SUPPLEMENTAL INFORMATION

The Specific α-Neurexin Interactor Calsyntenin-3 Promotes Excitatory and Inhibitory Synapse Development

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SUPPLEMENTAL FIGURES AND LEGENDS

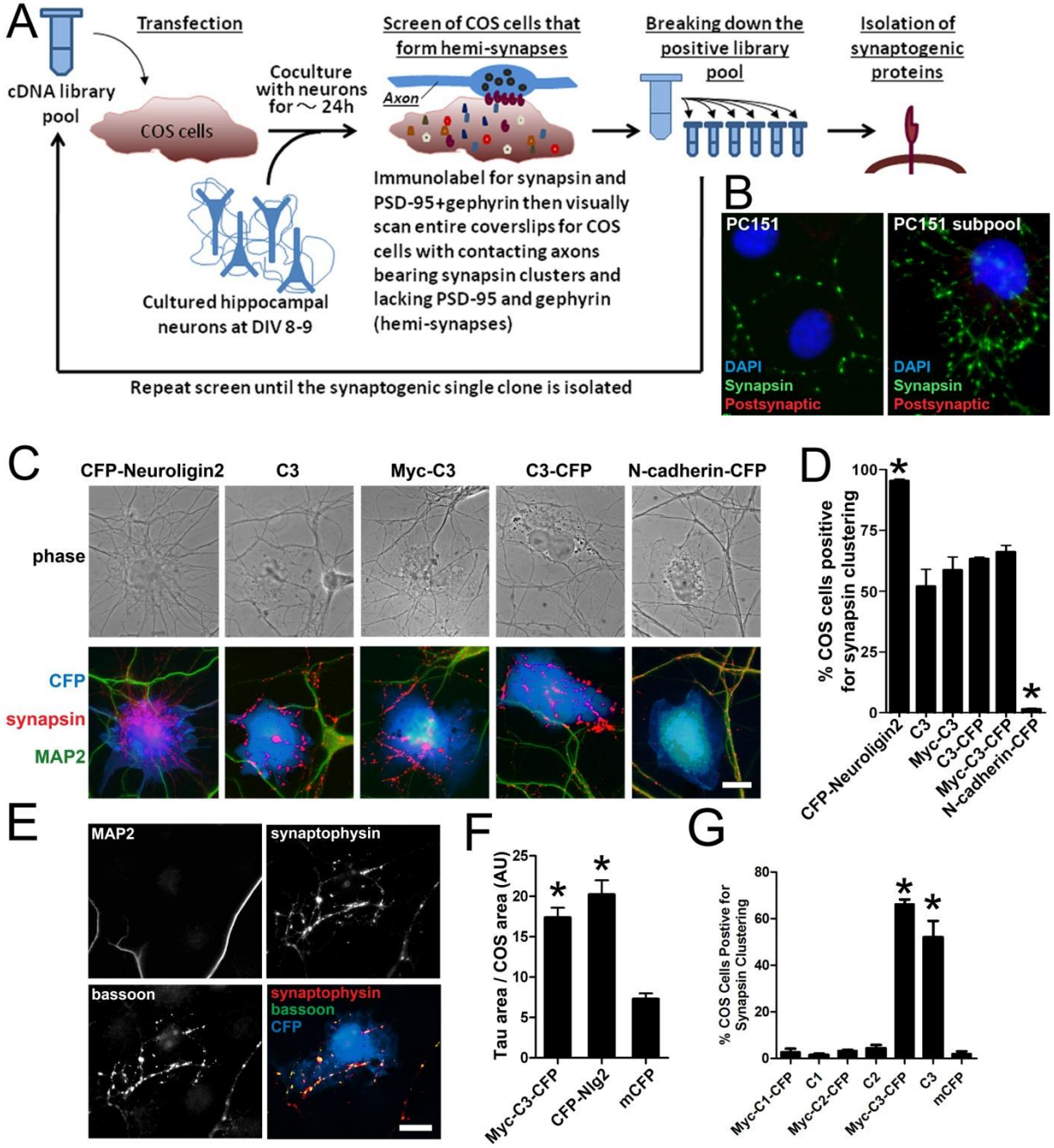

Figure S1: An Unbiased Screen Identifies Calsyntenin-3 as a Synaptogenic Factor, Related to Figure 1

(A) Flow diagram illustrating the experimental protocol for expression screen leading to discovery of calsyntenin-3. Pools of ~250 clones of a custom unamplified full-length size-selected cDNA expression library were transfected into COS cells in 12-well tissue culture plates, and transfected COS cells were then co-cultured with hippocampal neurons over a glial feeder layer for ~24 h. Co-culture coverslips were fixed and immunolabeled for synapsin, PSD95 and gephyrin. Coverslips were visually scanned on a fluorescent microscope for the presence of COS cells inducing presynaptic synapsin clustering without apposed postsynaptic PSD95 and gephyrin. Figure modified from (Takahashi et al., 2011).

(B) Image of the PC151 original pool and positive subpool co-culture showing clustering of synapsin (green) over a COS cell (nucleus stained blue with DAPI), without apposing PSD-95 or gephyrin (together in red).

(C) Calsyntenin-3 (C3) untagged or with an N-terminal Myc tag (Myc-C3) or C-terminal CFP tag (C3-CFP) expressed in COS cells induce synapsin clustering in contacting axons in hippocampal co-culture, like positive control CFP-neuroligin-2 and unlike negative control N-cadherin-CFP. Constructs that are not CFP fusions were co-expressed with CFP to mark transfected cells. Induced presynaptic sites are differentiated from endogenous axon-dendrite synapses by lack of apposing MAP2-positive dendrites.

(D) Quantitation of the percentage of COS cells expressing the indicated constructs that induced clusters of synapsin in contacting axons lacking associated MAP2-positive dendrites. ANOVA, $p < 0.0001$, $n \geq 3$ experiments counting ≥100 cells per experiment, *$P < 0.001$ compared to calsyntenin-3 plus CFP (C3 + CFP) by post-hoc Bonferroni multiple comparison test.

(E) Myc-calsyntenin-3-CFP (Myc-C3-CFP) expressed in COS cells induces clustering of synaptophysin synaptic vesicle protein and bassoon active zone protein in contacting axons in hippocampal co-culture. The induced presynaptic clusters lack apposed MAP-2 positive dendrites.

(F) Quantitation of tau-immunopositive axon area in contact with transfected COS cells expressing the indicated CFP fusion protein and co-cultured with hippocampal neurons. Both calsyntenin-3 and neuroligin-2 increase tau area compared to control mCFP. ANOVA, $P < 0.0001$, $n = 10$ cells each in three independent experiments; *$P < 0.001$ compared to mCFP by post-hoc Bonferroni test.

(G) Quantitation of the percentage of COS cells expressing the indicated constructs that induced clusters of synapsin in contacting axons lacking associated MAP2-positive dendrites. ANOVA, $p < 0.0001$, $n \geq 3$ experiments counting ≥100 cells per experiment, *$P < 0.001$ compared to mCFP by post-hoc Bonferroni multiple comparison test. Unlike Myc-calsyntenin-3-CFP, neither Myc-calsyntenin-1-CFP or Myc-calsyntenin-2-CFP nor untagged calsyntenin-1 or calsyntenin-2 were able to induce synapsin clustering in contacting axons (all $p > 0.05$ compared to negative control mCFP by post-hoc Bonferroni test and $p < 0.001$ compared to Myc-calsyntenin-3-CFP by post-hoc Bonferroni test).

Scale bars, 20 μm. Data are presented as mean ± SEM.

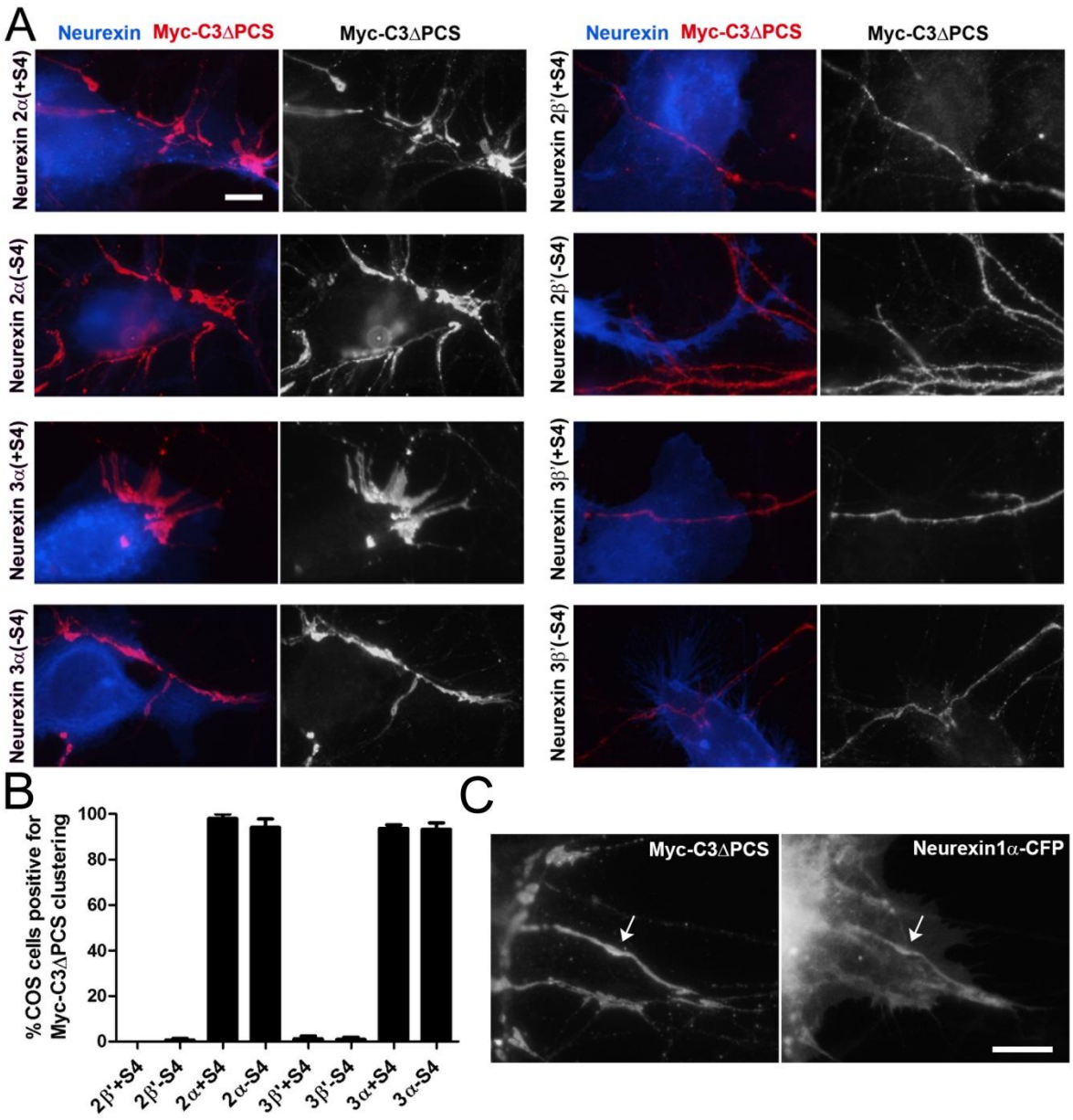

Figure S2: Neurexin-2 and Neurexin-3 α but Not β Isoforms Recruit Calsyntenin-3ΔPCS, Related to Figure 2

(A) In a recruitment assay, neurons expressing Myc-calsyntenin-3ΔPCS (Myc-C3ΔPCS) were co-cultured with COS cells expressing the indicated neurexin isoforms containing (+S4) or lacking (-S4) the insert at splice site 4 and tagged intracellularly with CFP. The α-neurexins but not β-neurexins in COS cells recruited Myc-C3ΔPCS to contact sites. Neurexin 2β' and 3β' contain the LNS domain from neurexin 2 or 3, respectively, fused to neurexin 1β flanking sequences.

(B) Quantitation of the percentage of COS cells expressing the indcated neurexin that induced clustering of Myc-C3ΔPCS in contacting neuronal processes. ANOVA, $p < 0.0001$, $n = 2$ experiments.

(C) In some of the recruitment assays, as shown here for neurexin 1α, the α-neurexin-CFP on COS cells was also observed concentrated at contact sites with neurons expressing Myc-C3ΔPCS (arrows).

Scale bars, 10 µm. Data are presented as mean ± SEM.

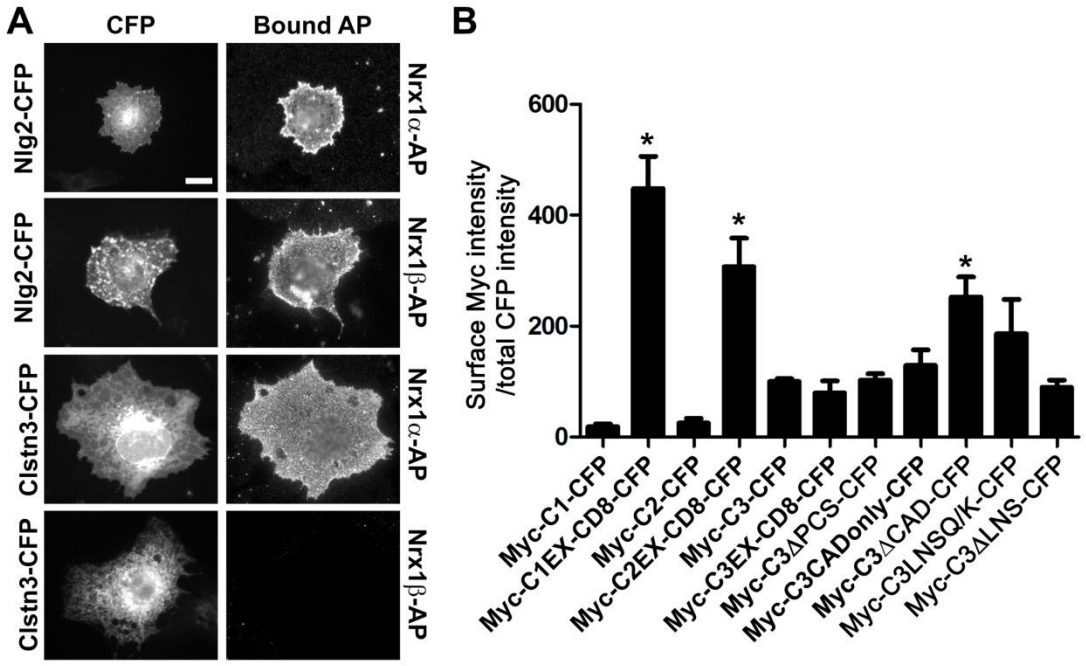

Figure S3: Confirmation of Selective Binding of Calsyntenin-3 to α Neurexin and Surface Expression of Calsyntenin Constructs, Related to Figure 4

(A) Neurexin α and β fusion proteins Nrx1α-AP and Nrx1β-AP bound to COS cells expressing neuroligin 2 (Nlg2-CFP), but only the neurexin α fusion protein Nrx1α-AP bound to cells expressing calsyntenin-3 (Clstn3-CFP).

(B) Surface expression of calsyntenin constructs in COS cells. Only Myc-C1-CFP and Myc-C2-CFP showed poorer surface expression than Myc-C3-CFP, all other constructs trafficked to the cell surface at least as well as Myc-C3-CFP. ANOVA, $p < 0.0001$, $n \geq 10$ cells each; *$p < 0.001$ compared to Myc-C3-CFP by post-hoc Bonferroni test..

Scale bar, 10 µm. Data are presented as mean ± SEM.

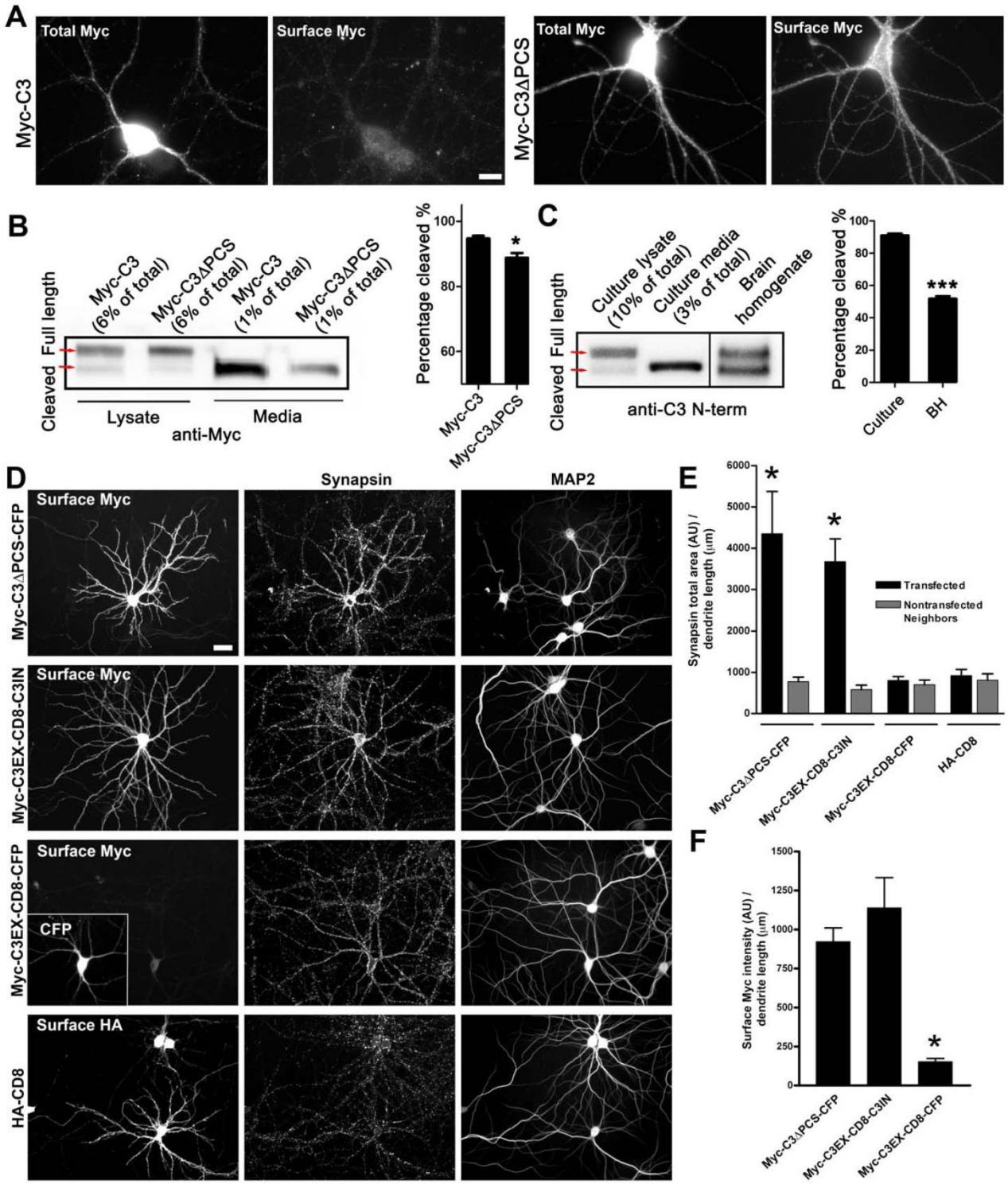

Figure S4: Overexpression and Cleavage Analysis of Calsyntenin Constructs in Cultured Neurons, Related to Figure 5

(A) Immunofluorescence of hippocampal neurons expressing Myc-C3 or Myc-C3ΔPCS from plating to 14 DIV for cell surface Myc before permeabilization and total Myc after permeabilization. Both constructs show high total epxression but Myc-C3 shows essentially no detectable surface expression (compare with untransfected neighbor neuron) whereas Myc-C3ΔPCS shows clear surface expression.
(B) Western blot of cell lysate and media of cortical neurons expressing Myc-C3 or Myc-C3ΔPCS from plating to 14 DIV. Percentage cleaved was calculated by adding the signal of cleaved bands from cell lysate (normalized to whole lysate volume) and culture media (normalized to whole media volume) and then dividing by the total expression level (sum of all normalized bands). The percentage cleaved was lower for Myc-C3ΔPCS as compared with Myc-C3. Student t test, * $p < 0.05$, n = 3 experiments. The level of full-length calsyntenin-3 in the lysate was also higher on average by 60% for Myc-C3-ΔPCS as compared with Myc-C3 but this difference was not significant ($p = 0.073$).
(C) Western blot and quantification of percentage of endogenous calsyntenin-3 present in cleaved form in cortical culture at 14 DIV and in adult brain homogenate (see Figure 6B for earlier developmental stages). Percentage cleaved was calculated as described as in panel (B). Student t test, *** $p < 0.001$, n=3 experiments.
(D) Hippocampal neurons transfected at 8–9 DIV with Myc-C3ΔPCS-CFP or Myc-C3EX-CD8-C3IN but not Myc-C3EX-CD8-CFP or HA-CD8 (negative control) showed increased inputs with presynaptic marker synapsin compared to non-transfected neighbor neurons at 14 DIV. Myc-C3EX-CD8-CFP trafficked very poorly to the neuron surface indicated by the lack of surface Myc immunoreactivity but was expressed as indicated by the CFP signal. Other constructs showed good surface immunoreactivity.
(E) Quantitation of synapsin-immunopositive area per transfected and neighbor non-transfected dendrite length. ANOVA, $p < 0.0001$, n = 20 cells each; *$p < 0.001$ compared to HA-CD8 by post-hoc Bonferroni multiple comparison test.
(F) Dendritic surface expression of Myc-tagged calsyntenin-3 constructs was also measured. Myc-C3ΔPCS-CFP and Myc-C3EX-CD8-C3IN had similar levels of surface expression. Myc-C3EX-CD8-CFP was expressed at a much lower level on the surface of dendrites. ANOVA, $p < 0.0001$, n = 20 cells each; *$p < 0.001$ compared to Myc-C3ΔPCS-CFP in post-hoc Bonferroni test.
Scale bars: 10 μm in A, 30 μm in D. Data are presented as mean ± SEM.

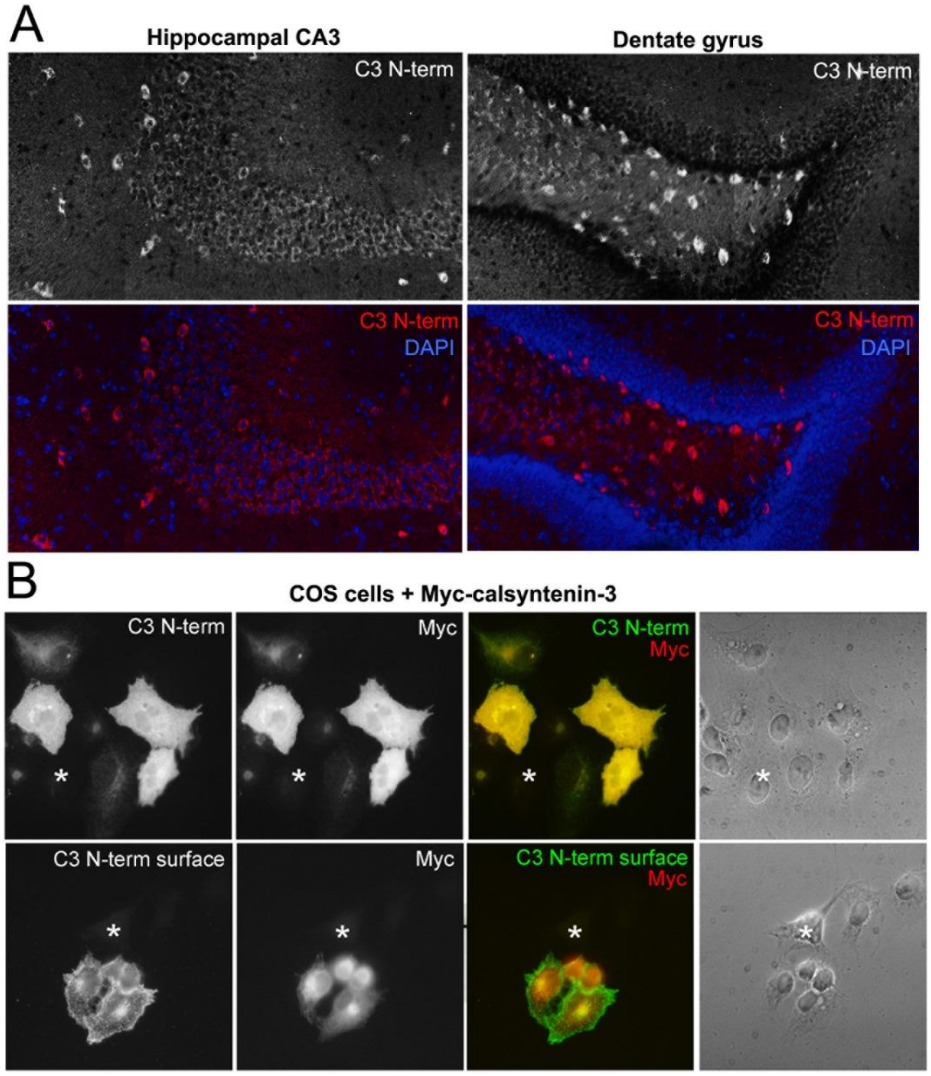

Figure S5: Calsyntenin-3 Distribution Assayed with an Independent N-terminal Antibody, Related to Figure 6

(A) The N-terminal antibody against calsyntenin-3 (C3 N-term) showed the same pattern of immunoreactivity as the C-terminal antibody (in Figure 6C-J). As shown here in images from hippocampus, calsyntenin-3 was most strongly detected in apparent interneurons, was also present in CA3 pyramidal neurons and dentate hilar neurons, but little or none was detectable in dentate granule cells. (B) This N-terminal antibody specifically recognized recombinant Myc-calsyntenin-3 expressed on the surface of COS cells (asterisks indicate untransfected cells).

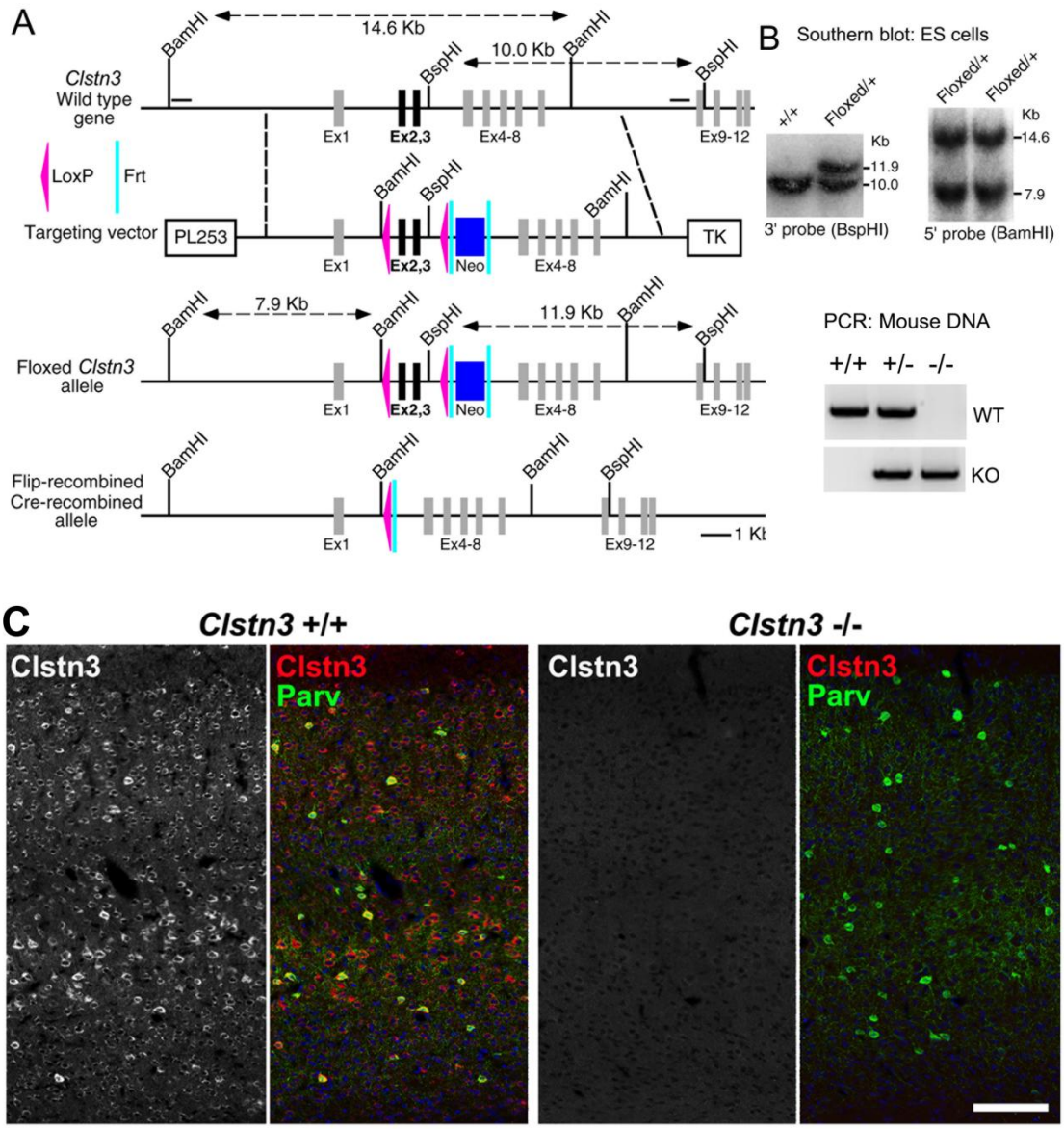

Figure S6: Generation of *Clstn3* -/- Mice, Related to Figure 7

(A) Targeting strategy for *Clstn3* locus. The restriction enzyme sites for BamHI and BspHI used for Southern blot analysis are included. Exons 2 and 3 were floxed and excised by crossing with EIIa-Cre mice resulting in a truncating frameshift.

(B) Top: Southern blot confirmation of homologous recombination in the ES cells. Bottom: PCR genotyping of mice with primer pairs specific for *Clstn3* +/+ (WT) or *Clstn3* -/- (KO).

(C) Immunofluorescence confirmation of loss of calsyntenin-3 immunoreactivity (using the C-terminal antibody) in cortex of *Clstn3* -/- mice. *Clstn3* -/- brains exhibited normal morphology and interneuron distributions indicated with the parvalbumin (Parv) co-stain. Sacle bar, 50 µm.

SUPPLEMENTAL EXPERIMENTAL PROCEDURES

DNA Constructs

Calsyntenin-3 cDNA was isolated from the screen, in the pcDNA3 vector under the cytomegalovirus (CMV) promoter. In order to measure surface expression levels and to track the N-terminal cleavage product, a Myc-tag (amino acids EQKLISEEDL) was inserted after the 20 amino acid signal sequence in full length calsyntenin-3 to make Myc-calsyntenin-3 (Myc-C3), using the QuikChange site-directed mutagenesis kit (Stratagene). In order to directly detect expression in cells, cyan fluorescent protein (CFP) was fused in-frame to the C-terminus of both calsyntenin-3 and Myc-C3 to produce C3-CFP and Myc-C3-CFP, respectively, by subcloning into the pECFP-N1 vector (Clontech).

The Myc-C3-CFP vector formed the base for the subcloning of a series of deletion constructs produced by overlap PCR methods or site-directed mutagenesis. These constructs were: Myc-C3secreted (Δ aa 851-957) in which the full length sequence was truncated just before the transmembrane (TM) domain; C3intracell-CFP (Δ aa 1-869) in which the entire extracellular and TM domains were removed; Myc-C3EXTM-CFP (Δ aa 870-957) in which only the intracellular domain was deleted and the C-terminus of the TM domain was fused to CFP; Myc-C3ΔPCS-CFP (Δ aa 805-824) PCS for "primary cleavage site" in which 20 amino acids flanking the reported cleavage site (Hata et al., 2009) were deleted; Myc-C3EX-CD8-CFP (Δ aa 815-957) in which the majority of the extracellular domain ending ~30 amino acids upstream of the cleavage site was fused to the TM domain of CD8 via a short linker sequence of three glycines which was fused to intracellular CFP; Myc-C3EX-CD8-C3IN (Δ aa 815-869) was subcloned from Myc-C3EX-CD8-CFP by replacing the CFP sequence with the calsyntenin-3 intracellular domain sequence resulting in a construct only lacking a short juxtamembrane extracellular sequence and the calsyntenin-3 TM domain. Calsyntenin-1 and calsyntenin-2 full length mouse cDNA sequences were obtained from Open Biosystems and subcloned into the pcDNA3 vector. Myc-C1 and Myc-C2 were generated in parallel methods as those used for calsyntenin-3. The plasmid encoding Clstn3-Fc was subcloned from C3-CFP into the pc4-sp-Fc1 vector (Takahashi et al., 2011) allowing for fusion of the extracellular domain of calsyntenin-3 (aa 21-847) between the signal sequence from neurexin1β and the human IgG Fc portion. The plasmid encoding Nrx1α-AP expresses rat nereuxin1α ectodomain (aa 1-1399) fused to PLAP-Myc/His in the pcDNA4 vector and was generated using the

PLAP-Myc/His vector previously used for LRRTM2 LRR-AP (Linhoff et al., 2009).

Previously described plasmids include CFP-neuroligin2 (CFP-Nlg2), N-cadherin-CFP, mCFP (membrane-associated CFP), neuroligin2-Fc (Nlg2-Fc), HA-CD8, HA-PTPσ, HA-PTPδ, HA-neurexin1α(-S4) (Gauthier et al., 2011; Graf et al., 2004; Linhoff et al., 2009; Takahashi et al., 2011; Takahashi et al., 2012) and CFP (Clontech). Neurexin-CFP (C-terminal CFP fusion) expression vectors for variants lacking (-S4) or containing (+S4) the splice site 4 insert corresponding to neurexin 1α, 2α, 3α, 1β, 2β' and 3β' have also been described (2β' and 3β' contain the LNS domains from 2β and 3β with flanking sequences from 1β) (Graf et al., 2004; Kang et al., 2008; Siddiqui et al., 2010).

Generation of Clstn3 -/- Mice

All animal experiments were performs in compliance with state and institutional guidelines. The targeting vector was generated by recombineering (Liu et al., 2003) from a 129S7 bMQ BAC clone from the Sanger Institute (Adams et al., 2005) and confirmed by sequencing. The targeting vector contained two loxP sites flanking exons 2 and 3, a Neomycin cassette flanked by frt sites in intron 3, and a herpes simplex virus thymidine kinase expression cassette for negative selection. Linearized targeting vector was electroporated into 129/Ola embryonic stem (ES) cells for homologous recombination (Augustin et al., 1999; Thomas and Capecchi, 1987). ES cells were selected in the presence of G418 (positive selection) and Ganciclovir (negative selection against random integration). Homologous recombination was verified by Southern blot analysis with probes 5' and 3' to the targeting vector region. Positive clones were injected into C57BL/6J blastocysts, which were transferred to female mice. Founder chimeras were backcrossed 7 generations with C57BL/6J mice. The conditional Clstn3 flox/+ line was first crossed with a FLP1-expressing deleter line [B6;SJL-Tg(ACTFLPe)9205Dym/J; Jackson Laboratory] to remove the Neomycin resistance cassette and subsequently crossed with the EIIa-Cre line that expresses Cre recombinase in early embryonic stages (Lakso et al., 1996) to generate Clstn3 +/- mice.

Production of Soluble Clstn3-Fc and Neurexin1α-AP and Binding Assays

Expression of Clstn3-Fc protein was performed by transfecting HEK293T cells with the encoding plasmid, and culturing in DMEM with 10% FBS and 0.5 mg/ml Zeocin (Invitrogen). After 21-day selection with Zeocin, medium was replaced with serum-free AIM V synthetic medium (Invitrogen). The

conditioned medium was collected every 2-3 days for three weeks and frozen at -80°C, for a total of 300-400 mL. Fc fusion protein was purified using protein-G sepharose 4 fastflow columns (GE Healthcare) and concentrated in PBS with Centricon filters (Millipore). Purified Fc fusion proteins were immunoblotted, visualized by chemiluminescence using a Bio-Rad gel documentation system, and quantified by densitometry relative to a human IgG standard curve. Nlg2-Fc fusion protein was prepared as described previously (Gauthier et al., 2011).

HEK293T cells stably expressing neurexin1α-AP were generated by antibiotic selection as above. Supernatant collected from expressing cells was concentrated using Centricon Plus-70 ultrafiltration units (30 kDa cutoff; Millipore). The neurexin1α-AP fusion protein also contained Myc and His tags and was purified using Ni-NTA agarose eluting with 200 mM imidazole. Imidazole was removed by overnight dialysis (Spectrapor). Purified neurexin1α-AP fusion proteins were quantitated by SDS-PAGE relative to a BSA standard curve using Sypro Ruby gel stain (Invitrogen), UV illumination, and a Bio-Rad gel documentation system.

To assess binding of fusion proteins, COS7 cells on coverslips were transfected with the indicated expression vectors and grown for 24 h. Live cells were incubated with fusion proteins (at 50 nM unless otherwise indicated) either for 1 hour at 20°C followed as appropriate by anti-HA antibodies (1:500; IgG2b; clone 12CA5; Roche) for 30 min, or the fusion proteins and anti-HA antibodies were incubated together for 45 min at 4°C. Binding was assayed in the following "binding buffer": 168 mM NaCl, 2.6 mM KCl, 10 mM HEPES, pH 7.2, 2 mM CaCl2, 2 mM MgCl2, 10 mM D-glucose, and 100 µg/ml BSA. Cells were fixed in parafix solution (4% paraformaldehyde and 4% sucrose in PBS pH 7.4) for 15 min at 20°C then incubated with blocking solution (PBS + 3% BSA and 5% normal goat serum) for 30 min at 37°C. This was followed by incubation with secondary antibodies FITC-conjugated donkey anti-human IgG (H+L) (1:100, Jackson ImmunoResearch) and Alexa-568 anti-IgG2b (1:1000, Invitrogen) for 1 h at 37°C to visualize bound Fc-fusion protein and surface HA, respectively. Binding of AP-fusion protein was visualized by incubation with anti-alkaline phosphatase primary antibody (1:500, Sigma) and Alexa-568 anti-IgG2a (1:1000, Invitrogen). Coverslips were mounted in elvanol (Tris-HCl, glycerol, and polyvinyl alcohol with 2% 1,4-diazabi-cyclo[2,2,2]octane).

Pull-Downs

Cow brain synaptosomes, prepared similarly to the method of (Villasana et al., 2006), were solubilized at final concentration 10 mg/ml in solubilization buffer (10 mM CAPS pH 10, 5 mM HEPES pH 7, 150 mM NaCl, 4% CHAPS, 0.5 mM PMSF, Roche protease inhibitor cocktail/EDTA free) by incubating for 90 min at 4°C and then centrifuging for 45 min at 16000 rpm to isolate the solubilized proteins.

For the pulldowns testing the interaction of neurexin 1α with calsyntenin 3, neurexin 1α-beads were first prepared by adding 1 mg of hexahistidine-tagged n1α L1L6(SS#1) expressed in insect cells (Chen et al., 2011) to 400 µl Talon resin (ClonTech, 50% slurry). The beads were incubated for 2 hr at 4°C and uncoupled protein was removed by washing the beads with 25 mM HEPES pH 8, 150 mM NaCl, 5 mM $CaCl_2$. As negative control, Talon resin with no protein coupled was taken along separately. For the pulldowns, 150 µl n1α L1L6(SS#1)-coupled beads (50% slurry) or beads alone were added to 150 mgr solubilized synaptosomes in a 30 ml volume in binding buffer (5 mM CAPS pH 10, 15 mM HEPES pH 7.4, 150 mM NaCl, 10 mM imidazole, 5 mM $CaCl_2$, 2% CHAPS, 0.25 mM PMSF, Roche Protease inhibitor cocktail/EDTA free) and incubated 2 hrs at 4ºC. The beads were washed 3x with 10 ml 25 mM HEPES pH 7.4, 150 mM NaCl, 5 mM $CaCl_2$, 0.5% CHAPS. The beads were eluted by incubating them 30 min at RT with 200 µl 20 mM CAPS pH 10, 1 M NaCl, 0.5% CHAPS, 5M Urea and recovering the flow-through (the 'wash' sample). Subsequently, the beads were eluted again by incubating them 30 min at RT with 100 µl 1x SDS-loading buffer (50 mM Tris pH 6.8, 100 mM DTT, 2% SDS, 10% glycerol, bromophenol blue, with or without 50 mM EDTA) (the 'elute' sample).

For the pulldowns testing the interaction of neurexin 1β with calsyntenin 3, neurexin 1β-GST-beads were first prepared by coupling 1.3 mg neurexin 1β-GST fusion expressed in E. Coli (Shen et al., 2008) to 1 ml glutathione resin (Sigma, 50% slurry). As negative controls, GST-beads were prepared containing 1 mgr GST expressed in E. coli coupled to 1 ml glutathione beads (50% slurry) as well as glutathione beads with no protein coupled. The beads were washed with 20 mM Tris pH 8, 150 mM NaCl, 5 mM $CaCl_2$ prior to use. For the pulldowns, beads with ca. 0.5 mgr neurexin 1β-GST, 0.5 mgr GST or beads alone were added to100 mgr solubilized synaptosomes in parallel in a 20 ml volume in binding buffer (5 mM CAPS pH 10, 15 mM HEPES pH 7.4, 150 mM NaCl, 5 mM $CaCl_2$, 2% CHAPS, 0.25 mM PMSF, Roche Protease inhibitor cocktail/EDTA free) and incubated 2 hrs at 4ºC. The beads were washed 3x with 10 ml 25 mM HEPES pH 7.4, 150 mM NaCl, 5 mM $CaCl_2$, 0.5% CHAPS. The beads were eluted by incubating them 30 min. at RT with 200 µl 20 mM CAPS pH 10, 1 M NaCl, 0.5%

CHAPS and recovering the flow-through (the 'wash' sample). Subsequently, beads were eluted again by incubating them 30 min. at RT with 200 µl 1x SDS-loading buffer (50 mM Tris pH 6.8, 100 mM DTT, 2% SDS, 10% glycerol, bromophenol blue).

To assess the presence of calsyntenin-3, samples were resolved on 8% or 10% SDS-PAGE gels and electroblotted to PVDF membranes. The membranes were blocked with 5% milk in TBST (10 mM Tris pH 7.5, 150 mM NaCl, 0.1% Tween-20), washed and probed with an antibody raised against the calsyntenin 3 C-terminus (diluted 1:1000 with 5% milk in TBST, o/n at 4ºC) as primary antibody and probed with goat anti-rabbit IgG HRP (Southern Biotech, diluted 1:8000 with 3% milk in TBST) as secondary antibody followed by visualization using the Immobilon chemiluminescent HRP detection kit (Millipore). Alternatively, to assess the presence of neuroligins, samples were electroblotted to nitrocellulose membranes and the membranes blocked with 5% milk in TBST (10 mM Tris pH 7.5, 150 mM NaCl, 0.1% Tween-20). Immunodetection was carried out with an anti-neuroligin antibody (Synaptic Systems, cat. no.129011) diluted 1:5000 with 5% milk in TBST. As a secondary antibody, goat anti-mouse IgG HRP (Invitrogen) diluted 1:2000 with 5% milk in TBST was used and the blots were developed with the Immobilon chemiluminescent HRP detection kit (Millipore).

Immunocytochemistry and Imaging

The additional polyclonal primary antibodies were used: rabbit anti-synapsin I (1:2000; Millipore; AB1543P), rabbit anti-VGlut1 (1:2000; Synaptic Systems; 135 302), rabbit anti-VGAT (1:1000; Synaptic Systems; 131 003). The following mouse monoclonal antibodies were used: anti-PSD-95 family (1:500; IgG2a; clone 6G6-1C9; Thermo Scientific; recognizes PSD-95, PSD-93, SAP102 and SAP97), anti-gephyrin (1:500; IgG1; mAb7a; Synaptic Systems), anti-HA (1:1000; IgG2b; clone 12CA5; Roche), anti-Myc (1:1000, IgG1; Invitrogen), anti-bassoon (1:1000; IgG2a; Stressgen; VAM-PS003) and anti-synaptophysin (1:1000; IgG1; BD Biosciences; 611880). For labeling dendrites, anti-MAP2 (1:4000, chicken polyclonal IgY; Abcam; ab5392) was used. For labeling axons, anti-tau-1 (1:2000; mIgG2a; clone PC1C6; Millipore; MAB3420; recognizes dephosphorylated tau) was used. Secondary antibodies used were raised in goat against the appropriate species and monoclonal isotype, highly cross-absorbed and conjugated to Alexa-488, Alexa-568 and Alexa-647 dyes (1:500, Invitrogen). AMCA (7-amino-4methylcoumarin-3-acetic acid)-conjugated anti-chicken IgY (donkey IgG; 1:200; Jackson

ImmunoResearch; 703-155-155) was used for visualizing dendrites.

For staining cells in culture, the following protocol was used. Cells were fixed in parafix solution (4% paraformaldehyde and 4% sucrose in PBS pH 7.4) for 15 min followed by permeabilization with PBST (PBS + 0.25 % Triton X-100) or in -20°C methanol for 10 min. They were then incubated with blocking solution (PBS + 3% BSA and 5% normal goat serum) for 30 min at 37°C, followed by incubation with primary antibodies in blocking solution (overnight, 20°C) and secondary antibodies (45 min, 37°C). Coverslips were mounted in elvanol (Tris-HCl, glycerol, and polyvinyl alcohol with 2% 1,4-diazabi-cyclo[2,2,2]octane).

For surface labeling of HA- or Myc- signals in neurons, the same protocol was followed except that cells were incubated with anti-HA (1:500; IgG2b; clone 12CA5; Roche) or anti-Myc (1:500; mIgG1; Invitrogen) antibodies for 1 h at 37°C following fixation in parafix solution, but prior to permeabilization with PBST. For determining surface expression in COS7 cells, anti-Myc antibody (1:500; mIgG1; Invitrogen) in "binding buffer" (168 mM NaCl, 2.6 mM KCl, 10 mM HEPES, pH 7.2, 2 mM CaCl2, 2 mM MgCl2, 10 mM D-glucose, and 100 µg/ml BSA) was incubated with cells for 30 min at 20°C. Cells were then fixed in parafix solution, permeabilized, blocked and incubated with primary and secondary antibodies as described above. For the synaptotagmin I antibody uptake assay, neurons were incubated live with antibodies to the synaptotagmin luminal domain (1:200; IgG1; clone 604.2; Synaptic Systems) for 30 min in culture media at 37°C in a 5% CO2 incubator.

For quantification, sets of cells were fixed and stained simultaneously and imaged with identical settings blind to the experimental condition. Cell culture images were acquired on a Zeiss Axioplan2 microscope with a 40X 1.30 NA oil objective, a 63X 1.4 NA oil objective or a 25X 0.8 NA oil objective and Photometrics Sensys cooled CCD camera using Metamorph imaging software (Molecular Devices) and customized filter sets. Images were initially acquired as 12 bit grayscale and were prepared for presentation using Adobe Photoshop (Adobe Systems).

Tissue Immunofluorescence and Imaging

For immunofluorescence studies, brain tissue samples were collected from P30 *Clstn3* -/- and littermate wild-type male mice. The mice were anaesthetized with 20% urethane and perfused transcardially with cold 0.1 M phosphate buffered saline (PBS) followed by 4 % paraformaldehyde + 4% sucrose in PBS (pH

7.4). Following perfusion, the brains were extracted and post-fixed in cold 4% paraformaldehyde overnight. The brains were then cryoprotected in 30% sucrose in PBS at 4˚C and frozen in OCT compound (Tissue-Tek; Sakura-Finetek) using dry ice. Coronal cryostat sections 20 µm thick were cut at hippocampal level and mounted on Superfrost Plus slides. For double staining with calsyntenin-3 and CaMKIIα/Parvalbumin antibodies, the sections were then incubated in 100% methanol for 10 minutes at -20°C followed by PBS wash and then incubated in blocking solution (5% BSA + 5% Normal goat serum + 0.25 % Triton X100 in PBS) for 1 hour. For staining with VGlut1 and GAD-65, the 4% PFA fixed sections were directly incubated in blocking solution for 1 hour. For all immunostaining, sections were incubated with primary antibodies diluted in the same blocking solution (overnight, 4°C) then with the appropriate secondary antibodies conjugated to Alexa 488, 568 or 647 (Molecular Probes/Invitrogen) (1 hour, 20°C). Sections were washed in PBS containing the nuclear counterstain DAPI (4',6 diamidino-2-phenylindole), and mounted in elvanol (Tris-HCl, glycerol, and polyvinyl alcohol with 2% 1,4-diazabi-cyclo[2,2,2]octane).

For studying the expression pattern of calsyntenin-3, tiled images were captured on a Zeiss LSM 700 confocal microscope using a 20x lens with 0.5x magnification followed by the use of a stitch macro for further processing. For synaptic density quantification images, sets of sections were fixed and stained simultaneously and imaged with identical settings blind to the experimental condition on the Zeiss confocal microscope using a 40x 1.4 numerical aperture lens with 2.5x magnification and sequential scanning with individual lasers and optimized filters.

Image Analysis

All image analysis was done blind to the experimental condition. For quantifying most co-culture experiments, a visual method was used. Transfected COS cells were selected for measuring based on moderate expression (based on CFP expression or co-expression), normal morphology, and contact with neurites (as viewed under phase contrast). A selected cell was then viewed under fluorescence to determine the presence of presynaptic protein clustering (synapsin, VGlut1, VGAT puncta), in the absence of either dendrites (by MAP2 staining) or postsynaptic clusters (by PSD95 and/or gephyrin puncta). If presynaptic clusters were present over the transfected cell without MAP2 or apposed postsynaptic clusters, the cell was scored as positive, whereas if presynaptic clusters were apposed to a

MAP2 positive neurite or postsynaptic clusters, the cell was scored as negative. Many hundreds of cells were scored across independent experiments.

For quantitation of tau and synapsin signals in co-culture assays, regions were created around the expressing COS cells that excluded MAP2-positive areas, and the total intensity and area of all puncta in the synapsin channel and all crossing axons in the tau channel were each thresholded and measured. The COS area measurements, created from the delineated COS cell region, were used to normalize measures to COS7 cell area. Measures were corrected for off-cell background.

To measure the binding of Clstn3-Fc to surface-expressed HA-neurexin-1α, regions were drawn around the perimeter of each COS cell, and the average intensity values of bound protein and expressed protein were measured within the region and corrected for average off-cell background.

For analysis of neurons in the overexpression experiments, neurons were chosen for imaging based on Myc / HA or CFP signal, as well as healthy morphology under phase contrast and MAP2 channels. Neighbouring cells for overexpression analysis were chosen based on similar MAP2 staining. During analysis, regions were created around single expressing or non-expressing dendrites and thresholded in the synapsin, VGAT or VGlut1, and gephyrin or PSD95 channels. Total number of puncta and area were measured for each channel. Average intensity for surface Myc signal was also measured in the selected region. Measures were corrected for off-cell background and normalized to dendrite length.

For quantification of excitatory (VGlut1) and inhibitory (GAD65) synaptic markers in brain sections, images were manually thresholded to define puncta and total integrated intensity of puncta per tissue area measured. Measures were performed on a minimum of six sections per animal for each region, nmeasuring the same regions for both VGlut1 and GAD65.

Analysis was performed using Metamorph (Molecular Devices), Excel (Microsoft) and GraphPad Prism (GraphPad Software). Statistical comparisons were made with Student's unpaired t-test or one-way ANOVA with post hoc Bonferroni's multiple comparison test, as indicated in figure legends. All data are reported as the mean ± SEM.

Western Blotting

For analysis of cleavage of various calsyntenin-3 constructs, COS7 cells were transfected as described above. After 24-48 hours of expression, the media was collected, treated with protease inhibitor cocktail

tablets (Roche) and placed on ice, and the cells were washed with phosphate buffered saline then scraped into lysis buffer (1% Triton X-100, 150mM NaCl, 20mM Tris pH 7.4, 1mM DTT, 1mM EDTA, plus protease inhibitor tablet). Lysates were centrifuged at 16000 x g for 15 min at 4°C and the supernatant was collected. The protein concentrations of both media and lysate fractions were determined using the Bio-Rad Protein Assay kit, using BSA as a standard (Sigma). Protein concentrations were normalized between samples and run on 10% polyacrylamide gels. Gel transfer was performed onto Immobilon P membranes (Millipore) which were blocked in 5% skim milk in Tris-buffered saline/0.05% Tween-20 (Sigma) and incubated with primary (anti-Myc mouse IgG1; Invitrogen), and secondary (Goat anti-mouse HRP conjugate; Millipore) antibodies. Immunoblots were detected using the SuperSignal Chemiluminescent kit (Thermo Scientific) and visualized by chemiluminescence using a Bio-Rad gel documentation system. Specificity of calsyntenin-3 antibody was assayed from transfected COS7 cell lysates in a similar manner.

To show the loss of calsyntenin-3 in *Clstn3* -/- animals, brain tissue was collected from P30 *Clstn3* +/+ and *Clstn3* -/- mice in cold homogenization buffer (320 mM sucrose, 5 mM Hepes pH 7.4). For developmental western, brain tissue was collected from P6, P21, P30, P60 wildtype mice and undergone the same procedure. The tissue samples were homogenized using a Dounce homogenizer in five volumes of homogenization buffer supplemented with protease inhibitor tablet. Homogenized tissues were centrifuged at 1000 x g for 10 min at 4°C, the supernatant collected, and protein concentration determined with the Bio-Rad Protein Assay kit. Protein samples (20 μg) were loaded on a 10% polyacrylamide gel and Western blot using calsyntenin-3 and β-actin antibodies was performed as described above.

To detect the expression level of neurexins in *Clstn3* -/- and *Clstn3*+/+ mice, brain tissue was collected from P42 mice and P2' fractions were prepared as described before (Linhoff et al., 2009). Western blotting were conducted as described above and membranes were probed with anti-pan neurexin (1:2000, Millipore) and anti-α-tubulin (1:10000, Thermo) antibodies.

Electrophysiology

For slice preparation, following cervical dislocation, mouse brains were rapidly removed and placed in ice-cold slicing solution consisting of (in mM): 120 NMDG, 2.5 KCl, 1.2 NaH_2PO_4, 25 $NaHCO_3$, 1.0

$CaCl_2$, 7.0 $MgCl_2$, 2.4 Na-pyruvate, 1.3 Na-ascorbate, 20 D-glucose with pH adjusted to 7.35 using HCl acid (unless stated, all chemicals and drugs were purchased from Sigma or BioShop, Canada). The hippocampus was dissected out and coronal brain slices (400 μm thickness) were cut using a manual chopper (Stoelting) and transferred to oxygenated, ice-cold slicing solution.

For whole-cell recordings, slices were subsequently maintained for 1 h at 30ºC in artificial cerebrospinal fluid (ACSF) containing (in mM): 124 NaCl, 3 KCl, 1.25 NaH_2PO_4, 1 $MgSO_47H_2O$, 2 $CaCl_2$, 26 $NaHCO_3$ and 15 D-glucose which was bubbled continuously with carbogen (95% O_2/ 5% CO_2) to adjust the pH to 7.3. Recordings were performed at room temperature in a submerged recording chamber perfused continuously with carbogenated ACSF (2-3 ml/min). Whole-cell recordings of CA1 neurons in brain slices were performed using the "blind" method with a MultiClamp 700B amplifier. For mEPSC recordings, CA1 neurons were voltage clamped at -60 mV. Recording pipettes were filled with solution containing (in mM): 122.5 Cs-methanesulfonate, 17.5 CsCl, 2 $MgCl_2$, 10 EGTA, 10 HEPES, 4 ATP(K), and 5 QX-314, with pH adjusted to 7.2 by CsOH. Prior to recording mEPSCs, tetrodotoxin (TTX; 500 nM; Ascent Scientific) and bicuculline methiodide (10 μM; Abcam) were added to block action potentials and $GABA_A$ receptor-mediated inhibitory synaptic currents, respectively. To verify that remaining responses were mEPSCs, CNQX (10 μM; Abcam) and DL-AP5 (50 μM; Abcam) were added towards the conclusion of some experiments to block remaining excitatory currents. For mIPSC recordings, CA1 neurons were voltage clamped at –70 mV and the recording pipette was filled with solution containing (in mM): 140 CsCl, 0.1 $CaCl_2$, 2 $MgCl_2$, 10 HEPES, 10 EGTA, 4 ATP(K), with pH adjusted to 7.2 using CsOH. Slices were treated with CNQX (10 μM), DL-AP5 (50 μM) and TTX (500 nM) to block excitatory synaptic transmission. Bicuculline methiodide (10 μM) was added towards the end of some experiments to confirm that the remaining responses were mIPSCs. mEPSCs and mIPSCs were recorded using WinLTP in continuous acquisition mode. Analyses for frequency and amplitude were conducted using MiniAnalysis software. Statistical analyses were completed using GraphPad InStat and SigmaPlot. Kolmogorov-Smirnov tests were used for determination of cumulative probability normality. Student's t-tests were conducted to test for differences between groups with n = number of cells. Data are presented as mean ± SEM.

For extracellular recordings, preparation and recording conditions were similar to whole-cell recording conditions except that recovery following slicing was for 2 h at room temperature and the

slicing solution was composed of (in mM): 124 NaCl, 3 KCl, 1.25 NaH_2PO_4, 10 $MgSO_47H_2O$, 2 $CaCl_2$, 26 $NaHCO_3$ and 15 D-glucose which was bubbled continuously with carbogen, pH 7.3. The perfusion solution was the same composition as the extracellular slicing solution except that $MgSO_47H_2O$ was reduced to 1 mM and $CaCl_2$ was increased to 2 mM. Both the recording electrode and a bipolar nickel-chromium stimulating electrode were positioned in the stratum radiatum of area CA1. fEPSPs were elicited by stimulating Schaffer collateral fibers projecting onto CA1 apical dendrites. For paired-pulse stimulation, two pulses were applied once every 30 s while incrementally decreasing the interpulse interval from 300 ms to 40 ms. Input/output plots were generated by increasing the stimulation intensity while monitoring the magnitude of fEPSP response. Student's t-test was used for statistical comparisons of mean fEPSP slopes between two groups. All values shown are mean ± SEM, with n = number of slices.

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