monitored under visual and electrophysiological control. Cells with morphological abnormalities or with alterations in resting potential and input resistance after the injection were discarded.

Intracellular injections of the MLCK peptide

In some experiments, the MLCK peptide (Calbiochem, Milan, Italy), a calmodulin inhibitory peptide, or the inactive MLCK control peptide (Calbiochem) were injected in C1 neurons following the same procedure used for mRNA injection, except for the timing of the injection, which was chosen according to the experimental requirements (see Results). The concentration of both peptides in the injection electrode was 50-100 μM.

Cell imaging

The cultured cells were observed under an Eclipse TE200 inverted microscope (Nikon Instruments, Japan) with phase contrast and epifluorescence optics. Micrographs were digitally acquired through a Monochrome QE camera (Mediacybernetics, Silver Spring, MD) connected to the microscope and processed using the Photoshop 6.0 software (Adobe Systems, San Jose, CA). Confocal images were acquired with a Fluoview 300 confocal microscope (Olympus, Hamburg, Germany) and processed as above.

Electrophysiology

Conventional electrophysiological techniques were used for intracellular recording of the synaptic activity of C1-B2 synapses, as previously reported (Fiumara et al., 2005). The synapses were electrophysiologically tested 24-48 h after cell-cell pairing of C1 and B2 neurons. Signals were digitally recorded through a Digidata 1322A interface (Axon Instruments, Union City, CA) and analyzed with Axoscope and Clampfit software (Axon Instruments). The induction and quantification of facilitation, augmentation and PTP at C1-B2 synapses was performed as previously reported (Fiumara et al., 2005).

Pharmacological treatments

In some experiments, the bath application of the protein kinase inhibitors KN-93 (Calbiochem) or Rp-cAMPS was required. Stock solutions of the inhibitors were prepared and stored at −20°C. PTP was evoked at C1-B2 synapses 24-48 hours after cell-cell pairing. To better detect possible reductions in PTP amplitude, only synapses with an initial PTP higher than 200% at 30 seconds after tetanus were used in these experiments. A small volume of stock solution of the inhibitor of choice was added to the culture medium in the recording chamber to reach a final concentration of 5 μM for KN-93 (Jin and Hawkins, 2001) and 500 μM for Rp-cAMPS (Ghirardi et al., 2004); 30 minutes later, PTP was tested again.

Electron microscopy

Cells were processed for electron microscopy as previously reported (Fiumara et al., 2005).

Statistics

The data are expressed as mean values ± standard error of mean (s.e.m.). Statistical analysis was performed with the Statistica software package (StatSoft, Tulsa, OK). Student's *t*-test, one-way and two-way ANOVA with one repeated measure (time) followed by post hoc tests, were used, where appropriate, to determine statistical significance. In all instances, a value of $P<0.05$ was considered as statistically significant.

Acknowledgments

We thank Mike Klymkowsky for the precious gift of the pCS2-mtGFP plasmid, and Luana Fioriti and Francisco Monje for critical reading of the manuscript. We are grateful to Claudio Franchino and Petra Kieslinger for technical assistance. This work was supported by the Italian Ministero dell'Università e della Ricerca (grants FIRB 2004 and MIUR-PRIN 2004-2006 to P.G.M. and F.B.) and the Austrian Science Foundation (grant P17874-B05 to G.L.).

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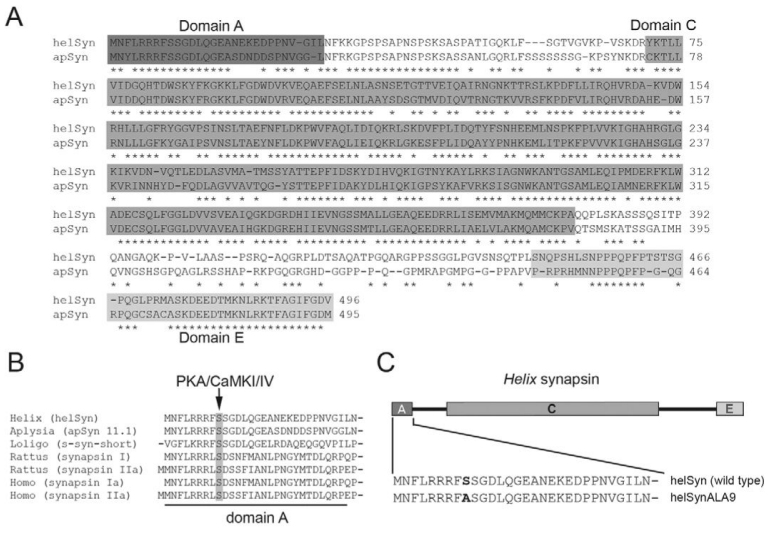

Fig. 1.

Cloning and mutation of *H. pomatia* synapsin. (A) Sequence alignment of the newly cloned *H. pomatia* synapsin (helSyn) and the closely related *A. californica* synapsin (apSyn) isoform 11.1. Asterisks indicate amino acid identities. The conserved structural domains A, C and E are highlighted. (B) Sequence alignment of the domain A of vertebrate and invertebrate synapsin isoforms. The highly conserved PKA/CaMKI/IV phosphorylation site (site 1) corresponding to Ser9 of helSyn is highlighted. (C) Schematic representation of the helSyn structure. To generate a mutant that could not be phosphorylated at site 1, Ser9 was substituted with Ala (helSynALA9).

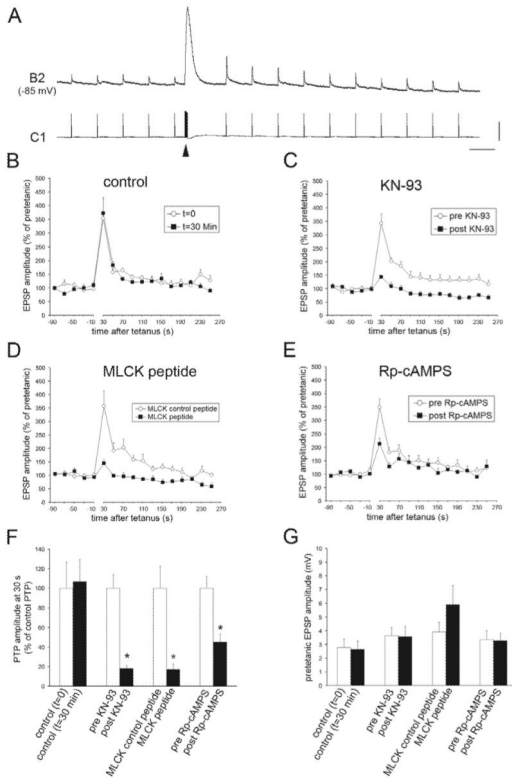

Fig. 2.

Inhibitors of CaMKs and PKA impair PTP at C1-B2 synapses. (A) Sample electrophysiological recording of PTP induction and decay at C1-B2 synapses in culture. Single action potentials in the C1 neuron are elicited at 0.05 Hz (lower trace) and the corresponding EPSPs evoked in the B2 neuron are simultaneously recorded (upper trace). After five basal stimuli, a tetanus (10 Hz for 2 seconds) is induced in the C1 neuron (arrowhead); 30 seconds later, the basal 0.05 Hz stimulation is resumed. The amplitude of the post-tetanic EPSPs is increased, reaching its peak 30 seconds after tetanus and progressively declining to pre-tetanic levels during the following 3-4 minutes. Bars, horizontal, 20 seconds; vertical, 8 mV upper trace, 60 mV lower trace. (B) Time-course of EPSP amplitude changes in two episodes of PTP evoked at a 30-minute interval in the same synapses. Values are normalized to the average amplitude of the last five pre-tetanic EPSPs. The peak amplitude and decay kinetics of PTP are nearly the same at t=0 (○) and at t=30 min (■) after tetanus. (C) Time-course of EPSP amplitude changes in two episodes of PTP evoked in the same synapses immediately before (○) and 30 minutes after bath application of the CaMKs inhibitor KN-93 (5 μM, ■). KN-93 nearly abolished the expression of PTP. (D) Time-course of the EPSP amplitude changes in episodes of PTP evoked in two distinct groups of synapses. In one group, the presynaptic C1 neuron was injected with the MLCK peptide (50-100 μM), an inhibitor of CaMKs (■). In the other group of synapses, the inactive MLCK control peptide was injected as a control (○). In both groups, PTP was evoked 30 minutes after injection of peptides. The MLCK peptide induces a dramatic impairment of PTP, similar to KN-93. (E) Time-course of EPSP amplitude changes in two episodes of PTP evoked in the same synapses immediately before (○) and 30 minutes after bath application of the PKA inhibitor Rp-cAMPS (500 μM, ■). (F) Mean peak PTP amplitudes measured at 30 seconds after tetanus in the experimental groups shown in panels B-E. Values are normalized to the mean peak potentiation measured under control conditions in each group. The peak amplitude of PTP is significantly reduced in the presence of KN-93, MLCK

peptide and Rp-cAMPS. (G) Mean pre-tetanic EPSP amplitude in the PTP episodes shown in panels B-E. The various inhibitors used did not significantly alter the basal EPSP amplitude with respect to control conditions.

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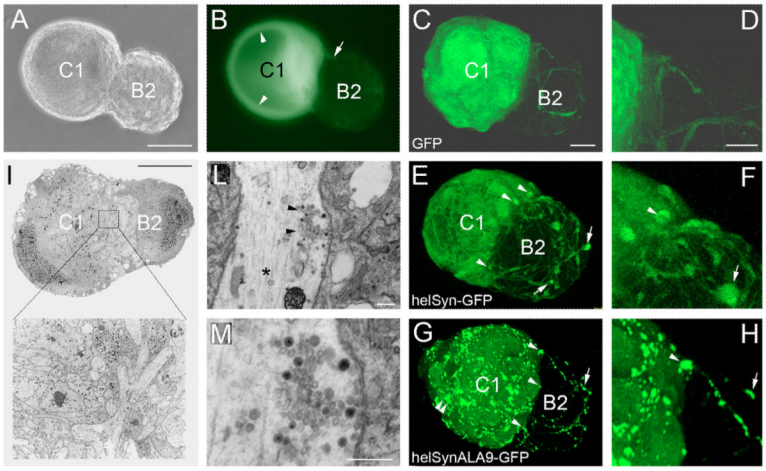

Fig. 3.

Presynaptic overexpression of GFP-tagged wild-type or mutant helSyn at C1-B2 synapses. (A) Phase-contrast micrograph of a C1-B2 soma-to-soma synapse in culture. (B) Epifluorescence micrograph of the same cell pair shown in A, 24 hours after the intracellular injection of mRNA encoding for GFP in the C1 neuron. GFP is expressed at high levels in the C1 neuron cytoplasm. At the focal plane of the picture, the dark profile of the C1 nucleus is visible (arrowheads), as well as the initial part of small neurites projecting from C1 onto the B2 surface (arrow). (C-H) Confocal stacks encompassing the whole volume of different C1-B2 cocultures overexpressing GFP (C,D), GFP-tagged wild-type helSyn (E,F) or GFP-tagged helSyn phosphorylation mutant (G,H). (C) GFP is distributed quite uniformly in the C1 cytoplasm and in the neurites growing onto the B2 surface. (D) Detail of the area of contact between the same C1 and B2 neurons shown in C. The arrow indicates a C1 neurite growing onto the B2 surface. (E) helSyn-GFP is more concentrated in discrete spots of increased fluorescence, which are particularly evident in the area of contact between the two cell bodies (arrowheads) and in varicose structures along the neurites (arrows). (F) Detail of the area of contact between the same C1 and B2 neurons shown in E. The arrow and arrowhead indicate sites of helSyn-GFP accumulation in the C1-B2 contact area and in neurites, respectively. (G) helSynALA9-GFP is strongly concentrated in numerous clusters widely distributed in the C1 cell body (double arrowhead) as well as in the C1-B2 contact area (arrowheads) and along C1 neurites growing onto B2 (arrow). (H) Detail of the area of contact between the same C1 and B2 neurons shown in G. The arrow and arrowhead indicate sites of helSynALA9-GFP accumulation in the C1-B2 contact area and in neurites, respectively. Note the higher fluorescence intensity of these helSynALA9-GFP puncta, as compared with the background fluorescence, with respect to the helSyn-GFP puncta shown in E and F. (I) Electron micrograph of a soma-to-soma C1-B2 synapse section encompassing the two cell bodies. The inset shows at higher magnification the meshwork of cellular processes in the contact area between the two somata. (L) Electron micrograph of a C1 neurite (asterisk) growing onto the surface of the postsynaptic B2 soma and containing a cluster of synaptic vesicles (arrowheads) at a putative synaptic site. (M) Detail of the synaptic site shown in L. Bars, 50 μm (A,B); 20 μm (C,E,G); 20 μm (D,F,H); 50 μm (I); 0.5 μm (L); 0.5 μm (M).

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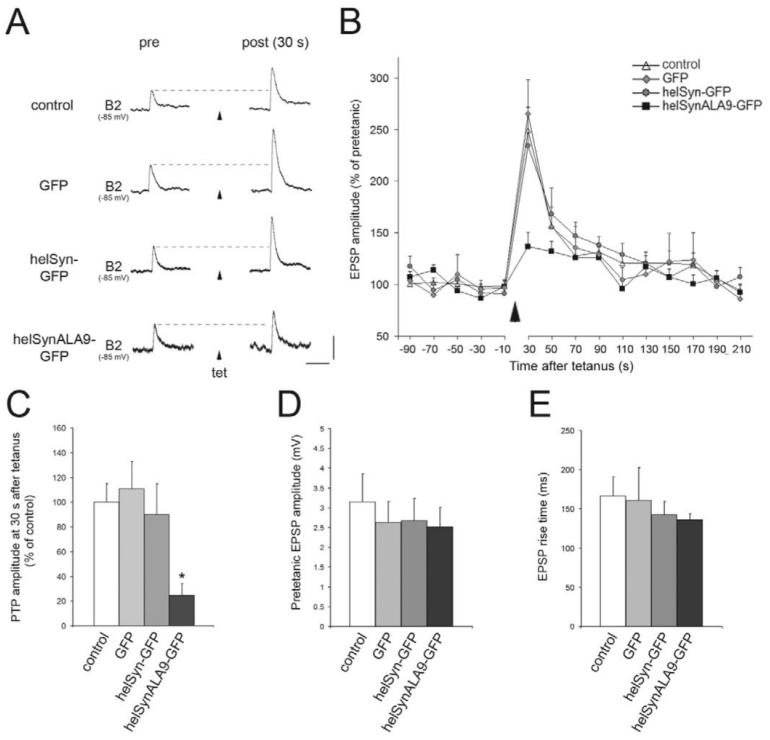

Fig. 4.

Presynaptic overexpression of the non-phosphorylatable helSynALA9 mutant impairs PTP at C1-B2 synapses. (A) Sample electrophysiological recording of EPSPs recorded in B2 neurons before (pre) and 30 seconds after (post) a tetanus was applied to presynaptic C1 neurons that were either untreated (control) or overexpressing either GFP (GFP), GFP-tagged wild-type helSyn (helSyn-GFP) or the GFP-tagged helSyn phosphorylation mutant (helSynALA9-GFP). Bars: horizontal, 2 seconds; vertical, 5 mV control, 4 mV GFP, 5 mV helSyn, 2.5 mV helSynALA9. (B) Time-course of EPSP amplitude changes in PTP episodes evoked by presynaptic tetanization (arrowhead) in the various experimental groups. Values are normalized to the average amplitude of the last five pre-tetanic EPSPs. The peak amplitude and decay kinetics of PTP are nearly the same in the GFP and helSyn group with respect to controls. Conversely, helSynALA9 overexpression determines a conspicuous impairment of PTP. (C) Mean amplitudes of the peak PTP measured at 30 seconds after tetanus in the experimental groups shown in B. Values are normalized to the mean value of peak potentiation measured in the control group. helSynALA9 overexpression dramatically reduces PTP to below 30% of control levels. (D,E) Amplitudes (D) and rise times (E) of pre-tetanic EPSPs in the experimental groups shown in B and C. No significant differences were detectable as a consequence of presynaptic GFP, helSyn-GFP or helSynALA9 overexpression.

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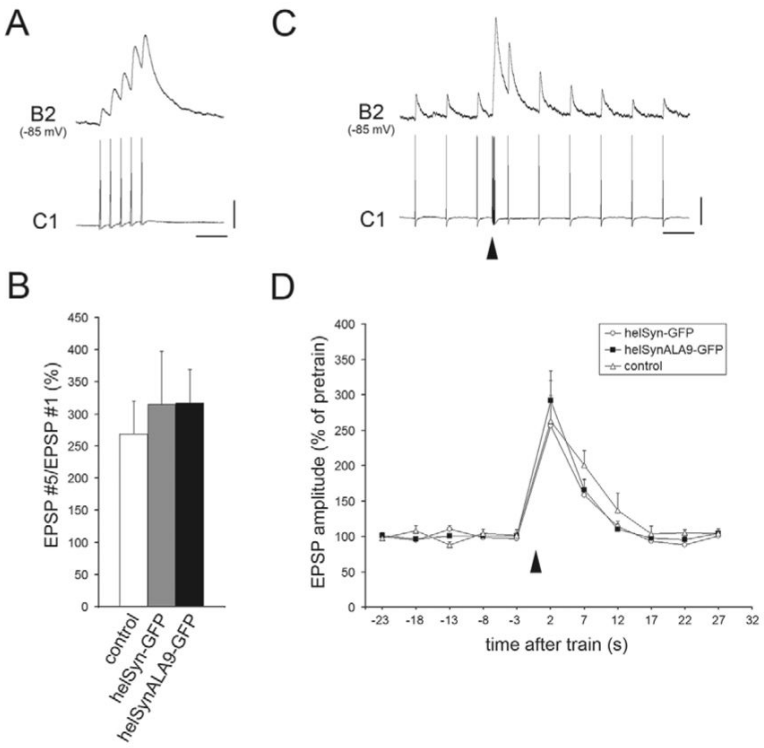

Fig. 5.

Presynaptic overexpression of either wild-type or mutant helSyn does not affect facilitation or augmentation at C1-B2 synapses. (A) Sample electrophysiological recording of frequency facilitation at C1-B2 synapses during a train of five action potentials induced at 2 Hz in the presynaptic C1 neuron (lower trace). Note the progressive increase in the amplitude of the summating EPSPs recorded in the B2 neuron (upper trace). Bars: horizontal, 1.5 seconds; vertical, 5 mV upper trace, 20 mV lower trace. (B) The graph shows the mean facilitation index (expressed as the percent ratio between the fifth and the first EPSP during 2 Hz trains) measured in the different experimental groups. No significant change with respect to control conditions is observed after either helSyn-GFP or helSynALA9-GFP overexpression. (C) Sample electrophysiological recording of augmentation induction and decay at C1-B2 synapses. Single action potentials in the C1 neuron are elicited by intracellular depolarizing stimuli delivered at a basal frequency of 0.2 Hz (lower trace) and the corresponding EPSPs evoked in the postsynaptic B2 neuron are simultaneously recorded (upper trace). After some basal stimuli, a train of five action potentials at 10 Hz is induced in the C1 neuron (arrowhead). Two seconds after the train, the basal 0.2 Hz stimulation is resumed. The amplitude of the EPSPs after the train is increased for about 10 seconds. Bars: horizontal, 5 seconds; vertical, 5 mV upper trace, 20 mV lower trace. (D) Time-course of EPSP amplitude changes in augmentation episodes induced in synapses overexpressing either wild-type or mutant helSyn and in control synapses. No significant change in augmentation is observed after either helSyn-GFP or helSynALA9-GFP overexpression with respect to control conditions.