Supporting Online Material

Materials and Methods

Generation of VGLUT1 knock-out mice

A mouse VGLUT1 genomic BAC clone from 129/SvJ mice was obtained from Incyte Genomics (Saint Louis, MO). Restriction and Southern analysis identified two independent ~6 kb BamHI fragments that contain the twelve protein-coding exons. The targeting vector was constructed by subcloning an ~4.4 kb Not1-Sal1 fragment that contains the first protein-coding exon (long arm, designated 5' arm in Figure 1) into the polylinker region flanking the 5' end of the neomycin resistance gene in pPNT (*1*). The ~1.3 kb BamH1-EcoR1 fragment (short arm, designated 3' arm in Figure 1) was subloned into the polylinker region flanking the 3' end of the neomycin resistance gene and the 5' end of the HSV TK gene of pPNT. The resulting targeting vector was confirmed by restriction mapping and sequencing, linearized by digestion with Not1, electroporated into JM1 embryonic stem cells (gift of Dr. L.F. Reichardt) and the resulting colonies selected for resistance to both neomycin and gancyclovir resistance as described (*2, 3*). Double resistant clones were analyzed by Southern blot to identify homologous recombination events using the 5' probe described in Figure 1. From more than 600 analyzed, six clones contained the 6.9 kb mutant fragment as well as the 9.2 kb wild type band. Two targeted clones were injected into mouse blastocysts to generate highly chimeric male mice that transmitted the mutation through the germline. Heterozygous animals derived from the two different ES cell lines were inbred to produce two independent lines of VGLUT1 knockout (-/-) mice, and were maintained as heterozygotes by continuous backcrossing with wild type C57/Bl6 mice. All experiments were performed on F2 littermates from F1 heterozygote matings. Mice were genotyped either by Southern analysis using the 5' probe described above or by PCR. One pair

of primers (5'-CCAAGCAAGGTTAAGCCTAG-3' and 5'-GGTGAATTTGGAAAAGAGC-3') was designed to amplify an ~650 bp fragment in the wild type but not the mutant allele. A second pair of primers (5'-TCGCAGCGCATCGCCTTCTA-3' and 5'-GCCACACGTTTCTTCTGAGG-3') was designed to amplify an ~1850 bp fragment in the mutant but not the wild type allele.

Transport assays

LP2 fractions were prepared from the brains of individual young adult wild-type (+/+) or VGLUT1 knockout (-/-) mice by differential centrifugation and resuspended in 0.32 M sucrose/10 mM HEPES-KOH, (pH 7.4) (SH buffer) containing protease inhibitors as previously described (*4, 5*). Using these fractions (25-50 μg protein per reaction), uptake of ^{3}H-glutamate and -serotonin was measured at 29^{o} C for 5 minutes as previously described (*4, 5*). FCCP-sensitive glutamate uptake was determined by subtracting the nonspecific accumulation of ^{3}H-L-glutamate (200 μM) in the presence of 46 μM FCCP. Reserpine-sensitive serotonin uptake was determined by subtracting the nonspecific accumulation of ^{3}H-serotonin (30 nM) in the presence of 5 μM reserpine. The measurements were performed in triplicate using LP2 preparations from at least 3 individual animals of each genotype.

Electrophysiology

Transverse hippocampal slices (300-400 μm) were prepared from VGLUT1-/-, heterozygous, and wild type littermates (3-75 days old) as previously described (*6*). The slices were maintained in a submerged chamber for both recovery (1 hour) and recording at room temperature (24-28° C). Perfusion medium contained (in mM): 119 NaCl, 2.5 KCl, 2.5 $CaCl_2$,

1.3 $MgSO_4$, 1 NaH_2PO_4, 26.2 $NaHCO_3$, 11 glucose, and 0.1 picrotoxin, saturated with 95% O_2-5% CO_2. The perfusion rate was 2 ml/min. A cut was made between CA1 and CA3 to prevent the propagation of epileptiform activity.

fEPSPs were evoked in CA1 stratum radiatum by stimulation of Schaffer collaterals with bipolar tungsten electrodes (FHC) or 1M NaCl filled glass pipettes, and were recorded with 1M NaCl-filled 3-5 MΩ glass pipettes using the Axopatch-1D amplifier (Axon Instruments). EPSPs were filtered at 2 kHz, digitized at 10 kHz, and stored on computers using IgorPro (Wavemetrics Inc.). Test stimuli consisted of 100 μs pulses of constant voltage delivered by stimulus isolation units (Digitimer Ltd). The baseline stimulation rate was 0.1 Hz for all experiments. For input-output experiments, 100 μM D-APV or 3 μM CPP was included in the external solution. The slices from knock-out mice and wild type littermates were interleaved, and the experimenter was blind to genotype (Figure 2A-C). Stimulus artifacts were blanked in sample traces.

Somatic whole cell voltage-clamp recordings in acute slices were obtained from visually identified CA1 pyramidal cells using 2.5-5 MΩ glass electrodes filled with (in mM): 107.5 Cs-gluconate, 20 Hepes, 0.2 EGTA, 8 Na-gluconate, 8 TEA-Cl, 4 Mg-ATP, 0.3 Na_3-GTP, 5 QX-314, pH 7.2, 280 mOsm. Cells in which the series or input resistance varied by 25% during the duration of the experiment were discarded. MK-801 experiments were recorded at +30 mV in 10 μM CNQX (*7*). mEPSCs were measured in the presence of 0.5 μM TTX and 100 mM sucrose to increase the frequency of events to reasonable levels, and were recorded and analyzed off line with customized software, using an amplitude threshold of 4 pA. Entries in cumulative distribution histograms ranged from 130-737.

Standard procedures were used to prepare and maintain cerebellar slices (*8*). Parasagittal slices (300 μm thick) from the cerebellar vermis of 3-4 week old mice were cut in ice-cold

Ringer's solution using a vibratome, kept in a holding chamber for at least 1 hour and then transferred to a superfusing chamber for recording, with recording and internal solutions the same as above. Stimulation of both parallel fibers and climbing fibers was provided by a glass patch pipette filled with 1 M NaCl, and stimulus intensities used in the fEPSP recordings were applied to whole cell measurements of input-output relations in the same slice without moving the stimulating electrode. Stimulus artifacts in sample traces were blanked.

Microdot cultures were prepared as described (*9*). For whole cell voltage-clamp recordings in autaptic culture (10-14 DIV), patch pipette solutions contained (in mM): 135 potassium gluconate, 10 Hepes, 1 EGTA, 4.6 $MgCl_2$, 4 NaATP, 15 creatine phosphate and 50 U ml^{-1} phosphocreatine kinase, pH 7.3, 300 mOsm. In microdot cultures, recorded cells were confirmed to be autaptic by filling with 0.05% Lucifer yellow included in the internal solution. The extracellular solution contained (in mM): 140 NaCl, 2.4 KCl, 10 Hepes, 10 glucose, 4 $CaCl_2$, 4 $MgCl_2$, pH 7.3, 300 mOsm. Cells were held at –60 mV and stimulated at 0.1 Hz with a 1-5 ms 70 mV depolarizing current pulse. Pyramidal cells and interneurons were distinguished based on the decay kinetics of the evoked current and by application of either 10 μM CNQX or 100 μM picrotoxin at the end of the experiment.

MK-801 was obtained from Tocris, TTX from Calbiochem, QX-314 from Precision Biochemicals and all other chemicals from Sigma.

In situ hybridization and immunofluorescence

cDNA fragments corresponding to the unique carboxy-termini and 3'-untranslated regions of the mouse cDNA's for VGLUT1 (nucleotides 1589-2510), 2 (nucleotides 1528-1834) and 3 (1511-1869) were amplified by PCR, subcloned into pBluescript II (Stratagene) and transcribed into ^{35}S-labeled single-stranded antisense- and sense-strand RNA probes at high specific activity (>10^9 cpm/μg). *In situ* hybridization was conducted as previously described on 20 micron brain sections at the level of the hippocampus from fresh frozen 4 day, 1, week, 2week, and adult (> 2 months) mice by post-fixation in 4% paraformaldehyde (PFA) and hybridization of ^{35}S-labeled single-stranded RNA probes in 50% formamide for 16-18 hr at 55^{0}C (*5*). The sections were then treated with RNase A (50 μg/ml for 60 min at 37-42^{0}C), washed at high stringency (0.1xSSC for > 3 hr at 50-55^{0}C), exposed to BioMax MS film (Kodak) for 4 days, dipped in NTB2 nuclear track emulsion (Kodak) and exposed for 2-4 weeks.

For the immunofluorescence, 40 μm coronal sections through the dorsal hippocampus of 4% PFA-perfused 8 day and adult mice (3 months) were immunostained with rabbit polyclonal anti-VGLUT1 antibody (1:2000) and a guinea pig anti-VGLUT2 antibody (1:2500). The immune deposits were detected with donkey anti-rabbit IgG conjugated to Cy3 (1:200) and donkey anti-guinea pig conjugated to FITC (1:200) and visualized with a confocal laser microscope.

Electron microscopy

Brain tissue was prepared for electron microscopy as described (*10*). Specimens of the hippocampus (middle of stratum radiatum in CA1) and cerebellum were treated by OsO_4, dehydrated in increasing concentrations of ethanol followed by propylene oxide, and embedded

in Drucupan ACM (Fluka). Ultrathin sections were contrasted with uranyl acetate and lead citrate and examined in a Philips CM10 electron microscope. Micrographs were taken randomly from at a primary magnification of 8400x and printed at a final magnification of 64680x. The stratum radiatum of the hippocampus CA1 and the cerebellar cortex were analysed quantitatively, assisted by the computer programs MORFOREL (*11*) and PALIREL (*12*). Brains from 7 -/- and 7 +/+ mice were prepared and analysed in pairs, 4 pairs from adult mice (age >7 weeks), 3 pairs from young mice (age 3 weeks). Distance from the active zone was assessed by counting the vesicles in consecutive 100 nm-wide rectangles layered on top of the synaptic membrane. Statistical analysis was by means of a paired sample t-test or the Wilcoxon signed-rank test. In Figure 5G, the results were obtained from photographs covering 9 μm^2 and 70 photographs were used for wild type hippocampus, 66 for knock-out hippocampus, 61 for wild type cerebellum and 74 for knock-out cerebellum. In Figure 5H-I, the number of synapses used to count vesicles and measure the postsynaptic density was 148 for wild type hippocampus, 128 for knock-out hippocampus, 125 for wild type cerebellum and 113 for knock-out cerebellum. In Figure 5J, 10 terminals were chosen for each animal. Since 3 wild type and 3 knock-out mice were used, the data derive from a total of 30 terminals.

Quantitative western analysis

Membranes were prepared from the hippocampi of 3 week old wild type and knock-out mice (n=3 for each genotype), separated by electrophoresis through Criterion Tris-HCl polyacrylamide gels (Bio-Rad), electrotransferred to Immobilon PVDF membranes (Millipore) and immunoblotted with primary antibodies directed against the proteins indicated in the text. After extensive washing, the membranes were incubated with infrared-labeled secondary

antibodies and the immune complexes were quantified by direct fluorescence detection using the Odyssey™ Infrared Imaging System (LI-COR Biosciences, Lincoln, NE), and Odyssey™ analysis software.

Supplemental Figure Legends

Figure S1. Generation of mice with a null mutation in the VGLUT1 gene.

(A) Schematic diagram of the wild-type VGLUT1 gene locus, the targeting vector, and the integrated VGLUT1 locus. The location of the 5' probe used for genotype analysis and the expected size of Xho I restriction fragments in the wild-type and mutant alleles are shown below. The twelve protein-coding exons are represented as filled boxes, numbered 1-12. The open regions of exons 1 and 12 represent untranslated sequence.

(B) Southern analysis of genomic DNA from VGLUT1 wild-type (+/+), heterozygous (+/-), and knock-out (-/-) mice. Tail genomic DNA was digested with Xho1, and the fragments were separated by electrophoresis, transferred to nylon membranes and hybridized with the 5' probe. The wild-type VGLUT1 allele generates a 9.2 kb Xho1 restriction fragment labeled by the 5' probe. Homologous recombination of the targeting vector with the VGLUT1 gene introduces an Xho1 restriction site at the 5' end of the neomyin resistance cassette, producing a 6.9 kb restriction fragment in the targeted allele. Heterozygous mice contain both the wild-type 9.2 kb and the mutant 6.9 kb alleles.

Figure S2. Heterozygous VGLUT1 knock-out mice contain half the VGLUT1 protein of wild type littermates but do not differ in excitatory transmission in the hippocampus.

(A) Input-output curve for basal synaptic transmission in three week old wild type and VGLUT1 +/- mice. fEPSPs were recorded from CA1 stratum radiatum of hippocampal slices at a range of stimulus intensities. Fiber volley amplitudes were binned and corresponding EPSP slopes were averaged between slices. EPSP slopes were not significantly different at every fiber volley

amplitude ($p > 0.1$). Each point represents the mean ± SEM for each bin (wild type, n=65; VGLUT1 +/-, n=69).

(B) Membranes prepared from 3 three week old hippocampi of VGLUT wild-type (+/+), heterozygote (+/-), and knock-out (-/-) mice were immunoblotted for the VGLUT1 protein, and the bound primary antibody visualized with a secondary conjugated to an infrared fluorophore (Licor Odyssey).

(C) Quantitative analysis revelaed that VGLUT1 heterozygous mice contain 44% of wild-type VGLUT1 levels. Data represent the mean ± SEM (n=3 for each genotype).

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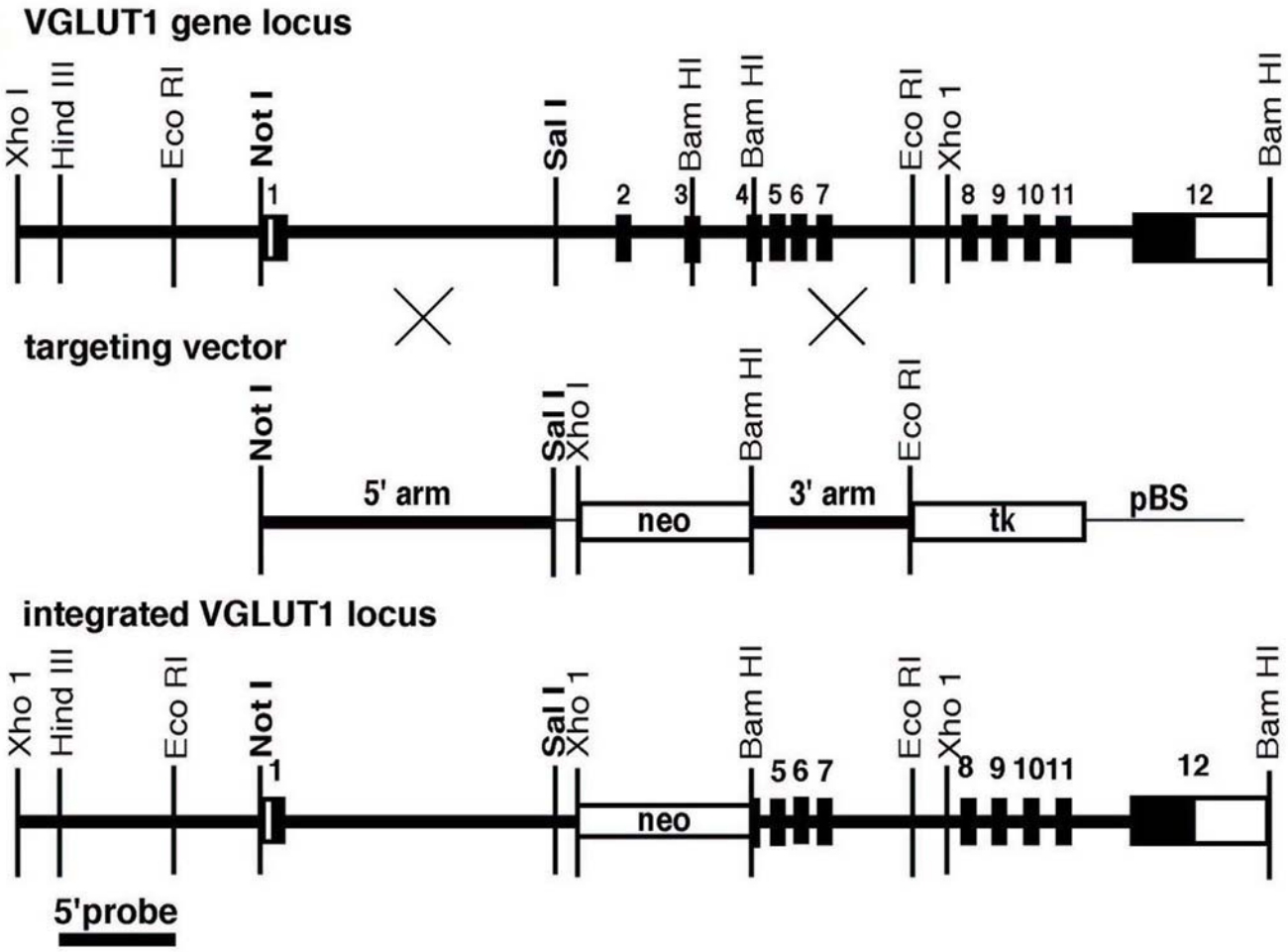

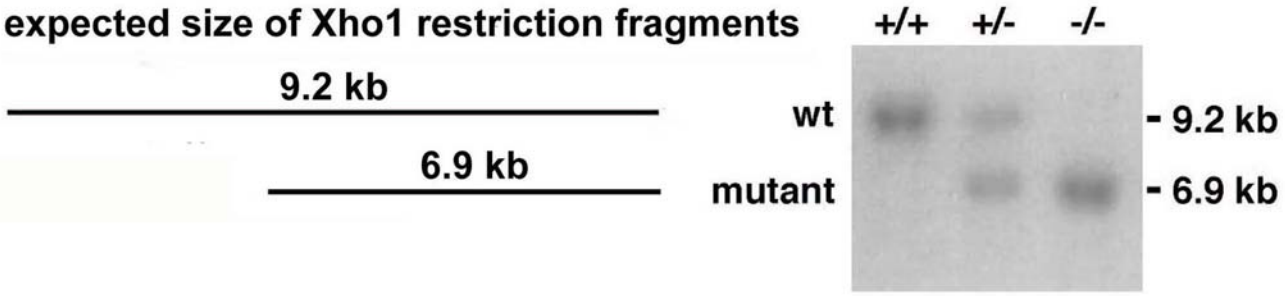

Supplemental Figure 1
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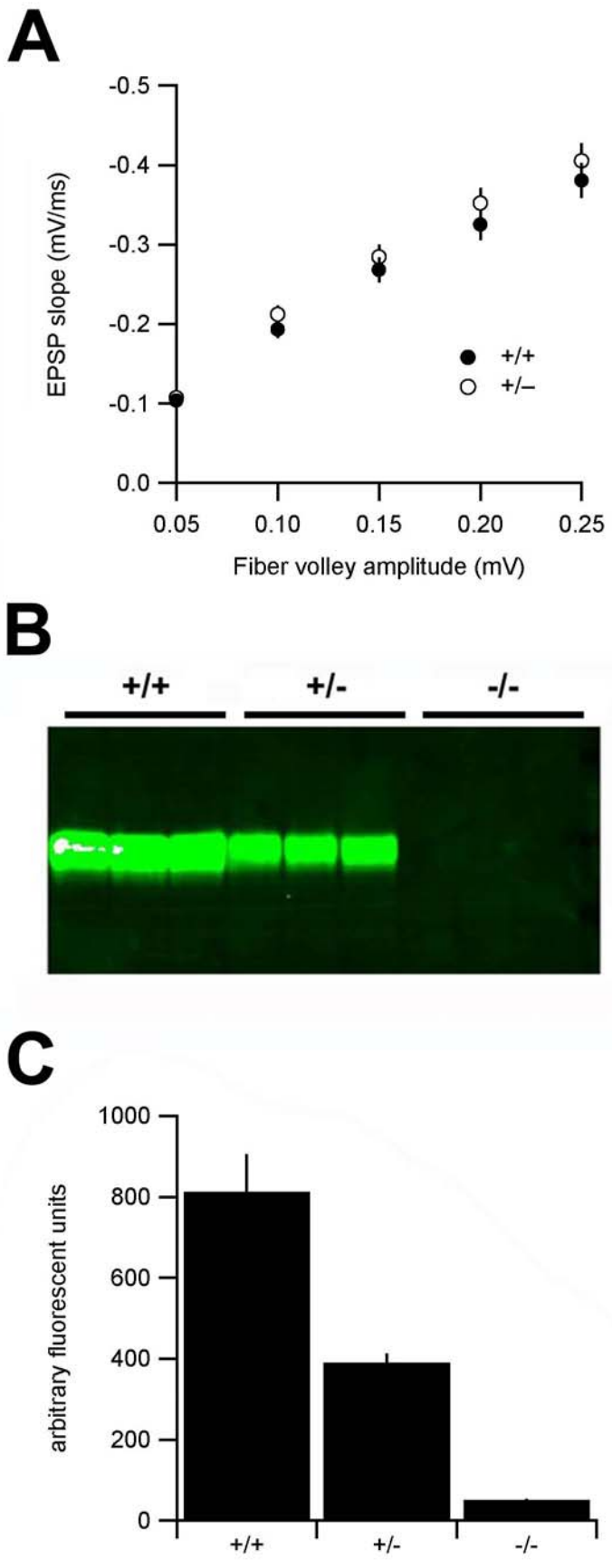

Supplementary figure 2
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