Synapsin dispersion and reclustering during synaptic activity

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Presynaptic modulation of synaptic transmission provides an important basis for control of synaptic function. The synapsins, a family of highly conserved proteins associated with synaptic vesicles, have long been implicated in the regulation of neurotransmitter release. However, direct physiological measurements of the molecular mechanisms have been lacking. Here we show that in living hippocampal terminals, green fluorescent protein (GFP)-labeled synapsin Ia dissociates from synaptic vesicles, disperses into axons during action potential (AP) firing, and reclusters to synapses after the cessation of synaptic activity. Using various mutated forms of synapsin Ia that prevent phosphorylation at specific sites, we performed simultaneous FM 4-64 measurements of vesicle pool mobilization along with synapsin dispersion kinetics. These studies indicate that the rate of synapsin dispersion is controlled by phosphorylation, which in turn controls the kinetics of vesicle pool turnover. Thus synapsin acts as a phosphorylation-state-dependent regulator of synaptic vesicle mobilization, and hence, neurotransmitter release.

Synaptic vesicle availability and mobilization are crucial elements in the regulation of synaptic transmission and synaptic plasticity. Synapsins, a family of highly conserved neuronal phosphoproteins that are specifically associated with synaptic vesicles[1], have been implicated in the regulation of neurotransmitter release by controlling the number of vesicles available for exocytosis.

Synapsins exist in all organisms with a nervous system, and are encoded by three distinct genes, synapsin I, II and III, in most vertebrates[2–6]. Synapsins are the most abundant synaptic vesicle proteins, with synapsin I alone accounting for 6% of total vesicle protein[1,7]. They are present in nearly all presynaptic nerve terminals, but different neurons have a distinct repertoire of different synapsins[1,6,8–10]. The high abundance, the specific association with synaptic vesicles, the highly conserved features, as well as the widespread distribution at nerve terminals, all signify synapsins as important and evolutionarily conserved regulatory proteins in synaptic transmission.

Biochemical studies have revealed multiple regulatory roles of synapsins. *In vitro* studies have shown that synapsins can interact with lipid and protein components of synaptic vesicles, as well as various cytoskeletal proteins, such as actin, spectrin and microtubules, in a phosphorylation-dependent manner[1,11–24]. These studies suggest that synapsins would move dynamically in response to physiological stimuli, and have led to the following hypothesis: binding of synapsins to synaptic vesicles prevents neurotransmitter release, and during synaptic activity, synapsins are phosphorylated, dissociate from synaptic vesicles and allow vesicles to mobilize and fuse with the plasma membrane[1,20,23,24].

Given the abundance and conservation of the synapsin family of proteins, it is interesting that mice with genetic perturbations to specifically remove synapsin genes are viable and have relatively limited behavioral phenotypes[25–27]. Although there are clear differences in synaptic physiology and presynaptic ultrastructure in the mutant animals, the lack of lethality and the modest effect on a number of learning protocols in the mutants have cast some doubt on the original hypothesis of synapsin function. However, as the primary role of synapsins that has been proposed is a regulatory one, it would be difficult to predict how loss of a layer of regulation would be manifested.

To more fully determine the mechanisms by which synapsins regulate synaptic function, we GFP-labeled synapsin Ia to examine the dynamic behavior of synapsin in response to action potential (AP) firing. We combined this approach with kinetic measurements of vesicle-pool turnover monitored by FM 4-64 to examine various mutants at the calcium-dependent phosphorylation sites of synapsin Ia in both wild-type and synapsin double knockout backgrounds. Here we demonstrate an activity-dependent dissociation of synapsin from synaptic vesicles and subsequent dispersion into the axon. The rate of the dispersion is regulated by calcium-dependent phosphorylation, which in turn controls the efficiency of vesicle pool turnover. These data thus provide a direct demonstration of the mechanism by which synapsins regulate the availability of synaptic vesicles for neurotransmitter release.

Results

Synapsin dissociates from vesicles during activity

A variety of *in vitro* biochemical studies imply that synapsins dynamically associate with and dissociate from synaptic vesicles in response to synaptic activity[1,20,21,24,28–30]. To study synapsin dynamics *in vivo*, we performed immunolocalization of synapsin and an integral synaptic vesicle protein, synaptophysin. Double immunostaining of synapsin and synaptophysin in hippocampal cell cultures at rest showed a typical

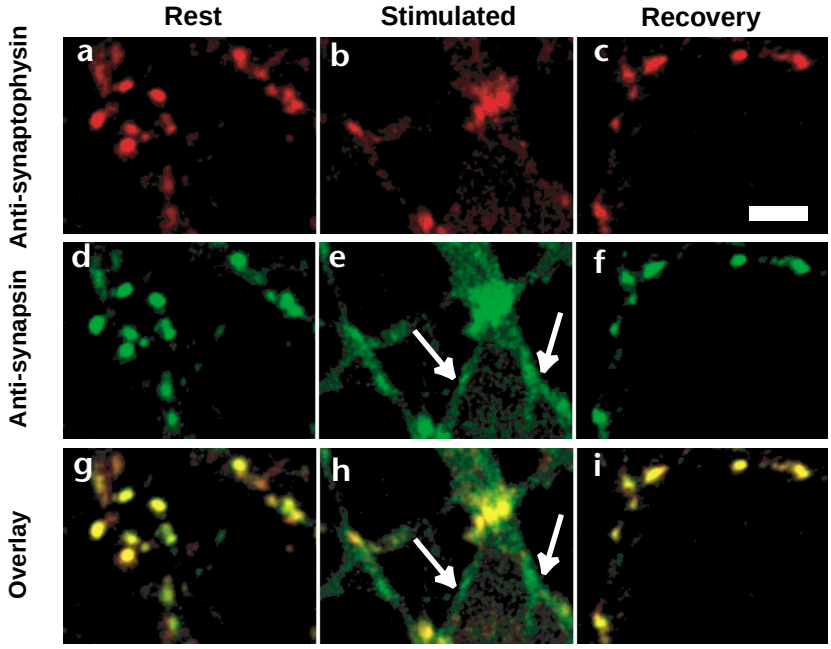

Fig. 1. Synapsin disperses from synaptic vesicles during activity. (**a**, **d**, **g**) Co-immunolocalization of synaptophysin (red; **a**) and synapsin (green; **d**) in hippocampal cell cultures fixed at rest show punctate staining for both markers (**g**). (**b**, **e**, **h**) In a parallel culture, fixed immediately after a train of a 900-AP stimulation at 10 Hz, synapsin staining (**e**, **h**) is much more diffuse than synaptophysin staining (**b**, **h**) indicating that synapsins dissociate from synaptic vesicles and redistribute into axons (arrows) in response to electrical stimulation. (**c**, **f**, **i**) In a different specimen fixed 10 min after stimulation, immunostaining of synapsin returns to a punctate pattern colocalized with synaptophysin (**c**, **i**), indicating a dynamic relocalization of synapsins from axons to synapses post-stimulation (**f**, **i**). Scale bar, 5 μm.

colocalized punctate staining pattern at the presynaptic nerve terminals (**Fig. 1a**, **d** and **g**). In parallel cultures fixed immediately following a train of 900 AP, the immunostaining of synaptophysin remained largely punctate, indicating that synaptic vesicles remained at nerve terminals (**Fig. 1b** and **h**). However, the immunostaining of synapsins became much more diffuse during stimulation (**Fig. 1e** and **h**), demonstrating a physical separation of synapsins from synaptophysin and suggesting that a substantial fraction of synapsins have dissociated from synaptic vesicles and redistributed into axons. The immunolocalization of synapsins returned to a punctate pattern in cultures fixed 10 minutes after stimulation (**Fig. 1f** and **i**), and it colocalized well with the immunostaining of synaptophysin (**Fig. 1c** and **i**). These observations demonstrate a dissociation of synapsin from synaptic vesicles and a dynamic movement in response to synaptic activity at nerve terminals.

Real-time measurements of synapsin dispersion

To measure the real-time dynamic movement of synapsin Ia at living hippocampal nerve terminals, we transfected neuronal cell cultures with a plasmid encoding GFP-labeled synapsin Ia. To test whether GFP-synapsin Ia behaves similarly to endogenous synapsins, we examined the localization of GFP-labeled synapsin Ia before, during and after synaptic activity. GFP-synapsin Ia, when expressed transiently in cultured rat hippocampal neurons, was targeted to nerve terminals (**Fig. 2a**), and appeared to have the same localization as native synapsins at rest (**Fig. 1d**). During a train of 900 AP stimulation, GFP-synapsin Ia fluorescence decreased at the nerve terminal and increased in the inter-bouton (axonal) region in a manner similar to that of endogenous synapsins (**Fig. 2a** and **b**). Ten minutes after stimulation, GFP-synapsin Ia reclustered within the nerve terminals, and appeared virtually indistin-

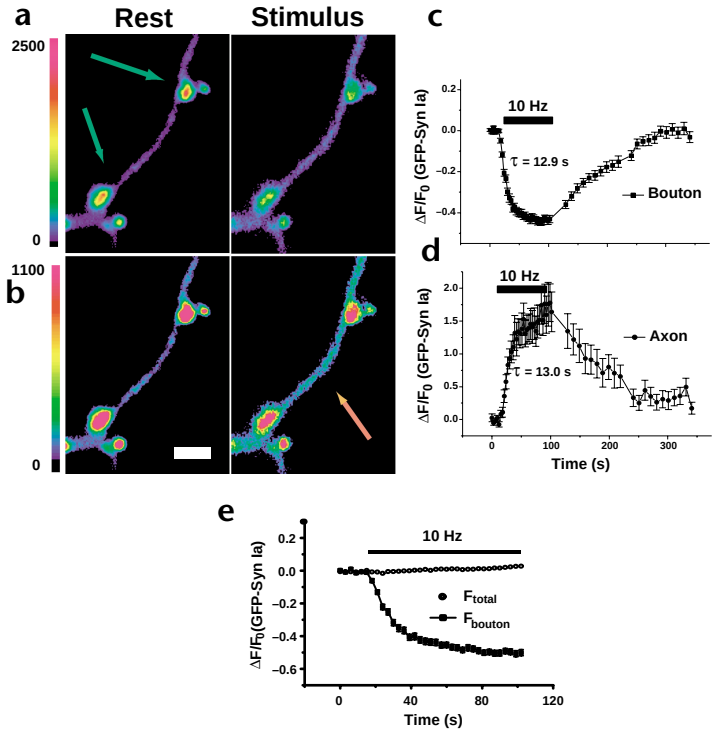

Fig. 2. Kinetics of GFP-synapsin Ia dispersion and recovery. (**a**) GFP-synapsin Ia fluorescence at synapses (green arrows) decreases upon stimulation. (**b**) GFP-synapsin Ia fluorescence in axons increases upon stimulation (red arrow). Same image as in (**a**) with different color scale to emphasize the fluorescence in the axon. The color scales show fluorescence intensity in arbitrary fluorescence units. Scale bar, 2 μm. (**c**, **d**) Time courses of fluorescence intensity at synapses (**c**) and in axons (**d**), during and following a train of 900 AP at 10 Hz (black bar). The decay of fluorescence intensity during stimulation, averaged over 43 boutons expressing GFP-synapsin Ia, is fit by a single exponential with τ = 12.9 s (**c**). During the same period of AP stimulation, GFP-synapsin Ia redistributes into axons with a similar rate constant, τ = 13.0 s (**d**). GFP-synapsin Ia reclusters within the terminal (**c**), and decreases in the axonal region (**d**) in approximately 4 min. (**e**) Time-lapse measurement of the total integrated fluorescence in the field of view and the fluorescence of GFP-synapsin Ia at synapses during a train of 900 AP field stimulation at 10 Hz (black bar). The total fluorescence remains relatively constant during stimulation, whereas the fluorescence intensity of GFP-synapsin Ia at synapses (n = 43) decreases.

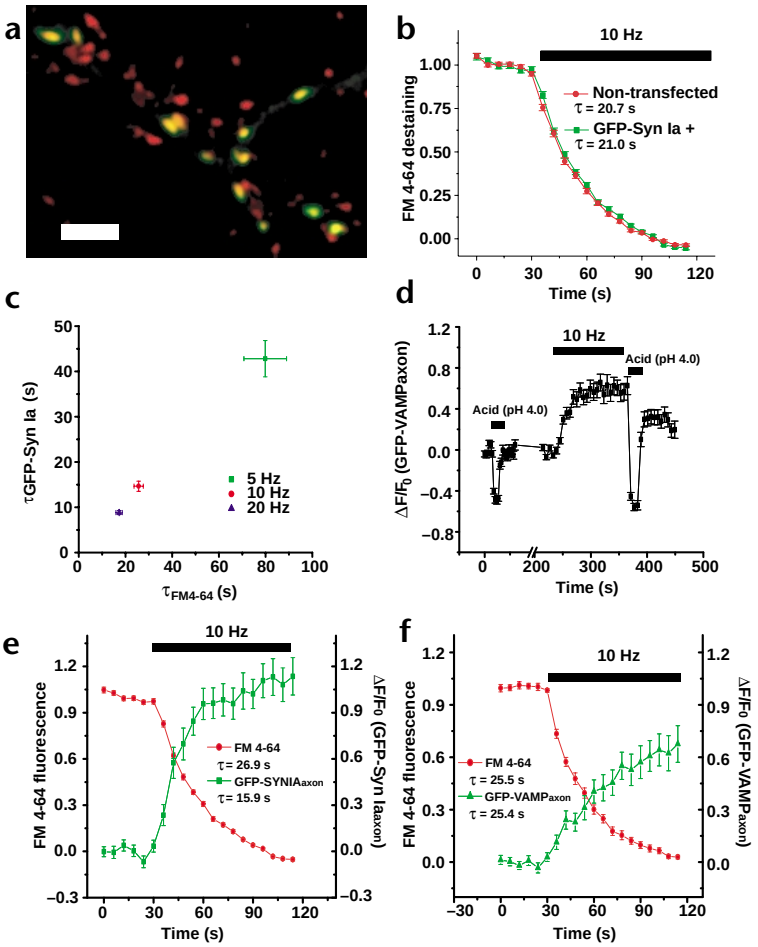

Fig. 3 Comparison of the dynamics of GFP-synapsin Ia dispersion and recycling vesicle turnover. (**a**) Colocalization of GFP-synapsin Ia-expressing boutons (green) with FM 4-64- loaded terminals (red), giving rise to yellow puncta, indicating that GFP-synapsin Ia-positive terminals functionally recycle vesicles. In addition, in the same field, there are boutons that do not express GFP-synapsin Ia, labeled by FM 4-64 only (red), which are used as internal controls in FM 4-64 destaining experiments. Scale bar, 5 μm. (**b**) Overexpression of GFP-synapsin Ia (wild type) does not affect the kinetics of vesicle pool turnover as assayed using FM 4-64 destaining. Destaining kinetics of FM 4-64-labeled synaptic vesicle pools during a train of 900 action potentials at 10 Hz (black bar) in GFP-synapsin Ia-positive (n = 40) and GFP-synapsin Ia-negative (n = 50) boutons are shown. Average time constants of FM 4-64 destaining in GFP-synapsin Ia-positive and negative boutons were τ = 21.0 s and τ = 20.7 s, respectively. Similar results were obtained in five of five experiments. (**c**) Frequency dependence of GFP-synapsin Ia dispersion and FM 4-64 destaining measured in GFP-synapsin Ia expressing nerve terminals. GFP-synapsin Ia dispersion kinetics and FM 4-64 destaining kinetics at different frequencies are well correlated over a 5–20 Hz stimulus frequency range (r = 0.99). Time constants of GFP-synapsin Ia dispersion and FM 4-64 turnover at different frequencies were averaged over 13–33 boutons pooled from 3 experiments. GFP-synapsin Ia disperses with statistically faster kinetics than FM 4-64 destaining at all frequencies tested (p < 0.002). (**d**) GFP-VAMP dispersion into the axon during AP firing is restricted to the plasma membrane. Before stimulation, a large fraction of GFP-VAMP in the axon is on the plasma membrane, and is quenchable by brief application of surface impermeant acid (pH 4.0). During stimulation (1,200 action potentials at 10 Hz), the concentration of GFP-VAMP increases at the axon. This additional stimulation-induced fluorescence remained quenchable with impermeant acid, indicating that the dispersion consisted entirely of surface GFP-VAMP and not vesicles simply dispersing into the axon. Axon regions analyzed, n = 51. (**e**) Comparison of GFP-synapsin Ia fluorescence (right y-axis) in the axon regions (n = 39) and simultaneous measurement of FM 4-64 turnover (left y-axis) at the GFP-synapsin Ia expressing nerve terminals (n = 40) during a train of 900 AP stimulation (black bar). The kinetics of the rise of GFP-synapsin Ia fluorescence in the axon (τ = 15.9 s) is much faster (p < 0.001) than that of FM 4-64 turnover (τ = 26.9 s), suggesting a physical separation of GFP-synapsin Ia from synaptic vesicles during activity. (**f**) Comparison of GFP-VAMP fluorescence (right y-axis) along the axon regions (n = 51) and simultaneous measurement of FM 4-64 turnover (left y-axis) at the GFP-VAMP-positive nerve terminals (n = 14) during a train of 900 AP stimulation (black bar). The kinetics of the rise of GFP-VAMP fluorescence along the axon (τ = 25.4 s) is very similar to that of FM 4-64 turnover (τ = 25.5 s). Similar results were obtained in three of three experiments.

guishable from GFP-synapsin Ia in a prestimulated culture (data not shown). GFP, when expressed alone, uniformly labeled the cell body, axons and dendrites, and showed no activity-dependent concentration change (data not shown). Quantitative analyses of the dynamic movement indicated that the concentration of GFP-synapsin Ia decreased by approximately 45% and reached a steady state at synaptic boutons during AP firing (**Fig. 2a** and **c**), coincident with a steep rise of GFP-synapsin Ia concentration in axonal regions (**Fig. 2b** and **d**); the total fluorescence in the whole imaging field remained relatively constant (**Fig. 2e**). Following stimulation, GFP-synapsin Ia reclustered within nerve terminals within approximately four minutes (**Fig. 2c**), with a gradual decrease of synapsin concentration in axons over the same time course (**Fig. 2d**). Stimulation in the absence of external calcium failed to disperse GFP-synapsin Ia (data not shown). These results indicate that this imaging approach provides a high-fidelity real-time measurement of the dynamic movement of synapsins in response to synaptic activity.

Synapsin Ia disperses faster than vesicle pool turnover

To examine the physiological relevance of the dynamic movement of synapsins at nerve terminals, we used FM 4-64 (ref. 31), a red-shifted variant of FM 1-43, to simultaneously monitor synapsin dispersion and synaptic vesicle turnover in GFP-synapsin Ia-expressing synapses. Loading of FM 4-64 by AP stimulation resulted in labeling all GFP-synapsin Ia-expressing synapses (**Fig. 3a**, yellow puncta), indicating that these synapses functionally recycle synaptic vesicles. The extent of FM 4-64 loading was similar in synapses either expressing or not expressing GFP-synapsin Ia (data not shown). Subsequent AP stimulation of FM 4-64-loaded nerve terminals resulted in dye destaining as synaptic vesicles fused with the plasma membrane and released the dye. GFP-synapsin Ia-expressing and non-expressing synapses in the same culture exhibited similar rates of FM 4-64 destaining (**Fig. 3b**), indicating that transient overexpression of GFP-synapsin Ia (wild-type) did not affect synaptic vesicle turnover at synapses. The kinetics of GFP-synapsin Ia dispersion at 10 Hz stimulation (τ = 12.9 s, **Fig. 2c**) was significantly faster than synaptic vesicle

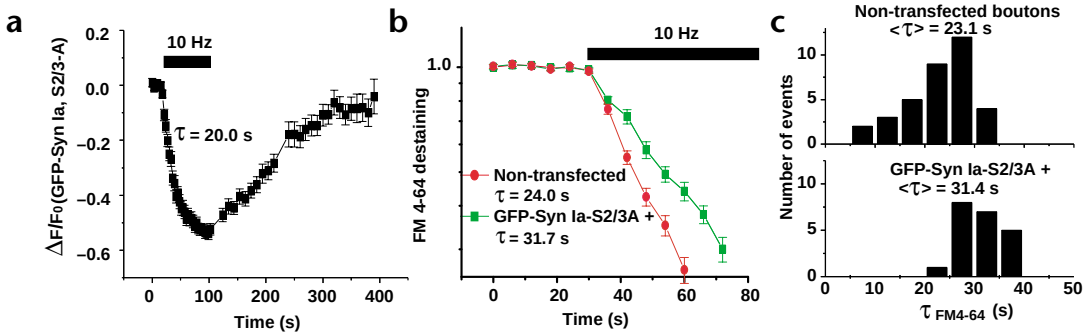

Fig. 4. Synapsin Ia is a phosphorylation-dependent negative regulator of vesicle pool turnover. (**a**) Mutations of serine to alanine at CaM kinase II sites 2 and 3 (S2/3A) slow the rate of GFP-synapsin Ia dispersion when expressed in rat hippocampal cell culture. Time course of fluorescence intensity at synapses, averaged over 20 boutons expressing GFP-synapsin Ia-S2/3A, during (dark bar) and following a train of 900 AP at 10 Hz. The dispersion kinetics of the GFP-synapsin Ia mutant during stimulation is much slower (τ = 20.0s) than wild type (**Fig. 2c**, τ = 12.9 s; $p < 0.001$). The time course of reclustering is similar to wild-type GFP-synapsin Ia (**Fig. 2c**). (**b**) Synaptic vesicle pool turnover monitored by FM 4-64 destaining is significantly slowed by the mutant form of GFP-synapsin Ia as compared to non-transfected boutons. A typical example is shown in this panel on a semi-log plot. The average time constant for FM 4-64 destaining during 1,200 AP (dark bar) in GFP-synapsin Ia-S2/3A-positive boutons (τ = 31.7 s, n = 21) is significantly larger than that in GFP-synapsin Ia-negative boutons (τ = 24.0 s, n = 35; $p < 0.001$). (**c**) Frequency distribution of FM 4-64 destaining time constants ($\tau_{FM\ 4\text{-}64}$) measured at individual GFP-synapsin Ia-S2/3A expressing and non-expressing boutons.

turnover assayed by FM 4-64 (τ = 21.0 s, **Fig. 3b**, $p < 0.001$). Stimulation at higher (20 Hz) or lower (5 Hz) frequency also revealed that kinetics of GFP-synapsin Ia dispersion were significantly faster than kinetics of FM 4-64 turnover (**Fig. 3c**, $p < 0.002$), and that the kinetics of synapsin Ia dispersion and vesicle pool turnover were well correlated in this frequency range (**Fig. 3c**). The difference in time scale between vesicle pool turnover and GFP-synapsin Ia dispersion provides additional evidence for a dynamic dissociation of synapsin Ia from synaptic vesicles during synaptic activity.

To further test the physical separation of synapsin from synaptic vesicles during stimulation, we specifically compared the increase of GFP-synapsin Ia fluorescence in axons with simultaneously monitored FM 4-64-labeled vesicle turnover. The kinetics of GFP-synapsin Ia appearing in axons (τ = 15.9 s) was much faster than that of FM 4-64 turnover (**Fig. 3e**, τ = 26.9 s, $p < 0.001$), indicating a physical separation of GFP-synapsin Ia from synaptic vesicles that precedes the fusion of synaptic vesicles with plasma membrane. We performed the same measurements for an integral-membrane protein of synaptic vesicles, VAMP, labeled with GFP on the lumenal domain along with FM 4-64 detaining kinetics. We previously showed that during stimulation, a small portion of the pool of VAMP disperses onto the axonal surface following stimulation[32]. Similarly, the GFP-VAMP signal increased to a small degree along the axonal region during stimulation. The kinetics of the appearance of GFP-VAMP in the axon, however, matched that of vesicle pool turnover very well (**Fig. 3f**), and was much slower than GFP-synapsin Ia dispersion ($p < 0.001$). Furthermore, the VAMP axonal signals measured in these experiments during stimulation were fully quenched by transient application of an impermeant acidic buffer (pH 4.0; **Fig. 3d**), in agreement with previous studies[32]. As similar applications of impermeant acidic buffer did not quench intracellular GFP (such as GFP-synapsin Ia fluorescence, data not shown), we conclude that the elevation in VAMP-GFP fluorescence along the axon during stimulation is confined to the plasma membrane and does not correspond to vesicles breaking away from presynaptic clusters. These findings, along with the differential immunolocalization of synaptophysin and synapsins in stimulated nerve terminals (**Fig. 1b**, **e** and **h**), strongly indicate that the measured synapsin Ia dispersion corresponds to dissociation of synapsin from synaptic vesicles and redistribution into the axon during activity in a step that precedes fusion of vesicles with the plasma membrane.

Phosphorylation regulates synapsin movement

Previous studies indicate that phosphorylation at site 1, a calcium-calmodulin-dependent kinase I/IV (CaM kinase I/IV) and protein kinase A (PKA) site, and at sites 2 and 3, CaM kinase II sites[33], leads to dissociation of synapsin Ia from synaptic vesicles[14,21,29]. Therefore, we specifically investigated the physiological functions of CaM kinase phosphorylation sites in synapsin Ia by directly mutating each of them from serine to alanine, an amino acid of similar size that cannot be phosphorylated. When both CaM kinase II sites were mutated to alanine (S2/3A), the kinetics of dissociation and dispersion of GFP-synapsin Ia-S2/3A from boutons during AP stimulation was slowed significantly ($p < 0.001$), with a τ of 20.0 s (**Fig. 4a**) as compared to a τ of 12.9 s observed with wild-type GFP-synapsin Ia (**Fig. 2c**). The kinetics of synaptic vesicle turnover as monitored by FM 4-64 was also significantly slowed in boutons expressing GFP-synapsin Ia-S2/3A (τ = 31.7 s) as compared to non-expressing boutons (τ = 24.0 s; **Fig. 4b** and **c**, $p < 0.001$). Similar observations were obtained from 7 other experiments with S2/3A; average $\tau_{dispersion}$ was 20.9 s and there was an approximately 22% slowing of FM 4-64 turnover (**Fig. 5a** and **b**, $p < 0.0001$). These results are consistent with studies that show decreased and increased synaptic transmission in squid giant synapse injected, respectively, with dephosphorylated synapsin Ia and CaM K II[34,35]. The time course of reclustering of GFP-synapsin Ia-S2/3A to nerve terminals was not significantly different from wild-type when averaged over eight experiments (data not shown).

To determine whether the different CaM kinase phosphorylation sites have differential effects on synaptic transmission, we measured the rate of synapsin dispersion and FM 4-64 destaining in rat hippocampal cultures transfected with various permutations of serine to alanine at the CaM kinase sites: S1A, S2A, S3A, S2/3A, and with all of sites 1, 2 and 3 mutated, S1/2/3A. All of these GFP-synapsin mutants except for S2A showed significant-

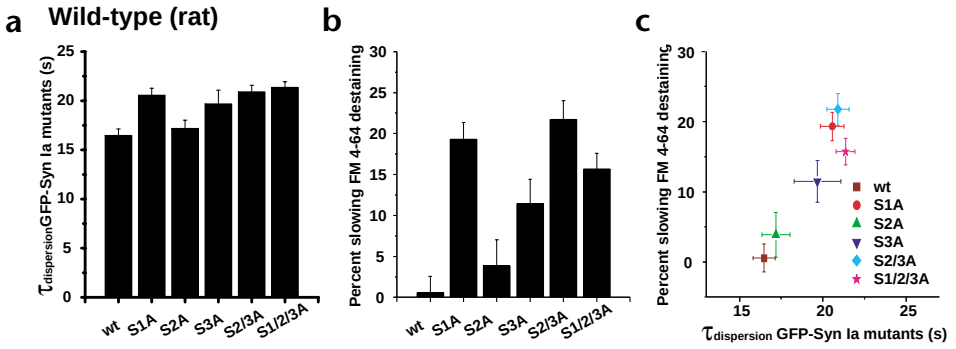

Fig. 5. Dispersion kinetics of GFP-synapsin Ia mutants regulate the efficiency of vesicle pool turnover in wild-type rat neurons. (**a**) Effect of different CaM kinase site mutants on the dispersion kinetics of GFP-synapsin Ia. Number of experiments for each specific mutation is as follows: wild type (wt), 6; site 1 (S1A), 6; site 2 (S2A), 3; site 3 (S3A), 3; sites 2 and 3 (S2/3A), 8; sites 1, 2 and 3 (S1/2/3A), 12. Except for S2A, all GFP-synapsin Ia mutants show statistically significant difference from wild-type GFP-synapsin Ia ($p < 0.02$). (**b**) Effect of various mutants on FM 4-64 destaining. The effect is calculated as the percentage increase of the time constant for FM 4-64 destaining of GFP-synapsin Ia-mutant-positive boutons compared to non-transfected boutons in the same experiment to reduce the effect of variability in different neuronal cultures. Typically, data from 15–30 transfected boutons and 40–50 non-transfected boutons were obtained in each experiment. Except for S2A, all other GFP-synapsin Ia mutants show statistically significant difference from wild-type GFP-synapsin Ia ($p < 0.03$). (**c**) Positive correlation between the dispersion kinetics and the effect on FM 4-64 destaining for various GFP synapsin Ia mutations.

ly decreased rates of dispersion and correspondingly decreased rates of FM 4-64 destaining compared to the wild type (**Fig. 5a** and **b**, $p < 0.03$ for each comparison). Furthermore, there was a strong correlation between how fast GFP-synapsin Ia and mutants dissociated from vesicles during AP firing and how fast the whole recycling vesicle pool could be mobilized to fuse with the plasma membrane; that is, as dispersion kinetics of the GFP-synapsin Ia mutant were slower, the effect was bigger on slowing synaptic vesicle turnover as monitored by FM 4-64 (**Fig. 5c**). These results strongly suggest that synapsin Ia is a negative regulator of neurotransmitter release, and that its function is controlled by calcium-dependent phosphorylation.

All three identified synapsins, I, II and III, can form homodimers[36]. Heterodimerization of synapsin I and II and of synapsin II and III (but not of synapsin I and III) have also been demonstrated by glutathione S-transferase (GST)-pull down or co-immunoprecipitation assays[36]. It seemed possible that some of the effects of GFP-synapsin Ia mutants observed in our transfection experiments in rat neuronal cultures might have been confounded by the presence of endogenous synapsins. To further determine the physiological function of synapsin Ia, we examined the CaM kinase phosphorylation site mutants in hippocampal cultures from synapsin I/II$^{-/-}$ mice in which interactions between transfected mutants and wild-type endogenous synapsins are minimal. Wild-type GFP-synapsin Ia dispersed in synapsin I/II (**Fig. 6a**) and wild-type mouse (data not shown) neuronal cultures with kinetics similar to those in rat cultures (**Fig. 5a**). The kinetics of vesicle pool turnover monitored by FM 1-43 was approximately 10% faster in synapsin I/II$^{-/-}$ synapses (unpublished data), consistent with the hypothesis that synapsin acts as a negative regulator of pool turnover. GFP-synapsin Ia-S2/3A and S1/2/3A mutants showed significantly slowed rates of dispersion and correspondingly decreased rates of FM 4-64 turnover in synapsin I/II$^{-/-}$ background (**Fig. 6a** and **b**, $p < 0.03$ for each comparison). The differential effect of various mutant synapsins on pool turnover kinetics does not seem to arise from differential expression levels or targeting efficiency of these mutant constructs, as the average fluorescence intensity of GFP-synapsin mutants at individual boutons was similar (data not shown).

The effect of either S1/2/3A or S2/3A mutations in GFP-synapsin Ia (**Fig. 6a** and **b**) was more pronounced when expressed in synapsin I/II$^{-/-}$ neuronal cultures than in wild-type mouse (data not shown) or rat cultures (**Fig. 5a** and **b**, $p < 0.0001$ for each comparison). This presumably reflects an interaction between GFP-synapsin Ia mutant and either endogenous wild-type synapsin I or endogenous wild-type synapsin II, when expressed in wild-type background. In contrast, the S1A mutation had a similar effect on FM 4-64 turnover in synapsin I/II$^{-/-}$ (**Fig. 6a** and **b**) and wild-type (**Fig. 5a** and **b**) neuronal cultures. This observation suggests that site 1 directly modulates binding affinities of synapsin Ia to synaptic vesicles, but not through interactions with endogenous synapsins, consistent with the observation that site 1 phosphorylation regulates the phospholipid binding of synapsins to synaptic vesicles[21]. Importantly, in the rat as well as in the synapsin I/II$^{-/-}$ mouse, there was a strong correlation between how fast GFP-synapsin Ia and its mutants dispersed and how fast FM 4-64 was released (**Figs. 5c** and **6c**). All of these observations not only point to synapsin Ia as a negative regulator of synaptic vesicle turnover, but also demonstrate two different levels of regulation exerted by different CaM kinase phosphorylation sites. The CaM kinase II sites seem to modulate the binding affinity of synapsin Ia to synaptic vesicles through interaction among synapsins, whereas the CaM kinase I/IV site (also a PKA site) seems to regulate the binding of synapsin Ia to vesicles directly.

Discussion

The studies presented here have allowed us to visualize the dynamic movement of a regulator of neurotransmission, synapsin Ia, during synaptic activity in living nerve terminals. This dynamic movement was regulated by the phosphorylation state of various CaM kinase sites of synapsin Ia, revealing the importance of the activities of CaM kinase I/IV and II on synapsin dynamics during well-defined periods of synaptic activity. Further, the correlation between the rate of dispersion of synapsin Ia mutants and the kinetics of vesicle pool turnover strongly suggest that synapsin Ia regulates neurotransmitter release via its dynamic interaction with synaptic vesicles.

Our observation of activity-dependent synapsin dispersion using immunohistochemistry corroborates a previous report[37]. However, in that study, it was concluded that dispersed synapsin remained associated with vesicle membranes. Here, using real-time measurements and detailed comparisons of synapsin dispersion, vesicle pool turnover and the redistribution of an integral membrane protein of synaptic vesicles (VAMP), we

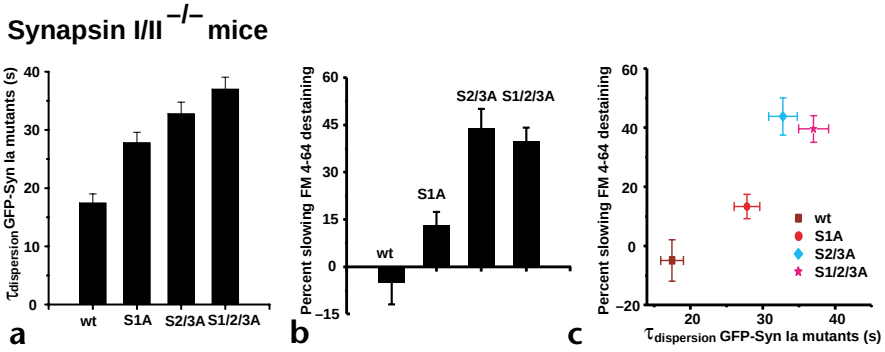

Fig. 6. Dispersion kinetics of GFP-synapsin Ia mutants regulate the efficiency of vesicle pool turnover in synapsin I/II$^{-/-}$ neurons. (**a**) Dispersion kinetics of GFP-synapsin Ia mutated at CaM kinase phosphorylation sites. The number of experiments for each mutation is as follows: wt, 2; S1A, 3; S2/3A, 4; S1/2/3A, 7. Abbreviations are as in Fig. 5. All GFP-synapsin Ia mutants tested showed significantly slower dispersion kinetics than wild-type GFP-synapsin Ia ($p < 0.001$). (**b**) Effect of different mutations on FM 4-64 turnover. All mutants show statistically significant difference from wild-type GFP-synapsin Ia ($p < 0.03$). (**c**) Positive correlation between the dispersion kinetics and the effect on FM 4-64 destaining kinetics for various GFP synapsin Ia mutations when expressed in the absence of endogenous synapsin I and II.

demonstrated that a large fraction of synapsin must dissociate from vesicles during activity.

The phenotypes of synapsin I-, II- and I/II-deficient mice characterized by postsynaptic electrophysiological measurements[25–27] are considered mild. Further detailed analyses of presynaptic vesicle recycling of synapsin I-deficient mice by FM 1-43 only reveal reduced vesicle pool turnover with brief trains of AP stimulation and reduced total recycling pool size, whereas other parameters including endocytosis and repriming are unaltered[38]. However, it is possible that the phenotypic changes in these mice are alleviated by functional redundancy of synapsin III[4] and by long-term compensatory changes in the levels of other nerve terminal proteins as demonstrated in synapsin I/II deficient mice[26].

Our studies demonstrate a link between dissociation of synapsin Ia and the ability of vesicles to mobilize and fuse with the plasma membrane during AP firing. However, several phenomena remain to be explained. First, it is not known if the dispersion and reclustering of synapsins following phosphorylation are driven by free diffusion or by an active transport process. Second, we do not understand why there is still dispersion in a fully mutated S1/2/3A form of synapsin when expressed in a synapsin I/II$^{-/-}$ background. This result suggests that in addition to the CaM kinase sites, an unknown activity-dependent modification of synapsin modulates its dissociation from vesicles. This could potentially be achieved through other phosphorylation sites, or through calcium-dependent binding of ATP[39,40]. Third, the finding that the concentration of GFP-synapsin Ia decreases only by approximately 45% before reaching steady state during AP firing at nerve terminals (**Fig. 2c**) suggests that a portion of synapsins do not dissociate from vesicles, as has been suggested by *in vitro* experiments[21]. Analysis of fluorescence intensity distribution of a freely diffusing cytoplasmic volume marker (cytoplasmically expressed GFP) suggests that if all GFP-synapsin dissociated, the extent of dispersion would be greater than approximately 75–80% (data not shown). What prevents the remaining GFP-synapsin Ia from dispersing? Whether this is correlated with a non-recycling pool of vesicles or whether there is something else in addition to calcium-dependent phosphorylation that is required for synapsin dispersion remains to be determined.

Although previous knockout analysis clearly demonstrated that synapsin I and II alone cannot account for the clustering of vesicles at active zones, the studies presented here indicate that synapsins control the availability of vesicles through their ability to dissociate from synaptic vesicles in a phosphorylation-dependent manner. Thus, synapsins provide a phosphorylation-mediated layer of control over presynaptic function by their ability to control vesicle availability at the nerve terminal during repetitive AP firing.

Methods

cDNA subcloning and site-directed mutagenesis. EGFP-synapsin Ia fusion protein was generated by subcloning the rat synapsin Ia cDNA into the pEGFP-C1 vector (Clontech, Palo Alto, California). Site-directed mutagenesis was done using the Stratagene (La Jolla, California) QuikChange site-directed mutagenesis kit.

Hippocampal cell culture and transfection. Synapsin I/II$^{-/-}$ mice were generated by homologous recombination[41]. Hippocampal CA3–CA1 regions were dissected from 3–4-day-old Sprague–Dawley rats, and 0–1-day wild type and synapsin I/II$^{-/-}$ mice. The dissected hippocampal regions were then dissociated, plated and cultured as described[42]. Transfections of GFP-synapsin Ia and its phosphorylation-site mutants as well as GFP-VAMP were done using calcium phosphate precipitation as described[43], and performed on 7–8-day-old neuronal cultures. All animal experiments and use were approved by the Institutional Animal Care and Use Committee of the Weill Medical College of Cornell University.

Immunocytochemistry. Individual dishes of 2–3-week-old rat hippocampal cultures were processed for immunofluorescence of both synaptophysin and synapsins after fixation in 4% paraformaldehyde (Electron Microscopy Science, Washington, Pennsylvania), 1× PBS, and 0.041% sucrose for 15 min. Dishes were separated into groups and subjected to 1 of 3 conditions immediately before fixation: control, 900 AP field stimulation, or 900 AP field stimulation followed by 10-min rest period. Cells were then permeabilized in the same fixative plus 0.25% Triton for 15 min, blocked in 10% BSA for an hour at 37°C and incubated overnight with both monoclonal anti-synaptophysin antibody (clone SVP-38, Sigma, St. Louis, Missouri) and affinity-purified anti-synapsin Ia, IIa and IIIa[10] polyclonal antibody (G-304). Cells were then incubated with an Alexa 488-labeled anti-mouse IgG and an Alexa 546 anti-rabbit IgG secondary antibody (Molecular Probes, Eugene, Oregon) for an hour, and mounted for observation. Image analysis was performed using a blinded procedure without knowledge of the experimental treatment.

Experimental conditions. Experiments were performed on 5–10-day post-transfected cultures. Coverslips were mounted in a perfusion chamber equipped with field stimulation electrodes on the stage of a custom-built laser-scanning confocal microscope as described[42]. Unless otherwise noted, cells were perfused at room temperature (~24°C) in a saline solution consisting of 119 mM NaCl, 2.5 mM KCl, 2 mM $CaCl_2$, 2 mM $MgCl_2$, 5 mM HEPES (pH 7.4), 30 mM glucose, 10 μM 6-cyano-7-nitroquinoxaline-2,3-dione (CNQX) and 50 μM D,L-2-amino-5-phosphonovaleric acid (APV). Acidic-solution with final pH

of 4.0 was prepared by replacing HEPES in the standard saline with MES (pK_a 6.1), all other components in the saline remaining unchanged. Synaptic vesicle pools were labeled by field-stimulating cultures for 30 s at 20 Hz in the presence of FM 4-64 in normal saline. An additional 60 s of dye exposure was allowed to ensure complete labeling of all recycling vesicles. The cultures were subsequently rinsed in dye-free solution for 10 min before dye destaining. Unless otherwise stated, all reagents were obtained from Sigma.

Optical measurements, microscopy and analysis. Laser-scanning fluorescence images were acquired as described[32]. Quantitative measurements of fluorescence intensity at individual boutons and neighboring axonal regions were obtained by averaging a 4×4 area of pixel intensities. FM 4-64 destaining data were normalized to the total loss of fluorescence during the train of AP, determined by subtracting the average of the final three time points from that of the first five time points before stimulation for each individual bouton. Time constants for FM 4-64 destaining were obtained by fitting the destaining curves to single exponential decays. The fluorescence change of GFP-synapsin Ia and its mutants either at synaptic boutons or in axons were normalized to the starting fluorescence (F_0) at individual selected region. The time constants for dispersion of GFP-synapsin Ia wild type and mutants were determined similarly by fitting the dispersion curve to single exponential decay, or in the case of minimal or very slow dispersion by directly taking the time point at 63% of the normalized fluorescence decrease.

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