Synapsin Regulates Basal Synaptic Strength, Synaptic Depression, and Serotonin-Induced Facilitation of Sensorimotor Synapses in Aplysia

Diasinou Fioravante, Rong-Yu Liu, Anne K. Netek, Leonard J. Cleary, and John H. Byrne
Department of Neurobiology and Anatomy, W.M. Keck Center for the Neurobiology of Learning and Memory, The University of Texas Medical School at Houston, Texas

Submitted 28 May 2007; accepted in final form 27 September 2007

Fioravante D, Liu R-Y, Netek AK, Cleary LJ, Byrne JH. Synapsin regulates basal synaptic strength, synaptic depression, and serotonin-induced facilitation of sensorimotor synapses in Aplysia. J Neurophysiol 98: 3568–3580, 2007. First published October 3, 2007; doi:10.1152/jn.00604.2007. Synapsin is a synaptic vesicle-associated protein implicated in the regulation of vesicle trafficking and transmitter release, but its role in heterosynaptic plasticity remains elusive. Moreover, contradictory results have obscured the contribution of synapsin to homosynaptic plasticity. We previously reported that the neuromodulator serotonin (5-HT) led to the phosphorylation and redistribution of Aplysia synapsin, suggesting that synapsin may be a good candidate for the regulation of vesicle mobilization underlying the short-term synaptic plasticity induced by 5-HT. This study examined the role of synapsin in homosynaptic and heterosynaptic plasticity. Overexpression of synapsin reduced basal transmission and enhanced homosynaptic depression. Although synapsin did not affect spontaneous recovery from depression, it potentiated 5-HT–induced de depressive. Computational analysis showed that the effects of synapsin on plasticity could be adequately simulated by altering the rate of Ca2+-dependent vesicle mobilization, supporting the involvement of synapsin not only in homosynaptic but also in heterosynaptic forms of plasticity by regulating vesicle mobilization.

INTRODUCTION

Heterosynaptic plasticity has received considerable attention as a means to induce and maintain learning-related synaptic modifications. Modulatory neurotransmitters have been postulated to provide the structural and molecular state for information processing and memory formation (Bailey et al. 2000; Walters and Byrne 1983). However, the cellular and molecular mechanisms mediating the effects of most of these modulators on synaptic plasticity and learning remain unclear. A well-established system for the study of heterosynaptic modulation of synaptic transmission and plasticity is the Aplysia sensorimotor synapse. In this system, serotonin (5-HT) engages several second messenger cascades, including cAMP/protein kinase A (PKA), to induce both short- and long-term facilitation of synaptic transmission (Byrne and Kandel 1996).

Extensive studies of 5-HT–induced plasticity have implicated broadening of the action potential as a mechanism mediating short-term synaptic facilitation (STF) (Eliot et al. 1993; Hochner and Kandel 1992; Hochner et al. 1986a). Other mechanisms, independent of spike duration, were also required for STF, especially when transmission was depressed before 5-HT treatment (Bráha et al. 1990; Ghirardi et al. 1992; Gingrich and Byrne 1985; Goldsmith and Abrams 1992; Hochner et al. 1986b; Sugita et al. 1997). Even though regulation of synaptic vesicle availability for release was proposed to be involved in STF (Byrne and Kandel 1996; Gingrich and Byrne 1985; Ghirardi et al. 1988), little is known about the molecular mechanisms underlying vesicle trafficking at Aplysia sensorimotor synapses.


Several studies have implicated synapsin in homosynaptic plasticity, albeit with conflicting results. Imaging studies of synapsin knockouts suggest that synapsin I is necessary for synaptic mobilization and exocytosis induced by high-frequency stimulation of hippocampal cells (Chi et al. 2003; Ryan et al. 1996). However, recent findings in synapses of thalamocortical cells from synapsin I/II knockouts suggested that synapsin is not essential for sustained, high-rate transmission (Kielland et al. 2006). Moreover, in hippocampal slices from synapsin I knockouts, paired-pulse facilitation (PPF) was enhanced (Rosahl et al. 1993), whereas in double synapsin I/II knockouts, no effect on PPF was observed (Rosahl et al. 1995). In addition, modulating levels of synapsin I dramatically affected posttetanic potentiation (PTP) at cholinergic synapses in Aplysia (Humeau et al. 2001) but not at the C1-M2 synapse in Helix (Fiumara et al. 2007) or in hippocampal slices (Rosahl

Address for reprint requests and other correspondence: J. H. Byrne, Dept. of Neurobiology and Anatomy, W. M. Keck Ctr. for the Neurobiology of Learning and Memory, The Univ. of Texas Medical School at Houston, PO Box 20708, Houston, TX 77225 (E-mail: John.H.Byrne@uth.tmc.edu).

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et al. 1993). Therefore a complete understanding of the role of synapsin in homosynaptic plasticity remains elusive. Some of this uncertainty could be attributed to gene redundancy, non-specific effects of synapsin deletion on synaptic integrity, developmental effects, and homeostatic compensation (Chin et al. 1995; Powell 2006). Other factors that may contribute to the apparent contradiction involve synapse-specific properties and differences in stimulation frequencies. Furthermore, little is known about the role of synapsin in heterosynaptic plasticity. Spillane et al. (1995) found that forskolin-induced plasticity was not affected in synapsin I/II knockout mice, whereas Menegon et al. (2006) recently showed that the effect of forskolin on the recovery of transmission from depression required synapsin phosphorylation. Other examples of heterosynaptic modulation have not been examined.

In this study, we directly investigated the role of *Aplysia* synapsin in basal neurotransmitter release and in two forms of synaptic plasticity. The *Aplysia* sensory-motor synapse in culture is an excellent system to study these processes, because transmission at this synapse has been thoroughly characterized (Byrne and Kandel 1996). Moreover, a single gene codes for synapsin in *Aplysia* (Angers et al. 2002). Therefore compensatory gene regulation is not expected to confuse interpretation of the experimental results.

**METHODS**

**Plasmid construction**

The sequence for *Aplysia* synapsin (apSyn) was obtained by PCR using a pGEX-apSyn vector (Angers et al. 2002) as template. The termination codon was mutated, and the purified PCR product was ligated into pNEXΔ expression vector upstream of the hemagglutinin (HA) tag sequence, using standard procedures (Maniatis et al. 1989). Automated sequencing (Seqwright, Houston, TX) of the plasmid was performed to confirm that the sequence was error-free and inserted properly into the vector. In pilot experiments, we also attempted to express a synapsin-EGFP construct for visualizing the expressed protein. However, for reasons that are not understood, the EGFP chimera required ≥12 days to be expressed as opposed to 2 days for synapsin-HA. Therefore we used the synapsin-HA vector for this study.

**Cultures and injections**

Sensorimotor neuron co-cultures were prepared as described previously (Angers et al. 2002; Rayport and Schacher 1986). On day 3, sensory neurons were injected with 0.5 μg/μl pNEXΔ-apSyn-HA or pNEXΔ-HA (empty vector, control) in injection solution (0.1% Alexa 488-dextran, 100 mM KCl). A pNEX3-EGFP expression vector (0.2 μg/μl) was co-injected to allow for assessment of injection efficiency. Co-cultures were returned to the incubator for 4 days, at which time experiments were performed. In order to confirm vector expression, co-cultures were processed for immunofluorescence analysis at the end of electrophysiological experiments. Specifically, immediately after the end of stimulation, co-cultures were rinsed with 5 volumes 50% isotonic L15-50% ASW and allowed to rest for a few hours before fixation. This rest period was necessary because 5-HT is known to induce synapsin dispersion, which lasts ≤2 h (Angers et al. 2002).

**Electrophysiology**

Recordings were made in 50% isotonic L15-50% artificial seawater (ASW). Sensory neurons were stimulated extracellularly with a patch electrode filled with 50% ASW—50% isotonic L15, and postsynaptic responses (excitatory postsynaptic potentials; EPSPs) were monitored intracellularly with sharp electrodes of 12- to 15-MΩ resistance, filled with 3 M KAc. The resting membrane potential of motor neurons was current clamped at −90 mV. Responses were recorded using an Axoclamp-2B amplifier and pCLAMP 8.2 software. The stimulation protocol consisted of a train of 10 stimuli at 1 Hz, at the end of which 5-HT (50 μM final concentration) or ASW (vehicle; control) was applied with a pipette. A 5-min rest period followed, at the end of which an additional stimulus was delivered to test for the extent of spontaneous recovery (in the ASW-treated group) or dedepression (in the 5HT-treated group). Off-line analysis of the magnitude of excitatory postsynaptic potentials (EPSPs) was performed using pCLAMP 8.2. EPSP kinetics were analyzed using the Statistics function of Clampfit 10.0, an analysis package that is part of pCLAMP. Rise time and rising slope were measured from 10 to 90% of peak. Decay time and decay slope were measured from 90 to 20% of peak.

**Immunofluorescence**

For fixation, 4% paraformaldehyde in 30% sucrose-PBS was applied to the cultures for 30 min. After incubation with blocking medium [Superblock medium (Promega) supplemented with 5% normal goat serum and 0.2% Triton-X 100] for 30 min, anti-apSyn (1:500), anti-αVAMP (1:500), and/or anti-HA (1:100; Abcam, Cambridge, MA) primary antibodies were added overnight. After three 15-min rinses with PBS, co-cultures were incubated with goat anti-rabbit and/or anti-mouse secondary antibodies coupled to Alexa 568 (Invitrogen, Carlsbad, CA) and Cy5 (Jackson ImmunoResearch, West Grove, PA), respectively. Anti-fade mounting medium (Invitrogen) was used for mounting of coverslips. Confocal images were collected using a 40× water-immersion lens (NA 0.8) of an Olympus upright microscope coupled to a Biorad 1024 MP confocal system. Excitation wavelengths of 488, 568, and 633 nm were used in sequential mode and z-stacks of 0.5-μm step size and/or single optical sections were collected. For imaging of isolated sensory neurons in Figs. 2, D and E, and 7, a 60× oil immersion lens (NA 1.4) was used. In experiments where two groups of cultures were compared, settings for image acquisition were kept the same. Off-line analysis was performed using Metamorph imaging system (Molecular Devices Corp., Sunnyvale, CA) and custom MATLAB (Mathworks) software. Signal colocalization was assessed using ImageJ 1.34n software (National Institutes of Health) or Adobe Photoshop CS2. Varicosities were defined as swellings located along neurites, at branch points, and at neurite terminals (Bailey and Chen 1988), with a diameter >1.5 times the diameter of the attached neurites (Bailey et al. 1979). Total neurite length for each image field was determined by the summed length of lines traced along neurites. For the analysis presented in Fig. 2, varicosities were confirmed as sites of vesicle accumulation by examining VAMP staining in overlaid images. Varicosities were counted only if VAMP staining was increased compared with the attached neurites.

**FM4-64 dye uptake experiments**

FM4-64 (8.5 μM final concentration; Molecular Probes) was used to label vesicle pools in control and synapsin-overexpressing sensory-motor co-cultures that co-expressed EGFP. Loading of vesicles was achieved through KCl stimulation (5 min, 100 mM) in the presence of FM4-64. KCl was washed out with 4 volumes modified L15 + FM4-64, and cultures were allowed to recover for 20 min. The dye was washed out with 5 volumes modified L15, and cultures were imaged immediately using the Biorad confocal system described above. The blue and green lines of a Kr/Ar laser were used sequentially at the minimal required power for excitation of EGFP and FM4-64, respectively. Imaging was restricted to the initial segment of the motor neuron axon contacted by sensory neuron processes (Glanzman et al. 1990), and z-stacks of 5 μm of optical sections at 0.5-μm
step size were collected. Analysis was performed off-line using the Metamorph software.

Pharmacology

5-HT (Sigma-Aldrich, St. Louis, MO) was applied to cultures at the indicated concentrations using a pipette. The PKA inhibitor KT5720 (Calbiochem) was bath-applied at a final concentration of 10 μM in 0.075% final DMSO concentration.

Computer simulations

To mathematically model the dynamics of transmitter mobilization and release from sensory neuron terminals, we used a model of the sensorimotor synapse that was previously developed (Gingrich and Byrne 1985, 1987) and confirmed by experimental results (Sugita et al. 1997). This model was based on a system of coupled ordinary differential equations. The equations described the action potential-evoked calcium influx (\( F_{Ca} \)), which stimulated release of transmitter from a release pool, and the mobilization of transmitter from a feeding pool to replenish the release pool. Repetitive stimulation of the modeled terminal led to gradual depletion of the releasable transmitter and depression of transmitter release to steady state. Recovery of transmitter release from depression occurred spontaneously with time after the end of stimulation. Decline of release to steady state with repetitive stimulation and the spontaneous recovery after the end of stimulation were accomplished through the process of transmitter mobilization from the feeding pool. Mobilization of transmitter could be driven by a concentration gradient between the release and feeding pools (\( F_{VT} \)), by elevation of cAMP in response to 5-HT treatment (\( F_{AMP} \)), or by the concentration of calcium in the cytosol. Calcium-dependent mobilization of transmitter, in turn, could take place in two different ways, which varied in their sensitivity to calcium concentration and in their rate constants (fast: \( F_G \); slow: \( F_S \)). The fast mobilization was primarily responsible for sustaining steady-state release during high frequencies of stimulation (i.e., 0.3–1 Hz), whereas the slow mobilization was primarily responsible for sustaining release at moderate frequencies of stimulation (0.03–0.3 Hz). Low-frequency release (0.01 Hz) was sustained by the concentration-driven mobilization.

The free parameters of the model by Gingrich and Byrne (1985) had been originally selected to match the empirically observed dynamics of the sensorimotor synapse of the abdominal ganglion (Byrne 1982). Because these dynamics are somewhat different from the dynamics of sensorimotor synapses in culture, we had to modify the parameters of the original model to fit the rates of synaptic depression and spontaneous recovery that were observed in cultures, under control conditions (see Fig. 4), as well as the effects of 5-HT on dedepression. The new parameters resulted from empirical observations and allowed the model to fit the data obtained from control cultures. The differences in the parameters between the original model of Gingrich and Byrne and the one used in this study are presumably caused by minor differences in synaptic morphology between cultures and ganglia. Despite these differences, results obtained from cultures can be generalized to ganglia, as previously shown (Dumitriu et al. 2006; Ghirardi et al. 1992; Martin et al. 1997; Sharma et al. 2006; Sugita et al. 1992).

To simulate the control depression, recovery, and dedepression data from cultured synapses, parameter optimization with PEST indicated that the release rate constant (\( K_{R} \)), the fast mobilization rate constant (\( K_{F} \)), and the volume of the feeding pool (\( V_{F} \)) were critical parameters to the fitting of the control depression kinetics. In addition, the rate constants of calcium diffusion (\( K_{Ca} \)) and of concentration-gradient-driven mobilization (\( K_{VT} \)) were critical for the recovery from depression. Finally, the cAMP-driven mobilization rate constant of stored transmitter (\( K_{FC} \)) was also adjusted to reproduce the empirically observed synaptic dedepression by 5-HT (Fig. 5). Because slow mobilization (\( F_S \)) did not contribute to this stimulation frequency, the slow rate constant (\( K_S \)) was fixed to 0.0 in order to enhance the efficiency of the optimization. The final parameter changes from Gingrich and Byrne (1985, 1987) for the cultured synapse dynamics (in ASW and 5-HT) were as follows: \( K_{R} \) +70%; \( K_{F} \) +128%; \( K_{S} \) +103%; \( K_{VT} \) -50%; \( V_{F} \) +65%; \( K_{FC} \) -16%. After the reparameterization process was complete, the error (average squared difference) between experimental and simulated EPSP peak amplitudes had been minimized to 2.65 (from 63.21 without reparameterization). The simulated effects of synapsin overexpression (see RESULTS) were not specific to the new set of free parameter values that we used to fit the synaptic dynamics of cultured synapses: when the original model parameters (Gingrich and Byrne 1985, 1987) were used, the simulated overexpression of synapsin had similar effects on synaptic depression and recovery as did the new, reparameterized model (data not shown). This observation suggests that the predicted effects of synapsin overexpression do not depend on the specific dynamics of cultured synapses.

RESULTS

Intracellular localization of overexpressed synapsin

To assess the role of Aplysia synapsin in homono- and heterosynaptic plasticity, we overexpressed full-length wild-type synapsin fused to a HA tag in sensory neurons co-cultured with motor neurons. This approach allowed us to increase the intracellular distribution of synapsin, enhancing its functions. In contrast to previous studies where mammalian synapsin was introduced in invertebrate systems (Dearborn et al. 1998; Humeau et al. 2001; Llinas et al. 1985), we overexpressed the Aplysia synapsin in Aplysia sensory neurons, minimizing the differences from endogenous synapsin. The extent of overexpression and the subcellular distribution of synapsin-HA were determined qualitatively using immunofluorescence (Angers et al. 2002). Sensory neurons were co-injected with a plasmid expressing enhanced green fluorescent protein (EGFP) to allow for visualization of sensory neuron processes and to control for the efficiency of expression. Cells were analyzed if they expressed EGFP and if levels of HA expression were greater than background.

Injection of the synapsin-HA construct resulted in increased levels of synapsin compared with injection of empty vector (control; Fig. 1A). The subcellular distribution of synapsin-HA appeared punctate throughout the sensory neuron processes, and thus resembled the distribution of endogenous synapsin (cf. Fig. 1, A and B). Co-localization studies using the anti-synapsin and anti-HA antibodies indicated that the HA tag can serve as an effective marker of the overexpressed protein (Fig. 1G). Using the anti-HA antibody, we found that synapsin-HA localized to presynaptic varicosities (Fig. 1F), defined as swellings along sensory neuron processes labeled with EGFP (Fig. 1D). These varicosities stained positive for the synaptic vesicle marker vesicle-associated membrane protein (VAMP) (Fig. 2.
A and B), suggesting that they constituted putative sites of synaptic contacts. Collectively, these results show that synapsin-HA was expressed in sensory neurons and localized at putative synapses, where it could affect synaptic transmission and plasticity.

Synapsin regulates the functional size, but not the number, of vesicle clusters

Because synapsin interacts with phospholipids and various synaptic molecules (Hilfiker et al. 1999) and is implicated in the formation and maintenance of synaptic structures (Chin et al. 1995; Ferreira et al. 1995; Lu et al. 1996), we examined the possibility that synapsin overexpression may affect the number of presynaptic vesicle clusters. To this end, we monitored the number of VAMP-containing varicosities per 100 μm neurite in control (Fig. 2D) and synapsin-overexpressing (Fig. 2E) sensory neurons. VAMP is an integral synaptic vesicle protein that has been used extensively as a marker of vesicle pools (Angers et al. 2002; Ryan 2006) and whose levels are affected by manipulations that perturb vesicle clusters (Gitler et al. 2004; Rosahl et al. 1995). We found no significant effect of synapsin overexpression on the number of VAMP-containing varicosities (P = 0.79; Fig. 2F), suggesting that our manipulation did not affect the number of presynaptic vesicle clusters. These results, along with previous observations that increasing synapsin levels does not have any deleterious effects on synaptic integrity (Fiumara et al. 2001; Humeau et al. 2001; Menegon et al. 2006), suggest that the overexpression approach is suitable for studying synapsin function.

Even though the results of the VAMP immunofluorescence experiment would argue against an effect of synapsin-HA on the total vesicle complement, synapsin overexpression may still affect the functional number of vesicles involved in neurotransmitter release. To study this hypothesis, we performed live-cell imaging experiments using the lipophilic dye FM4-64 (Fernandez-Alfonso and Ryan 2004; Gaffield and Betz 2006) (Fig. 3). Briefly, co-cultures that co-expressed EGFP and either synapsin-HA or control were stimulated with KCl (100 mM, 5 min), which is known to induce Ca^{2+}-dependent release of vesicles (Kavalali et al. 1999; Kuromi and Kidokoro 2003; Pyle et al. 1999) in the presence of FM4-64. After the end of stimulation, co-cultures were allowed to recover for 20 min before dye washout and imaging. The number of FM4-64 puncta that localized in presynaptic varicosities of control or synapsin-HA-overexpressing co-cultures was counted. These results revealed that synapsin-HA overexpression did not significantly change the number of FM4-64 puncta in synapsin-overexpressing co-cultures and normalized to 100 μm of sensory neuron process. Comparison between control and synapsin-overexpressing co-cultures revealed a significant decrease in the number of FM4-64 puncta that localized in presynaptic varicosities of control or synapsin-HA-overexpressing co-cultures and normalized to 100 μm of sensory neuron process.
and other cytoskeletal elements are thought to tether vesicles in activity-dependent synaptic depression (Kim et al. 2003). We used the term “feeding pool” as opposed to “reserve pool” to be consistent with the terminology used by Gingrich and Byrne (1985), whose computational model was used in this study. The feeding pool and RRP are thought to be in dynamic equilibrium (Gingrich and Byrne 1985; Murthy and Stevens 1999; Rizzoli and Betz 2004, but see Giritel et al. 2004), with vesicles from the feeding pool being recruited during periods of prolonged synaptic activity to sustain release (Brodin et al. 1997; Neher 1998; Rizzoli and Betz 2004; Zucker and Regh 2002). If these hypotheses are correct and synapsin tethers synaptic vesicles, overexpression of synapsin would be expected to sequester vesicles in an enlarged feeding pool, perhaps at the expense of the readily releasable pool, thus impeding their mobilization. This hypothesis is further supported by our FM4-64 experiment, which showed an apparent impairment of vesicle mobilization and/or fusion in synapsin-overexpressing cells (Fig. 3).

To further study the role of synapsin in synaptic transmission and plasticity, we performed electrophysiological experiments using sensorimotor co-cultures that co-expressed EGFP and either synapsin-HA or control. To induce synaptic depression, sensory neurons were stimulated with a 10-s train of electrical stimuli delivered at 1 Hz. This frequency was selected to challenge the release machinery, because it leads to significant homosynaptic depression (Byrne 1982) and partly depletes synaptic vesicle pools (Armitage and Siegelbaum 1998; Gingrich and Byrne 1985; Royer et al. 2000; Zhao and Klein 2004). Compared with empty vector-injected controls, sensory neurons expressing synapsin-HA displayed significantly reduced basal synaptic strength, assessed by the amplitude of the first EPSP in the train (Fig. 4, A and C; P < 0.05). This result could be explained by synapsin trapping vesicles in the feeding pool, effectively reducing the size of the RRP. This hypothesis would also be in agreement with the reduced FM4-64 loading of presynaptic boutons in synapsin-HA neurons.

To determine the specificity of the effect of synapsin overexpression on transmission, we analyzed the kinetics of postsynaptic responses from control and synapsin-overexpressing cultures. The two groups did not differ significantly in the rise time of the first EPSP during the 1-Hz train (means ± SE: control: 5.5 ± 0.2 ms, n = 15; synapsin-HA: 5.51 ± 0.19 ms, n = 13; t26 = 0.01, P = 0.99), suggesting that the duration of transmitter release is probably not affected by synapsin overexpression (Gingrich et al. 1988; Hochner et al. 1986a,b). The decay kinetics and the half-maximal width of the responses were also not significantly affected by synapsin manipulation [decay time (ms): control: 379.23 ± 71.32, n = 8; synapsin-HA: 258.87 ± 33.75, n = 10; t16 = 1.63, P = 0.12; decay slope (mV/ms): control: −0.04 ± 0.01, n = 8; synapsin-HA: −0.04 ± 0.01, n = 10; t16 = 0.11, P = 0.91; half-maximal width (ms): control: 46.22 ± 2.29, n = 13; synapsin-HA: 48.34 ± 5.59, n = 12; t23 = 0.36, P = 0.72]. In some cases, the decay phase of the EPSP was very slow, and the decay kinetics could not be measured. These results suggest that synapsin overexpression probably did not have postsynaptic

### Synapsin regulates basal synaptic strength and activity-dependent synaptic depression

The interactions of synapsin with synaptic vesicles, actin and other cytoskeletal elements are thought to tether vesicles in a “feeding pool” (Pieribone et al. 1995) (also referred to as “reserve pool”; Zucker and Regh 2002), which is morphologically and functionally distinct from the “readily releasable” pool (RRP) of vesicles docked on the presynaptic active zone (Rosenmund and Stevens 1996; Schikorski and Stevens 2001).
effects (Johnston and Wu 1995). In agreement with the effect of synapsin overexpression on basal synaptic strength (Fig. 4C), there was a significant decrease in the rising slope of the first EPSP (control: 4.65 ± 0.54 mV/ms, n = 15; synapsin-HA: 3.15 ± 0.56 mV/ms, n = 13; t26 = 1.93, P = 0.03). A decrease in the rising slope of the EPSP would be expected if the size of the releasable pool was reduced (Gingrich et al. 1988). These results suggest that synapsin predominantly regulates the rate of transmitter release.

Synapsin-HA–expressing neurons exhibited more pronounced depression than controls (Fig. 4B). The depression ratio between the second and the first EPSP was significantly smaller in the synapsin-HA group (P < 0.01; Fig. 4D). Moreover, the steady-state transmission was significantly decreased in the synapsin-HA group (P < 0.001; Fig. 4E), expressed as the amplitude of the steady state EPSP (average of last 3 EPSPs in the train) normalized to the first EPSP. These results were consistent with those obtained in other systems in which a presynaptic increase in synapsin concentration inhibited release (Hackett et al. 1990; Lin et al. 1990; Llinas et al. 1985, 1991; Nichols et al. 1992; but see Humeau et al. 2001). This inhibition of release could be explained by assuming that increased intraterminal concentration of synapsin tethered more vesicles away from the RRP and impaired their availability and mobilization, resulting in reduced synaptic efficacy and increased depression.

FIG. 3. Synapsin negatively regulates loading of synaptic vesicles with a lipophilic dye. A: control sensorimotor co-culture expressing EGFP presynaptically. B: stimulation of the same co-culture with KCl-induced uptake of FM4-64. C: FM4-64 puncta localized predominantly at presynaptic varicosities (arrowheads). D: different co-culture, injected with pNEX6-apSyn-HA, also expressing EGFP presynaptically. E: stimulation of the co-culture in D with KCl-induced uptake of FM4-64. F: fewer FM4-64 puncta were observed in presynaptic varicosities (arrowheads), suggesting that vesicle mobilization and/or fusion was impaired. Scale bar: 75 μm.

FIG. 4. Overexpression of synapsin decreases basal synaptic strength and enhances synaptic depression. A: representative traces of excitatory postsynaptic potentials (EPSPs) from control or synapsin-HA-expressing co-cultures during a train of 10 stimuli at 1 Hz. B: cumulative data from control or synapsin-HA–expressing co-cultures showed that overexpression of synapsin enhanced homosynaptic depression. C: overexpression of synapsin significantly reduced basal synaptic strength (mean amplitude ± SE; control: 29.1 ± 2.4 mV, n = 19; synapsin: 20.9 ± 2.6 mV, n = 16; t13 = 2.3, P < 0.05). D: synapsin overexpression significantly enhanced depression starting as early as the second stimulus [mean percent ratio (2nd/1st EPSP) ± SE; control: 40.6 ± 2.89, n = 11; synapsin: 27.6 ± 2.95, n = 15; t24 = 3.07, P < 0.001]. E: steady-state depression was also significantly enhanced in synapsin-HA–expressing neurons [mean percent steady state ± SE; control: 37.78 ± 2.5, n = 11; synapsin: 25.23 ± 1.8, n = 14; t23 = 4.11, P < 0.0001]. Successful expression of synapsin-HA was confirmed immunocytochemically in all sensory neurons.
Overexpression of synapsin enhances 5-HT–induced dedepression

We next examined the potential involvement of synapsin in heterosynaptic plasticity and, in particular, in 5-HT–induced facilitation of previously depressed transmission (dedepression), a form of heterosynaptic plasticity. 5-HT, which induces facilitation of release at the sensorimotor synapse, activates kinases that phosphorylate synapsin causing its subcellular redistribution (Angers et al. 2002). This redistribution could be explained by the dissociation of synapsin from synaptic vesicles, which occurs in response to 5-HT (Fioravante and Byrne, unpublished observations). Thus vesicles would become available for release, enhancing synaptic transmission. If overexpression of synapsin resulted in increased mobilization, we would predict that, after 5-HT, the synapsin-overexpressing sensory neurons would show enhanced dedepression compared with controls.

5-HT was applied immediately after the 10th EPSP and remained in the bath for 5 min, a protocol typically used to induce STF (Bartsch et al. 1998, 1995; Martin et al. 1997; Ormond et al. 2004; Phares et al. 2003; Sugita et al. 1997). At the end of the incubation period, a single stimulus was delivered to assess the magnitude of dedepression (Fig. 5A). In all co-cultures, 5-HT induced dedepression of the 11th EPSP in relation to the steady state of the previously induced depression (P < 0.001). However, the synapsin-HA group displayed significantly more dedepression than control (P < 0.05; Fig. 5B), suggesting that more vesicles were mobilized for release after 5-HT. This result was not caused by a “ceiling” effect in the control group because EPSPs can have values larger than the average amplitude of the control group, which was 38.6 ± 5.0 (SE) mV (Fioravante and Byrne, unpublished observations).

Overexpression of synapsin does not affect spontaneous recovery of release

The potentiating effect of synapsin overexpression on 5-HT–induced dedepression could be partly attributed to increased recovery of transmission, mediated by accumulation of intracellular calcium with repetitive activity. In that case, synapsin overexpression would be expected to affect spontaneous recovery of transmission. To address this question, we examined the recovery of transmission from depression in a subset of control and synapsin-overexpressing co-cultures after treatment with ASW (vehicle control) in lieu of 5-HT (Fig. 5C). No difference was observed between the two groups (P = 0.55; Fig. 5D), suggesting that the effect of synapsin overexpression on 5-HT–induced dedepression was not due to a covert effect on spontaneous recovery.

PKA-dependent redistribution of synapsin-HA

The enhanced dedepression observed in the synapsin-HA group could be explained by increased availability of vesicles to be mobilized from the feeding pool by 5-HT. For this increased availability to occur, inhibitory constraints such as synapsin tethering of vesicles must be removed. In the case of endogenous synapsin, 5-HT induces its phosphorylation, dissociation from synaptic vesicles, and subcellular redistribution (Angers et al. 2002). Does synapsin-HA also redistribute after 5-HT? To answer this question, synapsin-HA–expressing neurons were treated with 5-HT or ASW (control) for 5 min, fixed, and processed with an anti-HA antibody for immunofluorescence analysis. After control treatment, synapsin-HA staining appeared punctate throughout sensory neuron processes (Fig. 6, B and C), as observed previously (Figs. 1 and 2). However, fewer synapsin-HA puncta could be observed after 5-HT treatment (Fig. 6E). High-magnification confocal images of sensory neuron processes revealed that synapsin-HA displayed a more diffuse staining pattern along the processes of sensory neurons (Fig. 6F), which could result from its dissociation from synaptic vesicles.

We previously reported that the 5-HT–induced redistribution of endogenous synapsin depends critically on PKA (Angers et al. 2002). To examine whether the overexpressed chimeric synapsin-HA is subjected to similar regulatory control by PKA, we performed immunofluorescence experiments using an anti-HA primary antibody and an inhibitor of the PKA cascade (Fig. 7). We predicted that inhibition of PKA would block 5-HT–induced redistribution of synapsin-HA. Briefly, sensory neurons were pretreated with the PKA inhibitor KT5720 or vehicle (DMSO) for 1 h, followed by 5-min exposure to 5-HT or ASW (control). Cells were fixed imme-

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**FIG. 5.** Overexpression of synapsin potentiates 5-HT–induced dedepression of synaptic transmission without affecting spontaneous recovery. A: representative traces of the 10th (last EPSP in the 1-Hz train) and 11th (1st EPSP after 5-min treatment with 5-HT) postsynaptic responses in control and synapsin-overexpressing co-cultures. B: cumulative data showing potentiating effect of synapsin overexpression on 5-HT–induced dedepression (percent dedepression ± SE: control: 351.8 ± 39.9, n = 7; synapsin: 591.9 ± 95.5, n = 8; t13 = 2.20, P < 0.05). C: representative traces of the 10th (last EPSP in the 1-Hz train) and 11th (1st EPSP after 5-min rest period) postsynaptic responses in control and synapsin-overexpressing co-cultures. D: cumulative data showing that synapsin overexpression did not significantly alter the magnitude of spontaneous recovery of transmission in vehicle-treated [artificial seawater (ASW)] co-cultures (percent recovery ± SE: control: 205.4 ± 24.0, n = 4; synapsin: 245.9 ± 59.4, n = 4; t6 = 0.63, P = 0.55).
we adopted a computational approach. Specifically, we used a previously developed computational model of synaptic transmission in *Aplysia* (Gingrich and Byrne 1987) to fit the experimental data and identify processes that could potentially mediate the observed physiological effects.

The model (Fig. 8) consisted of a set of coupled ordinary differential equations that described activity-induced influx of calcium, regulation of intracellular calcium concentration, storage, trafficking, and release of synaptic vesicles. The effects of 5-HT were mediated in the model through recruitment of an additional vesicle mobilization process, which was cAMP-dependent. Using the parameterization software PEST, the model parameters were optimized to fit the data of both the control and synapsin-HA groups (see Methods for details). The decrease in basal synaptic strength, observed in the experimental data of the synapsin-HA group, could be reproduced in the model by decreasing the volume of the release pool (\(V_R\)) by 33%. There was a commensurate increase in the volume of the feeding pool by 3.3% to hold the total number of vesicles constant. With the release and storage vesicle pools clamped to their new optimized values, the effect of synapsin on enhancing the rate of activity-induced synaptic depression was best reproduced by a 63% reduction in the rate constant of the fast vesicle mobilization process (\(K_F\)). Because of this reduction in \(K_F\) and, consequently, in the rate at which vesicles were mobilized from the feeding pool to the release pool, at the end of the stimulus train, the feeding pool was larger in the synapsin-HA group than control. On application of 5-HT, the cAMP mobilization process (\(F_{cAMP}\)) acted on an enlarged feeding pool, thus leading to enhanced dedepression.

An alternative way to reproduce the synapsin-induced decrease of basal synaptic strength was by reducing the release rate constant (\(K_R\)) by 54%, a manipulation that would presumably reflect a change in release probability. However, when simulations were run with this reduced value of release rate constant, the error between experimental and simulated EPSP peak amplitudes increased more than six times (from 2.65 to 17.12), suggesting that the effect of synapsin on basal release is probably caused by a change in the volume of the release pool and not the probability of release. Collectively, the results of the computational simulations suggest that overexpression of synapsin leads to a considerable reduction in the number of readily releasable vesicles and in the rate at which they are replenished with activity, as well as a relatively small increase in the number of stored vesicles.

**Discussion**

In mammals, three genes have been identified thus far that code for five synapsin isoforms (Kao et al. 1999). Even though synapsin I and II isoforms present some structural and functional differences, they are thought to be functionally redundant. Synapsin III, on the other hand, seems to play distinct regulatory roles in synaptic transmission and development (Feng et al. 2002; Ferreira et al. 2000). Numerous studies have examined the role of synapsin in transmission and plasticity (Chi et al. 2001, 2003; Gültler et al. 2004; Li et al. 1995; Rosahl et al. 1993, 1995; Ryan et al. 1996; Spillane et al. 1995). However, none of these studies investigated the potential involvement of synapsin in short-term heterosynaptic plasticity.

**Mathematical model of the Aplysia sensorimotor synapse supports the role of synapsin in vesicle mobilization**

The experimental results presented above support a role for synapsin in the regulation of vesicle availability and trafficking. To identify cellular processes that could be regulated by synapsin and could result in the observed synaptic physiology,
To delineate the role of synapsin in heterosynaptic facilitation, we took advantage of the glutamatergic sensorimotor synapse in *Aplysia*, which exhibits homosynaptic plasticity and is heterosynthetically regulated by 5-HT (Antzoulatos and Byrne 2004; Byrne and Kandel 1996). A single gene codes for *Aplysia* synapsin, which shares many important similarities with its mammalian homologues in terms of domain arrangement, sequence conservation of the central C domain, and high sequence homology of the N-terminal PKA phosphorylation site (Angers et al. 2002). We found that overexpression of synapsin in sensory neurons negatively regulated vesicular uptake of FM4-64 dye (Fig. 3), impaired basal synaptic strength, and increased synaptic depression (Fig. 4). Moreover, synapsin overexpression potentiated 5-HT–induced recovery from depression without affecting spontaneous recovery of basal release (Hilfiker et al. 1998; Llinás et al. 1985, 1991). This discrepancy could be attributed to synapse-specific differences (Brodin et al. 1997; Gitler et al. 2004), such as initial release probability and level of tonic activation of various kinases. The latter possibility is particularly interesting because the biochemical properties of synapsin are modulated by phosphorylation (Bahringer and Greengard 1987; Bonanomi et al. 2005; Chi et al. 2001, 2003; Hilfiker et al. 1999; Jovanovic et al. 1996). PKA-mediated phosphorylation of synapsin regulates its association with synaptic vesicles and its subcellular distribution in several systems (Angers et al. 2002; Fiumara et al. 2004; Hosaka et al. 1999; Menegon et al. 2006) (also see Fig. 7). Moreover, in *Aplysia* basal phosphorylation of synapsin by ERK MAPK is required for the proper localization of synapsin on vesicles (Angers et al. 2002). If basal levels of kinase activity are synapse-specific (which seems to be the case at least for PKA, Hilfiker et al. 2001), experimental perturbation of synapsin levels might be expected to have differential effects on basal transmission, depending on the basal phosphorylation level of synapsin.

In contrast to the uncertainty around the involvement of synapsin in basal transmission, its role in sustaining release over a range of stimulation frequencies is better documented (Hilfiker et al. 1999). In general, genetic deletion (Gitler et al. 2004; Rosahl et al. 1995) or antibody-mediated neutralization (Humeau et al. 2001; Pieribone et al. 1995) of synapsin enhances synaptic depression, as does injection of recombinant synapsin peptides or protein (Hilfiker et al. 2005; Lin et al. 2000). This effect could arise from a decrease in release probability. However, such a change would be expected to result in less depression during the 1-Hz train caused by a surplus of vesicles, which was not observed in our study. Indeed, reducing the release probability in our simulations increased the error in fitting the experimental data.

The effect of synapsin on basal transmission is controversial. In some systems, interference with synapsin did not affect basal synaptic strength (Gitler et al. 2004; Humeau et al. 2001; Li et al. 1995; Menegon et al. 2006; Rosahl et al. 1995; Ryan et al. 1996), whereas in others, synapsin manipulation inhibited basal release (Hilfiker et al. 1998; Llinás et al. 1985, 1991). This discrepancy could be attributed to synapse-specific differences (Brodin et al. 1997; Gitler et al. 2004), such as initial release probability and level of tonic activation of various kinases. The latter possibility is particularly interesting because the biochemical properties of synapsin are modulated by phosphorylation (Bahringer and Greengard 1987; Bonanomi et al. 2005; Chi et al. 2001, 2003; Hilfiker et al. 1999; Jovanovic et al. 1996). PKA-mediated phosphorylation of synapsin regulates its association with synaptic vesicles and its subcellular distribution in several systems (Angers et al. 2002; Fiumara et al. 2004; Hosaka et al. 1999; Menegon et al. 2006) (also see Fig. 7). Moreover, in *Aplysia* basal phosphorylation of synapsin by ERK MAPK is required for the proper localization of synapsin on vesicles (Angers et al. 2002). If basal levels of kinase activity are synapse-specific (which seems to be the case at least for PKA, Hilfiker et al. 2001), experimental perturbation of synapsin levels might be expected to have differential effects on basal transmission, depending on the basal phosphorylation level of synapsin.

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pressing by 60% under control conditions (also see Chin et al. 2002). Because the stimulation frequencies with which the two systems were challenged are different, direct comparison of the two studies cannot be made. The increased depression observed at synapsin-overexpressing synapses can be explained if overexpressed synapsin traps additional vesicles and prevents them from being mobilized to the RRP, effectively decreasing the size of the RRP and increasing the size of the feeding pool.

If the feeding pool is increased by overexpressed synapsin, one might expect that synaptic depression would actually be decreased during synaptic stimulation. The additional synapsin molecules would be phosphorylated, freeing the trapped vesicles to be mobilized for release. Interestingly, such an enhancement of release was observed in the crayfish (Dearborn et al. 1998), and also in mice with constitutively active H-ras, which results in increased phosho-synapsin (Kushner et al. 2005). In our study, synapsin overexpression increased depression, suggesting that calcium accumulation during the stimulus train was not sufficient to regulate the interactions of synapsin with vesicles. This conclusion is also supported by the observation that synapsin does not redistribute after 1-Hz stimulation (Angers et al. 2002).

In our study, the 5-HT–induced facilitation of moderately depressed transmission (dedepression) was enhanced in synapsin-overexpressing neurons (Fig. 5). According to our simulations, this enhancement could result from enhanced mobilization of synaptic vesicles, which in turn stems from synapsin-mediated enlargement of the feeding pool. Phosphorylation of synapsin by kinases such as PKA would allow vesicles to transition from the feeding to the release pool, enhancing dedepression. Indeed, results from previous studies suggested that PKA is involved in facilitation of moderately depressed transmission (Dumitriu et al. 2006; Ghirardi et al. 1992). Consequently, one would predict that inhibition of PKA would impair synapsin-dependent enhancement of dedepression.

In addition to PKA, protein kinase C (PKC) has also been implicated in facilitation of depressed synapses (Dumitriu et al. 2006; Ghirardi et al. 1992; Manseau et al. 2001). Compared with PKA, the contribution of PKC to synaptic facilitation becomes more important as depression becomes more pronounced (Ghirardi et al. 1992). Because synapsin does not seem to be significantly phosphorylated by PKC despite the multiple putative PKC phosphorylation sites (Angers et al. 2002; Fiumara et al. 2004), we would predict that synapsin overexpression would not affect facilitation of highly depressed synapses, which depends on PKC, but it would enhance facilitation of nondepressed synapses, which depends on PKA.

Extending this line of reasoning, synaptic targets other than synapsin must be important for facilitation of moderately depressed synapses. As indicated by the work of Houeland et al. (2007), SNAP-25 and its phosphorylation by PKC is at least one other important modulator of synaptic facilitation. Extension of this model included consideration of the role of synapsin-dependent enhancement of dedepression (Fig. 6). According to our simulations, this enhancement could result from enhanced mobilization of synaptic vesicles, which in turn stems from synapsin-mediated enlargement of the feeding pool. Phosphorylation of synapsin by kinases such as PKA would allow vesicles to transition from the feeding to the release pool, enhancing dedepression. Indeed, results from previous studies suggested that PKA is involved in facilitation of moderately depressed transmission (Dumitriu et al. 2006; Ghirardi et al. 1992). Consequently, one would predict that inhibition of PKA would impair synapsin-dependent enhancement of dedepression.

The role of synapsin on recovery of transmitter release from depression is controversial. At hippocampal synapses, deletion of the synapsin I gene impaired recovery after 20- and 50-Hz stimulation (Li et al. 1995) but not after 10 Hz (Ryan et al. 1996). At the calyx of Held, deletion of synapsin I and II did not affect recovery after strong depolarization that depleted the RRP (Sun et al. 2006). Some of this uncertainty could be attributed to synapse-specific properties and the differences in

FIG. 8. A mathematical model of transmitter mobilization and release from sensory neuron terminals can capture observed effects of synapsin overexpression. A: schematic diagram of model, which was adapted from Gingrich and Byrne (1987). Text in red indicates elements modified to model effects of synapsin overexpression. B: repetitive stimulation of the modeled sensory neuron terminal led to homosynaptic depression (green and red lines), as was observed empirically (black and gray lines). Model could adequately reproduce observed effects of overexpressing synapsin on rate of activity-dependent synaptic depression. C: extent to which modeled terminal recovered from depression shown in B after 5 min of quiescence was very similar to the empirical observations, both for the control condition (left) and the synapsin overexpression condition (right). D: as with spontaneous recovery data shown in C, the mathematical model could adequately reproduce extent of 5-HT–induced dedepression, as well as potentiation of 5-HT effects by overexpression of synapsin.
stimulation protocols, which could trigger different recovery mechanisms. At the sensorimotor synapse, synapsin did not affect spontaneous recovery (Fig. 5). A similar observation was made in the crayfish after 3-Hz stimulation (Dearborn et al. 1998). This result suggests that, in several systems including *Aplysia*, the regulation of vesicles responsible for spontaneous recovery of release is synapsin-independent. In our model, spontaneous replenishment of the release pool occurs through a vesicle flux driven by a concentration gradient between feeding and readily releasable pools. We propose that a similar synapsin-insensitive process operates at the sensory-motor synapse and restores the RRP. This hypothesis is in agreement with the unchanged endocytosis observed in synapsin I knockout mice (Ryan et al. 1996).

In summary, we showed for the first time that synapsin contributes to not only homosynaptic but also heterosynaptic plasticity in *Aplysia*. Given the importance of heterosynaptic modulation in both the invertebrate and vertebrate nervous systems (Bailey et al. 2000; Marder 2006), our study raises the interesting possibility that synapsin may be an important mediator of the actions of neuromodulators in other systems.

**Acknowledgments**

We thank E. Antzoulatos, D. Baxter, and M. Byrne for help with computer simulations, E. Kartenkingerum and J. Liu for preparing the cultures, H. Vishwasa for advice on the FM4-64 experiment, N. Yadav for help with some of the data analysis, B. K. Kaang of Seoul National University for the pNEX6-HA vector, and K. Martin of UCLA for the anti-VAMP antibody.

**Grants**

This work was supported by National Institute of Neurological Disorders and Stroke Grants R01 NS-19895 and P01 NS-38310.

**References**


Synapsin mediates short-term facilitation


