Supplemental Information

Supplemental Methods

FM dye loading and confocal imaging

Neurons at DIV12 were loaded with 15 μm FM 4-64 (Invitrogen, San Diego, CA) by incubating in a high K^+ solution containing (in mM): 90 Na, 50 K, 2 Ca, 10 HEPES, 2 Mg, 8 Glucose and 0.5 Kynurenic acid. After incubation for 2 min, cells were transferred for quenching to the sulfonated β-cyclodextrin derivative ADVASEP-7 (125 μM; Biotium) for 4-5 min, followed by wash for 15 min with a Ca^{2+}-free solution containing (in mM): 145 Na, 3 K, 10 HEPES, 2 Mg, 8 Glucose. Images were collected using a Zeiss LSM 510 oil immersion 40X objective, at a resolution of 1024 X 1024 pixels, with similar zoom at 3. All settings were kept the same below saturation; images with maximal punctate staining were obtained by stacking 5 serial sectioning images spaced by 0.8 μm.

In vitro binding and co-immunoprecipitation assays

GST-fusion proteins were immobilized on glutathione-Sepharose beads (Amersham, GE Healthcare). The bead-bound proteins were then incubated with purified His-tagged proteins in TBST (50 mM TBS, pH: 7.4, 0.1% Triton X-100) with protease inhibitors (1 mM phenyl-methylsulphonyl fluoride, 1 mM Leupeptin, and 1 mM Aprotinin) and 1 mM Ca^{2+} or EGTA at 4°C for 3 hr. After washing with TBST, bound complexes were separated by SDS-PAGE and immunoblotted with antibody as indicated. HRP-conjugated secondary antibodies and ECL chemiluminescence (Amersham, GE Healthcare) were used to visualize proteins. For co-immunoprecipitation, proteins from P2 rat brain homogenates were solubilized with 1% Triton X-100 for 1 hr at 4°C, and insoluble material was cleared by centrifugation for 30 min at 4°C. Solubilized proteins (50 μg) were mixed with primary antibodies, or, as control, normal IgG (Invitrogen) and

2.5 mg Protein A-Sepharose CL-4B resin (GE Healthcare) in 0.5 ml TBST with protease inhibitor cocktail, and then incubated on a microtube rotator for 3 hr at 4°C, followed by three washes with TBST. Detection of immunoprecipitated proteins was performed by SDS-PAGE and immunoblotting. For sequential blotting, membranes were stripped between each antibody application in stripping buffer (62.5 mM Tris-HCl pH 7.5, 20 mM DTT, and 1% SDS) at 50°C for 20 min.

Electron microscopy and data analysis

Neurons were grown to DIV12-13 and fixed at room temperature with 2% glutaraldehyde, 2% paraformaldehyde in 0.1 N Na cacodylate buffer, and then stored at 4 °C over night. The samples were then treated with osmium tetroxide, en bloc mordanted with uranyl acetate, dehydrated through a series of graded ethanol, and embedded in epoxy resins. Thin sections were stained with uranyl acetate and lead citrate (EM Facility, National Institute of Neurological Disorders and Stroke, National Institutes of Health). The sections were examined on a JEOL (Akishima, Japan) 1200 EX electron microscope, and digital images were captured with a CCD camera system (XR-100; Advanced Microscopy Techniques, Danvers, MA). For samples from all genotypes, only cross-section synapses with clear electron-dense PSD and presynaptic vesicles were imaged at 40,000 magnification, and then analyzed by ImageJ 10.2 (NIH) with the same scaling system. Only vesicles that attached to the AZ were considered docked, and relative total vesicles were counted within the presynaptic boutons from the AZ to the edge of the terminal.

Supplementary Figure Legends

Suppl. Figure 1. Calcium channel density and function remain unchanged in *snapin*-deficient neurons.

(A) Upper, depolarization step from -40 mV to + 60 mV with 5 mV increment at a holding potential of -70 mV. Lower, representative calcium currents recorded from *snapin* (+/+) and (+/-) neurons. The corresponding currents at +20 mV are marked in black or green for (+/+) and (+/-) neurons, respectively.

(B and C) I/V curves from *snapin* (+/+) and (+/-) neurons. Peak currents were normalized to the cell capacitance (B). I-V relations of (+/-) neurons were normalized to overlap at the (+/+) peak (C). No change in current density or channel activation was observed in (+/-) neurons compared to (+/+) control.

(D) Calcium currents at +20 mV were isolated and the percent residual current relative to the peak (as in A, middle panel) was calculated for both (+/+) and (+/-) neurons. No significant difference (NS, $p>0.05$) was observed, suggesting similar inactivation rates at +20 mV.

Suppl. Figure 2. Immunoblot analysis of SNAREs and regulatory components of synaptic vesicle release machinery. Equal amounts of brain homogenates (20 μg) from E18 embryos of all genotypes from the same littermates were blotted with antibodies as indicated.

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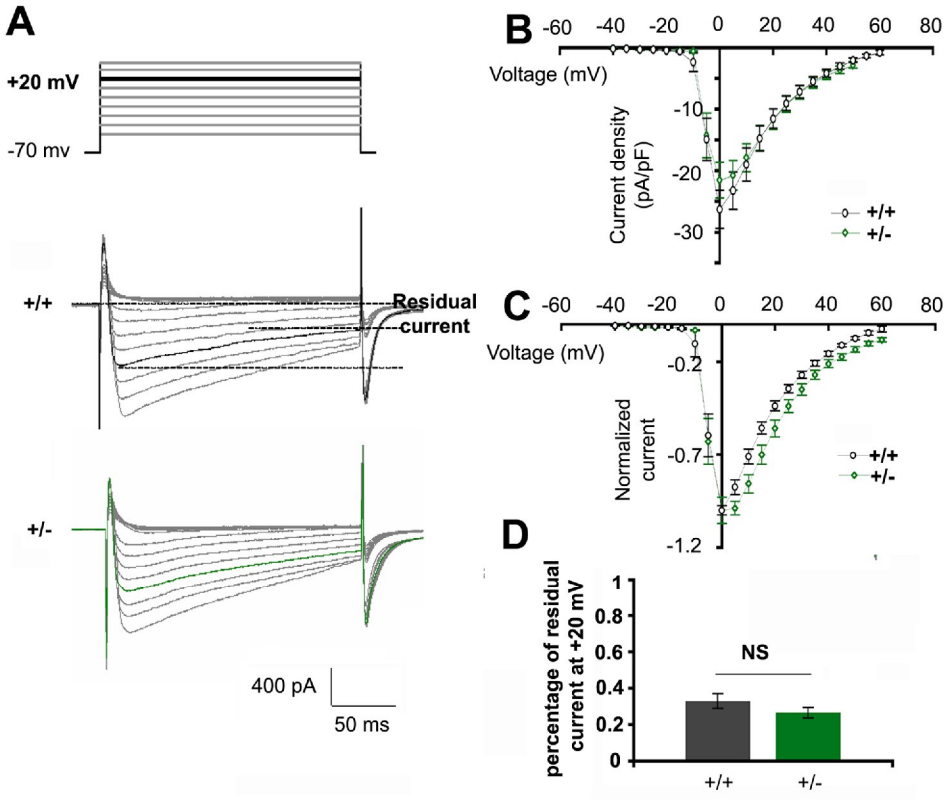

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