Supporting Information

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SI Methods

Antibodies. Antibodies used were as follows: rabbit anti-auxilin, Greene laboratory; rabbit anti-GAK, Greene laboratory; mouse anti-clathrin heavy chain and anti-AP2, Affinity BioReagents; mouse anti-clathrin heavy chain, BD Transduction Laboratories; rabbit anti-Eps15, gift from Linton M. Traub, Pittsburgh, PA; epsin, Santa Cruz Biotechnology; rabbit anti-synaptojanin 1, De Camilli laboratory or BD Transduction Laboratories; rabbit anti-dynamin, De Camilli laboratory or BD Transduction Laboratories; mouse anti-amphiphysin, De Camilli laboratory; rabbit anti-Hsc70, BioVision; VAMP-2 and β-actin, Abcam; secondary antibodies, Molecular Probes (Invitrogen) and Jackson ImmunoResearch Laboratories.

Targeting Scheme and Generation of Auxilin Knockout Mice. To screen the mouse auxilin genomic DNA from the BAC mouse library, an ~430-bp probe was subcloned from a mouse auxilin EST clone by using primers designed from the rat auxilin cDNA sequence. Three individual clones that have auxilin genomic DNA in the pBeloBacII vector were obtained by BAC mouse library screening (Incyte Genomics). Those clones were used as a template for PCR to obtain the targeting arms and the probes. Scrambler A, a 1.9-kb fragment containing the promoter region, exon 1, exon 2, partial exon 3, and scrambler B, a 6-kb fragment downstream of exon 3, were cloned into pKO scrambler NTKV-1903 (Stratagene) (Fig. S1*A*). For the conventional auxilin knockout, the linearized targeting construct was then introduced into the embryonic stem cell line GSI 129/SV. The transfectants were first selected by a G418 resistance cassette, followed by Southern blot analysis to obtain the positive clones (Fig. S1*B*). Genotyping of the offspring was performed by Southern blot analysis or PCR using DNA from tail snips.

For the experiments reported in the study, we backcrossed the S129 heterozygote mutant mice with wild-type C57BL/6 mice to completely transfer the genotype from the S129 mice strain to the C57BL/6 strain. After eight backcrossings, heterozygote mice were mated to obtain homozygote auxilin knockout mice.

Western Blotting. To investigate the expression level of proteins in brain or other organs, tissue samples were homogenized in PBS containing protease-inhibitor mixture (Roche Applied Science). The same amount of protein was loaded in each lane on 4–12% SDS/PAGE gels (Invitrogen). After electrophoretic separation, proteins were transferred to nitrocellulose membrane (Amersham) by using the Trans-Blot Semi-Dry system (Bio-Rad). Protein bands were detected by using infrared secondary antibodies (Molecular Probes) and the Odyssey infrared detection system (LI-COR Biosciences). Actin was always used as a loading control.

Immunofluorescence. Cortical neurons were fixed with 4% paraformaldehyde, 4% sucrose in 0.12 M phosphate buffer, rinsed several times in PBS, blocked, and permeabilized in 0.1% gelatin, 0.3% Triton X-100 in PBS. The samples were sequentially incubated with primary and fluorochrome-conjugated secondary antibodies (Invitrogen), and after several washes in PBS they were mounted on glass coverslips with Prolong Gold antifade reagent (Invitrogen) and analyzed by epifluorescence. Fluorescent puncta on cortical cultures were quantified as described previously (1). Briefly, fluorescent spots per 100 μm^2 were detected using the *Count Nuclei* application of MetaMorph v.7.1.2 (Molecular Devices).

For the immunostaining of deep cerebellar nuclei, mice were perfused transcardially with 4% paraformaldehyde in 0.1 M phosphate buffer. After several washes in PBS, tissues were infiltrated with a series of increasing concentrations of sucrose solution (up to 18%) in PBS, then infiltrated in optimum cutting temperature formulation and frozen in isopentane precooled in liquid nitrogen. Sagittal sections of 10-μm thickness were collected on glass slides and air-dried. The sections were incubated in blocking solution (2% goat serum, 1% BSA, 0.1% cold fish gelatin, 0.1% Triton X-100, 0.05% Tween 20, 0.05% sodium azide in 0.01 M PBS, pH 7.2) for 30 min and then incubated sequentially with primary and secondary antibodies diluted in blocking buffer. The intensity of the immunostaining for clathrin and synaptobrevin on brain sections was quantified using ImageJ (NIH).

Samples were examined with a Zeiss Axioplan2 microscope with a Plan-Apochromatic 40× objective, and the images were collected with a Hamamatsu ORCA II digital camera.

Electron Microscopy. Primary cortical neurons were fixed with 1.3% glutaraldehyde in 66 mM sodium cacodylate buffer, postfixed in 1% OsO_4, 1.5% $K_4Fe(CN)_6$, 0.1 M sodium cacodylate, *en bloc* stained with 0.5% uranyl magnesium acetate, dehydrated, and embedded in Epon. Ultrathin sections were contrasted with 2% uranyl acetate and Sato's lead solution and observed with a Philips CM10 microscope, and images were taken with a Morada 2k × 2k CCD camera (Olympus).

For brain tissue, mice were anesthetized deeply with chloral hydrate (0.4 mg/g) and perfused transcardially with ice-cold physiological saline for 3–4 min followed by a 0.1 M sodium cacodylate buffer containing 2% paraformaldehyde and 2% glutaraldehyde (pH 7.4). Brains were then stored overnight in fresh fixative at 4 °C and subsequently sliced in a sagittal rodent brain matrix (ASI Instruments). Regions of interest were cut into small pieces and postfixed in 1% OsO_4, 1.5% $K_4Fe(CN)_6$, 0.1 M sodium cacodylate and *en bloc* stained with 2% uranyl acetate in ethanol. Finally, samples were dehydrated and embedded in Epon. Ultrathin sections were observed with a Philips CM10 microscope at 80 kV and images were taken with a Morada 2k × 2k CCD camera (Olympus).

1. Hirst J, et al. (2008) Auxilin depletion causes self-assembly of clathrin into membraneless cages in vivo. *Traffic* 9:1354–1371.

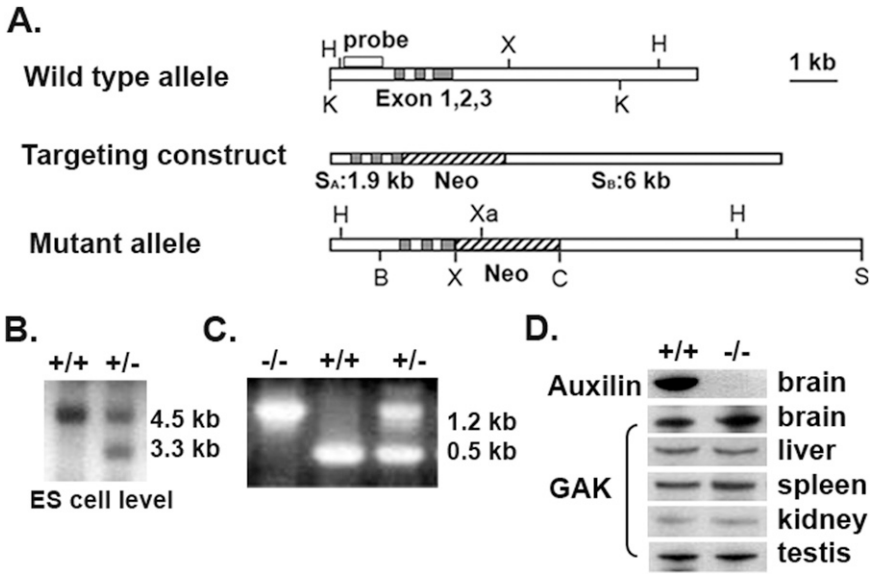

Fig. S1. Establishment of auxilin knockout mice cell lines. (*A*) Scheme of targeting construct and mutant allele used in generating the knockout mouse. Exons are indicated by filled boxes and neo is the neomycin resistance gene. Two insertion fragments (S_A, 1.9 kb containing exons 1, 2, and partial 3; S_B, 6 kb) were obtained by PCR to construct the targeting vector. Restriction enzyme sites are denoted as follows: S, SacII; H, HindIII; K, KpnI; X, XhoI; Xa, XbaI; C, ClaI; B, BglII. (*B*) Southern blot analysis of embryonic stem cells. The 5′ probe detects a 3.3-kb and a 4.5-kb band for the mutant and wild-type alleles, respectively, after digestion of the genomic DNA with HindIII and XbaI. (*C*) PCR genotyping from the mating of heterozygote mice to obtain auxilin knockout mice. PCR analysis showed 1.2-kb and/or 0.5-kb bands with and without insertion of the neo gene, respectively. (*D*) Western blot of GAK in different tissues from a wild-type mouse and auxilin knockout mouse. Western blot of GAK in cytosol prepared from brain, liver, spleen, kidney, and testes of mice at P21. Brain cytosol was also immunoblotted using anti-auxilin antibodies.

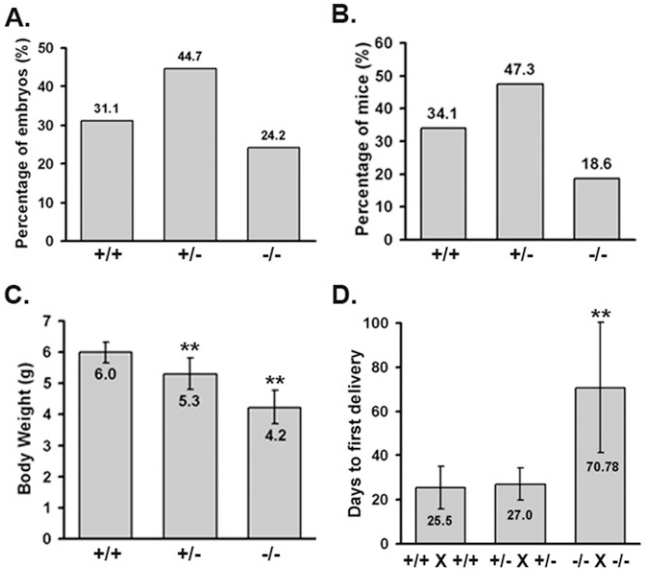

Fig. S2. Genotypes at embryonic stage and fertility defect of auxilin knockout mice. (*A*) Genotypes of mice obtained from heterozygotes mating at E18–E20 (n = 132 mice from 14 litters). χ^2 analysis shows that these results fit Mendelian genetics (P = 0.25775 statistical significance). (*B*) Genotypes of mice obtained from heterozygotes mating at P21. The litters from 30 auxilin heterozygote matings were genotyped by PCR at P21 (n = 220 mice from 30 litters). χ^2 analysis shows that these results do not fit Mendelian genetics (P = 0.0038 statistical significance). (*C*) Distribution of body weight of different genotype mice. The body weight of 1-week-old mice was measured for 16 mice for each genotype; $^{**}P < 0.01$, compared with wild-type using the Student's t test. (*D*) Virginal 8- to 10-week-old mice were mated to determine the length of time for them to deliver their first pups. Excluded from the analysis were auxilin knockout mice that did not have a litter in 100 days (n = 6–9 mice per genotype); $^{**}P < 0.01$ statistical significance compared to the wild-type determined with the Student's t test.

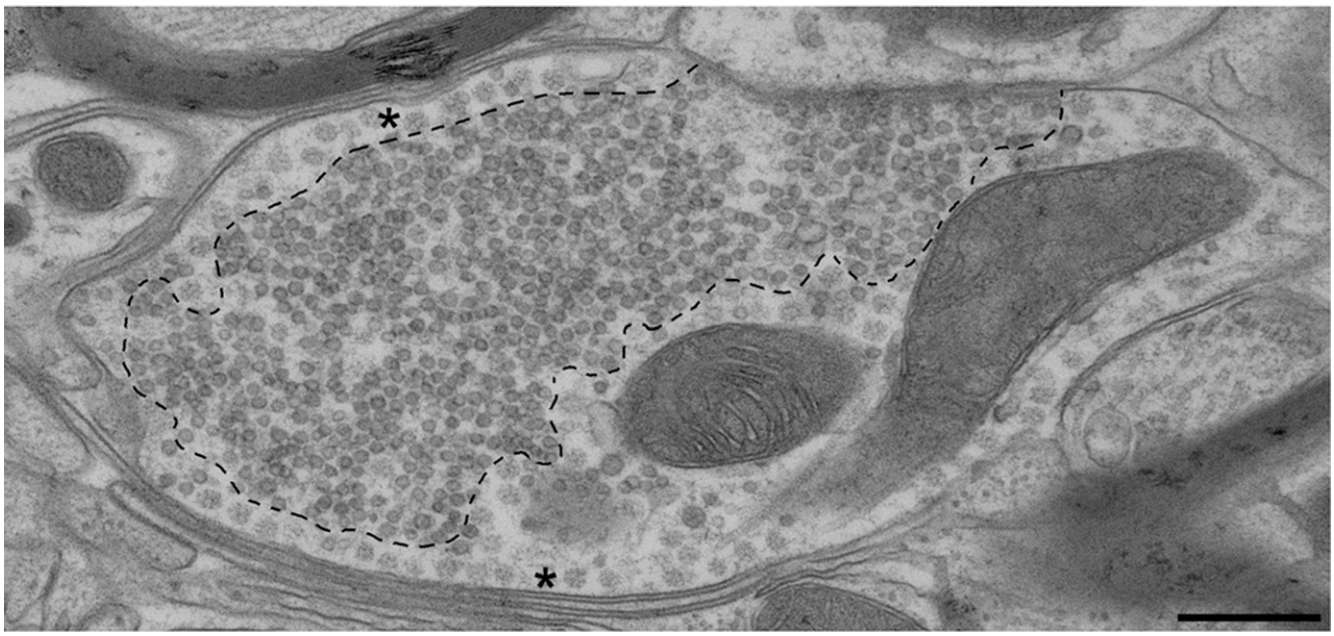

Fig. S3. Electron micrograph of a nerve terminal from an auxilin knockout adult mouse (deep cerebellar nuclei) showing accumulation of clathrin cages. CCVs and clathrin cages (asterisks) are located along the plasma membrane at the periphery of the synaptic vesicle cluster, which is delineated by a broken line. (Scale bar, 400 nm.)

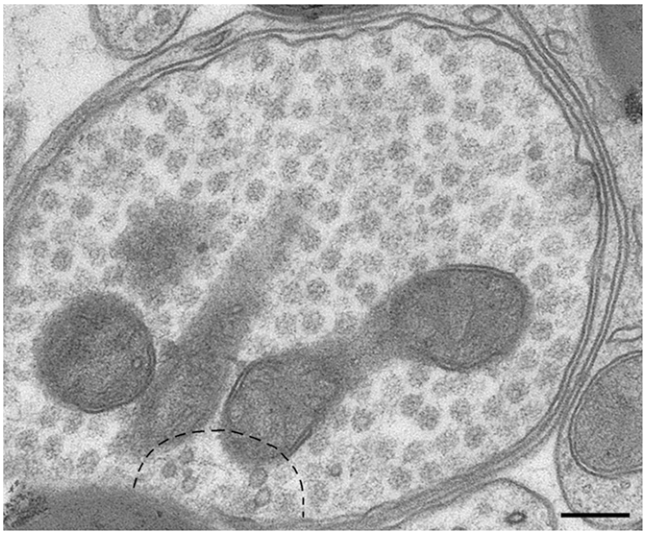

Fig. S4. Electron micrograph of a nerve terminal from an auxilin knockout adult mouse showing accumulation of clathrin cages. Note only a few synaptic vesicles (broken line line). (Scale bar, 200 nm.)

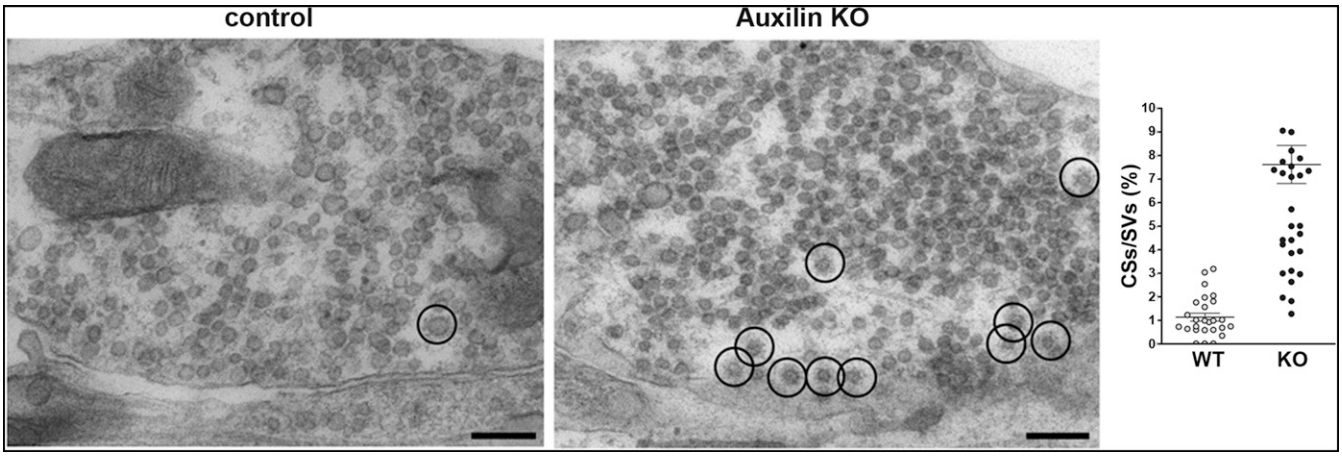

Fig. S5. Increased number of CCVs/cages at synapses of neuronal cultures from an auxilin knockout mouse as revealed by electron microscopy. Representative wild-type and knockout synapses are shown. At right is a quantification of the percentage of CCVs/cages (coated structures; CSs) relative to synaptic vesicles (SVs) in the two genotypes. (Scale bars, 200 nm.)