Synapsins regulate use-dependent synaptic plasticity in the calyx of Held by a Ca\(^{2+}\)/calmodulin-dependent pathway

Jianyuan Sun*†, Peter Bronk*, Xinran Liu*†, Weiping Han*§, and Thomas C. Südhof*†¶

*Center for Basic Neuroscience, †Department of Molecular Genetics, and §Howard Hughes Medical Institute, University of Texas Southwestern Medical Center, 6000 Harry Hines Boulevard, Dallas, TX 75390-9111

Contributed by Thomas C. Südhof, December 30, 2005

Synapsins are abundant synaptic-vesicle phosphoproteins that are known to regulate neurotransmitter release but whose precise function has been difficult to pinpoint. Here, we use knockout mice to analyze the role of synapsins 1 and 2 in the calyx of Held synapse, allowing precise measurements of neurotransmitter release. We find that deletion of synapsins did not induce significant changes in spontaneous release or release evoked by isolated action potentials (APs) and did not alter the size of the readily releasable vesicle pool (RRP), the kinetics of RRP depletion, or the rate of recovery of the RRP after depletion. Deletion of synapsins, however, did increase use-dependent synaptic depression induced by a high-frequency stimulus train (\(\geq 50\) Hz). The increased depression was due to a decrease in the fraction of the RRP, whose release was evoked by APs late in the stimulus train. The effect of synapsin deletions was occluded by intracellular application of the Ca\(^{2+}\)-calmodulin (CaM) inhibitor, suggesting that Ca\(^{2+}\)/CaM-dependent phosphorylation of synapsins 1 and 2 may regulate the vesicle \(P_r\) during high-frequency neuronal firing.

Results

Kinetics of Synaptic Transmission. Immunoblots revealed that synapsins are abundantly expressed in the medial nucleus of the trapezoid body (MNTB) (see Fig. 6, which is published as supporting information on the PNAS web site). To determine whether deletion of synapsins 1 and 2 leads to a change in individual quantal release events, we analyzed miniature EPSCs (mEPSCs) by whole-cell recordings from MNTB neurons at rest in the presence of extracellular bicuculline (10 \(\mu\)M), strychnine (10 \(\mu\)M), and D-APV (50 \(\mu\)M). We detected no significant difference between WT and synapsin DKO calyces in the mean frequency, amplitude or total electrical charge of mEPSCs (Fig. 1 A and D–F). Thus, deletion of synapsins does not appear to alter the size and neurotransmitter content of vesicles, the kinetics of single vesicle fusion, or the vesicles’ propensity to fuse spontaneously.

We next compared EPSCs induced by presynaptic APs in WT and synapsin-deficient terminals (Fig. 1C). We elicited orthodromic APs by afferent-fiber stimulation in transverse brain slices. The amplitude, charge, and quantal content of the EPSCs were not significantly different between WT and DKO neurons (Fig. 1 G and H), suggesting that deleting synapsins 1 and 2 from the calyx synapse does not significantly change the amount and kinetics of neurotransmitter release during low-frequency stimulation.

Short-Term Plasticity. We applied 40 APs at 2–200 Hz, a physiological stimulation frequency for the calyx synapse (15), and recorded evoked EPSCs from medial nucleus of the trapezoid body neurons. During the stimulus trains, the initially large EPSC amplitudes rapidly declined to a steady-state depressed value (Fig. 2 A–C). Steady-state synaptic depression was reached after \(\sim 6–10\) APs in both WT and DKO synapses, allowing us to quantify the degree of depression as the ratio of the 40th to the 1st EPSC (the EPSC\(_{40}\)/EPSC\(_1\) ratio), revealing that synaptic depression during the stimulus train was inversely proportional to the stimulation frequency (16).

Conflict of interest statement: No conflicts declared.

Abbreviations: AP, action potential; APe, AP equivalent; CaM, calmodulin; DKO, double knockout; EPSC, excitatory postsynaptic current; mEPSC, miniature EPSC; MLCK, myosin light-chain kinase; RRP, readily releasable vesicle pool.

†To whom correspondence may be addressed. E-mail: jianyuan.sun@utsouthwestern.edu or thomas.sudhof@utsouthwestern.edu.

‡Present address: Level 8, Centros, Singapore 138658.

© 2006 by The National Academy of Sciences of the USA

PNAS is a registered trademark of the National Academy of Sciences.
At low stimulation frequencies, the EPSC$_{40}$/EPSC$_1$ ratio was similar between WT and synapsin-deficient terminals (Fig. 2A and D). At higher stimulation frequencies (≥50 Hz), however, deletion of synapsins enhanced synaptic depression, and the EPSC$_{40}$/EPSC$_1$ ratio was decreased =2-fold (Fig. 2B and D). Thus, consistent with previous observations (7), the synapsin DKO strongly enhances synaptic depression during repetitive stimulation.

**Size and Refilling of the RRP.** To measure the size of the RRP, we voltage-clamped calyx terminals at a holding potential of −80 mV (in the presence of 1 μM tetrodotoxin and 20 mM tetraethylammonium extracellularly and 125 mM cesium gluconate intracellularly) and used step depolarizations from −80 mV to +10 mV for 1–30 ms to induce Ca$^{2+}$ influx and neurotransmitter release. We then monitored Ca$^{2+}$ currents and membrane capacitance as a function of the step depolarizations (Fig. 3A) (14, 17–19). In WT and synapsin DKO terminals, the size of the depolarization-induced capacitance jumps increased with the duration of the voltage step until the depolarization reached 10 ms. Step depolarizations of >10 ms induced no further capacitance increase, indicating that the RRP is depleted after 10-ms Ca$^{2+}$ influx (14). We thus define the 20-ms depolarization as the “RRP depletion pulse” that evokes a capacitance change corresponding to the RRP size. We found that all depolarizations evoked similar capacitance jumps in WT and synapsin-deficient terminals [e.g., for 10- to 20-ms depolarizations, WT, ΔC$_{RRP}$ = 341 ± 73 fF (n = 21); DKO, ΔC$_{RRP}$ = 343 ± 64 fF (n = 22); Fig. 3B] and that the relationship between Ca$^{2+}$ influx and capacitance changes was indistinguishable between WT and synapsin-deficient terminals (Fig. 3D). These data show that deletion of synapsins 1 and 2 had no effect on the size of the RRP, the Ca$^{2+}$ current, and the apparent vesicular Ca$^{2+}$ affinity for release. Moreover, because the amplitude of EPSCs in response to isolated APs is unchanged in synapsin-deficient terminals (Fig. 1), these data imply that deletion of synapsins also does not alter the $P_I$ of the calyx synapse.

We next tested whether deletion of synapsins impairs the refilling of the RRP. We applied sequential 20-ms step depolarizations that were separated by increasing interstimulus intervals (Fig. 4A). The first pulse was designed to deplete the RRP and the second pulse to test how much of the RRP was refilled during the interstimulus interval. The ratio of the second to the first capacitance change reflects the replenishment of the RRP. Plots of this ratio vs. the interstimulus interval revealed the time course of RRP refilling that can be fitted by a two-exponential function (Fig. 4B). The refilling time constants thus determined were very similar between WT and synapsin-deficient terminals [WT, $\tau_1$ = 0.57 s (53%); $\tau_2$ = 35 s (47%); DKO, $\tau_1$ = 0.55 s (54%); $\tau_2$ = 37 s (46%)], suggesting that deletion of
open symbols are covered by the identically place filled symbols). Data were short interstimulus intervals.

B traces). (A) Experimental protocol (Upper) and representative Ca\(^{2+}\)-current and capacitance traces (Lower). Calyces were stimulated with two successive RRP depletion pulses (20-ms depolarization from \(-80\) mV to \(+10\) mV) applied with a variable interval (500 ms in the example shown; time scale applies to all traces). (B) The ratio between the capacitance changes induced by the second and first RRP depletion pulse, plotted as a function of the interstimulus interval (WT, filled symbols, \(n = 7\); DKO, open symbols, \(n = 7\); note that the open symbols are covered by the identically place filled symbols). Data were fitted with a double-exponential function [WT: \(r_1 = 0.57\) s, \(r_2 = 35\) s (solid line); DKO, \(r_1 = 0.53\) s, \(r_2 = 37\) s (dotted line)]. (Inset) Capacitance ratio at very short interstimulus intervals.

**Fig. 4.** Time course of recovery from RRP depletion in WT and synapsin DKO neurons. (A) Experimental protocol (Upper) and representative Ca\(^{2+}\)-current and capacitance traces (Lower). Calyces were stimulated with two successive RRP depletion pulses (20-ms depolarization from \(-80\) mV to \(+10\) mV) applied with a variable interval (500 ms in the example shown; time scale applies to all traces). (B) The ratio between the capacitance changes induced by the second and first RRP depletion pulse, plotted as a function of the interstimulus interval (WT, filled symbols, \(n = 7\); DKO, open symbols, \(n = 7\); note that the open symbols are covered by the identically place filled symbols). Data were fitted with a double-exponential function [WT: \(r_1 = 0.57\) s, \(r_2 = 35\) s (solid line); DKO, \(r_1 = 0.53\) s, \(r_2 = 37\) s (dotted line)]. (Inset) Capacitance ratio at very short interstimulus intervals.

Synapsins does not alter the rate of synaptic-vesicle recycling and repriming.

**Unchanged \(P_r\) in Synapsin-Deficient Terminals.** At a synapse, the amount of evoked release depends on the size of the RRP and the \(P_r\) (20–24). In the mouse calyx synapse, a 1-ms depolarization to \(10\) mV provides a stimulus that is equivalent to a single AP in terms of vesicle release and is, thus, defined as the AP-equivalent (APE) stimulation (25). We determined in WT and synapsin-deficient terminals the APE-evoked (\(\Delta C_{\text{APE}}\)) and the RRP depletion pulse-evoked capacitance jumps (\(\Delta C_{\text{RRP}}\)). Because neither parameter was significantly different between WT and synapsin-deficient terminals [Fig. 3C; WT, \(\Delta C_{\text{APE}} = 30\pm 7\) fF (7); DKO, \(\Delta C_{\text{APE}} = 32.6\pm 9.1\) fF (9); \(\Delta C_{\text{RRP}}\), see above], deletion of synapsins does not appear to alter the \(P_r\) in the calyx synapse.

**Reduction in the \(P_r\) During Synaptic Depression.** We applied a train of 40 APes at 50 Hz, followed by an RRP depletion pulse, and monitored the membrane capacitance throughout the experiment, allowing us to measure exocytosis during the stimulus train, to determine the residual RRP size after the train and to calculate the \(P_r\) at the end of the train (Fig. 5A). Because synaptic depression decreases the size of responses late in the train, we increased the signal-to-noise ratio of the capacitance measurements by determining the average \(\Delta C_{\text{APE}}\) values for the last 10 APes (\(\Delta C_{\text{APE}} = \Delta C_{\text{last 10 APes}}\)) when synaptic depression is at steady state (Fig. 2), allowing us to calculate the \(P_r\) during constant synaptic depression as \(P_r = \Delta C_{\text{APE}}/\Delta C_{\text{RRP40}}\). This approximation was made possible, despite the ongoing endocytosis during the stimulus train, because the duration of the last 10 stimulations (0.2 s) is much shorter than the time constant of endocytosis (>2.7 s) (25) and because we observed no significant difference in the rate of endocytosis between WT and synapsin DKO calyces (data not shown).

Similar to the EPSC recordings, the capacitance recordings revealed rapid depression of synaptic responses during high-frequency stimulation, with capacitance responses declining to a steady-state level after \(\approx 6–10\) APes. Subsequent APes elicited constant capacitance responses that cause a linear increase in total terminal capacitance as a function of stimulus number (Fig. 5A). However, we observed significantly more depression in synapsin DKO terminals than in WT terminals, as evidenced by the decreased slope of the linear-capacitance increase during the plateau phase (DKO, \(240\pm 45\) fF/s; WT, \(342\pm 33\) fF/s; Fig. 5B). In WT terminals, APes at the end of the 50-Hz stimulus train evoked an average capacitance change of \(\Delta C_{\text{APE}} = 7.1\pm 2.2\) fF (Fig. 5C), and the residual RRP after the stimulus train was \(\Delta C_{\text{RRP40}} = 189\pm 31\) fF (Fig. 5D), corresponding to a residual release probability (\(P_r\)) of 3.7 ± 0.6% (n = 7; Fig. 5E). In synapsin DKO terminals, however, the APe-induced capacitance
measurements of small changes in calyx terminals, on the scale of synapses. Thus, although such an artifact might be important for peptide-sin-deficient calyx terminals (WT, control), probably due to a but, consistent with previous data (26), still allowed a limited indeed, largely blocked production of evoked EPSCs as expected calyx terminals that were intoxicated with intracellularly applied EGTA significantly decreased synaptic responses during 50 Hz APes was 4.3 ± 8.8 fF/s, ∼50 times lower than in control synapses. Thus, although such an artifact might be important for measurements of small changes in calyx terminals, on the scale of the changes observed here, this artifact can be neglected.

Intracellular Application of MLCK or EGTA Occludes the Effect of the Deletion of Synapsins on Synaptic Depression. All synapsins share a conserved N-terminal phosphorylation site for cAMP-dependent protein kinase and Ca2+/CaM-dependent protein kinase I (Ca2+/CaM kinase I) (27–29). In calyx terminals, high-frequency stimulation elevates the residual Ca2+ to concentrations as high as 1 μM (30), much higher than the Ca2+ concentrations required to activate CaM (31). To test whether CaM-kinase-dependent phosphorylation of synapsins is involved in maintaining a synapsin-boosted P, during high-frequency stimulation, we introduced high concentrations (20 μM) of a CaM inhibitor (the MLCK peptide) (32) into calyx terminals via the presynaptic patch pipette for ≥5 min and recorded the RRP sizes and the presynaptic membrane capacitance in response to 50-Hz APe stimulation.

Intracellular application of the MLCK peptide had no effect on the initial APe-evoked capacitive change in WT or synapsin-deficient calyx terminals (WT, control = 29.4 ± 5.3 fF; peptide = 30 ± 7.0 fF; DKO, control = 28.4 ± 3.3 fF; peptide = 32.6 ± 9.1 fF) but dramatically enhanced synaptic depression during repetitive stimulation in both types of terminals (e.g., decreased the P from 3.7 ± 0.6% to 1.3 ± 0.3% in WT terminals; Fig. 5 A and B). However, the difference in steady-state depression between WT and synapsin-deficient terminals was abolished by the MLCK peptide, suggesting that the CaM inhibitor efficiently occluded the synapsin-dependent enhancement of synaptic depression (see Fig. 8 A, which is published as supporting information on the PNAS web site; and see Figs. 5 B–E). To independently test whether CaM activation may be involved in the ability of synapsins to boost release during high-frequency stimulation, we examined the effect of suppressing the rise of residual Ca2+ during repetitive stimulation by injecting 5 mM EGTA and 50 μM of 1,2-bis(2-aminoephoxynoyl)ethane-N,N,N',N'-tetraacetate into the calyx terminals. Because 5 mM EGTA is high enough to prevent a rise in residual Ca2+ during repetitive stimulation (32), any activities induced by Ca2+-activated CaM would be blocked. Application of 5 mM EGTA significantly decreased synaptic responses during 50 Hz stimulation in WT and synapsin-deficient terminals and, again, occluded the difference in release between these two genotypes late in the stimulus train (Figs. 8 B and 5 B–E). Together, these data show that a Ca2+/CaM-dependent process is required for synapsins to boost the P during high-frequency stimulus trains.

Discussion
Deletion of Synapsins 1 and 2 Does Not Alter the Kinetics of Synaptic Vesicle Exocytosis. Previous studies on synapsins suggested that they control synaptic-vesicle availability, regulate vesicle fusion, and shape short-term synaptic plasticity (3–9, 33). However, these studies were carried out in preparations that do not allow accurate measurements of multiple synaptic parameters (e.g., kinetics of exocytosis, RRP size, P, and short-term plasticity), making it impossible to identify in these preparations the precise step at which synapsins act. For example, studies of synaptic transmission in hippocampal slices from synapsin-deficient mice revealed an impairment in short-term synaptic plasticity (7, 9). However, because parameters such as the RRP size and the P, could not be determined in these experiments, it was unclear from these studies whether synapsins directly mediate use-dependent changes in vesicle exocytosis or indirectly alter short-term plasticity, for example, by affecting the P, of synapses. Because similar limitations apply to other studies on synapsin function (2–5, 8–11), we have now sought to use the high resolution of analysis afforded by the calyx of Held synapse (12, 14, 16, 23) to pinpoint the precise step that is altered by deletion of synapsins. Our results demonstrate that, at least in this synapse, synapsins 1 and 2 are selectively required only for boosting the probability of neurotransmitter release during high-frequency stimulus trains.

We found that loss of synapsins 1 and 2 had no effect on the properties of spontaneous release (Fig. 1 A, B, D, and F); thus, synapsins are not essential for vesicle fusion as such. Although styryl FM-dye uptake and release measurements detected a significant decrease in the size of the recycling vesicle pool in synapsin 1-deficient hippocampal neurons (34), we observed no change in the RRP size or the kinetics of RRP depletion in synapsin-deficient calyx synapses (Fig. 3). In addition, we detected no difference in the kinetics of RRP replenishment between WT and synapsin-deficient synapses (Fig. 4). Injecting the C-terminal E domain of synapsin 1 into squid synapses alters the amplitude and kinetics of the EPSC, indicating that synapsins might change the availability, fusion, or apparent Ca2+ affinity of releasable primed vesicles (3, 33). In the calyx of Held, however, deletion of synapsins 1 and 2 did not alter either depolarization-evoked presynaptic Ca2+ influx or the basic properties of evoked EPSCs (e.g., amplitudes, synaptic charge transfer, or quantal content; Figs. 1 C and G–H and 3D), indicating that the fusion and apparent Ca2+ affinities of vesicles are also not controlled by synapsins under resting conditions. Viewed together, this evidence suggests that synapsins are not required for normal vesicle exocytosis and recycling in the calyx of Held synapse.

Synapsins Enhance the Vesicular P, During High-Frequency Stimulus Trains. When calyx terminals are stimulated at frequencies of 2–200 Hz, synaptic responses induced by the first ∼6–10 stimuli exhibit rapid depression that leads to a steady-state depressed level of subsequent EPSCs (Fig. 2 A–C). In both WT and synapsin-deficient terminals, the amplitude of the steady-state EPSCs during this use-dependent depression was inversely proportional to the stimulation frequency. At lower stimulation frequency, WT and synapsin-deficient terminals exhibited no difference, but at higher stimulation frequencies (∼50 Hz) synaptic depression was ∼2 times more severe in synapsin-deficient than in WT terminals (Fig. 2 B–D). The increase in synaptic depression in mutant terminals was equally observed by presynaptic capacitance and postsynaptic EPSC recordings, demonstrating that the depression was presynaptic (Fig. 5). Thus synapsins are selectively essential for boosting synaptic responses during the depressed plateau phase of synaptic responses elicited by high-frequency stimulation (Fig. 2 B–D).

We considered several possible causes for the enhanced depression in synapsin-deficient terminals. First, deletion of...
synapsins may increase spontaneous release, leading to tonic depletion of releasable vesicles. However, we found no significant difference in mEPSC frequency between WT and synapsin DKO neurons (Figs. 3 A and B). Second, deletion of synapsins may decrease the reserve pool, recycling pool, and/or RRP size and, thereby, cause a faster reduction of the number of releasable vesicles during sustained stimulation. We also ruled out this possibility because we found no significant decrease in the amplitude of the first EPSCs between synapsin-deficient and WT neurons (Fig. 1 C and G) and detected no significant difference in the size of the RRP at rest or after a high-frequency stimulus between synapsin-deficient and WT terminals (Figs. 3 and 5). Third, deletion of synapsins may decrease the rate of replenishment of the RRP. Our experiments also make this possibility unlikely, because measurements of the refilling kinetics of the RRP failed to uncover a significant difference between synapsin-deficient and WT synapses (Fig. 4), leaving us with a final possible explanation, namely, that synapsins are not required for basic elements of release in calyx terminals but are selectively required to maintain release at normal levels during the steady-state phase of high-frequency stimulus trains. To test this hypothesis, we estimated the average Pd for stimuli during the steady-state late phase of the high-frequency train. We found that, although the initial Pd, as described above, appeared normal in synapsin-deficient terminals, the Pd during the steady-state phase of depression was significantly reduced (~2-fold), confirming the hypothesis (Fig. 5 A and E).

Use-Dependent Synaptic Plasticity Is Probably Regulated by Ca2+/CaM-Dependent Phosphorylation of Synapsins. The N-terminal A domain of all synapsins includes a conserved phosphorylation site for cAMP-dependent protein kinase and Ca2+/CaM-dependent kinase I, the only conserved phosphorylation site in all synapsins (27–29). In calyx synapses, cAMP- and Ca2+/CaM-dependent kinases activate the recruitment of fast-releasing vesicles (32, 35), suggesting that synapsins may boost synaptic responses during the plateau phase of synaptic depression during high-frequency stimulation by a phosphorylation-dependent mechanism (Fig. 2 B–D). This hypothesis implies that Ca2+/accumulating during the high-frequency train stimulates Ca2+/CaM-dependent phosphorylation of synapsins, which, in turn, elevates the vesicular Pd. To test this hypothesis, we examined the effects of introducing a CaM inhibitor (MLCK peptide at 20 μM) (32) or Ca2+ chelators (50 μM EGTA and 50 μM 1,2-bis(2-aminophenoxy)ethane-N,N,N′,N′-tetraacetic) into calyx terminals. Both significantly reduced synaptic responses during the steady-state phase of synaptic depression during repetitive stimulation in WT and synapsin-deficient neurons (Figs. 8B and 5 B and E). Importantly, the difference in the steady-state EPSC amplitude during synaptic depression between un.injected WT and synapsin-deficient terminals was abolished by the injected MLCK peptide or the Ca2+ chelator. These observations suggest that the accumulated residual Ca2+ during high-frequency stimulation mediates Ca2+/CaM-dependent phosphorylation of synapsins, which, in turn, boosts the Pd during the plateau phase of use-dependent synaptic depression.

Previous studies showed that phosphorylation at the N-terminal Ca2+/CaM-dependent kinase I site of all synapsins modulates their binding to synaptic vesicles (36). It thus seems likely that the major mechanism by which synapsins boost release during sustained stimulus trains is the phosphorylation of the N-terminal A domain and subsequent dissociation of synapsins from synaptic vesicles (36). The fact that EGTA or blocking CaM caused stronger synaptic depression than deletion of synapsins, however, suggests that other CaM-dependent pathways, in addition to the Ca2+/CaM-dependent phosphorylation of synapsins, may be involved in regulating the Pd during sustained stimulation in the calyx synapse (37).

Materials and Methods

Mouse Breeds. Synapsin DKO mice (7) and unrelated WT control mice of a comparable genetic background were bred and genotyped by using standard procedures and analyzed on postnatal days 9–11.

Electrophysiology. Pre- or postynaptic recordings in the MNTP were performed in acute brainstem slices essentially as described in refs. 12–14. Whole-cell voltage-clamp recordings were made from both presynaptic terminals (with an EPC-10 amplifier, HEKA, Lambrecht, Germany) and postsynaptic cells (with Axopatch-200B amplifier, Axon Instruments) at room temperature (20–24°C). Series resistance in presynaptic recordings (<20 MΩ) was compensated to 60% (lag, 10 μs). Holding potential was −80 mV, and the potential was corrected for a liquid junction potential of −11 mV between the extracellular and the pipette solution (also applies to postsynaptic recordings). For AP-evoked EPSC recordings, only synapses in which the postsynaptic cells discharged an AP in response to afferent stimulation were included (12). Single afferent stimuli were applied via a bipolar electrode (3–30 V, 100 μs) placed at the midline of the trapezoid body (12, 13). Series resistance in postsynaptic recordings (<10 MΩ) was compensated to 95–98% (lag, 10 μs). Capacitance measurements were obtained with an EPC-10 amplifier together with a software lock-in amplifier (PULSE 886, HEKA). A sinusoidal stimulus was applied in addition to the DC holding potential (~80 mV). The peak-to-peak voltage of the sine wave was <60 mV to avoid activation of Ca2+ currents, and the sine wave frequency was 1 kHz (12–14). For additional details, see Supporting Methods, which is published as supporting information on the PNAS web site. Data were processed by IGOR 3.1 and SIGMAPLOT 2000 (ver. 6.00), using homemade programs for noise filtering, presynaptic and postsynaptic current integrations, and average mEPSC calculation and statistics. All data shown are means ± SD; statistical significances were analyzed by t test. Numerical data are listed in Table 1, which is published as supporting information on the PNAS web site.

We thank Drs. Ege Kavalali, Joy Gibson, Zhiping Pang, Ling-Gang Wu, and Ralf Schegner for critical comments on this manuscript. This work was supported by a Young Investigator Award of the National Alliance for Research on Schizophrenia and Depression (to J.S.).


Supporting Methods

Detailed Description of the Electrophysiological Methods. The preparation of brainstem slices and pre- or postsynaptic recordings in the median nucleus of the trapezoid body (MNTB) were performed essentially as described in refs. 1–3. Transverse or parasagittal slices of 200-µm thickness were cut from the auditory brainstem from WT and synapsins knockout (KO) mice (killed by decapitation; 7–11 days old) with a vibratome at 4°C in low-Ca²⁺ solution containing 125 mM NaCl, 2.5 mM KCl, 3 mM MgCl₂, 0.05 mM CaCl₂, 25 mM dextrose, 1.25 mM NaH₂PO₄, 0.4 mM ascorbic acid, 3 mM myo-inositol, 2 mM sodium pyruvate, and 25 mM NaHCO₃, pH 7.4, and gassed with 95% O₂ and 5% CO₂ (1–3).

Whole-cell voltage-clamp recordings were made from both presynaptic terminals (with an EPC-10 amplifier, HEKA, Lambrecht, Germany) and postsynaptic cells (with Axopatch-200B amplifier, Axon Instruments) at room temperature (20–24°C). In postsynaptic recording, the bath solution contained 125 mM NaCl, 2.5 mM KCl, 1 mM MgCl₂, 2 mM CaCl₂, 25 mM dextrose, 1.25 mM NaH₂PO₄, 0.4 mM ascorbic acid, 3 mM myo-inositol, 2 mM sodium pyruvate, and 25 mM NaHCO₃, pH 7.4, and was continually gassed with 95% O₂ and 5% CO₂ (1–3). The NMDA-receptor blocker D-APV (50 µM) was always added to isolate AMPA currents. In addition, miniature excitatory postsynaptic currents (mEPSCs) were recorded in the presence of bicuculline (10 µM) and strychnine (10 µM) in the bath solution. Postsynaptic pipettes (resistance of 1.5–2 MΩ) were filled with a solution containing 125 mM potassium gluconate, 20 mM KCl, 4 mM MgATP, 10 mM sodiumphosphocreatine, 0.3 mM GTP, 0.5 mM EGTA (Sigma), and 10 mM Hepes-KOH, pH 7.2. Presynaptic pipettes (resistance 3.5–5 MΩ) contained
125 mM cesium gluconate, 20 mM KCl, 4 mM MgATP, 10 mM sodium phosphocreatine, 0.3 mM GTP, 0.05 mM BAPTA, and 10 mM Hepes-CsOH, pH 7.2. Presynaptic recordings were obtained in a solution that pharmacologically isolated Ca\textsuperscript{2+} currents and contained 105 mM NaCl, 20 mM TEA-Cl, 2.5 mM KCl, 1 mM MgCl\textsubscript{2}, 2 mM CaCl\textsubscript{2}, 1.25 mM NaH\textsubscript{2}PO\textsubscript{4}, 25 mM dextrose, 0.4 mM ascorbic acid, 3 mM \textit{myo}-inositol, 2 mM sodium pyruvate, 0.001 mM tetrodotoxin (TTX), 0.1 mM 3,4-diaminopyridine, and 25 mM NaHCO\textsubscript{3}, pH 7.4, when gassed with 95% O\textsubscript{2} and 5% CO\textsubscript{2}.

Series resistance in presynaptic recordings (<20 M\(\Omega\)) was compensated to 60% (lag, 10 \(\mu\)sec). Holding potential was \(-80\) mV, and the potential was corrected for a liquid junction potential of \(-11\) mV between the extracellular and the pipette solution (also applies to postsynaptic recordings). For the action potential (AP)-evoked EPSC recordings, only synapses in which the postsynaptic cells discharged an AP in response to afferent stimulation were selected for recording (1). Single afferent stimuli were applied via a bipolar electrode (3–30 V, 100 \(\mu\)sec) placed at the midline of the trapezoid body (1, 2). Series resistance in postsynaptic recordings (<10 M\(\Omega\)) was compensated to 95–98% (lag, 10 \(\mu\)sec). The threshold amplitude for detection of mEPSCs was set at 5 pA. The mean mEPSC was obtained by aligning each individual mEPSC at its peak point. The capacitance was measured with the EPC-10 amplifier together with the software lock-in amplifier (PULSE v8.66, HEKA). A sinusoidal stimulus was applied in addition to the DC holding potential (−80 mV). The peak-to-peak voltage of the sine wave was <60 mV to avoid activation of Ca\textsuperscript{2+} currents, and the sine wave frequency was 1 kHz (1–3).
The acquired data were further processed by IGOR 3.1 and SIGMAPLOT 2000 (ver. 6.00). The homemade program was applied to statistics (expressed as means ± SD) and calculation of synaptic parameters:

- **average mEPSC**
  - align single mEPSCs to the onset, superimposing and averaging.
- **mEPSC or EPSC charge**
  - integral for 100 ms from the onset.
- **ΔC_{Ape} (fF)**
  - ΔC\textsubscript{31st-40th APe}/10.
- **Slope of ΔC_{Ape}**
  - Linear regression of C\textsubscript{m} during 11th–40th APes (C\textsubscript{m} = y\textsubscript{0} + k \times t), slope = k.
- **Release probability Pr**
  - ΔC_{Ape}/ΔC_{RRP}.

The numerical values for all electrophysiological results are shown in Table 1.

**Presence of Synapsins in the MNTB in Mice.** To ensure that synapsins were expressed in the MNTB, we performed immunoblotting analyses of proteins dissected from the MNTB in WT and synapsin double (D)KO mice (Fig. 6).

**Controlling for potential capacitance artifacts.** It was reported in calyx terminals intoxicated with the light chain of botulinum toxin E (BoNT/E) that postsynaptic EPSCs are abolished, but a stable presynaptic capacitance change can still be observed (4), a potentially troubling result, because it implies that there may be a nonvesicular artifact associated with the capacitance measurement in calyx terminals. We repeated the experiments of Yamashita et al. (4) in mouse calyx of Held terminals. We monitored
presynaptic capacitance changes and postsynaptic EPSCs in response to 40 APes induced at 50 Hz, followed by a readily releasable vesicle pool (RRP)-depletion pulse (see Fig. 5) in BoNT/E loaded terminals at 27–32 °C. BoNT/E largely abolished EPSCs after intracellular dialysis for 20 min or longer (Fig. 7 A and B). Of four terminals tested, we observed a minor capacitance artifact in one terminal (Fig. 7A) and a significant capacitance change with no visible corresponding EPSC, as reported by Yamashita et al. (4) in the other 3 terminals (Fig. 7B). The maximum of the BoNT/E-resistant capacitance change was <67 fF, with an average change of 33 ± 6.4 fF. The rate of capacitance increase was highest early during the stimulus train (APes 1–10) and gradually declined, demonstrating a capacity-limited process (Fig. 6C). We linearly regressed the capacitance change evoked during the last 20 APes and found that the slope of the capacitance change at this stage is 4.3 ± 8.8 fF/s. Because, in the absence of intracellular BoNT/E, the slope of the capacitance change is at least 129–342 fF/s (see text), we conclude that the possible artifact in the capacitance measurements obtained for the last 10 APes is negligible.

Figure S2
Figure S2

A  BoNT/E intoxicated terminals

B  

C  

Figure S2
Figure S3