

Synaptic Vesicle Recycling in Synapsin I Knock-out Mice

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Abstract. The synapsins are a family of four neuron-specific phosphoproteins that have been implicated in the regulation of neurotransmitter release. Nevertheless, knock-out mice lacking synapsin Ia and Ib, family members that are major substrates for cAMP and Ca²⁺/Calmodulin (CaM)-dependent protein kinases, show limited phenotypic changes when analyzed electrophysiologically (Rosahl, T.W., D. Spillane, M. Missler, J. Herz, D.K. Selig, J.R. Wolff, R.E. Hammer, R.C. Malenka, and T.C. Sudhof. 1995. *Nature (Lond.)*. 375:488–493; Rosahl, T.W., M. Geppert, D. Spillane, D., J. Herz, R.E. Hammer, R.C. Malenka, and T.C. Sudhof. 1993. *Cell*. 75:661–670; Li, L., L.S. Chin, O. Shupliakov, L. Brodin, T.S. Sihra, O. Hvalby, V. Jensen, D. Zheng,

J.O. McNamara, P. Greengard, and P. Andersen. 1995. *Proc. Natl. Acad. Sci. USA*. 92:9235–9239; see also Pieribone, V.A., O. Shupliakov, L. Brodin, S. Hilfiker-Rothenfluh, A.J. Czernik, and P. Greengard. 1995. *Nature (Lond.)*. 375:493–497). Here, using the optical tracer FM 1-43, we characterize the details of synaptic vesicle recycling at individual synaptic boutons in hippocampal cell cultures derived from mice lacking synapsin I or wild-type equivalents. These studies show that both the number of vesicles exocytosed during brief action potential trains and the total recycling vesicle pool are significantly reduced in the synapsin I-deficient mice, while the kinetics of endocytosis and synaptic vesicle repriming appear normal.

NEURONS use regulated secretion at specialized synaptic contacts to transmit information during patterns of electrical activity. Modulation of the probability of exocytosis during action potential firing is thought to underlie major forms of plasticity necessary for nervous system function. Determining the cellular processes that regulate vesicle exocytosis at presynaptic terminals is thus of central interest to neurobiology.

Although much progress has been made in identifying important components that participate in synaptic vesicle trafficking and secretion (for reviews see Scheller, 1995; Sudhof, 1995), the unambiguous assignment of these molecules to specific events in the presynaptic terminal remains a major challenge. Striking similarities have emerged upon comparison of molecular composition of presynaptic terminals with those of more general secretory pathways (Bennett and Scheller, 1993). This molecular homology suggests that many parallels exist between all secretory systems at the level of vesicle delivery, docking, fusion, and retrieval. Chemical synapses differ, however, in distinct ways from their secretory counterparts in other parts of the cell or in nonneural tissues. (a) Secretion is highly regulated, providing a tight coupling between action potential stimulation and neurotransmitter release.

(b) Synaptic vesicles mediating fast synaptic transmission are recycled locally after fusion with the plasma membrane. The recycling is accomplished in less than 1 min by a series of steps beginning with a very efficient endocytic retrieval of synaptic vesicle components and culminating with the regeneration of releasable synaptic vesicles (Heuser and Reese, 1973; Miller and Heuser, 1984; Valtorta et al., 1988; Ryan and Smith, 1995; for reviews see Betz and Wu, 1995; De Camilli and Takei, 1996).

In this report, we examine the roles of one of the most abundant phosphoproteins in the brain, synapsin I, by analyzing details of synaptic vesicle recycling in synaptic terminals from transgenic mice lacking this protein. The synapsins are specifically localized to synaptic vesicles in presynaptic nerve terminals (Valtorta et al., 1992). The absence of any members of this family in more ubiquitous secretory pathways suggests that synapsins participate in one or more vesicular trafficking events unique to synaptic terminals. Synapsins I and II are encoded by two distinct genes, each with two splice variants a and b (Sudhof et al., 1989). All four synapsins are good substrates for both cAMP-dependent protein kinase and Ca²⁺/Calmodulin (CaM) kinase I; however, synapsins Ia and Ib are additionally excellent substrates for CaM kinase II. Although a large body of evidence has implicated synapsin I in the regulation of neurotransmitter release (Greengard et al., 1993; Pieribone et al., 1995), recent studies of synapsin I-deficient mice using electrophysiological analyses of synaptic transmission revealed only limited phenotypic changes (Ro-

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sahl et al., 1993, 1995; Li et al., 1995). Electrophysiological assays of synaptic transmission generally measure the successful exocytotic events from a large and indeterminate number of synaptic inputs. As a result, many presynaptic details, such as the probability of release on a per terminal basis, as well as postexocytotic events in the synaptic vesicle cycle, remain hidden. In this report, we present measurements of several distinct steps in the synaptic vesicle cycle performed at individual synaptic terminals of both synapsin I-deficient mice and their wild-type counterparts based upon functional assays of synaptic vesicle recycling using the optical tracer FM 1-43.

This optical technique, originally developed by Betz and colleagues (Betz and Bewick, 1992, 1993; Betz et al., 1992), assays presynaptic function by quantitative fluorescence imaging of dye (FM 1-43) that is trapped in recycling synaptic vesicles (Henkel et al., 1996). The elegant studies of the Betz group demonstrated that this optical technique allows accurate measurement of activity-dependent exocytosis of vesicles at motor nerve terminals. Subsequent work established that FM 1-43 can be used in a similar fashion at synapses in hippocampal cell cultures (Ryan et al., 1993; Reuter, 1995; Ryan and Smith, 1995; Ryan et al., 1996), where it is also possible to measure several additional functional properties of synaptic vesicle recycling.

Here we demonstrate that both the number of vesicles caused to release their contents during very brief trains of action potentials and the total recycling vesicle pool are significantly reduced (60–70%) at synapses in hippocampal cultures derived from mice lacking synapsin I compared to their wild-type counterparts. The kinetics of endocytic reuptake of vesicle membrane externalized by brief trains of action potentials appear to be identical in the synapsin I knock-out and wild-type mice, with a $t_{1/2}$ of ~ 15 s, similar to that measured in rat hippocampal cultures (Ryan and Smith, 1995; Ryan et al., 1996). Finally, vesicle repriming, the minimum time required to return recently endocytosed membrane into a releasable synaptic vesicle, is unaltered in mice lacking synapsin I. These studies thus suggest that synapsin I plays a major role both in controlling the probability of vesicle exocytosis during action potential trains and in increasing the size of the entire vesicle pool. Our results do not support the notion that synapsin I is directly involved in the endocytic pathway of synaptic vesicle recycling or in the process of regenerating synaptic vesicles after endocytic retrieval (repriming).

Materials and Methods

Cell Culture

Synapsin I mutant mice were generated by homologous recombination (Chin et al., 1995). Offspring of littermates of wild-type and homozygous synapsin I mutant mice were used in all of the analyses, and the analyses were carried out by investigators without any knowledge of the genotype of the animal. Hippocampal CA1-CA3 regions were dissected from 2–3-d-old mice, dissociated and plated onto coverslips coated with Matrigel, and maintained in culture media consisting of minimal essential media (GIBCO BRL, Gaithersburg, MD), 0.6% glucose, 0.1 g/liter bovine transferrin (Calbiochem-Novabiochem Corp., La Jolla, CA), 0.25 g/liter insulin (Sigma Immunochemicals, St. Louis, MO), 0.3 g/liter glutamine, 5–10% FCS (Hyclone Labs, Logan, VT), 2% B-27 (GIBCO BRL), and 8 μ M cytosine β -D-arabinofuranoside. Cultures were maintained at 37°C in a 95% air, 5% CO₂ humidified incubator for 11–24 d before use. Results were

obtained from three litters, each of wild-type or synapsin I homozygous mutants.

Experimental Conditions

Coverslips were mounted in a rapid-switching, laminar-flow perfusion and stimulation chamber on the stage of a laser scanning confocal microscope. Test action potentials were evoked by passing 1-ms current pulses yielding fields of ~ 10 V/cm through the chamber via agar bridges and Ag-Ag-Cl electrodes. Except as otherwise noted, cells were continuously superfused at room temperature ($\sim 24^\circ\text{C}$) in a saline solution consisting of 119 mM NaCl, 2.5 mM KCl, 2 mM CaCl₂, 2 mM MgCl₂, 25 mM Hepes (buffered to pH 7.4), 30 mM glucose, and 10 μ M 6-cyano-7-nitroquinoxaline-2,3-dione (Research Biochemicals, Inc., Natick, MA). FM 1-43 (Molecular Probes, Inc., Eugene, OR) was used at a concentration of 15 μ M.

Optical Measurements, Microscopy, and Analysis

Scanning fluorescence images were acquired by averaging four frames obtained at a spatial sampling of 160 nm/pixel and a dwell time of 2 μ s/pixel through a 40X 1.3 NA objective using a modified laser scanning unit (model MRC 500; Bio-Rad Labs, Hercules, CA) coupled to a IM-35 inverted microscope (Carl Zeiss, Inc., Thornwood, NY). Quantitative measurements of fluorescence intensity at individual synapses were obtained by averaging a 4×4 area of pixel intensities centered about the optical center of mass of a given fluorescent punctum as illustrated in Fig. 2. Individual puncta were selected by hand, and the optical center of mass used to center the measurement box was computed over a slightly larger area (typically 6×6 pixels). All experiments and analyses were performed “blind” such that the experimenter had no knowledge of the genotype of the cells being used. Large puncta, typically representative of clusters of smaller synapses, were rejected during the selection procedure, as were any puncta that were not clearly discernible in all test episodes.

Results

FM 1-43 Loading and Unloading in Hippocampal Cultures from Synapsin I Knock-out and Wild-type Mice

To measure the turnover of synaptic vesicles induced by defined action potential trains, we used the fluorescent probe FM 1-43 and imaging and stimulation methods identical to those previously described (Ryan and Smith, 1995). Fig. 1 shows a schematic of the sequence of events used for most measurements of vesicle turnover. All measurements proceeded in two phases. The loading phase consisted of a period of vesicle exocytosis stimulated by a defined action potential train in the presence of FM 1-43. The FM 1-43 was left in the superfusate 1 min beyond the firing of action potentials. The extra 1-min exposure to dye ensured labeling of all of the vesicles retrieved during endocytosis (Ryan and Smith, 1995; Ryan et al., 1996). The unloading phase, performed after 5–10 min of rinsing in an FM 1-43-free solution, consisted of acquiring fluorescence images before (A) and after (B) a prolonged train of action potentials (90 s at 10 Hz) that released almost all of the fluorescence contained in vesicles (vesicular fluorescence). A measure of the vesicle turnover that occurred during the loading phase was obtained by calculating the difference in fluorescence intensity, ΔF , at individual boutons, between these two images. This two step approach corrected for possible nonspecific uptake of the dye by measuring only fluorescence of dye that was both taken up during a period of electrical activity and released during a subsequent one.

Fig. 2 A represents a dense matrix of axons, dendrites, and synapses in a preparation of cultured hippocampal

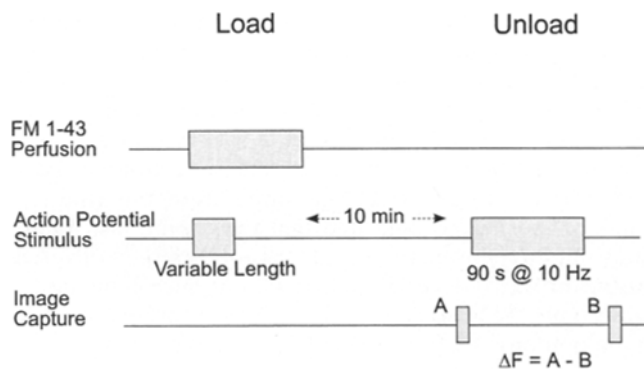


Figure 1. Schematic of protocols used to measure synaptic vesicle turnover. Vesicle turnover was measured during two phases. The protocol was designed to assay vesicle turnover evoked by varied lengths of field stimulation. Synaptic vesicles recycling in response to the stimulus were labeled by exposure to 15 μ M extracellular FM 1-43 (*Load*). Dye exposure continued for 60 s after the loading stimulus to allow ample time for the completion of vesicle endocytosis (Ryan and Smith, 1995). The second phase consisted of 90 s of 10-Hz stimulation in a dye-free solution to release the fluorescence from all vesicles stained during the preceding loading train (*Unload*). The datum we take to represent the synaptic vesicle release evoked by the loading-train action potentials (ΔF) was calculated from the difference between measurements before (*A*) and after (*B*) the train of action potentials used for unloading. This subtraction procedure was designed to distinguish fluorescence trapped in recycling vesicles from any nonreleasable background (see Fig. 2).

neurons from wild-type mice. Fig. 2 *B* is a fluorescence image (corresponding to *A* in Fig. 1) of the same field of view, acquired after the activity-dependent staining of synaptic boutons by a train of 50 action potentials (10 Hz for 5 s)

fired in an FM 1-43-containing perfusion solution (Fig. 1, *Load*) and subsequently rinsed in an FM 1-43-free solution for 10 min. Action potentials were evoked by uniform extracellular field stimulation. The glutamate receptor blocker 6-cyano-7-nitroquinoxaline-2,3-dione (10 μ M) was included in the perfusion solution to block recurrent excitatory activity and thus help assure that each stimulus pulse resulted in only a single action potential in each neuron.

We demonstrated previously that fluorescent puncta, like those in Fig. 2, *B* and *C*, correspond to focal accumulations of the synaptic vesicle marker synapsin I (Ryan et al., 1993) and therefore to presynaptic vesicle clusters. More recently, this identification has been confirmed using photoconversion methods (Henkel et al., 1996) and correlative electron microscopy (Ryan, T.A., and J. Buchanan, unpublished observations). Electron microscopy has also confirmed that small puncta like those shown in Fig. 2, *B* and *C*, correspond to single presynaptic active zones. Larger puncta, which are usually recognized by confocal imaging to consist of clusters of closely packed small puncta, were found by electron microscopy to encompass multiple active zones. To compare the properties of individual active zones between different genotypes more easily, such large puncta were excluded from analysis here. Usually, 15–50 measurable fluorescent puncta were discernible in each of the images acquired for the present study.

Fig. 2 *D* shows the same area as in Fig. 2 *C* after fluorescence was released by a long train of action potentials (90 s at 10 Hz) fired in an FM 1-43-free perfusion solution (corresponding to *B* in the *Unload* phase in Fig. 1). Note that almost all of the fluorescence (>90%) in the dye-loaded condition of Fig. 2 *B* has disappeared after this unloading stimulus train. The small, open squares drawn over the im-

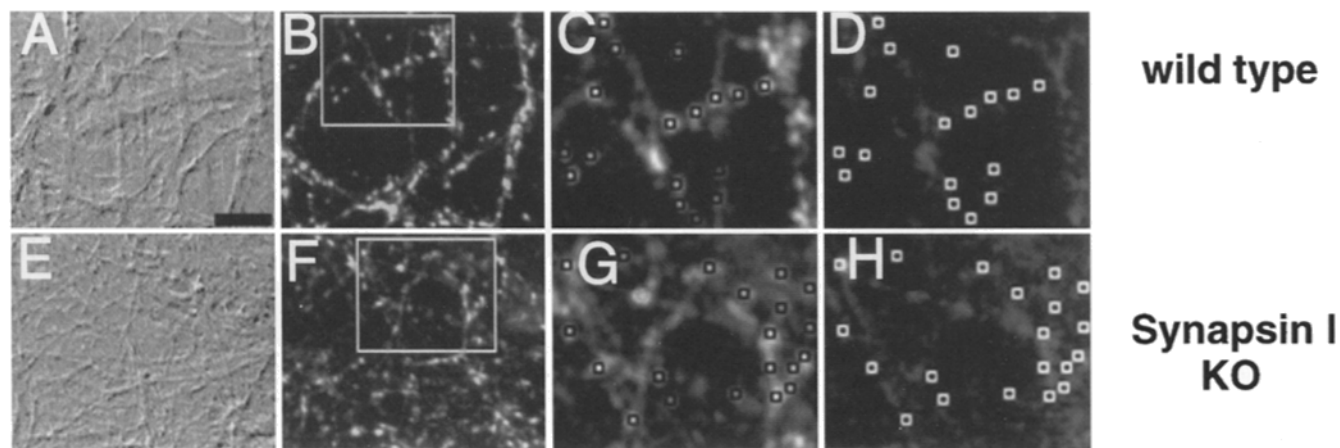


Figure 2. FM 1-43 uptake and release in hippocampal neurons cultured from synapsin I knock-out and wild-type mice. (*A*) A laser-scanned Nomarski DIC image showing a portion of one measurement field of a preparation of cultured hippocampal neurons from wild-type mice. As are typical of such preparations, fascicles of axons and dendrites form a dense mat against an astrocytic background. (*B*) Fluorescence image of the same field of view collected after a dye loading phase during which a train of 50 action potentials (at 10 Hz) was fired during a 1-min exposure to 15 μ M FM 1-43, followed by rinsing for 10 min in FM 1-43-free saline. Numerous discrete fluorescent puncta are clearly discernible. Earlier immunohistochemical work (Ryan et al., 1993) establishes that these puncta correspond to presynaptic active zone sites. (*C*) A magnified view (2X) of the area in *B* delineated by the rectangle. The boxes show typical measurement areas for fluorescence quantification, drawn over approximately half of the boutons in this field for illustration purposes. (*D*) The same field as in *C* after the dye was unloaded by firing a train of 900 action potentials (10 Hz) showing that almost all fluorescent dye has been released. (*E–H*) Same as in (*A–D*), but for synapsin I mutant mice. Note the reduced fluorescence intensity in *F* compared to *B*. Bar: (*A*, *B*, *E*, and *F*) 10 μ m.

ages of Fig. 2, *C* and *D*, indicate representative image measurement areas. To obtain a measure of vesicle turnover uncontaminated by the minor background staining that remains after maximal unloading stimuli, we calculated a fluorescence difference ΔF at individual boutons between measurements from images acquired just before (e.g., Fig. 2 *C*) and just after (e.g., Fig. 2 *D*) trains of action potentials in the unloading phase.

Note that since we measure ΔF by applying a very long train of action potentials to release virtually all of the fluorescence, the magnitude of this signal actually measures the efficiency of the loading phase rather than the efficiency of exocytotic release of vesicle content. Direct measurements of exocytotic release may also be obtained using a variation to the approach described. Previously published work (Ryan and Smith, 1995; and see below) demonstrated that extending the loading phase such that it includes a train of 900 action potentials effectively saturates the total recycling vesicle pool with FM 1-43. Direct quantitative measures of exocytotic release efficiency may then be obtained from terminals so labeled by measuring the loss of fluorescence evoked by unloading with action potential trains of various length.

A large body of previous work has established that synaptic vesicle exocytosis and endocytosis are closely coupled processes (Heuser and Reese, 1973; Miller and Heuser 1984; Valtorta et al., 1988). Recent work (Ryan and Smith, 1995) demonstrated that measurements of FM 1-43 release and uptake at hippocampal synapses provide quantitatively equivalent measures of vesicle turnover across a wide range of action potential stimulus conditions. It is therefore justifiable to choose either dye release or dye uptake as a measure of the number of synaptic vesicles caused to undergo recycling in response to electrical stimulation.

Fig. 2, *E–H*, represents measurements identical to those shown in Fig. 2, *A–D*, performed in this case on hippocampal cultures derived from synapsin I-deficient mice. Note the much dimmer fluorescence in Fig. 2, *F* and *G*, compared with that in Fig. 2, *B* and *C*. The results were consistent in all experiments performed comparing the two genotypes: the releasable fluorescence in the synapsin I-deficient mice was always ~60–70% of that obtained in wild-type mice for equivalent dye loading conditions (see below). The nonreleasable background, resulting from constitutive endocytic uptake of dye, was identical in the two genotypes and consisted of a fluorescence signal equal to ~7% of the total releasable fluorescence signal of a 50 action potential load in the wild-type synapses.

These results indicate that the magnitude of the vesicular turnover for equivalent trains of action potentials appears reduced in synapsin I-deficient mice compared to that for wild-type mice, although the overall recycling appears intact.

The Number of Vesicles Undergoing Exocytosis During a Train of 50 Action Potentials Is Diminished in Synapsin I-deficient Mice

Quantitative estimates of vesicular turnover evoked by trains of 50 action potentials confirm the qualitative results shown in Fig. 2. ΔF values were measured as de-

scribed above at a large number of individual presynaptic boutons in hippocampal cultures derived from both synapsin I-deficient and wild-type mice and presented in arbitrary fluorescence units. The frequency histograms of Fig. 3 *A* depict measurements from 135 wild-type and 145 synapsin I-deficient boutons. The data were normalized to the average value of ΔF measured from the wild-type mice. This analysis indicated that the overall distribution was shifted to lower values, i.e., the total amount of vesicle turnover was reduced in the knock-out mice compared to wild-type: the average amount of vesicle turnover in wild-type boutons is 1.0 ± 0.03 (SEM) and the median of the distribution was 0.89; the average amount of vesicle turnover in the synapsin I knock-out mice was 0.60 ± 0.02 (SEM) and the median of the distribution was 0.55.

Both to confirm that evoked vesicular release is reduced in knock-out mice and to verify that release and uptake measurements provide quantitatively similar measures of vesicle turnover, we used a direct release measurement protocol. Boutons were loaded by electrical stimulation with trains of 900 action potentials (at 10 Hz) in the presence of FM 1-43 and rinsed for ~10 min in dye-free solution. The amount of vesicular release was measured by acquiring two images, before and after partial unloading of the terminals with a train of 50 action potentials (at 10 Hz). Single bouton measurements of ΔF were performed as above. The results of experiments from both wild-type and synapsin I knock-out mice, depicted in Fig. 3 *B*, confirm that the amount of release in synapsin I knock-out mice is significantly diminished, as was concluded from the uptake measurements described above. Finally, to test the possibility that deficiency of release is apparent only at high stimulation frequencies, we repeated the release experiments from fully loaded terminals using a lower stimulation frequency (1 Hz for 50 s) to partially unload the terminals. No significant differences were found compared to the release measurements performed at 10 Hz (Fig. 3 *B*).

The Size of the Total Recycling Vesicle Pool is Reduced in Synapsin I Knock-out Mice

To determine the size of the total recycling synaptic vesicle pool, we measured the amount of FM 1-43 that can be loaded into individual presynaptic terminals during prolonged trains of action potentials. Previous measurements (Ryan and Smith, 1995) have shown that FM 1-43 uptake reaches steady state during trains of 900 action potentials in rat hippocampal boutons. Similarly, measurements of ΔF as a function of the length of loading train of action potentials indicate that the uptake of dye reaches steady state in 900 action potentials for both wild-type and synapsin I-deficient mice. Experiments were carried out as described in Figs. 1 and 2, with the following modifications: (a) Repeated measurements of dye loading were performed at the same boutons under saturating conditions (900 action potentials) as well as one or two other loads with shorter action potential trains. Uptake measurements for subsaturating loads (<900 action potentials) were normalized to the average of two bracketing runs of loads with 900 action potentials. Typically four different measurements were made from the same field. (b) Identical measurements were performed on boutons from syn-

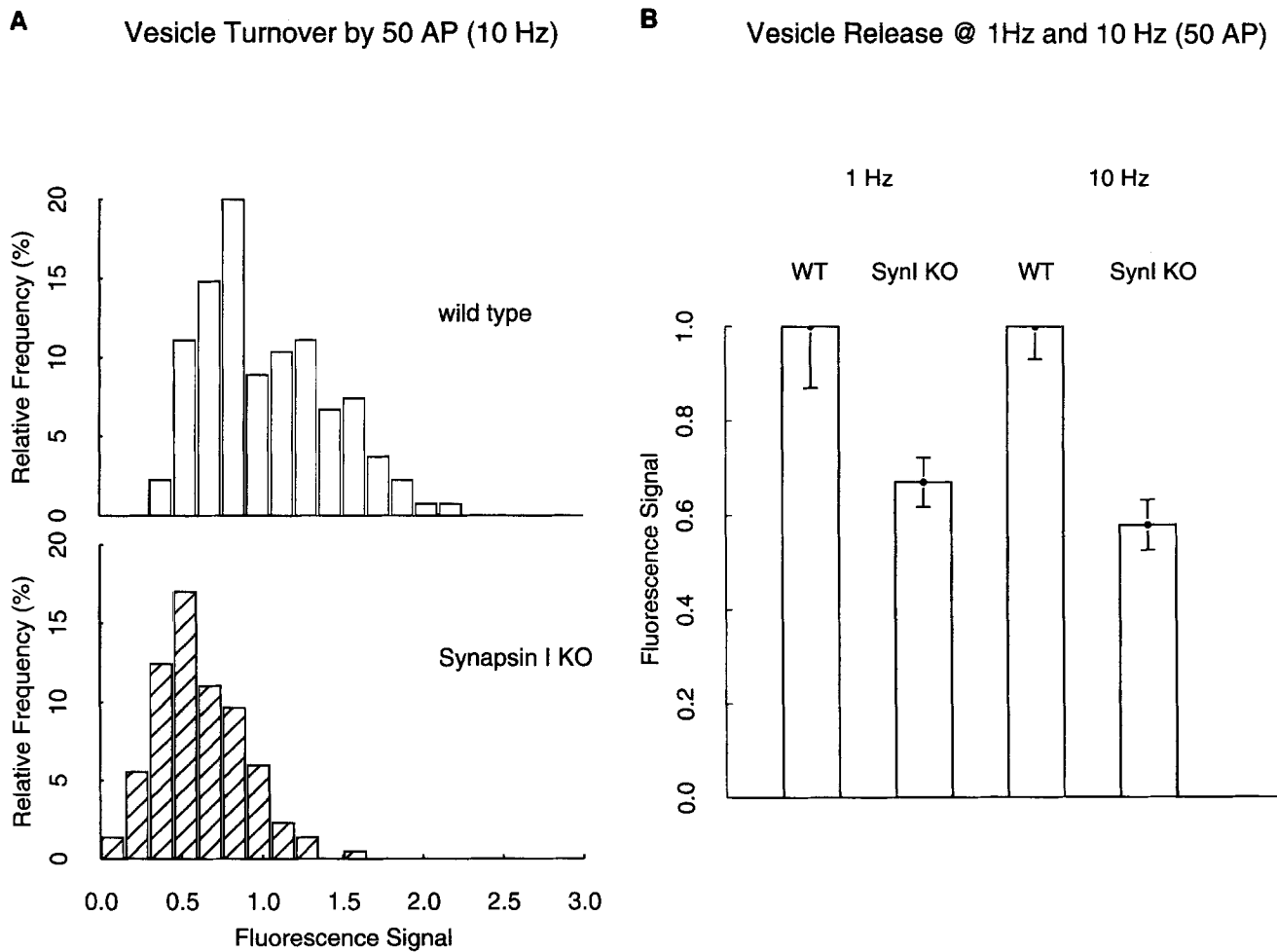


Figure 3. (A) Frequency distribution of vesicle turnover during a train of 50 action potentials measured at many individual synaptic terminals from wild-type and synapsin I mutant mice. The amount of vesicle turnover in a given synapse during the loading phase (50 action potentials in the presence of FM 1-43) was quantified by measuring the difference in fluorescence intensity between the two images (e.g., Fig. 2, C and D) before and after unloading by 900 action potentials. The fluorescence is averaged over a small area at individual puncta (see Fig. 2) over a large number of synaptic terminals in five different experiments for each genotype (one coverslip of cells for each experiment; $n = 135$, wild type; $n = 145$, synapsin I mutant). The mean values of the distributions are $\langle F \rangle = 1.0 \pm .033$ (SEM) and $\langle F \rangle = .60 \pm 0.021$ (SEM) for wild-type and synapsin I-deficient mice, respectively. (B) Measurements of FM 1-43 release during a train of 50 action potentials at 1 Hz and 10 Hz. Measurements were performed by loading the recycling vesicle pool to saturation with FM 1-43 during prolonged action potential trains in the presence of $15 \mu\text{M}$ FM 1-43 (900 action potentials at 10 Hz, see Fig. 4). After 5–10 min of washing in FM 1-43-free saline, two fluorescence images were captured before and after a train of 50 action potentials (1 Hz or 10 Hz), and the difference in fluorescence intensity at individual terminals was quantified. The data are presented as the amount of release normalized to the average fluorescence values obtained in the wild-type mice. The average amount of release measured at 1 Hz was $1.0 \pm .13$ (SEM, $n = 30$) and 0.67 ± 0.05 (SEM, $n = 50$) for wild-type and synapsin I-deficient mice, respectively. The average amount of release measured at 10 Hz was $1.0 \pm .07$ (SEM, $n = 30$) and 0.58 ± 0.05 (SEM, $n = 30$) for wild-type and synapsin I-deficient mice, respectively.

apsin I-deficient as well as wild-type mice, and the entire data set of the two genotypes was normalized to the average ΔF corresponding to the 900 action potential load of the wild-type terminals. These data are shown in Fig. 4, and indicate that although the kinetics of dye uptake are very similar, the magnitude of the steady state level of FM 1-43 uptake is significantly reduced in the knock-out compared to wild-type mice. For comparison, we have depicted two exponential curves (*dashed lines*) with identical time constants but different amplitudes. These data indicate that both the total pool size and the amount of vesicular release over a wide range of stimuli are reduced in syn-

apsin I-deficient mice to ~60–70% of those seen in wild-type.

Endocytosis Kinetics is Unaltered in Synapsin I Knock-out Mice

The kinetics of endocytic vesicle membrane reuptake after electrical stimulation was measured using a protocol identical to one previously published (Ryan and Smith, 1995; Ryan et al., 1996). As in Fig. 1, the measurement proceeded in two phases, a loading phase followed by an unloading phase in which maximal stimulation was used to

Vesicle Pool Size

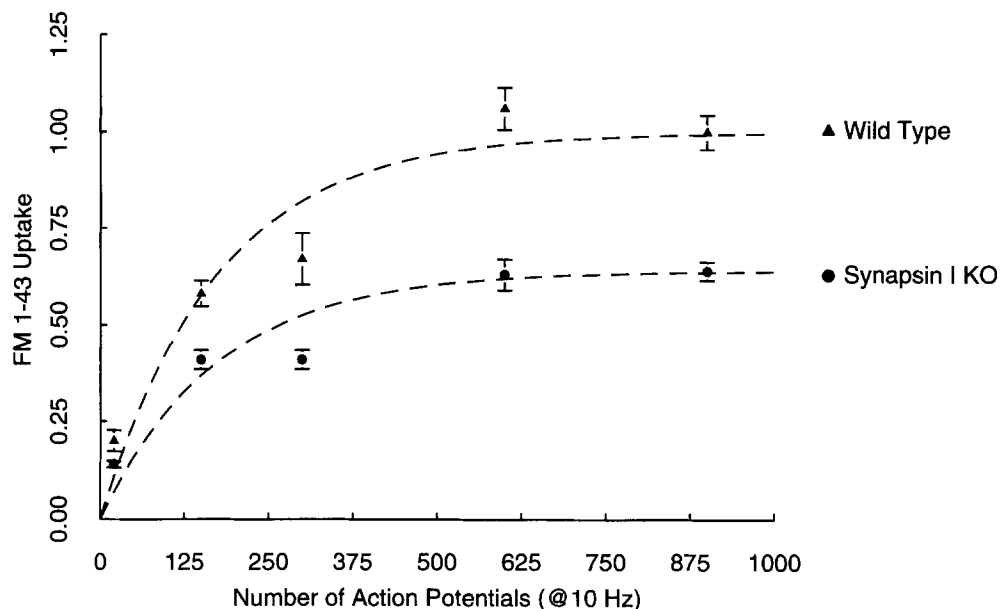


Figure 4. Functional synaptic vesicle pool size and vesicle turnover are reduced in synapsin I-deficient compared to wild-type mice. The kinetics of vesicle pool turnover were determined by measuring the amount of FM 1-43 taken up after vesicle release evoked by different length trains of action potentials fired at 10 Hz in wild-type mice (*triangles*) or synapsin I mutant mice (*circles*). Measurements were performed as in Figs. 1 and 2, with the exception that the number of action potentials fired during the loading phase was varied between 20 and 900. These data show that both the steady-state level of fluorescence loaded during prolonged trains as well as the amount loaded with the shortest trains are reduced to ~60–70% in synapsin I mutants compared to wild-type.

These results indicate that both the total recycling pool size as well as the average amount of vesicular release during brief trains is reduced in the absence of synapsin I. Vesicle turnover is normalized with respect to the wild-type, steady state, 900 action potential load. The dashed lines represent traces of curves derived from single exponentials with rise times of 175 action potentials plotted with an amplitude of either 1 or 0.64. The errors given are ± 1 SEM, where the number of boutons analyzed (n) for a given number of action potentials is as follows. Wild type: $n_{20} = 45$, $n_{150} = 69$, $n_{300} = 31$, $n_{600} = 67$, $n_{900} = 96$; synapsin-I knockout: $n_{20} = 62$, $n_{150} = 67$, $n_{300} = 66$, $n_{600} = 66$, $n_{900} = 114$.

quantify ΔF , the amount of dye taken up during the loading. Dye loading was performed by stimulating with a train of 50 action potentials (5 s at 10 Hz). To measure the kinetics of endocytosis, the amount of dye taken up at different times after the start of the stimulus train was measured by presenting FM 1-43 after a variable delay time Δt (Fig. 5, *Inset*) such that vesicles undergoing endocytosis during the variable delay period escape labeling while vesicles retrieved after the delay are labeled. Sequential measurements were performed, interspersing runs with $\Delta t \neq 0$ between bracketing runs with $\Delta t = 0$, correcting for possible fluctuations in the response. The relative amount of loading with $\Delta t \neq 0$ was normalized to the mean values of the bracketing runs, which measured the total amount of vesicle turnover for the given stimulus at a single bouton. The results, displayed in Fig. 5, show that the time course of synaptic vesicle endocytosis in wild-type and synapsin I-deficient mice are practically identical. Endocytosis lags exocytosis with a $t_{1/2}$ of 15 s, similar to that previously measured in rat hippocampal boutons (Ryan and Smith, 1995; Ryan et al., 1996). Here we have compared endocytosis rates for equivalent action potential trains in the two genotypes but different amounts of exocytosis (0.65 versus 1). Previous studies in rat hippocampal cultures (Ryan et al., 1996) have shown that the time course of endocytosis does not depend upon the magnitude of the exocytotic load over a much larger range (fivefold). These data thus imply that synapsin I does not directly participate in the endocytic retrieval of synaptic vesicles.

The Kinetics of Vesicle Repriming is Normal in the Absence of Synapsin I

A procedure for measuring the timescale of vesicle repriming, the interval that must elapse between vesicle membrane reuptake and the availability of stained vesicles for rerelease, has previously been described (Ryan and Smith, 1995). We repeated those measurements on synaptic boutons from both synapsin I-deficient and wild-type mice. The protocol is illustrated in the inset of Fig. 6. During the loading phase, FM 1-43 is introduced for a fixed period of time (30 s) coincident with the onset of a train of action potentials (10 Hz) that outlasts the exposure to the dye by a time interval Δt . Sequential runs with different periods Δt were measured at individual boutons to determine the gradual impact of the additional action potentials upon releasing the dye taken up during the dye exposure period. Single bouton measurements of ΔF , the net amount of dye remaining after the loading phase, were carried out as described in Fig. 1 by measuring the release of vesicular fluorescence in the unloading phase during a train of 900 action potentials. The unloading measurement was carried out after a 10-min rest after the loading phase. The data are normalized with respect to a run during which the stimulation was terminated precisely at the same time at which the extracellular dye was washed away ($\Delta t = 0$, ΔF_0). The ratio $(\Delta F_0 - \Delta F_{\Delta t})/\Delta F_0$ gives the fraction of fluorescence depleted by the action potentials fired during the period Δt . Fig. 6 depicts the results of such analyses per-

Endocytosis Kinetics

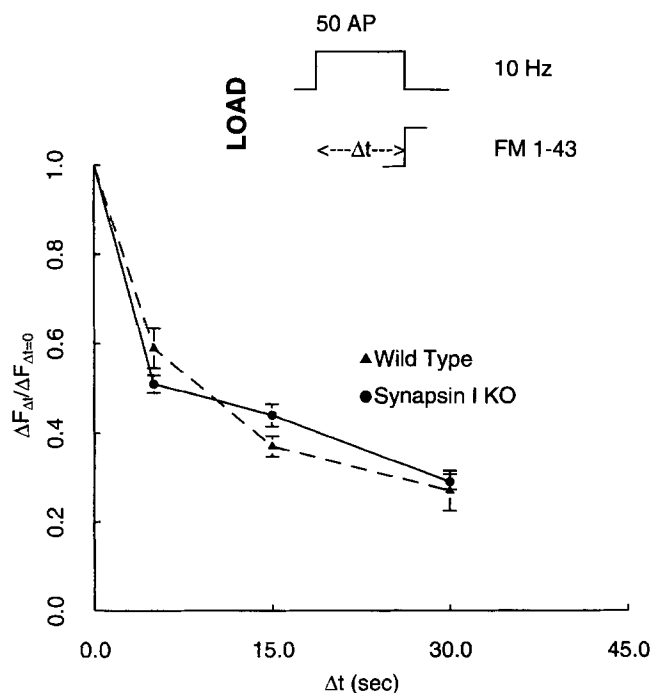


Figure 5. Synaptic vesicle endocytosis rates are unaffected in synapsin I mutants. The time course of endocytosis was determined by measuring the amount of dye uptake into synaptic terminals as a function of the delay time between the onset of a train of 50 action potentials (10 Hz) and the delivery of the dye (*inset*) for wild-type (*triangles*) and synapsin I mutant mice (*circles*). The data are normalized with respect to the amount of dye taken up for the $\Delta t = 0$ condition, i.e., in which all of the vesicles caused to fuse in response to the train of action potentials are labeled. Measurements were performed as in Figs. 1, 2, and 3 *A*, by first loading the terminals according to the protocol described and then quantifying the amount of fluorescence before and after a train of 900 action potentials used to unload the terminals (ΔF). A given time point was always measured between two bracketing runs with the $\Delta t = 0$ condition to compensate for possible fluctuations. Data were collected in three experiments for each genotype and represent averages \pm SEM for measurements of 30–100 boutons for each time point. The $t_{1/2}$ for endocytosis is ~ 15 s for both wild-type and synapsin I mutants, similar to that measured in rat hippocampal cultures (Ryan and Smith, 1995).

formed at synaptic boutons of cultured hippocampal neurons from both wild-type and synapsin I-deficient mice. The data for both mouse genotypes are similar to those previously reported for rat hippocampal cultures (Ryan and Smith, 1995). (*a*) During continuous action potential firing, very little, if any, fluorescence is depleted for at least 15 s after the dye has been rinsed away. (*b*) After the delay, $\sim 75\%$ of the dye taken up is released with kinetics similar to that of terminals after a period of inactivity. (*c*) The remaining $\sim 25\%$ is not releasable during continuous activity for up to at least 3 min but can be released after a 10-min period of inactivity.

Discussion

Synapsin I has been implicated in the regulation of neurotransmitter release in a variety of preparations (Llinas et al., 1985, 1991; Lin et al., 1990; Lu et al., 1992; Pieribone et al., 1995). Numerous *in vitro* studies have shown that synapsin I acts to cross-link synaptic vesicles with actin filaments in a phosphorylation-dependent manner (Bähler and Greengard, 1987; Petrucci and Morrow, 1987; Benfenati et al., 1993), a notion supported by electron microscopic observations of short bridging strands between synaptic vesicles and the cytoskeleton in nerve terminals (Landis et al., 1988; Hirokawa et al., 1989). Moreover, recent ultrastructural analyses have shown that the clustering of synaptic vesicles in synaptic terminals of synapsin I knock-out mice is dramatically reduced (Li et al., 1995; Takei et al., 1995) in the region >150 nm from the active zone.

Using the optical tracer FM 1-43, we have characterized several steps in synaptic vesicle recycling in synaptic terminals of cultured hippocampal neurons from both synapsin I-deficient and wild-type mice. This methodology has unique advantages over more conventional electrophysiological assays, as it allows the direct characterization of several subcellular processes critical to presynaptic function: (*a*) the single bouton release probability averaged over brief trains of action potentials, (*b*) the relative size of the total recycling vesicle pool, (*c*) the kinetics of endocytosis, and (*d*) the time scale of vesicle repriming.

We have shown that the total functional recycling vesicle pool size in synapses from synapsin I-deficient mice is reduced to $\sim 65\%$ of that in wild-type (Fig. 4). This is in agreement with ultrastructural analyses (Li et al., 1995; Takei et al., 1995) as well as studies of neurotransmitter release from synaptosomes (Li et al., 1995). Although it is also possible to explain our results by changes in the size, rather than the number, of recycling synaptic vesicles, morphometric analyses have shown that synaptic vesicle size is not altered in the knock-out mice (Li et al., 1995). Measurements of the kinetics of endocytosis (Fig. 5) as well as the time for vesicle repriming (Fig. 6) both indicate that the reduction in pool size is not the result of a deficiency in vesicle recycling. These results thus strongly support the notion that synapsin I plays an important role in maintaining the size of the total functional pool, perhaps by its ability to cross-link vesicles to each other (Benfenati et al., 1993) or to the cytoskeleton, maintaining them in clusters in apposition to zones of vesicle release.

We have shown that in individual synaptic boutons, the total number of synaptic vesicles which undergo exocytosis during brief trains of action potentials is reduced in the synapsin I knock out compared to wild-type mice (Figs. 3 and 4). This was true even for trains with as few as 20 action potentials (Fig. 4). This result was unexpected since stimuli in this range would be expected to draw solely upon the readily releasable pool of vesicles (<150 nm from the active zone), which is unaltered in the synapsin I knock out mice. Similar results were obtained at lower stimulation frequencies (1 Hz, Fig. 3 *B*).

Previous electrophysiological studies reported an increase in paired pulse facilitation in hippocampal synapses of mice lacking synapsin I (Rosahl et al., 1993) but no

Repriming Kinetics

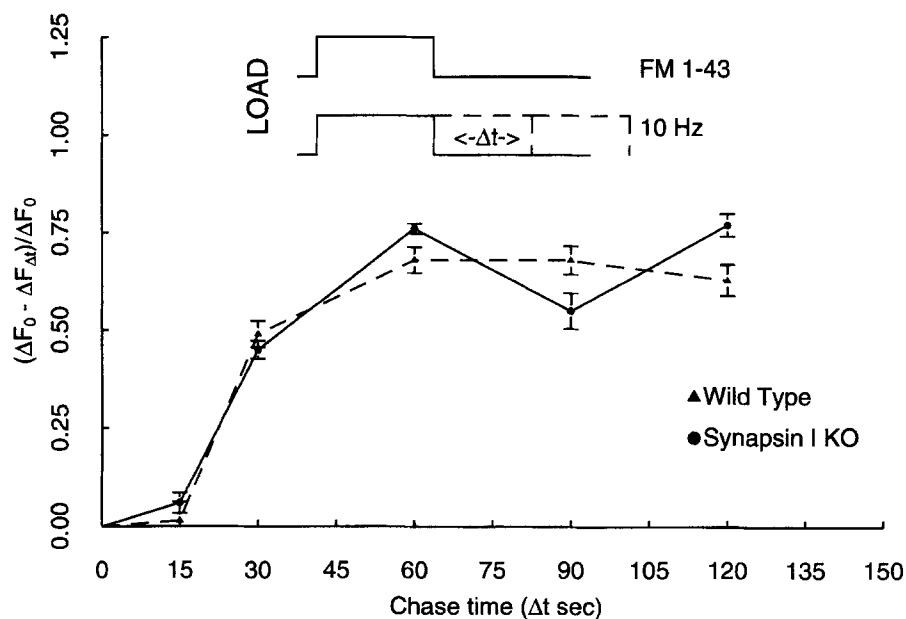


Figure 6. The kinetics of synaptic vesicle repriming is unaffected in synapsin I mutants. Repriming is defined as the time required to rerelease tracer dye taken up at the beginning of an action potential train during continuous stimulation. The time scale of this process reflects the minimum time required to return recently endocytosed membrane to a functional, releasable state. The measurements were performed according to the protocol depicted in the *Inset* in two phases. Synaptic terminals were loaded by a 30-s application of FM 1-43 simultaneously with a train of 10-Hz action potentials that exceeded the dye application by an amount of time Δt . The resulting loading in individual nerve terminals was assessed in an unloading phase by acquiring an image before and after a further electrical stimulation of 900 action potentials at 10 Hz after ~ 10 min of rest and washing. The difference in fluorescence intensity between these two images was measured at individual boutons. An estimate of the maximum amount loaded was obtained from the $\Delta t = 0$ run

(ΔF_0), whereas the impact of the additional action potentials interposed for a period Δt after the dye had been rinsed away was determined by measuring the remaining dye for these loadings ($\Delta F_{\Delta t}$). Collected data are shown for ($\Delta F_0 - \Delta F_{\Delta t}$) normalized to the value of ΔF_0 . Shown here are the results of three experiments for each of the synapsin I (circles) and wild-type (triangles) hippocampal cell cultures. Each time point consists of measurements from 45–65 individual boutons. Bars ± 1 SEM. The prominent feature of the repriming process is a minimum delay time of ~ 15 s during which no release occurs for recently endocytosed FM 1-43, in either the wild-type or synapsin I mutants, similar to that measured in rat hippocampal cultures (Ryan and Smith, 1995).

change in the rate of synaptic depression during continuous stimulation (Rosahl et al., 1995). Synaptosomal studies reported a decrease in neurotransmitter release using any of three different secretagogues (Li et al., 1995). Our data are consistent with all of these findings. An increase in paired pulse facilitation usually reflects a decrease in release probability for single action potentials, consistent with the observed depression of release during brief trains of action potentials (Figs. 3 and 4). The rate at which synaptic transmission is depressed during continuous stimulation is thought to be a function of both the release probability as well as the entire functional pool size. As reported here, both pool size and release probability decreased by similar amounts. Thus, the absolute amount of neurotransmitter released decreased, but the fractional release of the entire pool was unchanged (Fig. 4).

A model to explain the physiological data presented here is as follows: synapsin I acts to maintain the size of the reserve pool; the size of the releasable pool is directly related to the size of the reserve pool; in the absence of synapsin I, the reserve pool decreases in size, resulting in a commensurate decrease in the number of releasable vesicles, which is manifest as a decrease in release probability during action potential trains of varied duration. According to this model, the reserve and releasable pools are in equilibrium. This interpretation is consistent with the conclusions drawn from studies of synaptic transmission at neuromuscular junctions in the shibire mutant in *Drosophila* (Koenig et al., 1989). In that study, a temperature-

sensitive block in endocytic retrieval of synaptic vesicles was used to systematically reduce the size of the total vesicle pool. Analysis of the probability of vesicle exocytosis as a function of pool size revealed that the two are closely related in that system, suggesting an equilibrium between the total and immediately releasable pools. That model seems most consistent with the finding here that the amount of reduction in vesicular release in knock-out compared to wild-type mice is independent of the number of action potentials used to evoke the release (Fig. 4). At present, we can not completely reconcile this model with the ultrastructural data on wild-type and synapsin I knock-out mice. Previous analyses have shown that although the density of vesicles in a zone >150 nm from the active zone is significantly reduced (Li et al., 1995; Takei et al., 1995), the density in the zone proximal to the presynaptic membrane is only slightly diminished (Takei et al., 1995) or identical (Li et al., 1995) in synapsin I knock outs compared to wild-type mice. Thus, it appears to be possible to disperse the reserve pool without detectably altering the releasable pool. Clearly, further studies will be required to understand the dynamics of vesicle trafficking between the reserve and releasable pools.

Consistent with the idea that synapsins play a role in synapse-specific secretory pathways, we conclude that although synapsin I is not essential for neurotransmitter release, it plays an important role in maintaining the size of the total recycling vesicle pool and thereby in controlling the release probability during action potential trains. It

will be interesting to apply the comprehensive functional imaging methods used here to analyze various steps in the synaptic vesicle cycle in mice deficient in a variety of key molecular presynaptic components, including synapsin II.

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