Supporting Information

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SI Materials and Methods

Antibodies. Antibodies included the following: rabbit polyclonal antibodies to LGI1 (Abcam), ADAM22 (Abcam), ADAM23 (Abcam), HA (Santa Cruz Biotechnology), SAP102 (1), and $Kv_{1.1}\alpha$ (Sigma-Aldrich); mouse monoclonal antibodies to PSD-95 (MA1-046, Thermo scientific; K28/43, NeuroMab), FLAG (M2, Sigma-Aldrich), HA (HA.11, Covans), $Kv_{1.1}\alpha$ (K20/78, NeuroMab), $Kv_{1.2}\alpha$ (K14/16, NeuroMab), Na^+K^+ATPase α3 (XVIF9-G10, Novus Biologicals), N-cadherin (BD Biosciences). Rabbit polyclonal antibodies to ADAM22 and LGI3 were raised against GST-ADAM22 (aa 858–898) and His × 6-LGI3 (aa 31–110), respectively. Chicken polyclonal antibody to LGI1 was raised against a peptide, SKDNALCENARSIPRTVPPD (aa 49–68). Antibodies were affinity purified on CNBr-activated Sepharose 4B (GE Healthcare) column containing the immunizing antigen.

Cloning and Plasmid Constructions. The following cDNAs were cloned from rat brain total RNA by RT-PCR using primers based on the GenBank databases: rat LGI3 (NM_001107277), rat ADAM11 (NM_009613), rat $Kv_{1.1}\alpha$ (NM_173095), and rat $Kv\beta_2$ (NM_017304). All PCR products were analyzed by DNA sequencing. ADAM11, and $Kv_{1.1}\alpha$ were subcloned into a cytomegalovirus promoter-driven vector. $Kv\beta_2$ was subcloned into pEF-Bos-myc. To obtain LGI1-FH and LGI3-FH, LGI1, and LGI3 were subcloned into the EcoRI and BamHI sites of pcDNA3.1-FLAG-His × 6 (FH), which was generated by inserting a synthetic DNA fragment obtained by annealing sense synthetic nucleotide 5′-GATCCGAATTCGACTACAAGGATGACGACGACAAGGGAGGTCATCATCACCATCACCATTAGC-3′, and antisense nucleotide 5′-TCGAGCTAATGGTGATGGTGATGATGACCTCCCTTGTCGTCGTCATCCTTGTAGTCGAATTCG-3′ into BamHI and XhoI sites of pcDNA3.1 (+). Other constructs of LGI1 and ADAMs were described previously (2).

Generation of Knockout Mice. All animal studies were reviewed and approved by the ethic committees at our institutions and were performed according to the institutional guidelines concerning the care and handling of experimental animals. For construction of the targeting vector, a 11.2 kb region containing exon 1–2 was subcloned from C57BL/6 BAC clone (RPCI-23–127G7) into pSP72 backbone vector (Promega) using a homologous recombination-based technique. The Neo cassette replaces 1.9 kb of the region including exon 1–2, resulting in the long homology arm (7.5 kb long) on the 5′ side of exon 1 and the short homology arm (1.8 kb long) on the 3′ side of exon 2. The targeting vector was linearized and electroporated into iTL BA1 hybrid (C57BL/6 × 129/SvEv) embryonic stem cells. ES cell clones with the targeted *LGI1* locus were injected into C57BL/6 blastocysts by iTL Inc. The chimeras were crossed with C57BL/6 for germ line transmission. The genotypes were determined by Southern blotting or PCR using PCR primers: 5′-CCTCTTGCATGCCTGACCATTTGA-3′ and 5′-AGAAGGCTTATCCGAATACATGCC-3′ for the WT allele; 5′-AGCGCATCGCCTTCTATCGCCTTC-3′ and 5′-AGAAGGCTTATCCGAATACATGCC-3′ for the targeted alleles. Two lines (nos. 272 and 274) from two different ES clones were established, and both showed the same epileptic phenotype.

Generation of Transgenic Mice. For generation of transgenic mice, cDNAs of LGI1-FH and LGI3-FH were subcloned downstream of the Thy1 promoter (3, 4). Transgenic mice were generated by PhoenixBio. Transgenic founders were crossed with C57BL/6 mice to produce the LGI-FH transgenic animals. Genotyping was performed using PCR primers: 5′-GCTTGACCAGATTCATTGGCGACT-3′ and 5′- CTAATGGTGATGGTGATGATGACC-3′ for LGI1-FH and 5′- GTGCCTTAGCCGCTATATTGGTG -3′ and 5′- CTAATGGTGATGGTGATGATGACC-3′ for LGI3-FH. For the rescue experiment, the $LGI1^{+/-}$ mouse was crossbred with a LGI1-FH ($^{Tg1/+}$) or LGI3-FH ($^{Tg3/+}$) transgenic mouse. Obtained $LGI1^{+/-;Tg1/+}$ or $LGI1^{+/-;Tg3/+}$ was crossed with $LGI1^{+/-}$ to obtain $LGI1^{-/-;Tg1/+}$ or $LGI1^{-/-;Tg3/+}$.

Immunofluorescence Analysis. For cell surface-binding assay, COS7 cells were seeded onto three 12-mm coverslips in each well of a six-well cell culture plate (3×10^5 cells/well) and cotransfected with LGIs-FLAG and ADAMs-HA. At 24 h after transfection, cells were fixed with 2% paraformaldehyde at room temperature for 20 min and blocked with PBS containing 10 mg/mL BSA for 10 min on ice. The fixed cells were stained with anti-Flag antibody, followed by Cy3-conjugated secondary antibody. The cells were permeabilized with 0.1% Triton X-100 for 10 min, blocked with PBS containing 10 mg/mL BSA, and stained with anti-HA polyclonal antibody, followed by Alexa488-conjugated secondary antibody.

Cultured hippocampal neurons (5×10^4 cells) were seeded onto 12-mm coverslips. The neurons (DIV14) were transfected with Thy1:LGI1-FH or Thy1:LGI3-FH with ADAM22-GFP by Lipofectamine 2000 (Invitrogen). At DIV21, surface-bound LGI1 was "live"-labeled with anti-FLAG antibody by incubating neurons in conditioned medium for 15 min at 37°C. Neurons were then fixed with 4% paraformaldehyde/100 mM Hepes (pH 7.4) at room temperature for 10 min and blocked with PBS containing 10 mg/mL BSA for 10 min on ice. Surface-bound LGI1-FH was visualized by Cy3-conjugated secondary antibody. The neurons were then permeabilized with 0.1% Triton X-100 for 10 min, blocked with PBS containing 10 mg/mL BSA, and stained with anti-GFP antibody for ADAM22-GFP staining and anti-FLAG antibody for total LGIs-FH staining, followed by Alexa488- and Alexa647-conjugated secondary antibodies, respectively. Fluorescent images were taken with a confocal laser scanning microscopy system (LSM5 Exciter, Carl Zeiss) equipped with a Plan Apochromat 63×/1.40 NA oil immersion objective lens. Microscope control and all image analyses were performed with Carl Zeiss ZEN software.

Tandem Affinity Purification of LGI1-FH. For purification of LGI1-FH or LGI3-FH from the transgenic mouse, brains from one wild-type or transgenic mouse were homogenized with buffer A (10 mM Tris-HCl [pH 8.0], 5 mM EDTA, 320 mM sucrose, and 100 μg/mL PMSF). P2 membrane fractions were solubilized with buffer B (20 mM Tris-HCl [pH 7.4], 150 mM NaCl, 1% Triton X-100, and 50 μg/mL PMSF) for 1 h. After centrifugation at 100,000 *g* for 1 h, the supernatant was incubated with 75 μL of FLAG-M2 agarose (Sigma-Aldrich) for 2 h, washed with buffer B six times, and eluted with 375 μL of buffer B containing 0.25 mg/mL FLAG peptide and 20 mM imidazole. The eluate was mixed with 50 μL of Ni-NTA agarose for 1 h and washed with buffer B containing 20 mM imidazole four times. The final eluate was obtained with 250 μL of 250 mM imidazole in buffer B. Purified proteins were separated by SDS/PAGE and subjected to silver staining or Western blotting. For reprecipitation of ADAM22 or $Kv_{1.1}\alpha$, the eluate from FLAG-M2 agarose was incubated with anti-ADAM22 or anti-$Kv_{1.1}\alpha$ antibody, and then with protein A Sepharose (GE Healthcare). For quantitative Western blotting, blotted membranes were scanned with Light-

Capture II (ATTO), and the optimal specific band was analyzed with the CS Analyzer 3.0 software (ATTO).

For immunoprecipitation of endogenous ADAM22 or ADAM23, one mouse brain was homogenized in buffer C (20 mM Tris-HCl [pH 8.0], 2 mM EDTA, 320 mM sucrose, and 200 μg/mL PMSF). Homogenates were spun at 20,000 × *g* for 1 h and pellets were resuspended in buffer D (20 mM Tris-HCl [pH 8.0], 1 mM EDTA, 100 mM NaCl, and 1.3% Triton X-100). The lysates were spun at 100,000 × *g* for 1 h. Precleared lysates (5 mg protein) were immunoprecipitated with 5 μg antibodies. Immunoprecipitates were separated by SDS/PAGE and gels were subjected to Western blotting.

Mass Spectrometry. For the in-gel digestion, the specific protein bands were excised from a silver-stained gel, reduced with DTT, and alkylated with iodoacetamide. Band slices were digested with trypsin (12 μg/mL) at 37°C overnight and desalted with ZipTip C18 (Millipore). For shotgun analysis, the eluted sample was trypsin-digested in solution (5). Briefly, the eluates from tandem affinity purification were concentrated by trichloroacetic acid precipitation. The samples were reduced with DTT and alkylated with iodoacetamide. The S-carbamoylmethylated proteins were concentrated and digested with trypsin at 37°C overnight. The obtained peptides were then separated via nano-flow liquid chromatography (nanoLC) (Paradigm MS4, AMR) using a reverse-phase C18 column. The LC eluent was coupled to a nano ion spray source attached to a LCQ Advantage or Fleet mass spectrometer (Thermo Fisher Scientific). For protein identification and semiquantification, we used the score of the SEQUEST algorithm from BioWorks software (Thermo Fisher Scientific). The specificity was determined by subtracting the results obtained from wild-type mice. Three independent experiments were performed for statistical analysis.

Subcellular Fractionation. Brains from littermate mice (P16) were homogenized in buffer C. The homogenate was centrifuged for 1 h at 20,000 × *g* to produce a pellet (P2) as a crude synaptosomal fraction, and the pellet was homogenized in buffer D. The resuspended P2 fraction was centrifuged at 100,000 × *g* for 1 h to produce a supernatant (P2 sol) and a pellet. The pellet (P2 insol) was resuspended into equal volume with "P2 sol." Each fraction was subjected to quantitative Western blotting as described above. Three independent experiments were performed. For Fig. S3*A*, we followed the procedure as described previously (6).

Staining with AP Fusions. LGI1-AP, a secreted alkaline phosphatase (AP) fusion protein of LGI1, was prepared as described previously (2). COS7 cells transfected with ADAMs were washed with HBSS containing 0.5 mg/mL BSA and 20 mM Hepes, pH7.4 (HBH), and incubated for 90 min at 25°C with LGI1-AP. The cells were then washed with HBH, fixed with 4% paraformaldehyde for 15 min, and incubated for 100 min at 65°C to inactivate endogenous phosphatase activity. The cells were stained by 5-bromo-4-chloro-3-indolyl phosphate (BCIP) and nitroblue tetrazolium (NBT) solution (DAKO Cytomation) for several hours to visualize AP activity. The reaction was stopped by the addition of PBS.

Immunohistochemistry. Littermate mice (P17–19) were anesthetized by pentobarbital (60 mg/kg, i.p.) and perfused with 4% paraformaldehyde in 0.1 M phosphate buffer (PB, pH 7.4). The brains were removed and immersed in the same fixative for 12 h at 4°C and then cryoprotected in 20% sucrose in PBS overnight at 4°C. The 60-μm free-floating sections were cut on a cryostat (Leica CM1950). Sections were pretreated with 0.2 mg/mL pepsin (DAKO) for 10 min at 37°C (7). Sections were blocked for 1 h in PBS containing 3% normal goat or 5% normal horse serum and 0.3% Triton X-100 at room temperature and then incubated in the same buffer containing indicated antibodies (1 μg/mL) overnight at 4°C. Endogenous peroxidase activity was inactivated by incubating brain sections in 1% H_2O_2 for 30 min. Immunohistochemical staining was performed with an avidin/biotin/peroxidase system (ABC Elite; Vector Laboratories) and DAB (Vector Laboratories). Five independent experiments were performed.

For immunofluorescence analysis, the brain sections (P17) were blocked for 1 h in PBS containing 10% donkey serum and incubated in the mixtures of primary antibodies for 48 h at 4°C, followed by Alexa488- and Cy3-conjugated secondary antibodies. Fluorescent images were taken with a confocal laser scanning microscopy system (LSM5 Exciter, Carl Zeiss) equipped with a Plan Apochromat 63×/1.40 NA oil immersion objective lens. Three independent experiments were performed.

Postembedding Immunoelectron Microscopy. Mice were anesthetized and perfused with 25 mM PBS, pH 7.4, transcardially followed by fixative containing 4% paraformaldehyde, 0.5% glutaraldehyde, and 4% glucose in 0.1 M PB, pH 7.4, for 15 min. After perfusion, the brains were removed, and 200-μm thick coronal slices were cut on a vibratome. Small tissue blocks of the CA3/DG hippocampal area (0.5 × 1.0 mm) were trimmed from the 200-μm thick brain slices and cryoprotected in 10%, 20%, and 30% glycerol in 0.1 mM PB, pH 7.4 with 4% glucose, overnight. They were then frozen by being plunged into liquid propane (–185°C) in a cryofixation unit (EM CPC; Leica). The samples were immersed in 1.5% uranyl acetate dissolved in anhydrous methanol (–90°C, 32 h) in a cryosubstitution unit (EM AFS; Leica). The temperature was then raised (4°C/h) from –90°C to –45°C. The samples were washed three times with anhydrous methanol and infiltrated with Lowicryl HM20 resin (Polysciences) at –45°C with a progressive increase in the ratio of resin to methanol. Polymerization was performed with UV light (360 nm) at –45°C for 24 h and 0°C for 36 h. For LGI1 immunogold reaction, Lowicryl-embedded ultrathin sections (85-nm thickness) were picked up onto the nickel 400 mesh grids coated with coat-quick G medium (Daido Sangyo). The sections were briefly washed and incubated in blocking solution (2% human albumin sera in TBS with 0.1% Triton X-100) for 30 min. The sections were then incubated with the anti-rabbit LGI1 primary antibody (10 μg/mL) overnight at room temperature. After several washes with TBS for 30 min, the sections were incubated in 5 nm gold anti-rabbit IgG secondary antibody (British Biocell International) diluted (1:100) in blocking solution containing polyethylene glycol (molecular weight, 7,500 Da, 5 mg/mL) for 3 h. Then the sections were washed in ultrapure water, contrasted with uranyl acetate, and examined with a Philips EM208S electron microscope. Two independent experiments were performed.

Electrophysiology. Using a Leica vibratome, transverse hippocampal slices (300 μm) were obtained from P14-P15 mice in a high-sucrose cutting solution containing the following (in mM): NaCl, 50; KCl, 2.5; $CaCl_2$, 0.5; $MgCl_2$, 7.0; NaH_2PO_4, 1.0; $NaHCO_3$, 25; glucose, 10; and sucrose, 150. Slices were transferred to artificial cerebrospinal fluid (ACSF) containing the following (in mM): NaCl, 119; KCl, 2.5; $NaHCO_3$, 26.2; Na_2PO_4, 1; glucose, 11; $CaCl_2$, 2.5; and $MgCl_2$, 1.3, in an incubating chamber, and recovered at 35°C for 45 min. After at least 1 h at room temperature, slices were placed in a submersion chamber on an upright BX50WI Olympus microscope and perfused in ACSF with 100 μM picrotoxin, saturated with 95% O_2/5% CO_2. A cut was made between CA3 and CA1 to prevent epileptiform activity. Whole-cell recordings of synaptic currents were made with 3–5 MΩ borosilicate glass pipettes, following stimulation of Schaffer collaterals using a monopolar glass electrode filled with ACSF. The internal solution contained the following (in mM): $CsMeSO_4$, 135; NaCl, 8; Hepes, 10; Na-GTP, 0.3; Mg-ATP, 4; QX-314, 5; spermine, 0.1; and BAPTA, 10. Series resistance ranged between 10 and 20 MΩ, and cells in which the series resistance varied by

20% or more during recording session were discarded. For AMPA/NMDA ratios, the AMPA component was measured at –70 mV, and the NMDA component was determined at +40 mV by measuring the current 100 ms after the stimulation artifact, at which point the AMPAR-mediated EPSC has completely decayed. Paired-pulse ratios were determined at –70 mV by giving two pulses at a 40-ms interval and calculating the ratio of the two EPSC peaks (from an average of 50 sweeps). mEPSCs were acquired at –70 mV in the presence of 0.5 μM TTX and detected by offline analysis using in-house software in Igor Pro (Wavemetrics). Statistical data were analyzed using two-tailed, unpaired *t* tests. All error bars represent standard error measurements.

1. El-Husseini Ael-D, et al. (2002) Synaptic strength regulated by palmitate cycling on PSD-95. *Cell* 108:849–863.
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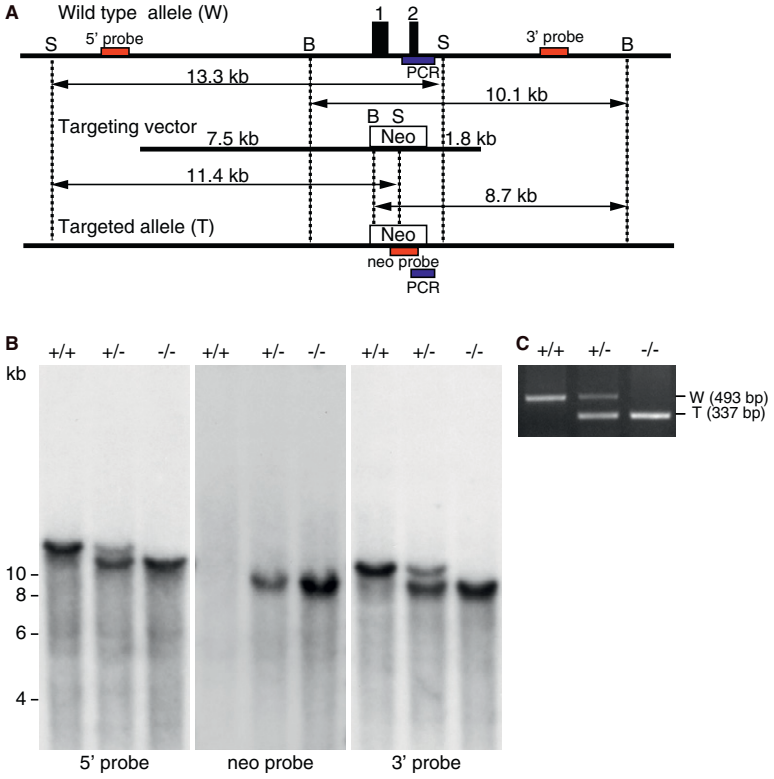


Fig. S1. Targeting construct and characterization of LGI1 knockout mice. (*A*) Restriction maps of wild-type murine LGI1 allele (*Upper*), pSP72 targeting plasmid (*Middle*), and targeted allele (*Lower*). Black filled bars indicate LGI1 exons; red filled bars denote Southern probes; and blue filled bars specify PCR genotyping fragments. B, BamHI; S, SpeI; Neo, neomycin. (*B*) Southern blots from wild-type ($^{+/+}$), heterozygote ($^{+/-}$), and null ($^{-/-}$) mice. Using a 5′ probe and SpeI digestion, the wild-type and the targeted loci generated 13.3- and 11.4-kb bands, respectively. The neomycin cassette probe recognized a 8.7-kb band in the targeted locus after BamHI digestion. Using a 3′ probe and BamHI digestion, the wild-type and the targeted loci generated 10.1- and 8.7-kb bands, respectively. (*C*) PCR genotyping. Wild-type (W) and targeted loci (T) gave 493- and 337-bp PCR products, respectively.

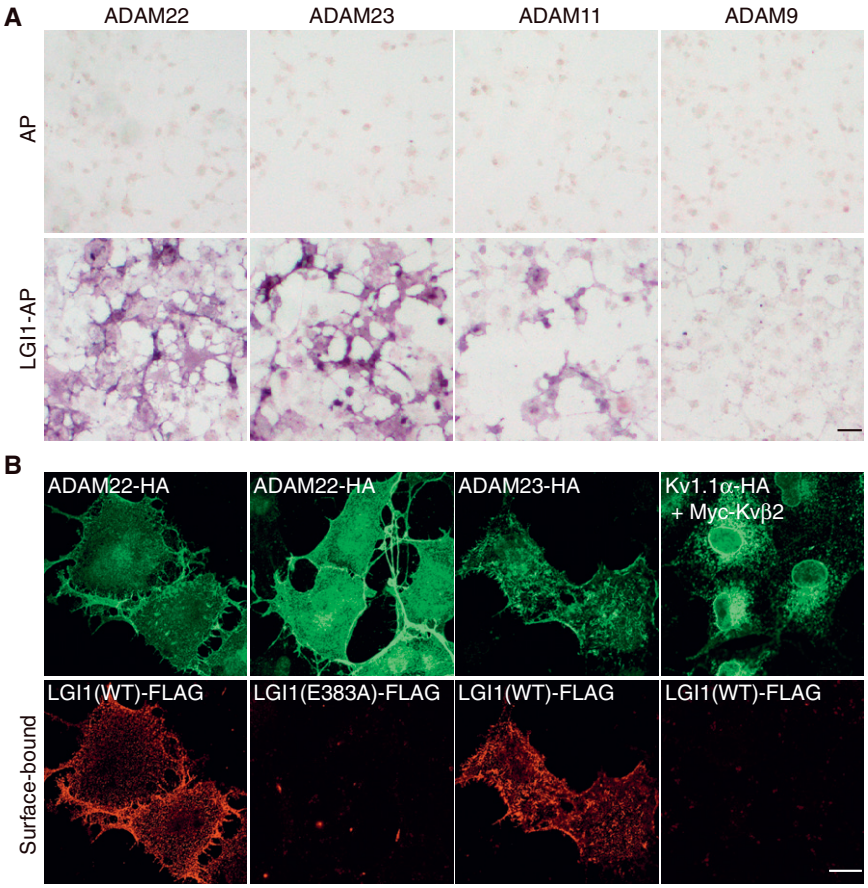


Fig. S2. ADAM22 subfamily members, but not Kv_1 channel, are LGI1 receptors. (*A*) Binding of LGI1-AP, a secreted alkaline phosphatase (AP) fusion protein of LGI1, to ADAM-expressing COS7 cells. LGI1-AP bound to the cell surface was detected by the AP reaction. Note that LGI1-AP is bound to the ADAM22 subfamily (ADAM22, ADAM23, and ADAM11) but not to a distant ADAM member, ADAM9. (Scale bar, 20 μm.) (*B*) Indicated cDNAs were cotransfected into COS7 cells. At 24 h after transfection, surface-bound FLAG-tagged proteins (red) were labeled before cell permeabilization, and then HA-tagged proteins were stained (green). Secreted LGI1 specifically bound to ADAM22 and ADAM23, but not to $Kv_{1.1}\alpha$ in the presence of $Kv\beta_2$. LGI1 E383A, an ADPEAF mutant, did not bind to ADAM22. (Scale bar, 10 μm.)

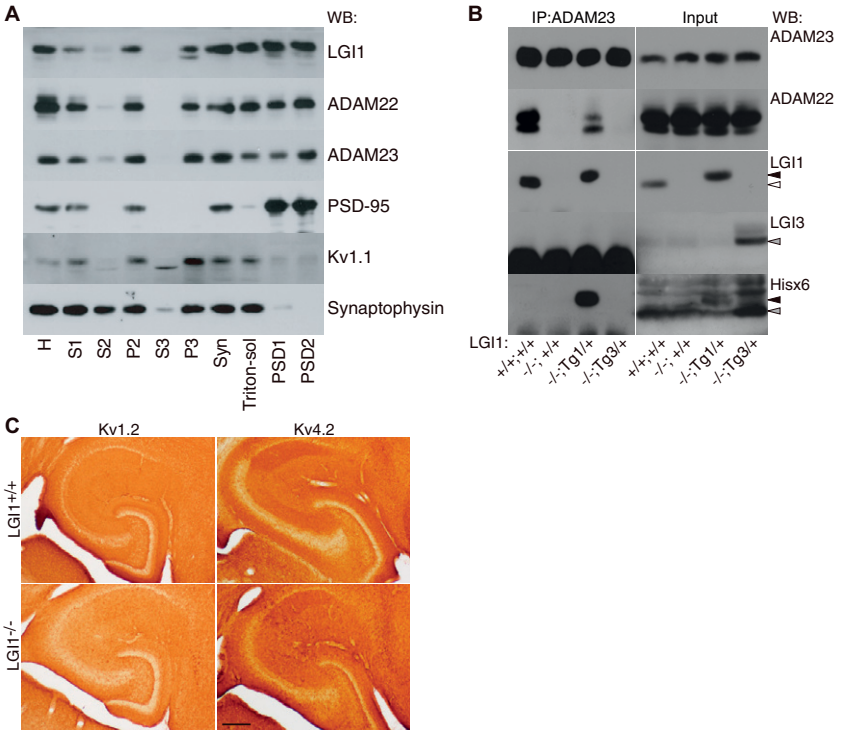


Fig. S3. LGI1, ADAM22, and ADAM23 distribute both pre- and postsynaptically. (*A*) LGI1, ADAM22, and ADAM23 displayed similar subcellular distribution. LGI1, ADAM22, and ADAM23 were enriched both in Triton X-100–soluble presynaptic and insoluble postsynaptic density (PSD) fractions. Presynaptic $Kv_{1.1}$ and synaptophysin were enriched in Triton X-100–soluble synaptic fraction, whereas postsynaptic PSD-95 was enriched in PSD fractions. These results suggest that the LGI1/ADAM22/ADAM23 complex is centered between presynapses and postsynapses. H, homogenate; S, supernatant; P, precipitate; Syn, synaptosome; Triton-sol, Triton X-100–soluble presynaptic; PSD, Triton X-100–insoluble postsynaptic density fractions. (*B*) Expression of LGI1-FH in $LGI1^{-/-}$ mouse restored the interaction between ADAM22 and ADAM23. When ADAM23 was immunoprecipitated from $LGI1^{+/+;+/+}$, $LGI1^{-/-;+/+}$, $LGI1^{-/-;Tg1/+}$, or $LGI1^{-/-;Tg3/+}$ mouse brain extracts, the tripartite complex composed of ADAM22, LGI1(-FH), and ADAM23 was specifically detected in $LGI1^{-/-;Tg1/+}$ mouse expressing LGI1-FH, but not in $LGI1^{-/-;Tg3/+}$ mouse expressing LGI3-FH. Note that LGI3-FH was not coimmunoprecipitated with ADAM23. Closed arrowheads, LGI1-FH; open arrowhead, endogenous LGI1; gray arrowheads, LGI3-FH. LGI3 antibody detects LGI3-FH and also weakly endogenous LGI3. (*C*) Reduction of $Kv_{1.2}$, but not $Kv_{4.2}$, was observed in the hippocampus of the $LGI1^{-/-}$ mouse. (Scale bar, 0.2 mm.)

Table S1. Growth failure of LGI1$^{-/-}$ mice at postnatal third week

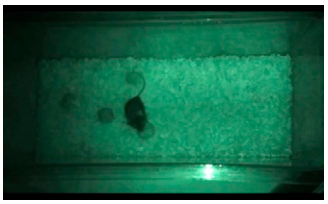
Postnatal day	LGI1 genotype body weight (g)		
	$^{+/+}$	$^{+/-}$	$^{-/-}$
P10	5.76 ± 0.89	5.51 ± 1.17 (P = 0.429)	5.01 ± 1.04 (P = 0.031)
P12	6.07 ± 0.94	6.11 ± 1.41 (P = 0.437)	5.51 ± 0.99 (P = 0.014)
P14	6.69 ± 0.53	6.83 ± 0.89 (P = 0.545)	5.82 ± 0.96 (P = 0.015)
P17	7.77 ± 1.29	7.70 ± 1.01 (P = 0.866)	5.29 ± 0.99 (P < 0.001)

$^{+/+}$ Wild type;
$^{+/-}$ heterozygote; $^{-/-}$ homozygote,

Table S2. Mass spectrometry (gel-based) analysis

Isolated protein*	Protein[†]	Probability[‡ (protein)]	Score[§]	Hits[‖]
P100	ADAM22	1.82E-08	50.21	6
	PSD-95	2.62E-05	20.19	2
P90	ADAM22	2.10E-12	110.25	13
P80	ADAM22	1.50E-06	78.20	8
	ADAM23	8.11E-08	50.20	5
P65	LGI1	3.56E-09	144.23	23
P55	Tubulin β2	6.78E-06	44.16	5
	Tubulin β6	4.68E-05	48.21	6
	Tubulin α1	1.85E-04	20.15	2
P30	14-3-3zeta	4.45E-07	20.13	2

*p100, p90, p80, p65 p55, and p30 were major bands copurified during the TAP (FLAG-His purification). p100 is a reproducible smear band just above p90. They were found in both FLAG and His eluates.
[†]Proteins with probability value (<1.0E-04) and score (>20) are listed.
[‡]Probability (protein) of finding a match as good as or better than the observed match by chance.
[§]SEQUEST scores.
[‖]Number of unique parent peptides found.



Movie S1. Epileptic phenotype of LGI1 knockout mice. A P19 LGI1$^{-/-}$ mouse that looks fine and behaves normally at first during the observation shows a spontaneous generalized seizure with a sudden onset of wild running and jumping, followed by full tonic limb extension.

Movie S1