

Essential Role of Phosphoinositide Metabolism in Synaptic Vesicle Recycling

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Summary

Growing evidence suggests that phosphoinositides play an important role in membrane traffic. A polyphosphoinositide phosphatase, synaptojanin 1, was identified as a major presynaptic protein associated with endocytic coated intermediates. We report here that synaptojanin 1-deficient mice exhibit neurological defects and die shortly after birth. In neurons of mutant animals, PI(4,5)P₂ levels are increased, and clathrin-coated vesicles accumulate in the cytomatrix-rich area that surrounds the synaptic vesicle cluster in nerve endings. In cell-free assays, reduced phosphoinositide phosphatase activity correlated with increased association of clathrin coats with liposomes. Intracellular recording in hippocampal slices revealed enhanced synaptic depression during prolonged high-frequency stimulation followed by delayed recovery. These results provide genetic evidence for a crucial role of phosphoinositide metabolism in synaptic vesicle recycling.

Introduction

In the 1950s, studies by Hokin and Hokin on pancreatic slices (1953) provided the first evidence that stimulation of exocytosis induces an increased turnover of phosphatidylinositol (PI) and its metabolites. Similar findings were subsequently extended to other secretory systems, including brain synaptosomes (reviewed in Hawthorne and Pickard, 1979). Early interpretations of this phenomenon included a direct involvement of PI metabolism in membrane traffic. Subsequently, however, research in this field focused mainly on the role of phosphoinositides in intracellular signaling.

In recent years, a potential function of PI metabolism in membrane traffic has returned to center stage (De Camilli et al., 1996; Martin, 1998). Studies on broken cell preparations have shown that synthesis of PI(4,5)P₂ is necessary for regulated secretion from neuroendocrine cells (Eberhard et al., 1990; Hay et al., 1995) and that the PI transport protein (PITP) stimulates the formation of secretory vesicles from the *trans*-Golgi network (Ohashi et al., 1995). Furthermore, genetic studies in yeast have demonstrated that enzymes which participate in PI metabolism are implicated in a variety of vesicular transport reactions (Cleves et al., 1989; Bankaitis et al., 1990; Schu et al., 1993; Odorizzi et al., 1998; Guo et al., 1999). The participation of specific phosphoinositides in membrane trafficking may reflect, at least in part, the ability of their phosphorylated inositol groups to bind specific proteins and protein modules. For example, PI(4,5)P₂ and PI(3,4,5)P₃ bind to PH domains with various degrees of affinity (Kavran et al., 1998; Irvine, 1998), whereas PI(3)P specifically binds to the FYVE motif, which is present in a variety of proteins implicated in membrane traffic, including endosome–endosome fusion (reviewed in Wurmser et al., 1999).

Several proteins that play a key role in the synaptic vesicle cycle were shown to be phosphoinositide-binding proteins. These include the clathrin adaptor AP-2 (Gaidarov and Keen, 1999), the protein AP180 (Hao et al., 1997), the GTPase dynamin (Zheng et al., 1996), and the intrinsic protein of the synaptic vesicle membrane, synaptotagmin (Mikoshiba et al., 1999). Based on biochemical, cell-free, and transfection studies, these interactions appear to be physiologically important (Hao et al., 1997; Jost et al., 1998; Achiriloaie et al., 1999; Gaidarov and Keen, 1999; Lee et al., 1999; Vallis et al., 1999).

Recently, a polyphosphoinositide phosphatase, synaptojanin 1, was shown to be highly concentrated in nerve terminals (McPherson et al., 1996). Synaptojanin 1 has a central inositol 5-phosphatase domain, which can act on both PI(4,5)P₂ and PI(3,4,5)P₃, and an NH₂-terminal Sac1-like inositol phosphatase domain, which can hydrolyze to PI *in vitro* PI(3)P, PI(4)P, and PI(3,5)P₂ (Guo et al., 1999). Its COOH-terminal domain interacts with several accessory factors implicated in clathrin-mediated endocytosis, including amphiphysin (David et al., 1996), endophilin (de Heuvel et al., 1997; Ringstad et al., 1997), DAP160/intersectin (Roos and Kelly, 1998), syndapin (Qualmann et al., 1999), and Eps15 (Haffner et al., 1997). Synaptojanin is concentrated on endocytic intermediates of nerve terminals (Haffner et al., 1997) and undergoes stimulation-dependent dephosphorylation in parallel with other endocytic proteins (Slepnev et al., 1998). Homologs of synaptojanin in yeast were shown to play a role in endocytosis and actin function (Singer-Kruger et al., 1998). Based on these considerations, synaptojanin 1 was proposed to participate in synaptic vesicle endocytosis, possibly by regulating the interactions between coat proteins and the lipid bilayer or by regulating a pool of actin implicated in endocytosis (Cremona and De Camilli, 1997).

In addition to this function, synaptojanin 1 may have

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a role in intracellular signaling (Sakisaka et al., 1997). In transfected cells, it binds Grb2 (McPherson et al., 1994) and forms complexes with the EGF receptor and Grb2 (Sakisaka et al., 1997). These effects may underlie the pleiotropic role of phosphoinositides in the control of signaling, membrane traffic, and actin cytoskeleton in all cells (De Camilli et al., 1996; Martin, 1998).

The identification of synaptojanin 1 offers the possibility of testing the hypothesis that phosphoinositide metabolism is implicated in synaptic vesicle traffic by a genetic approach. For this purpose, we inactivated synaptojanin 1 gene by homologous recombination in mice and studied the effect of this mutation on presynaptic function.

Results

Targeted Disruption of the Synaptojanin 1 Gene

Inactivation of the synaptojanin 1 gene in ES cells from 129SV/J mice was achieved by targeted disruption of its first coding exon. To this aim, 103 bp from the 3' portion of this exon and 1571 bp of the adjacent intron were replaced by the neomycin cassette (Figure 1A). Chimeric male mice were crossed with C57BL/6 females to obtain germline transmission of the mutant allele. Heterozygous animals were phenotypically normal and fertile. Matings between heterozygous mice produced a number of pups with a typical Mendelian distribution (+/+ : +/- : -/- = 25:53:25 in 103 analyzed pups from 12 litters), indicating that disruption of the synaptojanin 1 gene does not result in embryonic lethality. Correct gene targeting in ES cells and transmission of the mutant allele to the progeny were verified by Southern blot analysis (Figure 1B). Western blotting of brain tissue from homozygous mutant mice revealed that neither synaptojanin 1 nor fragments of the protein were expressed (Figure 1C and data not shown).

Synaptojanin 1 Is an Essential Protein

Immediately after birth, knockout mice were indistinguishable from heterozygous and wild-type mice in weight, size, external morphology, histological structure of major organs, including brain, as well as in apparent behavior. However, in a few hours, knockout mice became recognizable due to severe reduction of milk in their stomachs. Approximately 85% of the mutant mice died within 24 hr. The remaining 15% failed to thrive and invariably died within 15 days. Their growth rate fell far behind that of their littermates with a 3-fold difference in weight at 10 days (Figure 1D and data not shown; $n = 20, p < 0.01$). Furthermore, they progressively developed severe weakness, ataxia, and displayed generalized convulsions that could be evoked by the tail flick test. Fostering with CD-1 mothers or reduction of possible competitors in the cage did not increase their life span.

The absence of synaptojanin 1 expression did not affect the expression level of a large variety of nerve terminal proteins. These included interactors of synaptojanin 1 (amphiphysin 1 and 2, Grb2, SH3P4, SH3P8, and SH3P13, also referred to as endophilins), proteins with a putative role in synaptic vesicle endocytosis (dynamitin 1, clathrin, AP-2, AP180, Eps15, epsin 1, auxilin,

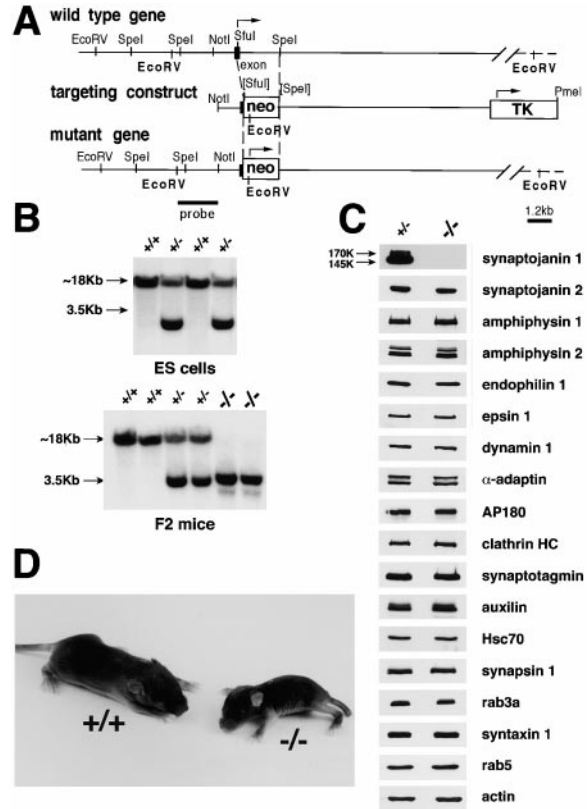


Figure 1. Generation of Synaptojanin 1 Knockout Mice

(A) Schematic representation of the synaptojanin 1 genomic locus, of the targeting construct, and of the recombinant mutant allele. The locations of the EcoRV restriction sites and of the probe used for Southern blot analysis are indicated. The expected fragments generated by EcoRV digestion are 18 kb and 3.5 kb for the wild-type and the mutant alleles, respectively.

(B) Southern blot analysis of EcoRV-digested DNA from two wild-type (+/+) and two recombinant (+/-) ES cell clones (top) and tail DNA from pairs of homozygous wild-type (+/+), heterozygous (+/-), and homozygous knockout (-/-) mice (bottom).

(C) Western blot analysis of postnuclear supernatant from wild-type (+/+) and knockout (-/-) mouse brain for the indicated proteins. Synaptojanin 1 is not expressed in knockout mice, as demonstrated by the lack of both 170 kDa and 145 kDa isoforms.

(D) Ten-day-old wild-type and knockout mice.

Hsc70), intrinsic membrane proteins of synaptic vesicles (synaptotagmin, synaptophysin, and synaptobrevin), plasmamembrane t-SNAREs (syntaxin, SNAP25), additional proteins implicated in the synaptic vesicle cycle (synapsin 1, rab3a, rab5, actin), and enzymes involved in PI metabolism [PI(4)P 5-kinase type II, PI 3-kinase (P110 subunit), and the inositol 5-phosphatase OCRL]. No compensatory increase in the expression of another synaptojanin family member, synaptojanin 2b, was observed (Figure 1C and data not shown).

Abnormal Phosphoinositide Metabolism in Synaptojanin 1-Deficient Mice

We investigated whether the absence of synaptojanin function correlated with increased steady-state levels of specific phosphoinositides in living cells. To this aim, cortical neurons in primary culture (after 10 days in vitro)

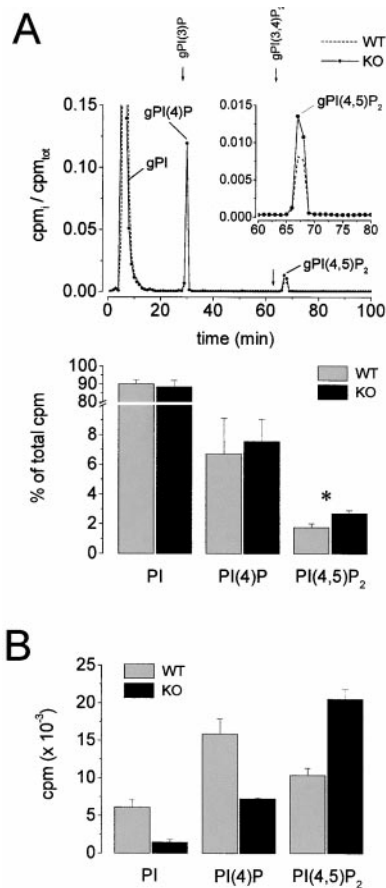


Figure 2. Abnormal Phosphoinositide Metabolism in Synaptotagmin 1-Deficient Mice

(A) Levels of phosphoinositides in cultures of cortical neurons derived from newborn knockout and wild-type mice. ³H-inositol labeled cells were quenched, extracted, and analyzed by HPLC. Typical chromatographic tracings are shown in the upper panel. Data are expressed as radioactivity in each fraction (cpm) normalized to the total radioactivity of each run (cpm_{tot}). The identity of each peak was ascertained by using standards of [³H]PI, [³H]PI(4)P, and [³H]PI(4,5)P₂. Elution times of internal standards, [³²P]gPI(3)P and [³²P]gPI(3,4)P₂, are indicated by arrows. The inset shows the chromatography of gPI(4,5)P₂ on an expanded scale. Lower panel: levels of phosphoinositides (mean ± SD [n = 5]); *, p < 0.001.

(B) PI(4,5)P₂ phosphatase activity of wild-type and knockout brain cytosol. Cytosols from wild-type and knockout mice were incubated with [³H]inositol-labeled PI(4,5)P₂ for 10 min at 37°C. The lipid products were deacylated and separated by HPLC. Fractions (1 ml) were collected and counted for radioactivity. Values shown are the mean ± SD (n = 3).

were metabolically labeled with [³H]inositol for 48 hr. Lipid extraction followed by HPLC analysis revealed a selective increase (1.6-fold, p < 0.001) of PI(4,5)P₂ in knockout neurons with no major differences in PI(4)P (Figure 2A). Other phosphoinositide species were undetectable in our assay. To confirm that the accumulation of PI(4,5)P₂ was the result of impaired phosphoinositide phosphatase activity, we measured this activity in brain cytosol using [³H]PI(4,5)P₂ as a substrate. The dephosphorylation of PI(4,5)P₂ to PI(4)P after 10 min of reaction at 37°C was drastically decreased in knockout cytosol compared to wild type (Figure 2B).

Increased Number of Clathrin-Coated Vesicles in Nerve Terminals of Mutant Mice

To gain some insights into the effect of synaptotagmin 1 absence on the synaptic vesicle cycle, we performed electron microscopy of the anterior horn of the spinal cord, a region intensively immunoreactive for synaptotagmin 1. In mutant nerve terminals, clathrin-coated vesicles were frequently observed in contrast to their rare occurrence in wild-type nerve terminals. They represented 8.2% of all small vesicles in axon endings of 10-day-old mutant mice (in 74 synapses) and only 2.3% in endings of wild-type mice at the same age (in 73 synapses) (data not shown).

To rule out the possibility that the structure of synapses of 10-day-old mutant mice may be altered by a metabolic impairment which correlates with their postnatal growth retardation, we performed electron microscopy on cultured cortical neurons from newborn animals. Cultures were grown in vitro for 10 or 20 days to allow for the maturation of synaptic contacts. Mutant neurons did not show any obvious alteration in the pattern of dendritic and axonal growth or of synaptogenesis (data not shown). However, their synapses contained an increased number of clathrin-coated vesicles, which was very striking in some neurons, such as those shown in Figure 3. These vesicles were typically localized around the synaptic vesicle cluster. They were less densely packed than synaptic vesicles as if they were suspended in the actin-rich matrix surrounding the active zone (Gustafsson et al., 1998) (Figure 3). Relative to the total pool of small vesicles in nerve terminals, coated vesicles represented 10.9% (SD ± 3.5%) and 1.9% (SD ± 0.9%) in mutant and wild-type neurons, respectively (p < 0.01) (inset of Figure 3). Ruthenium red staining and serial sectioning of several nerve terminals confirmed that the great majority of the coated profiles visible in knockout axon endings were bona fide isolated vesicles, anatomically separated from the plasma membrane (data not shown). However, they could be labeled by incubation of the cultures with the extracellular tracer horseradish peroxidase prior to fixation, indicating their endocytic nature (data not shown).

Considering the phosphoinositide binding properties of clathrin coat proteins, we hypothesize that the increased number of clathrin-coated vesicles in nerve terminals of mutant neurons may be explained, at least partially, by the accumulation of PI(4,5)P₂.

Increased Number of Clathrin Coats on Liposomes Incubated with Mutant Brain Cytosol

To assess more directly the effect of synaptotagmin 1 deficiency on the dynamics of endocytic coats, we compared the effect of wild-type and knockout brain cytosol in a cell-free assay (Takei et al., 1998). Protein-free liposomes composed of a crude brain lipid extract were incubated for 15 min at 37°C with brain cytosol plus nucleotides (ATP and GTP) and then analyzed morphologically and biochemically. By electron microscopy, numerous clathrin-coated vesicular profiles were observed with both cytosolic preparations (Figures 4A–4C). However, the cytosol of knockout animals produced a 4-fold

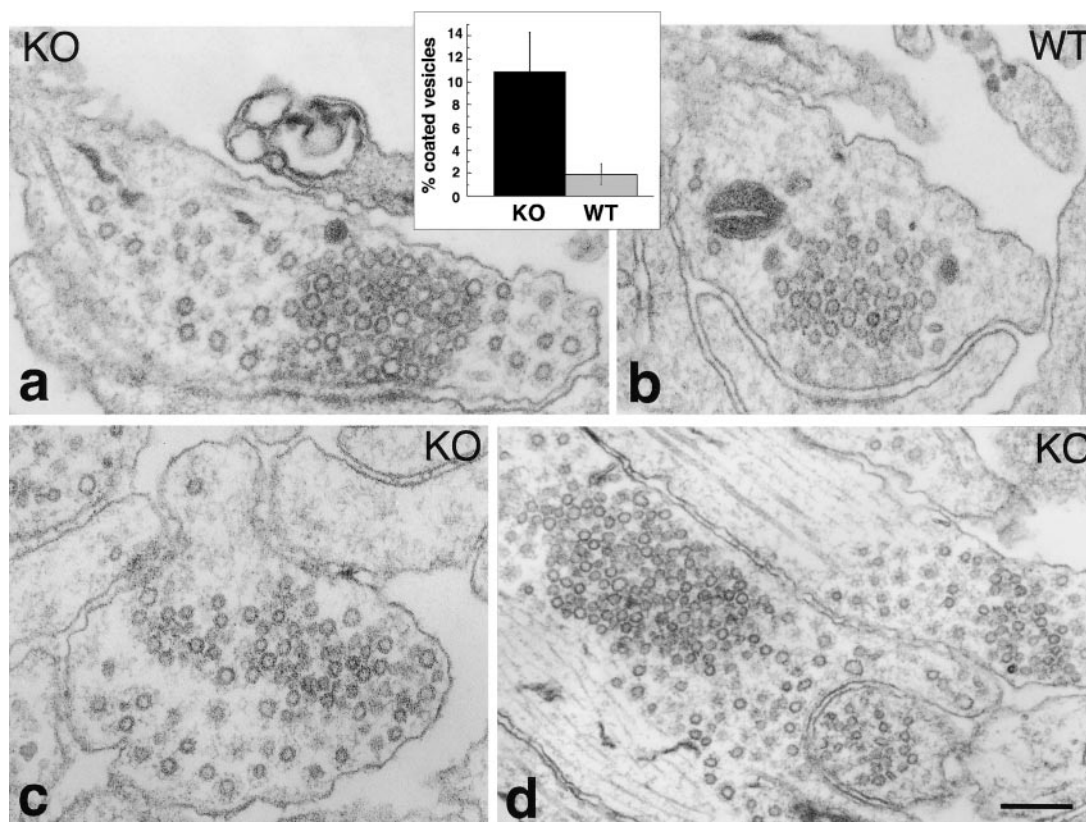


Figure 3. Accumulation of Clathrin-Coated Vesicles in Nerve Terminals of Knockout Animals

(a–d) Electron microscopy of synapses in 20-day-old cultures from cortical neurons of knockout (a, c, and d) and wild-type (b) mice. In synapses from knockout animals, the typical cluster of tightly packed synaptic vesicles is surrounded by less tightly packed vesicles that appear suspended in a cytoskeletal matrix. Many of these vesicles have a clearly recognizable clathrin coat, while others are only partially coated. Very few clathrin-coated vesicles are visible in wild-type synapses and often not visible in each section. Bar, 160 nm in (a) and (b), 170 nm in (c), and 230 nm in (d).

(Inset) Morphometric analysis demonstrating the percentages of coated vesicles relative to the total number of vesicles in nerve terminals of cortical neurons after 20 days *in vitro*. They represent the means \pm SD of data ($n = 5$, $p < 0.01$).

higher number of coated vesicular profiles than wild-type cytosol (Figure 4D). Comparable results were obtained with cytosols depleted of synaptojanin by immunoadsorption (data not shown). When GTP γ S was used instead of GTP, a similar increase was observed, although in this case the total number of clathrin-coated profiles was higher (data not shown). Furthermore, dynamin-coated tubules were also present under these conditions, and their cumulative length was about 4-fold higher in the knockout cytosol preparation (data not shown).

Biochemical analysis of coat proteins associated with liposomes under the same assay conditions strongly corroborated morphological observations (Figure 5). SDS-PAGE followed by immunoblotting revealed a larger pool of clathrin (heavy and light chain) and AP-2 (α -adaptin) bound to liposomes incubated with knockout cytosol (Figure 5A). An increase was also observed in the level of bound amphiphysin, while no obvious difference was observed for tubulin (Figure 5A). The difference between knockout and wild-type cytosol was counteracted by addition to the knockout cytosol of purified synaptojanin, but not of the control protein GST (Figure 5B). Furthermore comparison of synthetic lipid

mixtures that contained or did not contain PI lipids revealed that the difference observed between the two cytosols in this assay was dependent upon the presence of PI lipids in the liposomes (Figure 5C).

Given the presence of ATP, the phosphorylation state of PI during these cell-free incubations is expected to reflect a dynamic equilibrium between the action of inositol kinases and inositol phosphatases. The absence of a major inositol phosphatase should result in an increased level of phosphorylated inositol species. Accordingly, when liposomes were incubated under the same conditions in the presence of [γ - 32 P]ATP, lipid analysis by HPLC demonstrated an increase in the 32 P labeling of PI(4,5)P $_2$ and PI(3,4,5)P $_3$ with knockout cytosol (Figure 6).

We conclude that the dynamics of coat assembly and disassembly on lipid bilayers are different with wild-type and knockout cytosol and that this difference correlates with an alteration in phosphoinositide metabolism.

Enhanced Synaptic Depression in Hippocampal Synapses of Mutant Mice

We performed electrophysiological experiments on 10-day-old animals in order to determine whether the phe-

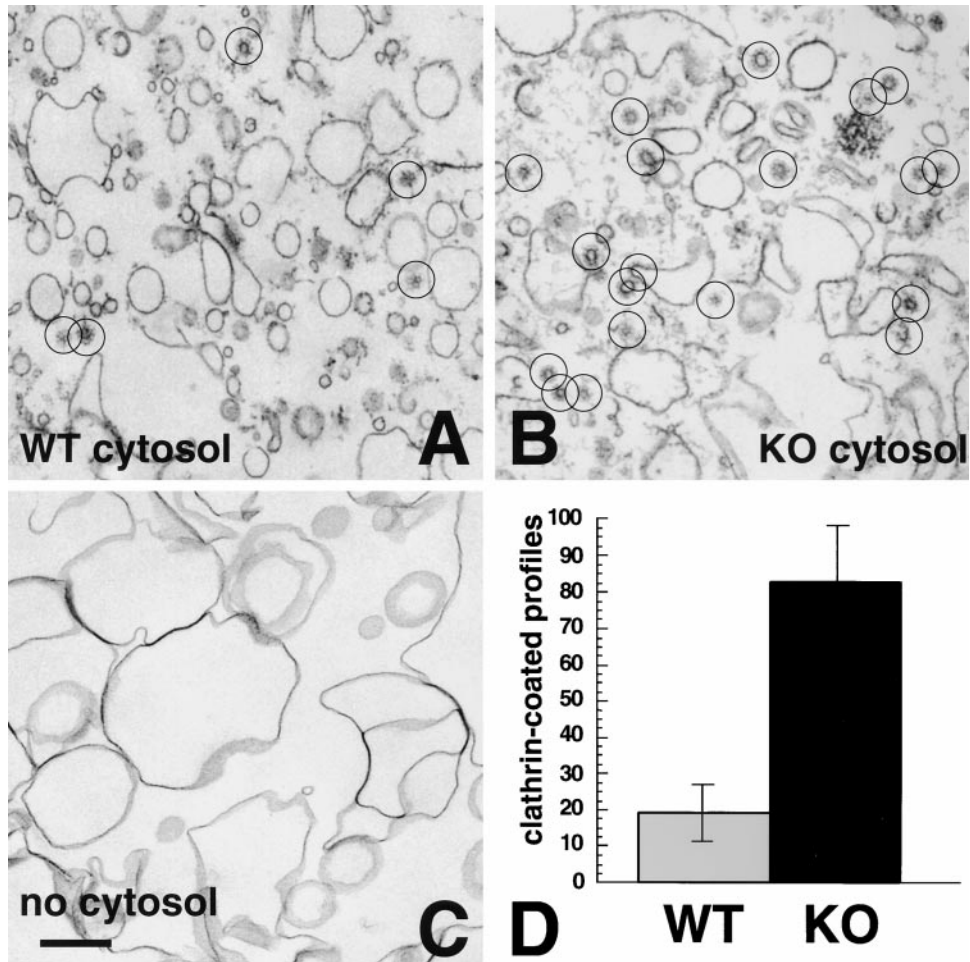


Figure 4. Generation of Coated Intermediates of Clathrin-Mediated Endocytosis on Liposomes Incubated with Brain Cytosol in the Presence of Nucleotides

(A–C) Electron micrographs of liposomes composed of a crude brain lipid extract incubated with brain cytosol of wild-type (A) and knockout (B) mice or with buffer alone (C). Cytosol, as previously reported, produces a drastic change of liposome morphology (Takei et al., 1998). Clathrin-coated profiles are scattered throughout the preparation (circles in [A] and [B]) and more abundant in the knockout sample. Bar, 343 nm.

(D) Morphometric analysis of the number of clathrin-coated vesicular profiles in the preparations shown in (A) and (B). Values represent the average number of coated profiles per unit field (\pm SD) (field of $40 \mu\text{m}^2$; $n = 22$ fields for each condition).

notypic changes described so far in mutant mice are associated with alterations in synaptic function. Glutamatergic synaptic transmission was examined in hippocampal slices via recording of postsynaptic responses of CA1 pyramidal neurons to stimulation of the Schaffer collaterals.

To test the functional integrity of the presynaptic release machinery, paired-pulse facilitation (PPF) was examined (Figure 7A). No defect was observed in synaptotagmin 1 knockout animals at all interstimulus intervals assayed (20–500 ms), thus suggesting that the basal properties of synaptic transmission were unchanged in mutant mice.

Next, we analyzed the response of hippocampal synapses to long trains of action potentials that represents a stimulatory condition under which synaptic vesicle release becomes rate limited by a variety of factors, including the regeneration of a releasable pool by membrane recycling. A stimulation at 10 Hz for 200 s induced

an initial pronounced facilitation of synaptic responses that lasted for about 25 s, followed by a persistent depression that leveled off to a steady-state level of $\sim 45\%$ of the initial responses. Mutant mice, while displaying a level of facilitation similar to that of control mice, developed a significantly stronger depression following ~ 530 stimuli that reached a steady-state level of $\sim 25\%$ of initial responses (Figure 7B).

Recovery from synaptic depression was investigated by suddenly changing stimulation frequency from 10 to 0.33 Hz after the application of 2000 pulses (Figure 7C). Control mice demonstrated a rapid recovery of EPSC amplitude that was complete within 15 s, whereas in knockout mice the recovery was complete only after 50 s.

Discussion

This study demonstrates that a polyphosphoinositide phosphatase enriched in nerve terminals is essential for

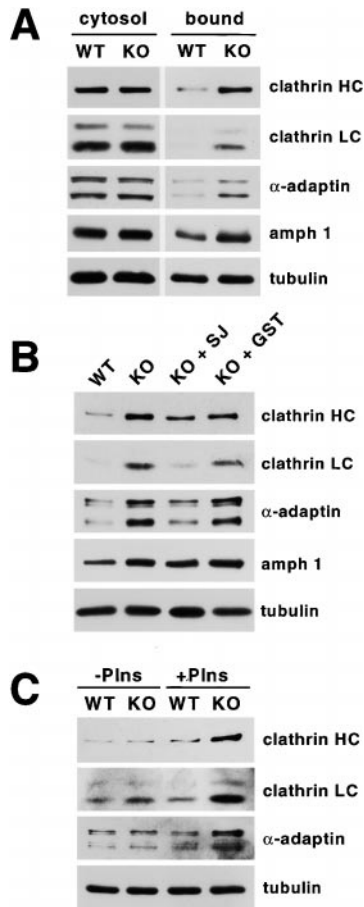


Figure 5. Binding of Coat Proteins to Liposomes Incubated with Wild-Type and Knockout Cytosol in the Presence of Nucleotides

(A) Western blot analysis of wild-type and knockout brain cytosol and of protein binding to liposomes composed of a crude brain lipid extract after incubation for 15 min at 37°C in the presence of ATP and GTP. Higher levels of AP-2 (α -adaptin), clathrin (heavy and light chains), and amphiphysin 1, but not of the control protein tubulin, are associated with liposomes following the incubation with knockout cytosol.

(B) Addition of purified synaptojanin 1 (SJ) (20 nM), but not of the control protein GST, to knockout cytosol decreases coat binding to levels obtained with wild-type cytosol.

(C) Coat protein binding to liposomes that contain (+PIns) or do not contain (-PIns) inositol phospholipids. Increased coat binding occurs only when knockout cytosol is incubated with inositol phospholipids-containing liposomes.

growth and development in postnatal life and provides evidence for an important role of its enzymatic activity in synaptic vesicle recycling. Neurological symptoms are the most obvious phenotypic manifestations observed in synaptojanin-deficient mice. The lack of obvious defects at birth are consistent with the nonessential role of synaptic transmission in prenatal life (Verhage et al., 1997). Given the expression of synaptojanin 1 outside the nervous system (Ramjaun and McPherson, 1996) and the importance of phosphoinositide metabolism for a variety of cellular processes, the possible abnormal function of other tissues, which do not affect embryonic development, may contribute to failure to thrive. In this first analysis of synaptojanin 1 knockout mice, we focused on changes in PI metabolism in brain and on

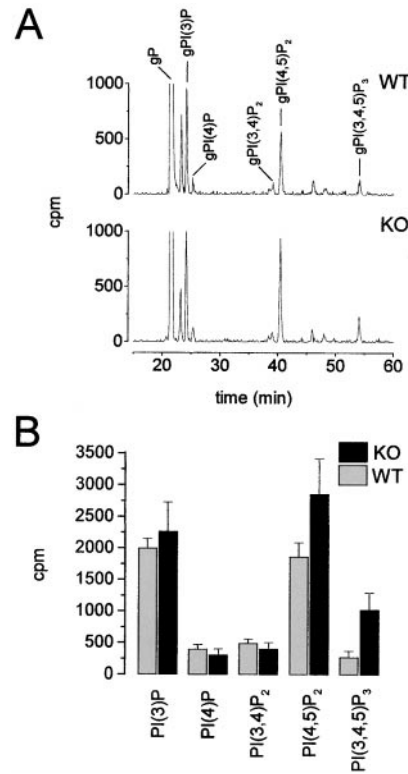


Figure 6. ^{32}P Labeling of Phosphoinositides with Cytosols from Wild-Type and Knockout Mice in the Presence of $[\gamma\text{-}^{32}\text{P}]\text{-ATP}$

(A) Analysis of lipids separated by HPLC following the incubation for 15 min at 37°C of liposomes with cytosols from wild-type and knockout mice in the presence of $[\gamma\text{-}^{32}\text{P}]\text{-ATP}$.

(B) Quantification of ^{32}P incorporation. Values shown are the mean \pm SD ($n = 4$; $p < 0.01$ for PI(4,5)P₂ and PI(3,4,5)P₃).

the effect of these changes on synaptic structure and function.

Key Role of Synaptojanin 1 in the Regulation of Phosphoinositide Levels in Brain

Synaptojanin 1 deficiency resulted in a significant increase in the steady-state level of PI(4,5)P₂ in differentiated neurons, which correlated with a substantial reduction of cytosolic inositol 5-phosphatase activity. Other phosphoinositide species, which are known to represent only a small fraction of cellular phosphoinositides, were not detectable in our analysis of cortical neurons. However, our study of ^{32}P incorporation in liposomes incubated with cytosol and nucleotides demonstrated that the absence of synaptojanin 1 leads to enhanced labeling of PI(3,4,5)P₃ in addition to PI(4,5)P₂, in agreement with the previous demonstration that synaptojanin 1 can act as an inositol 5-phosphatase both for PI(4,5)P₂ and for PI(3,4,5)P₃ (Woscholski et al., 1997). It is therefore possible that even in situ the absence of synaptojanin 1 may result in enhanced levels of PI(3,4,5)P₃ in specific neuronal microcompartments or in response to specific stimuli. Recently, the recombinant Sac1 domain of synaptojanin 1 was shown to act as a broad specificity inositol phosphatase for PI(3)P, PI(4)P, and PI(3,5)P₂ in vitro (Guo et al., 1999). However, in intact neurons and

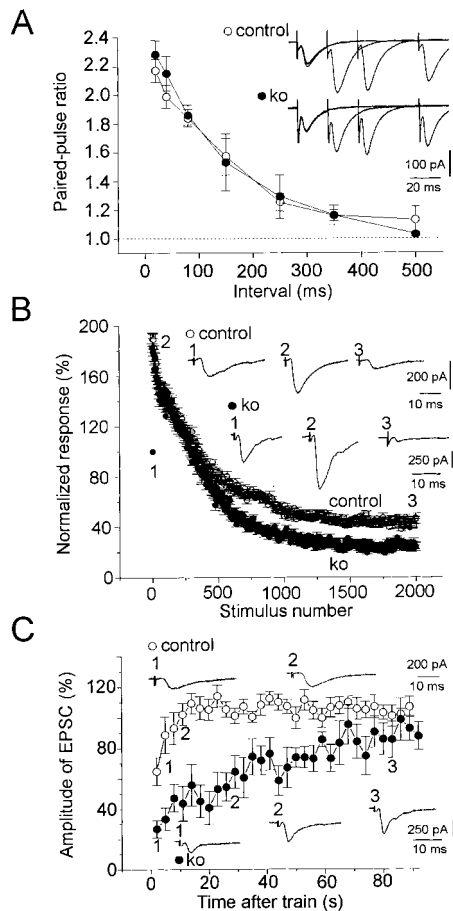


Figure 7. Analysis of Synaptic Transmission in Control and Synap-tojanin 1 Knockout Hippocampal Slices

(A) Magnitude of paired pulse facilitation as a function of interstimulus interval. The paired pulse ratio was determined as the ratio of amplitudes between the second and the first EPSC from control ($n = 32$, 13 animals) and knockout ($n = 14$, 6 animals) mice. Sample EPSCs recorded from control (top) and knockout (bottom) mice in response to paired stimuli delivered at interpulse intervals of 20, 40, and 80 ms are shown in the inset. Holding potential was -70 mV.

(B) Synaptic depression during repetitive stimulation at 10 Hz (2000 pulses). Responses were normalized to the first response in the train. Amplitudes of successive EPSCs were plotted as a function of stimulus number, with five consecutive EPSCs averaged (starting from the EPSC in response to the second stimulus) and presented as one data point. The difference between control ($n = 19$, 6 animals) and knockout ($n = 18$, 6 animals) mice was statistically significant ($p < 0.05$, paired t test for each data point) starting with the 528th response. Data from each day were averaged and considered as one experiment. Responses from individual experiments are shown in the inset. Stimulation artifacts are truncated.

(C) Time course of recovery from synaptic depression following prolonged stimulation (10 Hz, 2000 pulses). Recovery was assessed by applying single stimuli at 0.33 Hz starting 2 s after the end of the 10 Hz train. Recovery in the control animals was complete within ~ 15 s ($n = 11$, 6 animals), whereas recovery in the knockout animals was decelerated and complete within only ~ 50 s ($n = 9$, 5 animals). Responses from individual experiments (same as those presented in [C]) are shown in the inset. Stimulation artifacts are truncated. Responses are statistically different ($p < 0.05$) until the data points at 50 s.

liposomes, we did not observe clear changes in the levels of phosphoinositides other than $PI(4,5)P_2$ and $PI(3,4,5)P_3$. It would appear therefore that the inositol 5-phosphatase activity of synaptojanin 1, encoded by its central domain, mediates the main action of this protein on PI metabolism.

Perturbation of Vesicle Traffic in Nerve Terminals of Mice Lacking Synaptojanin 1

The absence of synaptojanin 1 produced structural changes in synapses that are consistent with a role in synaptic vesicle recycling. Knockout animals contained a higher number of clathrin-coated vesicles in the cytomatrix-rich area (Gustafsson et al., 1998) surrounding the synaptic vesicle cluster (i.e., the site where clathrin-mediated internalization of synaptic vesicle membrane occurs physiologically [Heuser and Reese, 1973]). This localization, together with the similarity of their size to synaptic vesicles, and with their property to become labeled by extracellular horseradish peroxidase, indicate that these vesicles represent intermediates in synaptic vesicle recycling.

An impairment in synaptic vesicle recycling could explain the enhanced depression of synaptic transmission observed in the mutant mice after prolonged high-frequency stimulation and the corresponding delayed recovery after interruption of the stimulus. Based on the average number of synaptic vesicles in CA1 hippocampal synapses (a few hundred) (Harris and Sultan, 1995) and on the known release probability of these synapses (in the range of $p < 0.5$) (Rosenmund et al., 1993), a significant reduction in the releasable pool of synaptic vesicles should occur only after several hundred stimuli.

Phosphoinositides Regulate the Interaction of the Clathrin Coat with Membranes

The increased potency of knockout brain cytosol in generating clathrin coats on liposomes fully supports our observations made at synapses *in situ*. The differential effect of knockout and wild-type cytosol on liposomes was directly dependent on the presence of synaptojanin 1, required the presence of PI lipids, and correlated with an enhanced phosphorylated state of PI lipids in the knockout cytosol samples. Collectively, these findings demonstrate that the enzymatic activity of synaptojanin 1 decreases the pool of coat proteins assembled on membranes. In cell-free experiments, the changes observed with the knockout sample may result both from enhanced coat recruitment and from decreased coat dissociation. In a living synapse, where coat assembly is triggered by synaptic vesicle exocytosis, synaptojanin 1 may function physiologically in the uncoating process. It was reported that PIP_2 generation is a key step in the cascade of reactions which lead to Ca^{2+} -dependent exocytosis in neuroendocrine cells (Eberhard et al., 1990; Hay et al., 1995; Wiedemann et al., 1998). If this was the case, phosphoinositides are already present on the synaptic vesicle membrane as it fuses with the plasmamembrane, and spatial segregation or other regulatory mechanisms may prevent their interaction with coat proteins.

The demonstration that synaptojanin 1 acts as a negative regulator of coat-membrane interaction complements

previous biochemical and transfection experiments, suggesting a critical importance of phosphoinositides in clathrin-mediated endocytosis (reviewed in Martin, 1998). More specifically, mutagenesis experiments have shown that the interaction of AP-2 with PI(4,5)P₂ is essential for AP-2 recruitment into membrane-associated clathrin coats (Gaidarov and Keen, 1999). The addition to permeabilized cells of PH domains that bind PI(4,5)P₂ inhibits both early and late stages of clathrin-mediated endocytosis (Jost et al., 1998). Finally, PI(4,5)P₂ synthesis was found to be required for AP-2/clathrin coat assembly on lysosomal membranes (Arneson et al., 1999).

A Hypothetical Model for Synaptojanin 1 Function

The formation of a clathrin coat is triggered by the binding of clathrin adaptors to the membrane in a process implicating both phosphoinositides and membrane proteins (Zhang et al., 1994; Haucke and De Camilli, 1999). Once the coat is formed and stabilized by interactions among its components, the affinity of the adaptors for the membrane must be decreased so that catastrophic uncoating can occur after fission of coated vesicle. We propose that synaptojanin 1, which dephosphorylates phosphoinositides and interacts with proteins of the endocytic machinery, may play a role in uncoating. This model provides a potential mechanism for adaptor dissociation, a process that could not be explained by the effect of the "uncoating ATPase" alone (Schmid, 1997). Strong evidence suggests that small GTPases act as the switches that provide directionality to the coating-uncoating reaction (Ostermann et al., 1993; Springer et al., 1999), and the phosphoinositide switch may act in parallel with a GTPase switch. It is of interest in this connection that at least some of the actions of Arf, a small GTPase implicated in clathrin coat recruitment, appear to be mediated by the generation of PI(4,5)P₂. It was proposed that synaptojanin 1 may act at a step closely related to dynamin site of action in the synaptic vesicle cycle (McPherson et al., 1994, 1996). The data reported here suggest that synaptojanin 1 acts downstream of dynamin. Although the PH domain of dynamin binds phosphoinositides (Zheng et al., 1996), the hydrolysis of phosphoinositides may not be crucial for its function.

The regulation of coat-membrane interactions may not be the only function of synaptojanin 1 in endocytosis. Phosphoinositides are important regulators of the actin cytoskeleton (reviewed in Janmey, 1998; Martin, 1998), and growing evidence strongly implicates actin in endocytosis (Munn et al., 1995), including clathrin-mediated endocytosis (Lamaze et al., 1996; Witke et al., 1998; Qualmann et al., 1999). In nerve terminals, actin is enriched at hot spots of endocytosis (Gustafsson et al., 1998) (i.e., the region where clathrin-coated vesicles accumulate in nerve terminals of synaptojanin 1 mutant mouse). Decreased catabolism of PI(4,5)P₂ and PI(3,4,5)P₃ due to absence of synaptojanin 1 may affect actin dynamics with a resulting increase of assembled actin in this cellular compartment. This actin matrix, in turn, may trap clathrin-coated vesicles on their journey from sites of endocytosis to the vesicle cluster and contribute to the observed phenotype.

In conclusion, our results provide evidence for a critical role of phosphoinositide metabolism in the reformation of synaptic vesicle after exocytosis. They suggest the existence of a phosphoinositide cycle nested within the synaptic vesicle cycle. Given the pleiotropic roles of phosphoinositides, it is likely that the effects of the catalytic action of synaptojanin 1 are not limited to the regulation of interactions between endocytic membranes and cytosolic components. It will now be of interest to determine the interplay in presynaptic function of these effects of synaptojanin 1 with other effects on the cytoskeleton and on signaling pathways.

Experimental Procedures

Synaptojanin 1 Gene Targeting

A phage clone containing the first coding exon of synaptojanin 1 was isolated from a λFIXII mouse genomic library (129SV/J strain, Stratagene) by hybridization screening. A targeting vector that replaced the 3' end of the first coding exon and part of the following intron was assembled in pBluescript KS+ vector (Stratagene) as shown in Figure 1A. The linearized vector was electroporated into ES cells, which were then selected as described (Yang et al., 1997). For Southern blot screening, genomic DNA was digested with EcoRV, transferred onto nylon filters and hybridized with a radioactively labeled Spel-NotI fragment from the original phage clone (see Figure 1). Targeted ES cells were microinjected into E3.5 C57BL/6 blastocysts, which were then implanted into pseudopregnant foster mothers. Heterozygous and homozygous animals were identified by Southern blot analysis.

Lipid Biochemistry

For phosphoinositide phosphatase analysis, a solution of [³H]-inositol]PI(4,5)P₂ in organic solvent (NEN Dupont, Boston, MA; specific activity 3500 μCi/mmol, 0.05 μCi/sample) was supplemented with 10 μg of crude brain phosphoinositides (Sigma, St. Louis, MO), dried under a stream of nitrogen, resuspended in buffer A (25 mM HEPES-KOH [pH 7.4], 25 mM KCl, 2.5 mM magnesium acetate, 10 μM CaCl₂, 150 mM K-glutamate) containing 0.2% (w/v) Triton X-100, and sonicated for 5 min. The resulting micelles were incubated in a final volume of 50 μl with brain cytosol (50 μg protein) for 10 min at 37°C. Reactions were stopped by addition of 400 μl of methanol:water:HCl (20:20:1 v/v). Lipids were subsequently extracted by addition of chloroform (two times 700 μl) and vigorous vortexing. The organic phases were pooled and dried under nitrogen. For metabolic labeling, cultured neurons were incubated for 48 hr in medium supplemented with 10 μCi/ml [³H]-myo-inositol, prior to lipid extraction by a methanol-chloroform procedure. All lipids were then deacylated and analyzed by HPLC according to Kirk et al. (1990) and Auger et al. (1990), respectively. Radioactivity was assayed in 1 ml fractions or with an on-line counter (Packard, Meridian, CA).

Liposome Experiments

Liposomes from either a bovine brain extract (type I Folch fraction I; Sigma, St. Louis, MO) or synthetic lipids (Avanti Polar Lipids, Alabaster, AL) were prepared as described (Takei et al., 1998). PI-containing liposomes were composed of 20% cholesterol, 40% phosphatidylcholine, and 40% PI mixture, while PI-free liposomes contained 40% phosphatidylglycerol instead of PI. Liposomes (0.1 mg/ml) were incubated for 15 min at 37°C with dialyzed brain cytosol (6.5 mg/ml) in 0.5 ml of buffer A, which was supplemented with 0.2 mM GTP, 2 mM ATP, and an ATP regenerating system (Takei et al., 1998). For electron microscopy, incubations were stopped by the addition of an equal volume of 2× concentrated (Takei et al., 1998). For biochemical analysis of proteins, samples were loaded on a bed of 0.5 M/2 M sucrose and centrifuged at 150,000 × g in a Beckman TLA100.2 rotor for 30 min at 4°C. Liposomes collected at the interphase were washed in 0.5 ml buffer A, resuspended in sample buffer, and processed for immunoblotting. For lipid analysis, incubations were performed in the presence of 10 μCi [³²P]ATP, stopped, and processed for HPLC as described above.

Electron Microscopy and Morphometry

Cortical neuron cultures were fixed with 2% glutaraldehyde in 0.1 M sodium phosphate/2% sucrose. The number of synaptic vesicles and coated vesicles in nerve terminals was determined on electron micrographs at a final magnification of 54,600 \times . Synaptic vesicles were defined as the small homogeneously sized vesicles forming large clusters in the terminal. Coated vesicles were defined as vesicles that had a complete or partial clathrin coat and were localized in close proximity to the synaptic vesicle cluster. Ninety-nine wild-type synapses and 115 knockout synapses were analyzed from five independent sets of cortical neuron cultures. The average number of total vesicles (synaptic vesicles plus coated vesicles) per synapse was 65.2 ± 6.9 for wild-type neurons and 55.6 ± 15.8 for knockout neurons. The morphometric analysis of liposomes incubated with brain cytosol was performed as previously described (Takei et al., 1998) with minor modifications. The number of clathrin-coated vesicular profiles was counted in fields of 40 μm^2 each ($n = 22$ fields counted for each condition and from two independent sets of experiments). Statistical significance was calculated by Student's *t* test.

Electrophysiology

Coronal hippocampal slices (400 μm) were prepared from 10-day-old mice and maintained in a chamber that was continuously superfused with a solution containing (in mM): NaCl 126, KCl 2.5, MgSO_4 2, NaH_2PO_4 1.25, CaCl_2 2, NaHCO_3 26, dextrose 10. Solutions were aerated with 95% O_2 , 5% CO_2 to pH 7.4 at 33.5°C–35°C. One hundred micromolar picrotoxin was added to block inhibitory GABA_A synapses, and 100 μM DL-AP5 (DL-2-amino-5-phosphonovaleic acid) and 0.3 μM CNQX (6-Cyano-7-nitroquinoxaline-2,3-dione) were added to prevent epileptiform discharges. Whole-cell patch clamp recordings from hippocampal pyramidal CA1 cells (1–2 cells per slice) were performed with electrodes filled with (in mM): K-glucuronate 110, KCl 10, HEPES 10, EGTA 1, MgCl_2 2; Na-ATP 2, Na-GTP 0.2 adjusted to an osmolality of 290 mOsm, to a pH of 7.25, and to a pCa of 7. Electrode resistance was 3–5 M Ω . The membrane holding potential was -70 mV. Afferent fibers in the stratum radiatum were stimulated with a tungsten bipolar electrode at 1.5- to 2-fold intensity above threshold. In paired pulse experiments, 10–15 pairs of EPSCs were recorded at each interstimulus interval. For prolonged repetitive stimulation (2000 \times , 10 Hz), maximally two trains were applied per cell and averaged. Data were acquired at 5 Hz through an Axopatch-1D amplifier and collected through a multichannel encoding device (Neurodata Inc., Pasadena, CA) onto videotape. For off-line analysis, data were digitized at 20 kHz with the use of an IBM Pentium computer equipped with pClamp6 (Axon Instruments, Inc., Foster City, CA), Axotape, and Origin software. No clear differences were observed between wild-type and heterozygous mice, which were therefore pooled in a same group, referred to as control animals. Statistical analysis was performed using paired or unpaired *t* test as appropriate.

Miscellaneous Procedures

Tissue homogenization, SDS-PAGE, and immunoblotting were performed as described (Haffner et al., 1997). Brain cytosol was prepared as described by Takei et al. (1998). Primary cultures of cortical neurons were prepared as described (Banker and Goslin, 1991; Ryan et al., 1996) and maintained up to 3 weeks in Neurobasal/B27 medium (GIBCO, Gaithersburg, MD). Synaptojanin was purified from rat brain cytosol by immunoadsorption on an antibody column generated by cross-linking mouse anti-synaptojanin mAb (Haffner et al., 1997) to Ultralink Biosupport Medium (Pierce, Rockford, IL).

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