Supplementary Materials for

Elfn1 Regulates Target-Specific Release Probability at CA1-Interneuron Synapses

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Materials and Methods

Plasmids
The full length mouse clone for Elfn1 (Accession# BC059029) was obtained from Open Biosystems (Thermo Fisher Scientific, Huntsville, AL) and subcloned into the expression vector pEGFPN1 (Accession# U55762) to yield a C-terminal GFP fusion construct. For knockdown experiments, an shRNA was obtained from Open Biosystems containing nucleotides 2440-2460 of Elfn1 (gacatcctagactactggaaa), which is 100% conserved between mouse and rat. The U6 promoter and shRNA was subcloned into the lentiviral plasmid FUGW (Addgene), which contains a ubiquitin promoter driving eGFP expression. The Elfn1 rescue construct had a single point mutation in the shRNA target sequence (gacatccttgactactggaaa).

In Situ Hybridization
In situ hybridizations were performed as described in (31), using P7, P14, or P21 mouse or rat brain cryosections. Digoxigenin-labeled cRNA probes were generated from linearized cDNA templates. Full length mouse Elfn1 was subcloned in the reverse orientation into pcDNA3.1(-) to generate anti-sense riboprobes from the coding region using T7 RNA polymerase. For experiments combining in situ hybridization with immunohistochemistry, tissue was prepared using an immunoperoxidase detection kit prior to dehydration (Vectastain Elite ABC; Vector Labs, CA).

Hippocampal Culture
Hippocampal neurons were cultured from P0 Long-Evans rats (Charles River, Wilmington, MA) and plated on poly-D-lysine (Millipore, Temecula, CA), and laminin (Invitrogen, Carlsbad, CA) coated chamber slides (Nalge Nunc International, Rochester, NY). Neurons were maintained in Neurobasal-A medium (Invitrogen) supplemented with B27, glucose, glutamax, and penicillin/streptomycin (Invitrogen).

Mixed Culture Assay
Mixed-culture assays were performed as described in (32). HEK 293T cells were grown in DMEM (Invitrogen) supplemented with 10% FBS (Invitrogen) and penicillin/streptomycin. Cells were transfected with eGFP or Elfn1-GFP using Fugene6 (Roche). After 24 hours, cells were mechanically triturated and replated on hippocampal neurons (7 DIV) for 2 days and immunostained for synapsin and GFP.

Immunocytochemistry
Neurons were fixed at P14 in 4% paraformaldehyde in phosphate buffered saline (PBS) and processed for immunofluorescence according to standard procedures. For synapse density experiments, primary antibodies were: rabbit anti-VGlut1, rabbit anti-VGAT, mouse anti-gephyrin, (Synaptic Systems, Goettingen, Germany); mouse anti-PSD-95 (Affinity BioReagents, Rockford, IL); goat anti-GFP, chicken anti-Map2 (Abcam, Cambridge, MA). For mixed culture assay, primary antibodies were: rabbit anti-synapsin (Millipore) goat anti-GFP, (Abcam, Cambridge, MA). For cell type determination, primary antibodies were: mouse anti-GAD6 (Developmental Studies Hybridoma Bank, Iowa City); mouse anti-CamKII (Chemicon, Ramona, CA); rabbit anti-Elfn (anti-lrrc62, Prestige Antibodies-Sigma, St. Louis, MO), rat anti-somatostatin (Millipore), goat anti-mGlur1a (Frontier Institute), sheep anti-PV (R&D Systems).
Fluorophore-conjugated secondary antibodies were from Jackson ImmunoResearch (West Grove, PA) or Invitrogen.

Image Acquisition and Analysis
Images were captured on a Leica SP5 confocal microscope (Leica Microsystems, Bannockburn, IL). Z-stacks were collapsed in a maximum projection and analyzed using NIH ImageJ software. Images were thresholded using constant settings per experiment and the density of colocalizing pre- and postsynaptic puncta was measured per length of GFP-positive dendrite of transfected neurons. For quantification of mixed-culture assays, images were thresholded and the total area of synapsin puncta was measured and normalized to the total GFP-positive area per cell. For experiments to determine cell-type, z-stack images were collapsed and images were thresholded to determine colocalization of cell type markers. Fields of view at 20x were then counted for each cell type.

Mice
Somatostatin-IRES-Cre mice were created by the Huang lab (17) and Parvalbumin-IRES-GFP mice were generated by the Arber lab (15). Both are available through Jackson Labs. LSL-CAG-tTomato mice were created by the Allen Institute for Brain Science and acquired from Jackson Labs (16).

Lentivirus Production
For lentivirus production, 293T cells were transfected with control or shElfn1 containing FUGW vector plasmids and helper plasmids MDL, RSV-REV and VSVG using polyethylenimine (PEI). Supernatant was collected 48 hrs after transfection, spun at 2000 rpm to remove debris and filtered through a 0.22 µm filter (Millipore). Viral particles were pelleted using two centrifugation steps at 19500 rpm for 2 hrs each. The final pellet was resuspended in 100 µl PBS and stored at -80°C in 10 µl aliquots.

Electrophysiology
Postnatal day 5-6 mouse pups were anaesthetized with isoflurane and received a subcutaneous injection of bupivacaine prior to craniotomy. Lentivirus injections were targeted to the CA1 region of the hippocampus using stereotaxic coordinates. At postnatal day 13-16, 300 µm slices were maintained in a sucrose substituted solution: 83 NaCl, 2.5 KCl, 1 NaH2PO4, 26 NaHCO3, 22 glucose, 72 sucrose, 0.5 CaCl2, 3.3 MgCl2. Slices were moved to the recording chamber and perfused with an ACSF that consisted of (in mM): 119 NaCl, 2.5 KCl, 26 NaHCO3, 1 NaH2PO4, 2 MgCl2, 2 CaCl2, 11 glucose, 0.1 picrotoxin and bubbled constantly with 95% O2/5% CO2. Somatostatin positive oriens interneurons were identified by tdTomato and eGFP epifluorescence microscopy (Zeiss Axioskop 2). Whole-cell voltage clamp recordings were made under visual guidance aided by infrared differential interference. Neurons lying near the alveus that expressed both eGFP and tdTomato were recorded from using ~3MΩ pipettes pulled on a horizontal micropipette puller (Sutter P-97) and filled with an internal solution that contained (in mM): 130 Cs-methanosulfonate, 5 NaCl, 10 EGTA, 10 HEPES, 10 phosphocreatine, and 2 Mg-ATP, pH 7.3 with CsOH, 280-290 mOsm. Synaptic responses were evoked every 15 s with a bipolar cluster electrode (FHC) placed in the alveus near the subiculum. The signals were low-pass filtered at 2 kHz, digitized at 10 kHz (Molecular Devices
Multiclamp 700B) and analyzed with pClamp 9 (Molecular Devices). Series resistance and input resistance were monitored throughout the experiment by a test pulse.

Short-term facilitation was measured at a holding potential of -70mV by delivering a train of 5 stimuli at 5, 10, 20, and 40 Hz. At least 10 sweeps were averaged at each frequency and the amplitude was calculated as the average 1 ms around the peak. The amplitudes were normalized to the amplitude response to the first stimulus in a train to determine the facilitation ratio. To determine rectification of synaptic currents, D-APV (50µM) was added to the perfusion solution. The alveus was stimulated once as described above, then the holding potential was increased from -70mV to +70 mV by 20 mV increments, and repeated 10 times to calculate an average. For MK801 experiments, DNQX (20µM) was used to block non-NMDA currents. The cell was held at +40mV and after a steady baseline was attained, MK801 (40µM) was washed in and the alveus was repetitively stimulated at 5 s intervals. The decrease in NMDA-mediated current was plotted over time and the number of stimuli needed to reach half the initial amplitude was calculated.

For IPSC recordings, cells were held at 0mV and the alveus was stimulated at 20Hz. Picrotoxin (0.1mM) was washed in to block GABA-mediated currents. The resulting trace was subtracted from the baseline to calculate the GABA mediated current. For presynaptic kainate and NMDA experiments, a stable baseline recording was performed and a pre-drug average was calculated from at least 10 sweeps. The antagonist (20µM NS102, 15µM UBP302, or 50µM APV) was washed in and then a second average was calculated. The two averages were each normalized to the amplitude of their first EPSC and a facilitation ratio was calculated for each subsequent EPSC.

For mEPSC recordings, Gabazine (SR 95531 hydrobromide), D-APV, and TTX were added at the time of recording. mEPSCs were identified and isolated using ClampFit software. Using a control culture, a template was created from a composite average of manually identified mEPSCs and was used with a broad threshold for deviation to ensure all events in subsequent recordings were captured. These events were then manually inspected to discard any non-mEPSC traces. Cumulative fractions were calculated by randomly selecting 125 events and plotting the distributions.
Elfn1 mRNA colocalizes with horizontal Sst (OLM) interneurons. (A) Colocalization of Elfn1 mRNA and tdTomato protein in Sst::tdTomato mice. Stained cells were classified by location (CA1 or hilus) and morphology (horizontal, oval cell soma with horizontal proximal dendrites; multipolar, circular cell soma with radial dendrites). Colors indicate the percentage of all stained cells that contain the corresponding mRNA or protein. (B) Colocalization of Elfn1 mRNA and tdTomato protein in PV::tdTomato mice. “Horizontal CA1” data points in (A) and (B) are reproduced from Figure 1D for ease of comparison.
Fig. S2
Characterization of Elfn expression and antibody characterization. (A) In situ hybridization of Elfn1 and Elfn2 probes on rat hippocampal sections at P7, P14, and P21. (B) HEK cells transfected with GFP, Elfn1-GFP, or Elfn2-GFP and immunostained using an antibody that recognizes Elfn1 and Elfn2. (C) Western Blot of HEK cell lysates from cells transfected with GFP, Elfn1-GFP, or Elfn2-GFP and blotted using an antibody that recognizes Elfn1 and Elfn2. For (B), scale bar = 20µm.
**Fig. S3**

**(A)** Dissociated hippocampal cultures immunostained for Elfn1 and CamKII, GAD6, somatostatin, parvalbumin or mGluR1a. 69% of Sst cells contain Elfn1, which is comparable to the 75% by in situ/immuno in Fig 1C,D. PV colocalization is much lower than with the in situ (4%), which may be due to lower levels of Elfn1 protein that fall below the detection threshold of the antibody. CamKII, n=350 cells; GAD6, n=104 cells; Sst, n=42 cells; PV, n=108 cells; mGluR1a, n=42 cells. **(B)** P14 rat hippocampal sections stained for Elfn, somatostatin, and Hoechst. Upper panels, low magnification images of CA1. Lower panels, high magnification images of CA1 stratum oriens area from dotted box above. For (A), scale bar = 10µm. For (B), scale bar = 20µm.
**Fig. S4**
Strategy to target OLM cells for electrophysiological recording. (A) Genetic cross labeling somatostatin-containing interneurons *in vivo*. (B) Hoechst staining of hippocampal slices from Sst:tdTomato mice. Sst interneurons can be seen in the stratum oriens and hilus (arrows). Sst cells are well labeled and electrophysiological recording was targeted to tdTomato cells adjacent to the alveus, corresponding the OLM cell type. The axons of these OLM cells can be seen as the band of tdTomato fluorescence in the stratum lacunosum moleculare.
Elfn1 knockdown does not affect postsynaptic properties (A) Example traces from OLM interneurons in acute slices infected with GFP or shElfn1 in the presence of TTX, APV and Gabazine to isolate AMPA mediated EPSCs. (B) Cumulative distribution of mEPSC amplitudes. (C) Quantification of average mEPSC amplitude for GFP control and shElfn1 infected neurons. GFP, n=8; shElfn1, n=9. p=.11, t-test. (D) Peak AMPA- and NMDA-mediated currents following a stimulus to the alveus. For AMPA EPSC, cells are voltage clamped at -70mV. For NMDA EPSCs, cells are held at +40mV in 20 µM DNQX. GFP,
n=8; shElfn1, n=10. p=.36, t-test. (E) Decay kinetics of AMPA EPSC. Tau calculated from a fit to a single exponential curve. GFP, n=22; shElfn1, n=15; p=.45, Mann-Whitney U test. (F) Decay kinetics of NMDA EPSC. Tau calculated from a fit to a single exponential curve. GFP, n=7; shElfn1, n=7. p=.89, t-test. (G) Examples traces and IV curve for AMPA-mediated currents. (H) The proportion of GluR2-lacking AMPA receptors controls rectification at CA1-OLM synapses and has been suggested to produce short-term facilitation (33,34). To test this, the holding potential was varied between -70mV and +70 mV and the evoked response from alvear stimulation was recorded in control and shElfn1-expressing cells. Spermine is included in the patch pipette. (I) Rectification index in control and shElfn1-expressing cells was calculated by the ratio of the slope of a line fit from +10mV to +70 mV to the slope of a line fit between -70 mV to -10mV. GFP, n=6; shElfn1, n=7. p=.96, t-test.
Fig. S6
Elfn1 overexpression does not alter synapse density. Neurons were electroporated at plating with GFP and Elfn1-myc and fixed at 14DIV. (A) Left, neurons were stained for Vglut and PSD95 to visualize excitatory synapses at 14DIV. Right, quantification of the size and density of excitatory synapses. GFP, n= 3372 synapses on 21 cells; Elfn1, n= 4006 synapses on 21 cells. Synapse density, p=.22 by t-test; synapse size, p=.88, Mann-Whitney U-test. (B) Left, neurons were stained for VGAT and gephyrin to visualize inhibitory synapses. Right, quantification of the size and density of inhibitory synapses. GFP, n= 902 synapses on 15 cells; Elfn1, n= 1508 synapses on 15 cells. Synapse density, p=.29 by Mann-Whitney U-test; synapse size, p=.45 by t-test. (C) Left, example traces of mEPSCs recorded at 14DIV in the presence of Gabazine, APV and TTX to isolate AMPA-mediated currents. Right, quantification of mEPSC amplitude and frequency. Error bars represent S.E.M.
Elfn1 is not a synapse-inducing molecule. (A) HEK cells expressing GFP, Elfn1, or LRRTM2 were co-cultured with dissociated hippocampal neurons from 7-9 DIV, fixed and stained for synapsin. (B) Fractional area of the HEK cell stained with synapsin, normalized to GFP transfected cells. Asterisk indicates p<.01; ANOVA. Scale bar is 20µm. Error bars represent S.E.M.
CA1-OLM synapses do not have GluR5- or NMDAR-dependent mechanisms of short-term facilitation. (A) Average postsynaptic response of control uninfected OLM neurons to 20Hz stimulation of the alveus, normalized to the amplitude of the first response. Black and red points signify before and after application of the NS102 drug vehicle DMSO, respectively. (B) Average postsynaptic response of GFP infected OLM neurons to 20Hz stimulation of the alveus, normalized to the amplitude of the first response. Black, baseline response; Red, after application the GluR5 containing kainate receptor antagonist of UBP302 (15µM). (C) Average postsynaptic response of GFP infected OLM neurons to 20Hz stimulation of the alveus, normalized to the amplitude of the first response. Black, baseline response; Red, after application of 50 µM APV.

References and Notes


