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Decreased Synaptic Vesicle Recycling Efficiency and Cognitive Deficits in Amphiphysin 1 Knockout Mice

Gilbert Di Paolo,[1,2] Sethuraman Sankaranarayanan,[5] Markus R. Wenk,[1,2] Laurie Daniell,[1,2] Ezio Perucco,[6] Barbara J. Caldarone,[4] Richard Flavell,[1,3] Marina R. Picciotto,[4] Timothy A. Ryan,[5] Ottavio Cremona,[6,7] and Pietro De Camilli[1,2,8]

[1]Howard Hughes Medical Institute
[2]Department of Cell Biology
[3]Sections of Immunology
[4]Department of Psychiatry
Yale University School of Medicine
New Haven, Connecticut 06510
[5]Department of Biochemistry
The Weill Medical College of Cornell University
New York, New York 10021
[6]Dipartimento di Scienze Mediche
Università del Piemonte Orientale "A. Avogadro"
28100 Novara
[7]Università Vita-Salute San Raffaele
Milano, San Raffaele Scientific Institute
20132 Milano
Italy

Summary

The function of the clathrin coat in synaptic vesicle endocytosis is assisted by a variety of accessory factors, among which amphiphysin (amphiphysin 1 and 2) is one of the best characterized. A putative endocytic function of amphiphysin was supported by dominant-negative interference studies. We have now generated amphiphysin 1 knockout mice and found that lack of amphiphysin 1 causes a parallel dramatic reduction of amphiphysin 2 selectively in brain. Cell-free assembly of endocytic protein scaffolds is defective in mutant brain extracts. Knockout mice exhibit defects in synaptic vesicle recycling that are unmasked by stimulation and suggest impairments at multiple stages of the cycle. These defects correlate with increased mortality due to rare irreversible seizures and with major learning deficits, suggesting a critical role of amphiphysin for higher brain functions.

Introduction

Neurotransmission relies on a tightly regulated balance between synaptic vesicle exocytosis and endocytosis to maintain a pool of functional synaptic vesicles. A major pathway through which synaptic vesicle membranes can be recycled is clathrin-mediated endocytosis (Cremona and De Camilli, 1997; Brodin et al., 2000). Following depolarization-mediated synaptic vesicle fusion at active zones, membrane retrieval occurs either by the formation of individual clathrin-coated vesicles or by bulk invagination of the plasmalemma to generate endosome-like structures from which clathrin-coated vesicles originate. Once clathrin-coated vesicles have pinched off, they rapidly shed their coat and are directed back to the cluster of synaptic vesicles, from where they can be mobilized for subsequent rounds of release. Recently, a variety of accessory factors thought to assist the clathrin coat in its function have been identified. These include proteins that affect membrane dynamics by direct physical interaction, lipid metabolizing enzymes, regulatory components of the actin cytoskeleton, signaling proteins, and adaptor proteins that link these factors to each other and to the clathrin coat (reviewed in Marsh and McMahon, 1999; Owen and Luzio, 2000; Slepnev and De Camilli, 2000).

In nerve terminals, clathrin-mediated endocytosis occurs at sites that are spatially segregated from sites of exocytosis (Heuser and Reese, 1973; Gad et al., 1998; Teng and Wilkinson, 2000). These endocytic zones form a belt around the active zones of secretion and are regions where components of the endocytic machinery are concentrated (Roos and Kelly, 1999). They are also enriched in actin (Gad et al., 2000; Dunaevsky and Connor, 2000), consistent with evidence that several clathrin accessory factors may be components of protein scaffolds that regulate the actin cytoskeleton (reviewed in Geli and Riezman, 1998; Qualmann et al., 2000; Slepnev and De Camilli, 2000). For instance, the GTPase dynamin, which plays a critical role in fission, also interacts with actin binding proteins and may affect actin dynamics at the synapse through these interactions (Witke et al., 1998; Qualmann et al., 1999; McNiven et al., 2000; Ochoa et al., 2000). The polyphosphoinositide phosphatase synaptojanin 1 hydrolyzes a pool of phosphatidylinositol-4,5-bisphosphate [PI(4,5)P_2] that participates both in clathrin coat recruitment and in actin polymerization at endocytic zones in nerve terminals (Cremona et al., 1999; Gad et al., 2000; Harris et al., 2000). Syndapin/pacsin and DAP160/intersectin, which bind dynamin 1 and synaptojanin 1, are also involved in actin function via an interaction with regulatory components (WASP and Cdc42, respectively) of the Arp2/3 actin nucleating complex (Roos and Kelly, 1998; Qualmann et al., 1999; Modregger et al., 2000; Hussain et al., 2001; Wasiak et al., 2001).

One of the best characterized clathrin accessory factors is amphiphysin (reviewed in Wigge and McMahon, 1998). The amphiphysin protein family is conserved from yeast to man and has a three domain structure (see Figure 3A): an NH_2-terminal BAR (Bin-Amphiphysin-Rvs) domain (Elliott et al., 1999), which mediates binding to acidic phospholipids (Takei et al., 1999; Farsad et al., 2001) and homo/heterodimerization (Wigge et al., 1997a; Slepnev et al., 1998; Ramjaun et al., 1999), a central variable domain, and a COOH-terminal Src homology 3 (SH3) domain that interacts with dynamin 1 and synaptojanin 1 (David et al., 1996; McPherson et al., 1996). Two isoforms of amphiphysin, amphiphysin 1 and 2, are expressed in mammals. Amphiphysin 1 is primarily expressed in brain and is the dominant target of autoimmunity in human paraneoplastic Stiff-man syndrome (De Camilli et al., 1993). Amphiphysin 2 is more widely ex-

[8]Correspondence: pietro.decamilli@yale.edu

pressed as alternatively spliced isoforms, with the highest levels found in brain and striated muscle (Butler et al., 1997; Leprince et al., 1997; Ramjaun et al., 1997; Wechsler-Reya et al., 1997; Wigge et al., 1997a). The predominant neuronal isoforms of amphiphysin 2 form heterodimers with amphiphysin 1 and the heterodimer is concentrated in nerve terminals (Wigge et al., 1997a; Ramjaun et al., 1997), where it is localized in the presynaptic cytomatrix and is further enriched on coated endocytic intermediates (Bauerfeind et al., 1997). Both components of this heterodimer contain in their central region binding sites for clathrin and AP-2 (McMahon et al., 1997; Ramjaun and McPherson, 1998; Slepnev et al., 2000), while the muscle isoform of amphiphysin 2 lacks the clathrin and AP-2 binding sites, suggesting functions for these isoforms independent of the clathrin coat. Similarly, *Drosophila* amphiphysin lacks clathrin coat binding sites (Razzaq et al., 2000; Leventis et al., 2001). Additional proteins that contain a BAR domain, but are otherwise different from amphiphysin in their domain structure, are expressed in mammals and other organisms (Crouzet et al., 1991; Ge and Prendergast, 2000; Routhier et al., 2001).

A function for amphiphysin in synaptic vesicle endocytosis is supported by several lines of evidence in addition to its localization and biochemical interaction with other endocytic proteins. Expression or microinjection of amphiphysin fragments that compete for the binding of this protein to any of its major partners, such as dynamin, AP-2, and clathrin, strongly inhibits clathrin-mediated endocytosis, possibly by titrating out these factors, thus demonstrating that these interactions can occur in vivo (Shupliakov et al., 1997; Wigge et al., 1997b; Slepnev et al., 2000). More importantly, presynaptic microinjection of peptides that bind the SH3 domain of amphiphysin, and therefore compete its SH3-dependent interactions, also severely impair synaptic vesicle endocytosis (Shupliakov et al., 1997). The interaction of amphiphysin with dynamin affects dynamin's enzymatic activity and oligomeric state (Wigge et al., 1997a; Owen et al., 1998; Takei et al., 1999). Furthermore, the interaction of amphiphysin with dynamin and other endocytic partners in enhanced by nerve terminal depolarization via Ca^{2+}-dependent dephosphorylation, when a burst of endocytic activity is needed (Bauerfeind et al., 1997; Slepnev et al., 1998). Finally, amphiphysin binds and deforms lipid bilayers and may, therefore, facilitate membrane deformations that accompany vesicle budding (Takei et al., 1999; Farsad et al., 2001).

Additional evidence for a role of amphiphysin in endocytosis comes from studies in *Saccaromyces cerevisiae*. In this organism, the homolog of amphiphysin, Rvs167, forms a heterodimer with Rvs161—a protein that comprises a BAR domain only (Crouzet et al., 1991; Lombardi and Riezman, 2001). Although neither gene is essential, mutations in either one of them produce endocytic defects that correlate with a disruption of the actin cytoskeleton (Sivadon et al., 1995). Collectively, these observations suggest that amphiphysin may have an important role in the physiology of endocytic zones of synapses. It may act as a multifunctional adaptor that links cytosolic proteins to the membranes and coordinates the function of clathrin coat proteins, other accessory factors, and the actin cytoskeleton.

In this study, we have used a genetic approach to investigate the role of amphiphysin in synaptic physiology. We have generated mice that lack the expression of amphiphysin 1 and, as an unexpected consequence, also lack brain amphiphysin 2. These mice exhibit synaptic vesicle recycling defects that are compatible with the basic function of the nervous system. However, the mice are prone to seizures, which dramatically decrease their viability, and exhibit severe learning deficits.

Results

Inactivation of the Amphiphysin 1 Gene and Resulting Loss of Both Amphiphysin 1 and 2 in Brain

The amphiphysin 1 gene was disrupted by insertion of a gene encoding a selection marker (neo cassette) in the first coding exon (Figure 1A). The occurrence of correct recombination events was confirmed by Southern blot analysis of ES cells and F2 mice (Figure 1B). Homozygous mutant animals were born, and their genotypes had normal mendelian distribution, revealing lack of embryonic lethality. They did not exhibit any obvious anatomical or histological changes and appeared to develop and reproduce normally, although they displayed an increased mortality rate after reaching adulthood (see below). The expression of the amphiphysin 1 protein was completely abolished in homozygous mutant animals, as shown by Western blotting analysis of brain extracts using a panel of poly- and monoclonal antibodies directed against either the COOH- or the NH_2-terminal region of amphiphysin 1 (Figure 1C and data not shown; see also Figure 2B).

Interestingly, Western blot analysis revealed that expression of amphiphysin 2 was also nearly abolished in the brain of amphiphysin 1-deficient animals (Figure 1C), regardless of the developmental stage of mutant animals (data not shown). No change in the expression level of a variety of other synaptic proteins was observed, including the major interacting partners of amphiphysin such as dynamin 1, synaptojanin 1, clathrin, and the clathrin adaptor AP-2. Levels of intrinsic membrane proteins of synaptic vesicles were also unchanged (Figure 1C and data not shown). The parallel disappearance of amphiphysin 1 and 2 was confirmed by immunofluorescence staining of brain frozen sections (data not shown) and of cortical neurons in culture (Figure 1D). While amphiphysin 1 and 2 colocalize with the synaptic marker synaptophysin in wild-type neurons, the immunoreactivity for both proteins, but not for synaptophysin, is absent in knockout neurons (Figure 1D and data not shown). By Northern blot analysis expression levels of the 2.2 kb transcript encoding amphiphysin 2 were comparable in wild-type and knockout brain (Figure 2A), suggesting that transcriptional mechanisms do not account for the downregulation of amphiphysin 2 in mutant brain. In skeletal muscle, where a shorter isoform of amphiphysin 2 is expressed and where amphiphysin 1 is undetectable (Figure 2B and see also Butler et al., 1997; Wigge et al., 1997a), amphiphysin 2 protein levels were not affected (Figure 2B). These data suggest that amphiphysin 1 is required for the stability of amphiphysin 2 only in neurons, where the two isoforms are primarily found as heterodimers and colocalize accordingly (Figure 2C; see also Ramjaun et al., 1997; Wigge et al., 1997a; Slepnev et al., 1998).

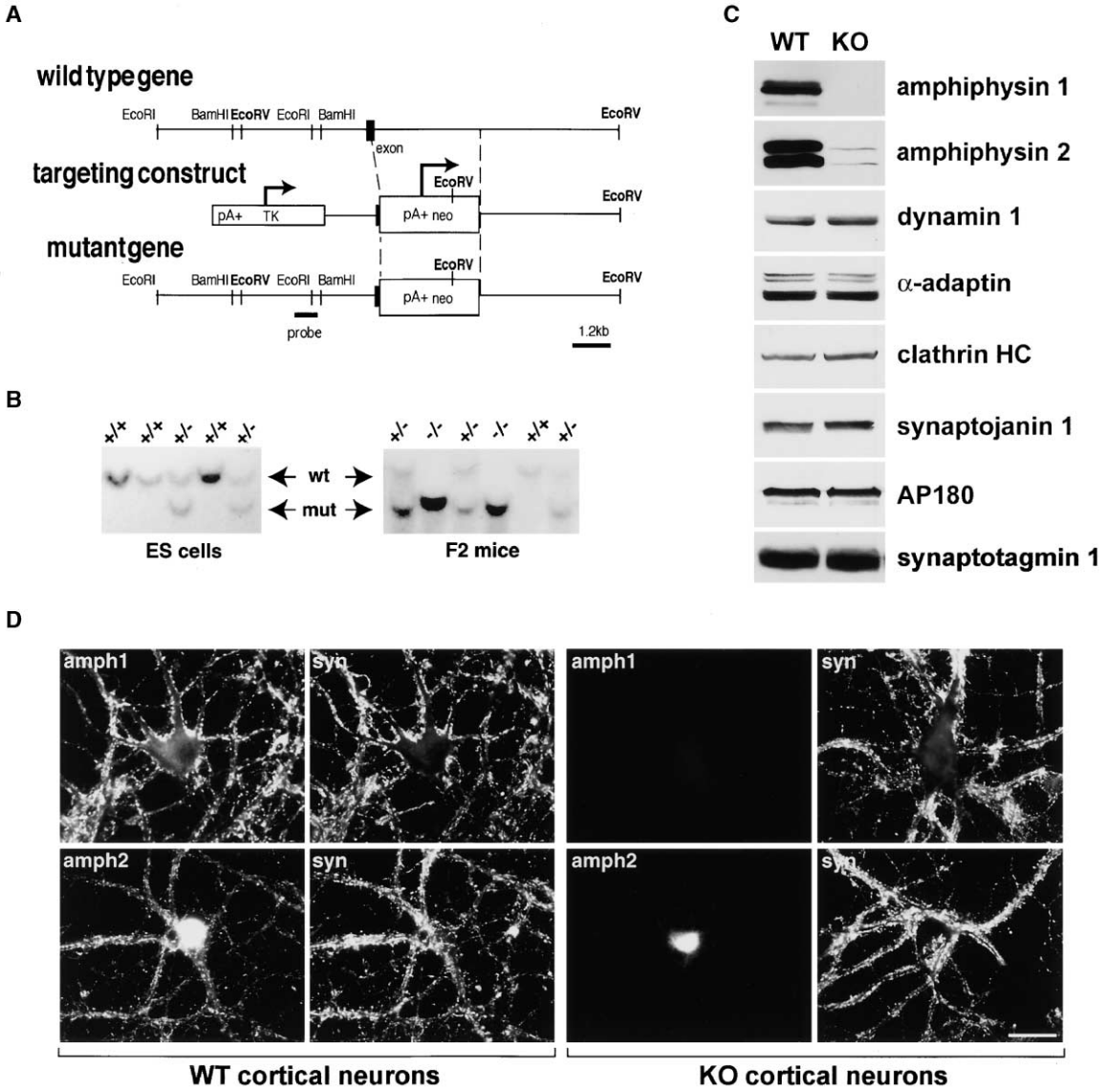

Figure 1. Generation of Amphiphysin 1 Knockout Mice and Downregulation of Amphiphysin 2 Expression

(A) Schematic diagram showing the amphiphysin 1 gene locus, the targeting vector, and the mutant allele following homologous recombination. The neo cassette substitutes part of the first coding exon and part of the following intron. The EcoRV restriction sites and the probe used for the Southern blot analysis are indicated.

(B) Southern blot of EcoRV-digested genomic DNA from wild-type (+/+) and heterozygous (+/−) ES cell clones and from wild-type (+/+), heterozygous (+/−), and homozygous mutant mice (−/−) of the F2 generation.

(C) Western blot of brain extracts from wild-type and knockout mice with antibodies directed against either amphiphysin 1 or 2 or several other synaptic proteins, including major interacting partners of amphiphysin 1 (HC, heavy chain). The knockout extract contains no amphiphysin 1 and drastically reduced levels of amphiphysin 2.

(D) Double immunofluorescence of primary cultures of cortical neurons prepared from the brain of wild-type and knockout mice. Sections were stained for either amphiphysin 1 or 2 (amph) and counterstained for the presynaptic marker synaptophysin (syn). Lack of amphiphysin 1 expression correlates with loss of amphiphysin 2 immunoreactivity in knockout neurons. Residual immunoreactivity found in the nucleus may represent a nuclear pool of amphiphysin 2 (Wechsler-Reya et al., 1997). Scale bar = 15 μm.

Defective Cell-Free Assembly of Endocytic Protein Complexes in Brain Extracts of Amphiphysin 1 Knockout Mice

The properties of the amphiphysin 1/2 heterodimer to bind and deform lipid bilayers (Takei et al., 1999) and to interact with several endocytic proteins (Figure 3A; see also Wigge and McMahon, 1998) suggest that it functions as a multifunctional adaptor at the membrane. We tested this putative function of amphiphysin by determining in cell-free reactions whether the formation of complexes among these proteins, or between these proteins and lipid bilayers, was affected by the lack of the amphiphysin 1/2 heterodimers from brain cytosol. Liposomes composed of total brain lipids were incubated with cytosol from wild-type and mutant brain in the presence of nucleotides (Cremona et al., 1999; Gad et al., 2000). Proteins bound to membranes were then isolated by ultracentrifugation and analyzed by quantita-

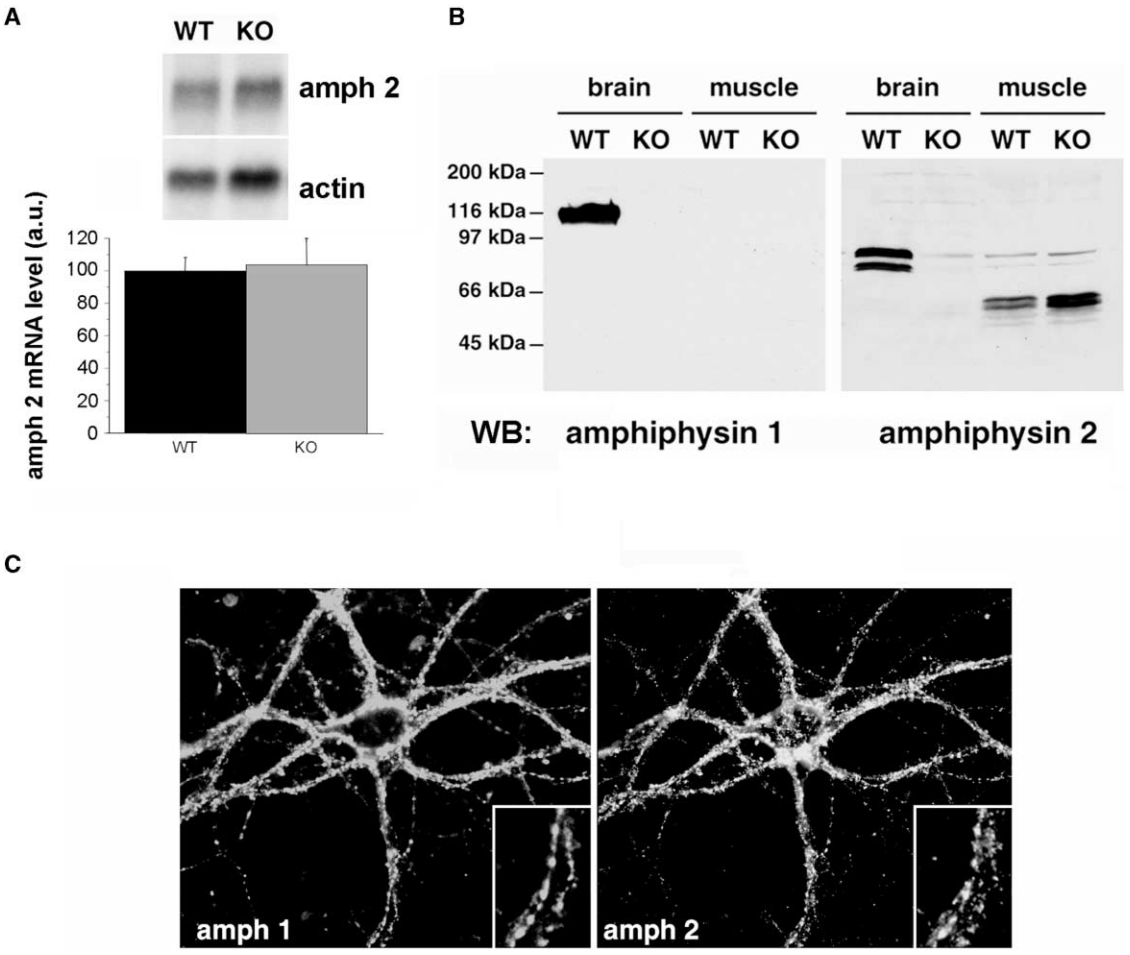

Figure 2. Selective Loss of Amphiphysin 2 in Brain where Amphiphysin 1 and 2 Form Heterodimers and Colocalize

(A) Northern blot analysis showing normal levels of amphiphysin 2 mRNA in knockout brain. Actin mRNA was used as a control for loading. mRNA levels were quantified by phosphoimaging, and amphiphysin 2 mRNA levels were normalized to those of actin mRNA ($n = 2$).

(B) Western blot analysis of brain and skeletal muscle extracts from wild-type and knockout mice using anti-amphiphysin 1 and anti-amphiphysin 2 antibodies. While amphiphysin 1 is only detected in brain, amphiphysin 2 is expressed at high levels in both brain and skeletal muscle, but as isoforms of different molecular weight. In mutant mice, the downregulation of amphiphysin 2 expression levels occurs selectively in brain.

(C) Double immunofluorescence of cultured cortical neurons from wild-type mice showing precise colocalization of amphiphysin 1 with amphiphysin 2 at synaptic sites. Inset: higher magnification.

tive immunoblotting along with proteins in the starting cytosols. While levels of all proteins examined were the same in the starting cytosol (data not shown), the recovery of clathrin (heavy chain) and AP-2 (α-adaptin) in the "bound" fraction was reduced by 40% and 25%, respectively, in mutant extract relative to wild-type extract (Figures 3B and 3C). A 40% decrease in the recovery of the polyphosphoinositide phosphatase synaptojanin 1 was also observed. Accordingly, incubation of the pelleted liposomes in the presence of ^{32}P-labeled ATP after the binding reaction resulted in an increased level of [^{32}P]-PI(4,5)P_2, most likely reflecting altered PI(4,5)P_2 turnover due to decreased synaptojanin 1-mediated PI(4,5)P_2 hydrolysis (Figure 3D). The recovery of dynamin 1 in the "bound" fraction of liposomes was not affected by the lack of amphiphysin 1 (Figure 3B and 3C). This observation most likely reflects, at least in part, the direct binding of dynamin to lipid membranes via both its pleckstrin homology (PH) domain and the recently identified additional lipid binding site (Burger et al., 2000). As it was shown previously, although amphiphysin does not enhance dynamin recruitment to liposomes, it affects the morphology and the properties of dynamin oligomers at the surface of liposomes (Takei et al., 1999).

We further investigated the potential adaptor function of amphiphysin in the assembly of endocytic proteins in brain, by performing affinity chromatography of a detergent brain extract from wild-type and knockout mice on the COOH-terminal proline-rich tail of dynamin 1 (GST-PRD)—i.e., the amphiphysin binding domain of dynamin. The use of wild-type extracts for these experiments leads to the affinity purification not only of amphiphysin 1 and 2 and other direct interactors of dynamin's tail, such as endophilin and intersectin, but also of clathrin and AP-2 (Figure 3A; see also Slepnev et al., 1998). Immunoblotting of the affinity-purified material revealed a reduction in AP-2 (α-adaptin) and clathrin

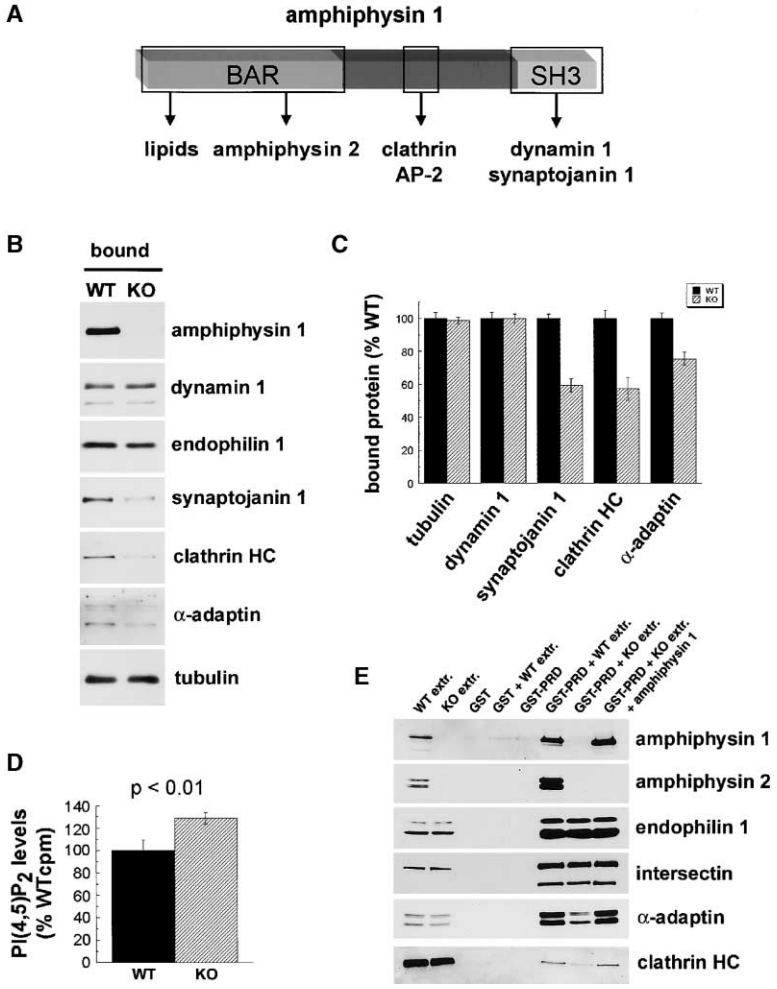

Figure 3. Evidence for a Role of Amphiphysin as an Adaptor Protein in the Recruitment of Cytosolic Endocytic Complexes

(A) Schematic drawing depicting the structure of amphiphysin 1 and its interactors.

(B and C) Recovery of endocytic proteins on liposomes following their incubation with brain cytosol from wild-type and knockout mice in the presence of ATP and GTP. Representative Western blots are shown in (B), and a quantification by phosphoimaging of results from three experiments is shown in (C). Note the absence of amphiphysin 1 and the selective decrease of clathrin, AP-2 (α-adaptin subunit), and synaptojanin 1 in the "bound" material from knockout animals.

(D) Quantification of ^{32}P incorporation into $PI(4,5)P_2$ in liposomes that were first incubated with brain cytosol in the presence of GTP and cold ATP, washed, and subsequently incubated with [$\gamma^{32}P$]ATP. Decreased recovery of synaptojanin 1 in membranes shown in (B) and (C) correlates with increased labeling of $PI(4,5)P_2$.

(E) Western blot analysis of a representative affinity chromatography experiment from wild-type and mutant brain extracts on GST alone, or GST fused to the proline-rich domain (PRD) of dynamin 1. The first two lanes show starting material from both genotypes. Mock affinity purifications (lack of extract) are included as indicated. The knockout brain extract used for the last lane was supplemented with recombinant amphiphysin 1 at physiological concentration. The PRD of dynamin affinity purifies less clathrin and AP-2 from knockout cytosol and exogenous amphiphysin 1 rescues the effect.

Values in (C) and (D) denote means ± SD. The p value from a Student's t test analysis is indicated.

binding, but not in that of endophilin and intersectin, when brain extracts from knockout animals were used (Figure 3E), consistent with a bridging function of amphiphysin. This decrease was rescued by the addition to the knockout extract of recombinant amphiphysin 1 at physiological concentration prior to affinity purification. Collectively, these results provide strong evidence for a role of amphiphysin in the assembly of endocytic complexes at the membrane.

Decreased Stimulus-Dependent Labeling of Synaptic Vesicles Following a "Pulse" Incubation with an Endocytic Tracer

In spite of the biochemical results described above, no obvious ultrastructural differences were observed by electron microscopy in synapses of wild-type and mutant neurons both in brain tissue or in cultures. No accumulation of endocytic intermediates was observed in mutant synapses and the number of synaptic vesicles per synapse was the same in the two genotypes (data not shown). To assess the possible occurrence of synaptic vesicle recycling defects that could be unmasked by stimulation, we used two distinct and complementary approaches.

First, we performed experiments on cerebral cortex synaptosomes. This preparation allows for the analysis of exo- and endocytosis on a global synaptic population, thus avoiding possible bias due to uneven neuronal sampling. Furthermore, synaptosomes, in contrast to neuronal cultures (see below), reflect the properties of synapses in the adult brain. Using a well established fluorimetry-based glutamate release assay (Nicholls and Sihra, 1986), we found no difference in the kinetics or amount of glutamate release between wild-type and knockout synaptosomes upon high K^+ stimulation, which points to a normal exocytic function in knockout synapses (Figures 4A and 4B). Fluorescent amphipathic styryl dyes were then used to study synaptic vesicle recycling. We employed FM2-10 for these experiments, because it has the fastest "off rate" among commonly used styryl dyes and was shown to give a good signal-to-noise ratio in synaptosomes (Cousin and Robinson, 1998; Marks and McMahon, 1998; Cousin and Robinson, 2000). We started by determining the ability of synaptosomes to internalize the dye into vesicular compartments in a stimulation-dependent fashion. Briefly, synaptosomes were preincubated with FM2-10 and depolarized for 90 s by the addition of high K^+ in the continued presence of dye. Then, after a brief wash, synaptosomes were hypotonically lysed, subjected to low-speed centrifugation, and the fluorescence trapped

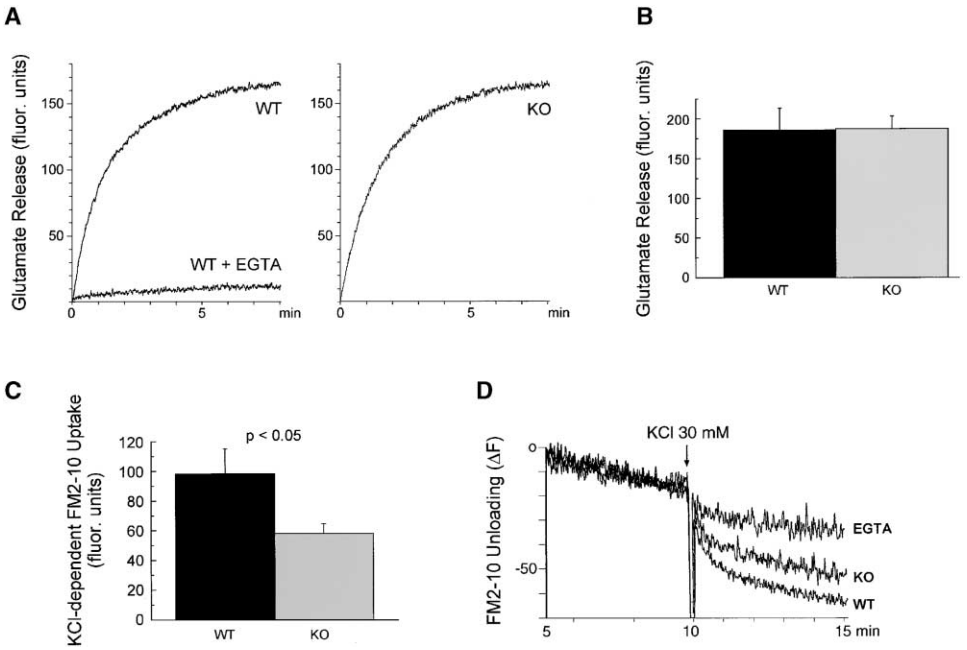

Figure 4. Normal Glutamate Release but Defective Recycling of FM2-10 in Knockout Mice

(A) Fluorimetric tracings of wild-type and knockout synaptosomes showing comparable Ca^{2+}-dependent glutamate release for both genotypes after stimulation with 30 mM KCl.

(B) Quantification of total glutamate release after stimulation with 30 mM KCl for 8 min.

(C) Recovery of FM2-10 in a low speed supernatant of lysed synaptosomes after a first round of stimulation with 30 mM KCl in the presence of dye. The amount of dye trapped in this fraction (endosomes and small vesicles) was smaller in mutant synaptosomes.

Values in (B) and (C) denote means ± SEM (n = 3). The p value from a Student's t test analysis is indicated.

(D) Fluorimetric tracing of wild-type and knockout synaptosomes showing high K^+-dependent unloading of FM2-10 following a first round of stimulation in the presence of dye and a 10 min incubation in dye-free low K^+ to allow for recycling of the dye into the releasable vesicle pool. A tracing showing dye unloading of wild-type synaptosomes in the presence of 2 mM EGTA is also shown.

within membranous organelles of the supernatant, which include primarily endosomes and small vesicles, was measured by fluorimetry. This assay revealed a 40% decrease in the depolarization-dependent internalization of dye in knockout synaptosomes, compared to wild-type synaptosomes (Figure 4C). In the next series of experiments, synaptosomes were stimulated for 90 s in the presence of dye, further incubated for 10 min in dye-free medium to allow for chasing of the dye into releasable synaptic vesicles, and finally exposed to a second high K^+ incubation to induce exocytotic release of the dye. Under these assay conditions, knockout synaptosomes released significantly less FM2-10 than wild-type synaptosomes (Figure 4D), suggesting that synaptic vesicle recycling efficiency is also reduced in amphiphysin 1-deficient nerve terminals.

Second, we used electron microscopy in combination with the fluid-phase uptake of the endocytic marker horseradish peroxidase (HRP) to monitor formation of endocytic intermediates in cultured cortical neurons (Heuser and Reese, 1973). Neurons were grown in culture for at least 2 weeks to allow for the formation of numerous synaptic contacts (see Figure 1D). They were then preincubated with HRP for 3 min, stimulated with high K^+ for 90 s (or mock-incubated in low K^+) in the continued presence of the tracer, fixed, and processed for electron microscopy analysis. High K^+ stimulation produced a significant increase in the HRP labeling of synaptic vesicles and endosomal structures as expected (Figure 5A). However, morphometric analysis indicated that the fraction of HRP-labeled synaptic vesicles and endosome-like structures was significantly reduced in knockout synapses relative to wild-type after stimulation, demonstrating the occurrence of a recycling defect in knockout animals (Figures 5B and 5C). No statistical difference in the number of labeled organelles was found under resting conditions (Figures 5B and 5C).

Smaller Recycling Vesicle Pool Size, Slower Destaining Kinetics, and Delayed Vesicle Repriming in Mutant Nerve Terminals

The reduced stimulus-dependent dye uptake into vesicular compartments described above could be explained by a delayed endocytic uptake of the tracers but also by a smaller size of the recycling pool of synaptic vesicles. Although the number of vesicles per synapse appears to be normal, the functional recycling pool could be smaller. To determine the possible contribution of a smaller functional recycling vesicle pool and to validate the results described above with a more physiological stimulus, we performed styryl dye uptake and release studies in paired cortical cultures from wild-type and mutant animals using action potential trains of variable duration at 20 Hz. Cultures were first exposed to an initial dye-staining step with FM1-43 using a variable number (x) of action potentials (where x ranges from 20

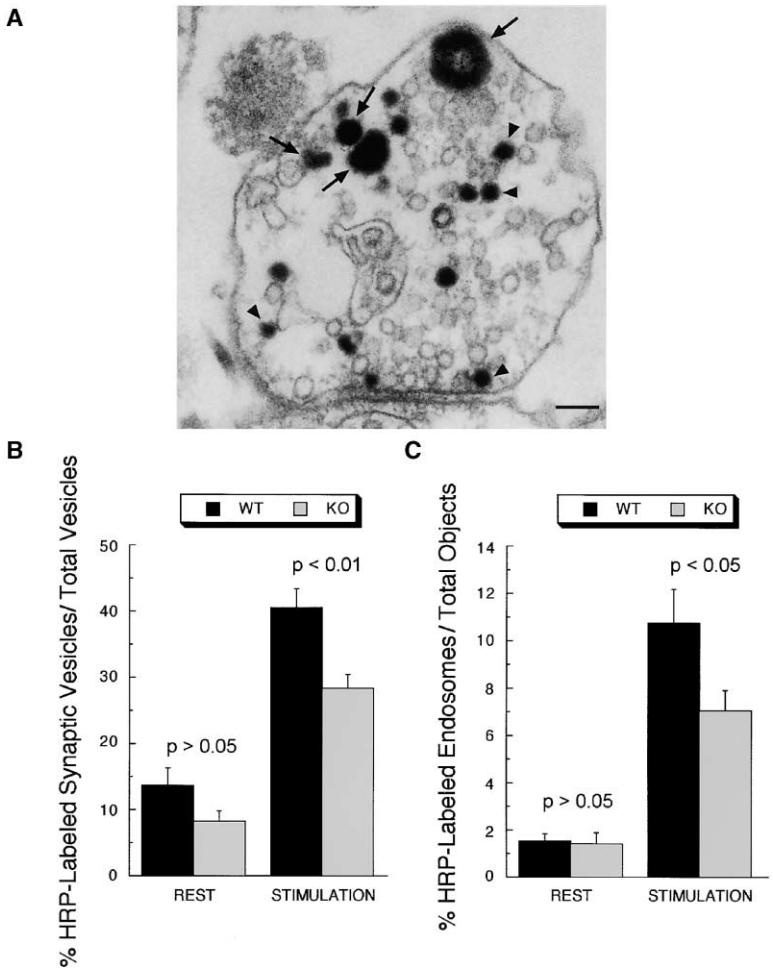

Figure 5. Decreased Labeling of Synaptic Vesicles and Endosome-like Structures after a "Pulse" Incubation with the Fluid Phase Tracer HRP in Synapses of Mutant Cortical Neurons in Culture

(A) Electron micrograph of wild-type synapse stimulated with 90 mM KCl for 90 s in the presence of tracer. A fraction of synaptic vesicles is labeled by HRP (arrowsheads). HRP-labeled objects defined as endosome-like structures are indicated by arrows. Scale bar = 100 nm.

(B) Morphometric analysis indicating the percentage of total small vesicles that are labeled by HRP. The stimulation-dependent increase in the labeling of synaptic vesicles is greater for wild-type synapses than for knockout synapses.

(C) Percentage of HRP-labeled endosomal structures over total number of objects (small vesicles plus labeled endosomal structures). Even in this case, the stimulation-dependent increase in the labeling of endosomes is less pronounced in the case of mutant synapses. Values denote means ± SEM, and p values according to ANOVA are indicated.

to 800) followed by an additional 90 s incubation in the continued presence of the dye in order to label endocytic vesicles whose formation lags behind the interruption of the stimulus. Then, after a 10 min washing period to allow for the chasing of the dye into the releasable pool vesicles, internalized dye was released by a stimulus protocol previously shown to result in maximal destaining (Ryan, 1999). The amount of released dye gives an estimate of the dye taken up in the staining period and thus an estimate of the fraction of synaptic vesicles that underwent exocytosis during a particular action potential stimulus in the dye-loading step.

The total amount of dye uptake with different number of action potentials was normalized to the uptake at 600 action potentials in wild-type terminals and plotted as a function of action potential numbers (Figure 6A). As expected, there was clearly an increase in the size of the vesicle pool that is mobilized with a longer stimulus train in both wild-type and knockout cultures. However, the recycling vesicle pool was 25%–30% smaller in the knockout cultures compared to the wild-type cultures over the entire range of action potential stimuli.

The experiments described in Figure 6A, which showed a smaller total recycling vesicle pool size in knockout cultures, also allowed us to determine whether there was a difference in the fraction of the recycling pool that is engaged by a given duration of action potential train in knockout cultures. To this aim, each of the FM1-43 uptake values from Figure 6A was normalized to the uptake at 600 action potentials for both wild-type and knockout cultures. Comparison of the two sets of values revealed a statistically significant defect in the fractional pool of vesicles mobilized at intermediate AP stimuli in knockout synapses. The fractional pool mobilized at 200 and 300 AP was 0.61 ± 0.02 and 0.74 ± 0.02 for wild-type cultures (n = 265 boutons, three experiments) and 0.54 ± 0.01 and 0.61 ± 0.02 for knockout cultures (n = 288 boutons, three experiments).

We also measured the destaining kinetics of synaptic terminals loaded with FM1-43 during a 600 action potential stimulus. A delay in the delivery of newly internalized vesicles to the releasable vesicle pool would be reflected in a greater time constant for dye release. Onset of the stimulus was associated with a decline in fluorescence in both wild-type and knockout synaptic terminals (Figure 6B). The decline in fluorescence was identical until 100 action potentials, the fifth time point measured. However, at later time points (>200 action potentials), there was a statistically significant delay in the destaining kinetics of knockout terminals relative to wild-type terminals. The time constant of destaining was 17.6 ± 0.6 s in wild-type compared to 21.1 ± 0.5 s in knockout terminals ($p < 0.02$, n = 3).

The slower destaining kinetics of FM1-43-loaded syn-

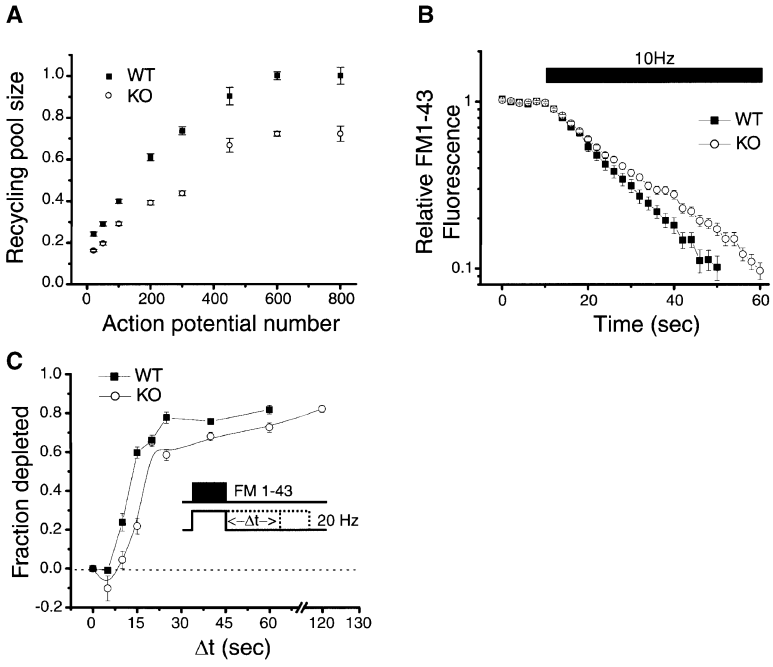

Figure 6. Defects in Synaptic Vesicle Dynamics Revealed by FM1-43 Studies in Synapses of Knockout Mice

(A) Measurement of synaptic vesicle functional pool size in response to action potential (AP) trains of variable duration at 20 Hz. Cortical cultures were first loaded with FM1-43 during a train of APs (abscissa), then, after a 10 min wash-out period, they were subjected to two rounds of AP trains at 20 Hz (separated by a 2 min rest period) to ensure complete dye release. The total amount of dye released during the destaining period gives an estimate of dye uptake into synaptic vesicles during the staining period, and, indirectly, of the vesicle pool size engaged in exo-endocytosis during the same period. Values of FM1-43 uptake at different AP stimuli for both wild-type and mutant synapses were normalized to the uptake at 600 APs in wild-type synapses. Mutant cultures have a smaller vesicle pool size. The total FM1-43 uptake at 600 AP stimuli was 3969 ± 73 (a.u.) in wild-type cultures (n = 635 boutons, five experiments) compared to 2867 ± 45 (a.u.) in knockout cultures (n = 725 boutons, six experiments), statistically significant at $p < 0.001$, Student's t test.

(B) Following FM1-43 loading with 600 APs at 10 Hz, the time course of destaining differs between wild-type and mutant nerve terminals. Destaining of knockout synapses lags behind wild-type. Comparable results were obtained in six other experiments.

(C) Time course of vesicle repriming. The inset shows the experimental protocol. Nerve terminals were stimulated, during a 5 s exposure to FM1-43, by a train of action potentials at 20 Hz that exceeded the period of dye exposure by an amount of time Δt. The resulting dye uptake was measured during a subsequent destaining stimulus at 20 Hz after a 10 min rest. The dye uptake at $\Delta t = 0s$ (F_0) represents the total number of releasable vesicles engaged by the stimulus. The dye uptake measured in runs where $\Delta t > 0s$ ($F_{\Delta t}$) provides an estimate of vesicle depletion, due to fusion of reprimed vesicles, during the additional action potential stimulation after dye washout. The data points represents the fraction depleted ($F_0 - F_{\Delta t}/F_0$) as a function of Δt. The data are from a total of 80–165 boutons for each time point in both wild-type and knockout cultures. Error bars are ± SEM.

aptic terminals could be due to a delay in repriming time, defined as the time that elapses before newly endocytosed vesicles become available for a new round of release (Ryan and Smith, 1995). We tested this hypothesis by directly measuring the repriming time course in cultured cortical neurons (Figure 6C). Nerve terminals were loaded with FM1-43 for a fixed period of 5 s, during a train of action potentials at 20 Hz that exceeded the period of dye exposure by an amount of time Δt (inset in Figure 6C). The total fluorescence incorporated into vesicles was measured 10 min later using a maximal destaining stimulus ($F_{\Delta t}$). These data were normalized to the average fluorescence in bracketed control and recovery runs, when the stimulation was terminated at exactly the same time as the washout of dye ($\Delta t = 0$, F_0). The ratio ($F_0 - F_{\Delta t}/F_0$) represents the fraction of the fluorescence that was depleted during the additional period of stimulation and reflects the time course of gain in rerelease-competence of recently endocytosed vesicles. Any delay in the endocytic journey of the vesicle will prolong this time. Results of such analyses show that both wild-type and amphiphysin 1-deficient nerve terminals released about 80% of the endocytosed vesicles in the next 120 s of continuous stimulation. However, mutant nerve terminals showed a significant delay in dye depletion compared to wild-type terminals (at $\Delta t = 15$ s, wild-type was 60% depleted compared to 20% in knockout cultures, $p < 0.001$). The time to deplete 40% of dye taken up was 12 s in the wild-type terminals compared to 17 s in the knockout cultures. Thus, a delay in repriming of vesicles may at least partially account for the slower destaining kinetics observed in knockout cultures (Figure 6B).

Collectively, these results demonstrate the occurrence of complex recycling defects in amphiphysin knockout terminals, which range from a decreased functional recycling pool size to delayed recycling and repriming rates.

Reduced Viability and Increased Susceptibility to Seizures in Amphiphysin 1 Knockout Mice

Given the cell biological defects described above at synapses of knockout animals, we further investigated changes at the organismal level. A significant decrease in survival rate was observed in the amphiphysin 1 knockout colony, such that only 50% of the animals survived to 10 months of age, as compared with 95% for wild-type mice (Figure 7A). Animals were found dead, mainly between 2 and 5 months of age, with no previous decrease in body weight or other noticeable health problems. To document what appeared to be sudden death, continuous digital camera monitoring of a small group of animals was performed. This approach revealed the occurrence of rare spontaneous seizures. Death resulted from seizures that reached a tonic extension and failed to resolve. Such an observation suggested that amphiphysin 1 knockout mice may exhibit a lower threshold to seizures compared to wild-type animals.

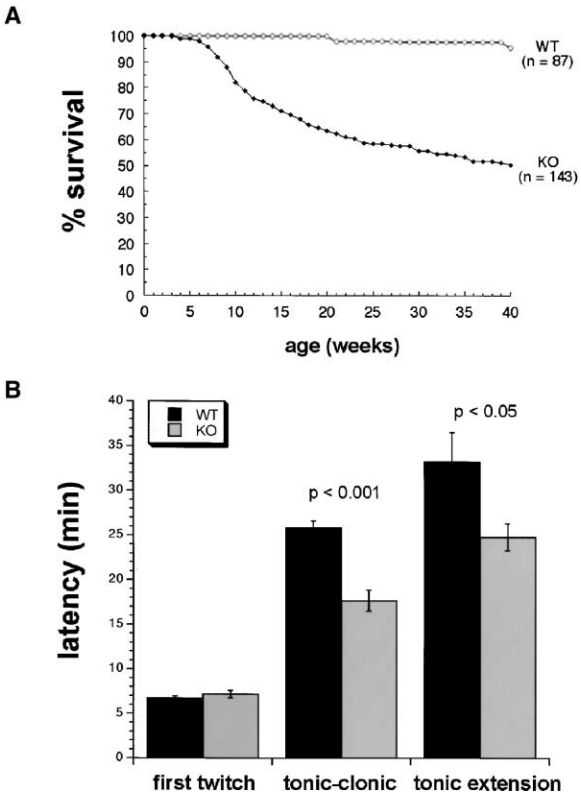

Figure 7. Amphiphysin 1 Knockout Mice Exhibit Increased Mortality and a Higher Propensity to Seizures

(A) Survival curves showing reduced viability of knockout mouse colonies at adult age. Animals die from rare irreversible seizures.

(B) Time (latency) required for wild-type and knockout mice to develop three phases of epilepsy after repeated intraperitoneal injections of pentamethylenetetrazole (metrazol), an epileptogenic $GABA_A$ receptor antagonist. No difference is observed for the first phase (first twitch), but amphiphysin 1 knockout mice progress significantly faster to the hyperkinetic tonic-clonic phase and finally to tonic extension and death. Values denote means ± SEM (n = 7 for wild-type; n = 10 for knockout). P values from a Student's t test analysis are indicated.

To test this hypothesis, we investigated the response of the animals to intraperitoneal injections of pentamethylenetetrazole (metrazol), an epileptogenic $GABA_A$ receptor antagonist (Tecott et al., 1995). Increasing blood levels of the drug results in a series of well-defined responses that start with intermittent body twitches, leading to hyperkinetic tonic-clonic seizures and finally to a tonic extension and death. Whereas the time lag to the first neurological symptoms (first twitch) was comparable in both genotypes, knockout mice progressed significantly faster to the next two phases (tonic-clonic and tonic extension, respectively), demonstrating an increased propensity to seizures (Figure 7B).

Amphiphysin 1 Knockout Mice Exhibit Cognitive Defects

Considering the synaptic vesicle recycling defects revealed by our studies, the lack of impairment in nervous tissue function, besides occasional seizures, was puzzling. Behavioral tests were therefore performed to assess higher brain function. We first examined the spatial learning ability of amphiphysin 1 knockout mice using the Morris water maze task (Morris, 1989). Mice were trained on a task in which they searched for a hidden submerged platform in order to escape from water, using visual cues from the environment. The escape latency, recorded over several sessions, revealed a progressive improvement of the performance in all animals. However, a striking learning deficit in the mutant animals was detected (Figure 8A). After training in the hidden platform test, the transfer test in which mice were allowed to swim freely for 60 s following removal of the platform, was performed. A measurement of quadrant occupancy revealed that wild-type mice spent significantly more time in the quadrant where the platform had been originally located, whereas mutant mice did not (Figure 8C). Moreover, crossings of amphiphysin 1 knockout mice over the original platform location were significantly lower compared to wild-type animals (Figure 8D). No difference in path length was observed between the two genotypes (Figure 8E), indicating that swimming ability is not impaired in knockout mice. To rule out any differences in visual acuity, locomotor activity, or motivation, mice were trained on a visible platform test, in which the platform was placed above the surface of the water with a prominent "flag" attached to it. The escape latency was recorded over the course of 4 days. During this time, the performance of mice from both genotypes improved, although less dramatically in the case of mutant mice (Figure 8B). These results strongly suggest that amphiphysin 1 knockout mice exhibit deficits in learning that may not be only restricted to spatial learning.

To assess whether other forms of learning are impaired in mutant mice, we performed a fear-conditioning test (Paylor et al., 1994), which measures an amygdala-dependent form of associative emotional learning (reviewed in LeDoux, 2000). In this paradigm, an innocuous conditioned stimulus (tone) elicits fear response after being associatively paired with an aversive unconditioned stimulus (footshock). The fear response is measured by the frequency of freezing behaviors, which is defined as a stereotyped motionless crouching posture. Amphiphysin 1 knockout mice exhibited very mild fear responses in both the context test and the auditory cue test compared to their wild-type littermates. In contrast, the fear response generated by a novel context was comparable for both genotypes (Figure 8F). As a control, nociceptive reactions to footshocks were also investigated for both genotypes and were found to be comparable (data not shown). Altogether, our data indicate that amphiphysin 1 knockout mice exhibit major learning deficits, which are not restricted to spatial learning deficits.

Discussion

The results of this study show that in mammals amphiphysin function is required for efficient synaptic vesicle recycling. The data further demonstrate that a partial impairment of this process, and possibly of other membrane recycling pathways at the synapse, is compatible with the basic operation of the nervous system, although

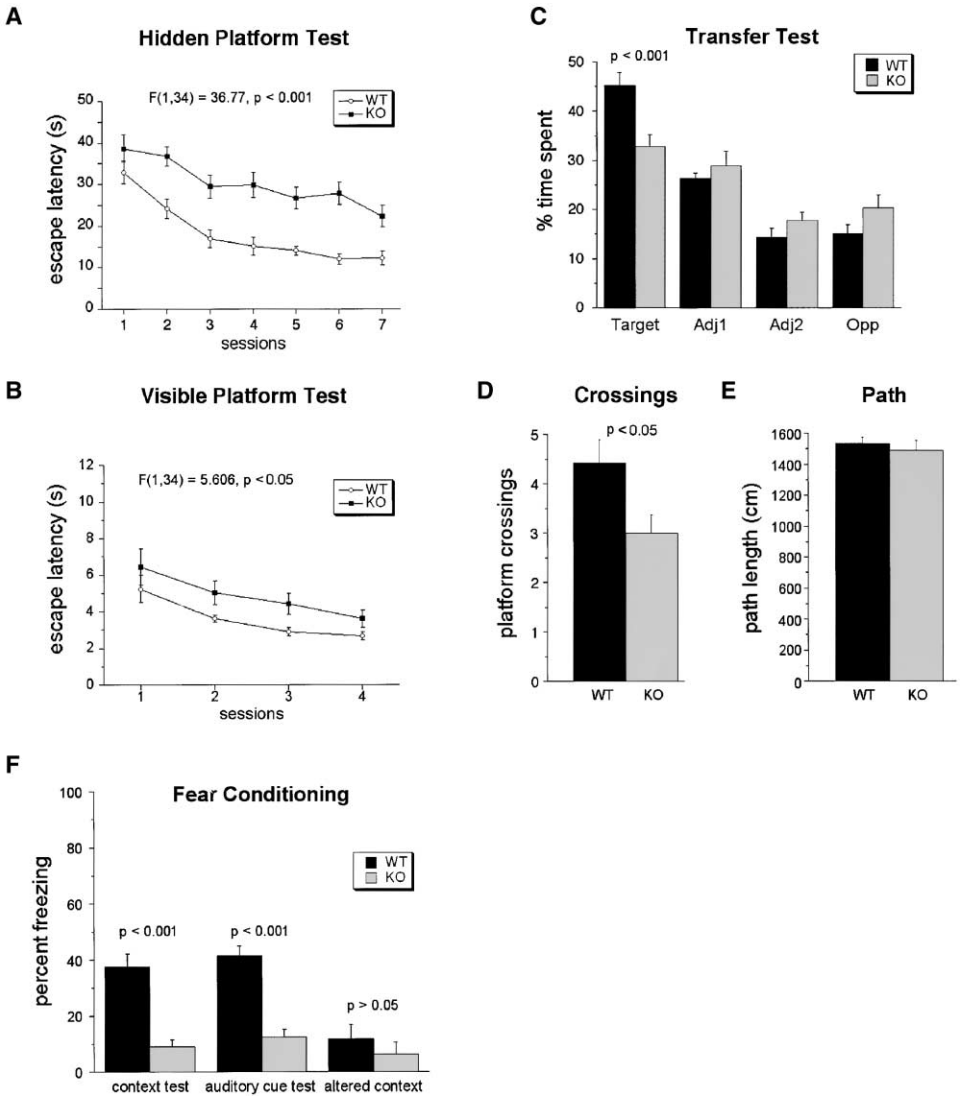

Figure 8. Amphiphysin 1 Knockout Mice Exhibit Learning Deficits in the Morris Water Maze Task (A–E) and in the Fear Conditioning Task (F)

5- to 7-month-old wild-type or knockout animals were used. All values denote means ± SEM.

(A) Learning curve indicating the time required to reach the hidden platform over seven days (n = 18 for each genotype). Statistical analysis was performed by two-way ANOVA for repeated measures.

(B) Latency to find the visible platform over the course of four sessions.

(C–E) Transfer test performance after the acquisition trials of the hidden platform task.

(C) Bars represent the time spent in the four quadrants (target, adjacent 1, adjacent 2, and opposite). Wild-type animals spent significantly more time in the quadrant that originally contained the platform than in each of the other quadrants. This was not the case for knockout animals.

(D) Number of crosses over the area that originally contained the platform during the transfer test were significantly lower for knockout animals relative to wild-type. P values from Mann-Whitney *U*-test are indicated.

(E) Path length (cm) during the transfer test. No statistical difference was observed between the two genotypes.

(F) Freezing responses in the fear conditioning test (n = 9 for each genotype). Amphiphysin 1 knockout mice exhibited significantly less freezing behavior than wild-type mice in both the context test and the auditory cue test. Comparable responses to altered context were observed for both genotypes. Statistical analysis was performed according to Student's t test, and p values are indicated.

it is associated with decreased viability and with defects in higher brain function.

At the molecular level, a striking consequence of the lack of amphiphysin 1 expression in brain was the nearly complete disappearance of amphiphysin 2. Since levels of amphiphysin 2 mRNA are unchanged in the brain of mutant mice, this phenomenon is likely to result from decreased stability of amphiphysin 2 and is consistent with the reported occurrence of amphiphysin 1 and 2 primarily as stable heterodimers in brain (Wigge et al., 1997a; Ramjaun et al., 1997; Slepnev et al., 1998). No downregulation of amphiphysin 2 occurs in skeletal muscle where this protein is normally expressed at very high levels without amphiphysin 1 (Butler et al., 1997). It remains to be determined whether the stability of this skeletal muscle isoform, which differs from the major

brain amphiphysin 2 isoform (Butler et al., 1997), is accounted for by its greater intrinsic resistance to proteolytic degradation or different proteolytic machinery in muscle, by heterodimerization with another BAR domain-containing protein, or by its localization in a subcellular compartment which protects it from high turnover.

The property to form dimers, which resides in the BAR domain, is a conserved feature in the amphiphysin family of proteins, since it also applies to yeast Rvs167, which heterodimerizes with Rvs161 (Navarro et al., 1997; Lombardi and Riezman, 2001). Furthermore, deletion of either the *RVS167* or the *RVS161* gene leads to a significant decrease in the half-life of the protein encoded by the other gene. Interestingly, overexpression of the Rvs161 protein in the *RVS167* knockout strain aggravates, rather than rescues the abnormal phenotype, indicating that the stoichiometry of interaction between related amphiphysin isoforms is a critical parameter for their function (Wigge et al., 1997a; Lombardi and Riezman, 2001).

Our biochemical results strongly support the hypothesis that one of the functions of brain amphiphysin is to act as a multifunctional adaptor connecting various cytosolic components of the endocytic machinery to each other and to the lipid bilayer (Takei et al., 1999). They suggest that amphiphysin may contribute to the recruitment of clathrin, AP-2, and synaptojanin 1 to the membrane. Moreover, although binding of dynamin to lipid bilayers was not dependent upon amphiphysin under our in vitro conditions, affinity purification experiments on the proline-rich tail of dynamin demonstrated that amphiphysin can indeed act as a bridge between clathrin coat components and dynamin. Thus, amphiphysin may help to coordinate the function of all these proteins at the membrane. Furthermore, the direct binding of dynamin to membrane in situ may undergo regulation, and a contribution of amphiphysin to dynamin recruitment in vivo cannot be ruled out.

The defects revealed by our cell-free approaches did not correlate with obvious morphological changes in mutant synapses. However, changes in the efficiency of synaptic vesicle recycling were demonstrated by a variety of physiological approaches after synapse stimulation. These include decreased uptake of extracellular tracers into small vesicles after an endocytic labeling pulse and a slower recycling time as demonstrated by a delay in styryl dye unloading, both during a prolonged stimulus (minimum repriming time) and after recovery from a stimulus (destaining kinetics). Consistent changes were observed both in synaptosomes and in synapses of cultured cortical neurons. Based on FM1-43 experiments in cultured neurons, the pool size of recycling synaptic vesicles also appeared to be smaller. This finding cannot merely be accounted for by a difference in the number of synaptic vesicles per synapse, based on both biochemical (comparable levels of synaptic proteins in brain homogenates) and morphometric results. Thus, the lack of amphiphysin seems to affect several stages of recycling. It seems likely, that although the kinetics of endocytosis is not perturbed (data not shown), the fate of vesicles endocytosed in the absence of amphiphysin is altered such that capacity for subsequent reuse of synaptic vesicles is diminished. This could arise for example if amphiphysin plays a role in mediating the correct sorting of components necessary for efficient postendocytic trafficking to the budding membrane. Although some data could also be explained by the occurrence of a partial defect in exocytosis (for example, the delay in dye unloading after stimulation), the normal glutamate release observed in synaptosomes argues against a significant impairment of this process. It remains possible that the upregulation of a putative "kiss-and-run" recycling pathway partially compensates for recycling defects in knockout animals and contributes to glutamate release stability during prolonged stimulation.

The complex recycling defects may be the results of adaptive changes triggered by an endocytic defect. They may also depend on actions of amphiphysin that are linked to its proposed, albeit not yet understood, function in actin cytoskeleton dynamics. Such a function is suggested by the severe disruption of actin cytoskeleton that is observed in *rvs167* and *rvs161* yeast mutants, in addition to endocytic defects (Sivadon et al., 1995; Lombardi and Riezman, 2001). So far, evidence for a role for amphiphysin in actin dynamics in mammalian cells is only indirect (Mundigl et al., 1998), but the putative role in actin function of some of its partners, dynamin and synaptojanin, support this possibility (Sakisaka et al., 1997; Gad et al., 2000; McNiven et al., 2000). Furthermore, the occurrence of amphiphysin 2 isoforms that lack clathrin and AP-2 binding sites points to functions of amphiphysin that are unrelated to the clathrin coat.

Interestingly, acute disruption of amphiphysin function at synapses of stimulated giant lamprey axons by microinjection either of peptides that bind specifically the SH3 domain of amphiphysin (Shupliakov et al., 1997) or of anti-lamprey amphiphysin antibodies (N. Tomilin, M. Marcucci, H. Gad, P. Löw, V. Slepnev, L. Brodin, P.D.C., and O. Shupliakov, unpublished data) produce more severe vesicle recycling defects than those reported here. Compensatory changes dependent upon the presence of proteins whose function is partially redundant with that of amphiphysin may explain the lack of a dramatic cellular phenotype in neurons of mice that develop in the absence of amphiphysin. It is of note that an acute antisense-mediated inhibition of amphiphysin 1 expression in cultured neurons results in major defects of neurite outgrowth (Mundigl et al., 1998), whereas this process occurs normally in mutant neurons. A puzzling observation is the lack of biochemical evidence for an interaction between amphiphysin and dynamin in *Drosophila* (Razzaq et al., 2001), where the synaptic dynamin interacting network seems to be at least partially different from the corresponding mammalian one (Roos and Kelly, 1998). In *Drosophila*, the single amphiphysin gene lacks clathrin and AP-2 binding sites and is abundantly expressed in skeletal muscle (Razzaq et al., 2001). Accordingly, amphiphysin-deficient flies predominantly show a muscle phenotype (Razzaq et al., 2001; Zelhof et al., 2001). In mammals, the presence of two neuronal isoforms of amphiphysin (amphiphysin 1 and the neuronal form of amphiphysin 2) in brain underlines their importance for nervous system function.

A striking observation is that the subtle vesicle recycling defects observed in amphiphysin 1 knockout mice produce important defects at the organismal levels.

While amphiphysin 1 knockout mice develop and reproduce normally, suggesting a basic normal functioning of the nervous system, some important neurological and behavioral defects were also observed. The death rate of mutant animals is significantly increased compared to normal littermates, in particular during the first few months of adulthood. Death was caused by rare irreversible seizures and, accordingly, mutant mice have a lower threshold to seizures. It was shown that synaptic depression during high-frequency stimulation occurs much faster at excitatory synapses than at inhibitory synapses, and this difference may be a critical mechanism protecting the nervous system from seizures (Galarreta and Hestrin, 1998; Varela et al., 1999; Kraushaar and Jonas, 2000). In turn, the remarkable stability of inhibitory synapses during ongoing stimulation is critically dependent upon the vesicle cycle, as shown by the loss of this stability in synaptojanin 1 knockout mice (Cremona et al., 1999; Lüthi et al., 2001). Thus, an increased susceptibility to seizures is consistent with the occurrence of a recycling defect in amphiphysin 1 knockout mice.

Another consequence of the lack of amphiphysin is a cognitive deficit, as defined by a significant impairment in learning tasks. Mutant animals exhibited severe learning disabilities in the Morris water maze test as well as in the fear-conditioning task, which rely on distinct neuronal circuitries. Although we cannot rule out that subthreshold seizures interfered with these experiments, it appears unlikely that seizure activity accounted for the defects observed. First, other knockout mouse models which experience severe seizures, such as synapsin 1 knockout mice, do not show impaired learning (Silva et al., 1996). Second, we used 5- to 7-month-old animals for our behavioral experiments, and we found that in this age category as well as in older animals, the death rate drops dramatically compared to younger adult animals. Since knockout animals die from seizures, we may have selected for our experiments a category of animals that is less prone to epilepsy.

Many mouse models with abnormal function of signaling pathways show learning deficits (Mayford and Kandel, 1999). A role of amphiphysin in signaling pathways cannot be excluded given the strong interconnections between endocytosis and signaling (Di Fiore and De Camilli, 2001; McPherson et al., 2001). Indeed some interactions of amphiphysin with signaling proteins have been reported (Kadlec and Pendergast, 1997; Leprince et al., 1997). Furthermore, in view of growing evidence for a critical role of clathrin-mediated endocytosis in the internalization of postsynaptic receptors (Turrigiano, 2000; Carroll et al., 2001; Kittler et al., 2000), it is possible that postsynaptic vesicle recycling changes may contribute to the cognitive defects of amphiphysin 1 knockout animals. However, we can at least conclude from our study that amphiphysin is indeed an effector in synaptic vesicle recycling and in the physiology of endocytic zones of mammalian synapses and that a perturbation of this function is likely to play a role in the cognitive deficits observed in mutant animals.

Several human conditions involve substantial cognitive defects that are not associated with major neurological impairments. In most cases, responsible genes have not yet been identified. An inherited mental retardation syndrome was mapped to the gene encoding a Rab GDP-dissociation inhibitor (GDI), a protein that controls the Rab cycle, and therefore the vesicle cycle, in nerve terminals and possibly also postsynaptically (D'Adamo et al., 1998). The learning defects observed in amphiphysin 1 knockout mice provide another example of a gene which functions in vesicle recycling and whose mutation underlies learning defects in a mammalian organism. Other human psychiatric conditions may be due to defects in membrane trafficking at the synapse.

Experimental Procedures

Generation of Amphiphysin 1 Knockout Mice

A λFIXII genomic library from 129SV/J mice was screened with a 250 bps DNA fragment corresponding to the 5′ end of the rat amphiphysin 1 cDNA. Phage clones containing the first coding exon of amphiphysin 1 gene were isolated and identified. A replacement targeting vector was constructed as shown in Figure 1A. In the targeting vector, most of the 3′ end of the first coding exon and a fragment of the following intron were replaced by the selection cassette (neomycin resistance gene under the PGK promoter [neo cassette]). The herpes simplex virus-1 thymidine kinase gene under the PGK promoter (TK cassette) was added at the end of the short arm of the construct. Both the neo and the TK cassette have a transcription direction that is the same as the amphiphysin 1 gene. The vector was linearized and electroporated into embryonic stem (ES) cells (129/Sv agouti ES cells) that were then selected according to standard procedures (Yang et al., 1997). Recombination events were identified by Southern blot screening using a PCR-generated probe corresponding to a fragment outside the recombination site but inside the restriction fragment generated by EcoRV digestion (Figure 1A). Chromosomal counting on recombinant ES cell clones was performed before blastocyst microinjection, in order to increase the chances of germline transmission. Positive clones were independently microinjected into blastocysts, which were then reimplanted into pseudopregnant foster mothers. Several chimeras were generated and independently bred with C57BL/6 mice. Germline transmission was achieved, and heterozygous animals were produced. Knockout mice were generated by successive breedings and identified by Southern blot analysis using the same screening strategy as for the identification of recombinant ES cell clones (Figure 1B). For Northern blot analysis, probes consisting of full length amphiphysin 2 and actin sequences, respectively, were obtained by PCR amplification from a mouse brain cDNA library and were labeled by random priming.

Antibodies, Immunoblotting, and Immunofluorescence

Brains from wild-type and knockout mice were homogenized in a lysis buffer containing 25 mM HEPES (pH 7.4), 150 mM KCl, 2 mM EGTA supplemented with a cocktail of proteinase inhibitors. Postnuclear supernatants (50 μg) were processed for SDS-PAGE and immunoblotting using ECL procedures (Amersham Pharmacia Biotech, Inc., Piscataway, NJ). The following antibodies were generated in our lab: rabbit antisera to synaptojanin 1, amphiphysin 1 and 2, endophilin 1, synaptophysin, as well as mouse mAb to amphiphysin 1 and AP180. Other antibodies are from commercial sources: Bin 1 (Upstate Biotechnology, Lake Placid, NY), dynamin 1 (Transduction Laboratories, Lexington, KT), α-adaptin (Affinity BioReagents, Golden, CO), a mouse hybridoma producing antibodies to clathrin heavy chain (ATCC, Rockville, MD), and α-tubulin (Sigma, St. Louis, MO). The mAb to synaptotagmin was a kind gift from Dr. R. Jahn (University of Göttingen, Germany). Immunofluorescence was performed on 2- to 3-week-old cultures of cortical neurons as previously described (Mundigl et al., 1998).

Biochemical Procedures

The experiment using liposomes was performed as previously described (Cremona et al., 1999; Gad et al., 2000). Quantification was performed using a phosphoimaging. For the lipid experiment, liposomes (0.25 mg/ml) were incubated with brain cytosol (0.5 mg/ml) for 15 min at 37°C in the presence of 2 mM ATP and 0.2 mM GTP.

Liposomes were then isolated by ultracentrifugation, washed three times, and incubated in the presence of 20 μCi [γ^{32}P]ATP for 10 min at 37°C. Lipids were extracted and processed for analysis by thin layer chromatography, and the identity of lipids was confirmed by high performance liquid chromatography as described previously (Cremona et al., 1999). Using phosphoimaging, counts for PI(4,5)P2 were normalized to total counts, which mainly included those of phosphatidic acid. The affinity chromatography using GST fused to the proline-rich region of dynamin was performed as in Slepnev et al. (1998), with slight modifications. 15 mg detergent extracts from wild-type and knockout brains were incubated for 4 hr at 4°C in the presence of 0.8 mg GST or GST fused to the proline-rich region of human dynamin 1 prebound to 150 μl Sepharose 4B beads (Amersham Pharmacia Biotech, Inc., Piscataway, NJ) in a buffer containing 25 mM HEPES (pH 7.4), 150 mM KCl, 1 mM EGTA, 1% Triton X-100, and a cocktail of protease inhibitors. In one experimental condition, 15 mg knockout extract were supplemented with 30 μg purified recombinant amphiphysin 1 prior to affinity chromatography. Following five washes with the same buffer, proteins were eluted in sample buffer and processed for immunoblotting.

FM2-10 Uptake in Synaptosomes

Synaptosomes were prepared from four mouse cortices as previously described (Marks and McMahon, 1998). For each experiment, 500 μg synaptosomes were used. In the "unloading" experiment, synaptosomes were incubated for 5 min at 37°C in low K^+ saline solution prior to transfer to the 1 ml cuvette preheated at 37°C in a Hitachi F-3010 fluorimeter. Synaptosomes were preincubated for 3 min with FM2-10 (Molecular Probes, Inc., Eugene, OR) at 100 μM final concentration and stimulated with 30 mM KCl for 90 s. Synaptosomes were washed twice with 1 ml low K^+ saline solution containing 1 mg/ml BSA, resuspended in low K^+ buffer, and transferred back to the cuvette. Following a 10 min incubation, a second stimulation with 30 mM KCl was applied, and fluorescence was measured at 467/550 nm as a function of time. In the FM2-10 internalization experiment, a similar protocol was used except that, following the first stimulation with 30 mM KCl and the washes, synaptosomes were lysed in a hypotonic solution containing 20 mM HEPES (pH 7.4), vortexed for 10 s, supplemented with KCl to 150 mM final concentration, and centrifuged for 2 min in a microfuge at maximal speed. Fluorescence associated with the supernatant, containing FM2-10 labeled internal membranes, was subsequently measured with the fluorimeter. The glutamate release was performed as previously described (Nicholls and Sihra, 1986).

Electron Microscopy and Morphometry of HRP-Labeled Cortical Neurons in Culture

Primary cultures of cortical neurons were prepared according to procedures previously described (Banker and Goslin, 1991; Cremona et al., 1999). Cells were maintained up to 3 weeks in Neurobasal/B27 medium (Life Technologies/Gibco-BRL, Gaithersburg, MD) at 37°C in a 5% CO_2 humidified incubator. Neurons were labeled with the fluid-phase marker HRP (Sigma, St Louis, MO) at 10 mg/ml for 3 min at 37°C before stimulation in a low K^+ saline solution containing 130 mM NaCl, 5 mM KCl, 2 mM $CaCl_2$, 2 mM $MgCl_2$, 25 mM HEPES (pH 7.33), 30 mM glucose, 10 μM CNQX, and 50 μM AP-V (Research Biochemicals, Inc., Natick, MA). Neurons were then either kept in low K^+ for another 90 s ("rest") or stimulated for 90 s with a solution containing 90 mM KCl and 45 mM NaCl in the presence of HRP ("stimulation"). Cells were fixed in 2% glutaraldehyde/2% sucrose in 0.1M sodium phosphate buffer (pH 7.4) for 2 hr at 4°C and processed for electron microscopy as described (Cremona et al., 1999). For morphometry, 37–54 synapses were analyzed at a final magnification of 63,000×. For each condition, 2000–4000 objects were scored. They included labeled or unlabeled synaptic vesicles and clathrin-coated vesicles as well as labeled endosome-like structures. Endosome-like structures were defined as HRP-labeled compartment larger than synaptic vesicles. Statistics were performed using ANOVA. Results of two independent experiments were pooled.

FM1-43 Uptake and Release in Cultured Cortical Neurons

Cortical cultures were generated from 1- to 2-day-old amphiphysin 1 knockout and wild-type mice. Brains were dissected; cerebral cortices were separated from the hippocampus and basal ganglia and dissociated using protocols similar to those used for hippocampal cultures (Ryan, 1999). Confocal laser scanning microscopy, electrical field stimulation, FM1-43 uptake, and release experiments were performed as described (Ryan, 1999) in 2- to 3-week-old cultures. FM1-43 (Molecular Probes, Eugene, OR) was stored at 4°C as 3 mM aliquots and used at a final concentration of 15 μM.

Metrazol Injection

12-week-old mice were injected intraperitoneally with a solution of pentamethylenetetrazole at 15 mg/kg every 5 min until observation of the last phase of seizure. The time required to reach the various phases of seizure was recorded. The first twitch was generally detectable in the ear; the tonic-clonic phase was defined as repeated hyperkinetic movements of forelimbs followed by a loss of postural control and falling, after which both the forelimbs and hindlimbs were characterized by clonic movements; the tonic extension phase began when animals arched their body and their limbs, after which, in most cases, they stopped breathing and died. Seven wild-type and ten knockout mice were used for the experiment. Statistics were performed using ANOVA.

Behavioral Paradigms

The Morris water maze test was performed as described previously (Morris, 1989; Wickman et al., 2000). In total, 18 mice (nine males, nine females) of 5–7 months of age were used for the experiments for both wild-type and knockout genotypes. A circular white plastic tub 1 min in diameter was filled with water (21°–22°C) and divided into four quadrants of equal surface. Swim times, path length, and all other measurements were made using the Poly-track Video Tracking System (San Diego Instruments). For the hidden platform test, a 10 cm^2 transparent Plexiglas platform was submerged 0.5 cm beneath the surface and positioned consistently over the course of the test. Mice were allowed to swim until they encountered the platform or for a maximal duration of 60 s, after which they were placed on the platform for 15 s. Mice began each of the four daily trials from a different quadrant, and the time required to find the platform was recorded and averaged. The intertrial interval was 3.5 min. The test was performed for 7 consecutive days, always in the afternoon. On day 8, the transfer test was performed. Briefly, the platform was removed, and mice were allowed to swim for 60 s, starting from the center of the pool. The time spent in every quadrant, the number of entries into the area where the platform had been originally located, and the swimming distance were measured. On days 9–12, in the visible platform test, trials were performed like in the hidden platform test, except that the platform was flagged.

The fear-conditioning test was performed as described (Paylor et al., 1994; Caldarone et al., 2000). In total, nine mice (five males, four females) of 5–7 months of age were used for the experiments for both wild-type and knockout genotypes. On the first day of training, each mouse was placed in a training chamber and freezing behavior was assessed every 10 s. After 2 min, the conditioned stimulus, a tone, was applied for 30 s and terminated by a 2 s unconditioned stimulus, a footshock of 0.5 mA. This sequence was repeated a second time, after which animals were kept for 30 s in the chamber and returned to their original cages. On the second day, animals were first tested for contextual fear. Each mouse was placed in the training chamber, and freezing behavior was assessed for 5 min in the absence of tone. One hour later, animals were tested for their fear response to the auditory stimulus. Each mouse was placed in a different training chamber and exposed to a smell of orange extract. During the first 3 min, freezing to altered context was measured, after which the tone was continuously presented for another 3 min, and freezing behavior to the auditory stimulus was scored.

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