Supporting Information

Takayasu et al. 10.1073/pnas.1015553107

SI Materials and Methods

Drugs. (*RS*)-3,5-dihydroxyphenylglycine (DHPG), D-2-amino-5-phosphonopentanoic acid (D-AP5), (S)-(+)-α-amino-4-carboxy-2-methylbenzeneacetic acid (LY367385), 2-methyl-6-(phenylethynyl) pyridine (MPEP), anisomycin, rapamycin, 1,4-diamino-2,3-dicyano-1,4-bis[2-aminophenylthio]butadiene (U0126), and picrotoxin were purchased from Tocris Cookson. Stock solutions were made in DMSO or H_2O, stored at –20 °C, and diluted to final concentrations in external solution immediately before use. Lidocaine N-ethyl bromide (QX-314, a quaternary form of lidocaine; Sigma Aldrich) was used in the internal solution for whole-cell patch-clamp recording.

Mice and Slice Preparation. All procedures involving animals were carried out in accordance with the guidelines of the National Institute of Health for the care and use of laboratory animals and were approved by the Animal Institute of the Albert Einstein College of Medicine. Acute hippocampal slices were prepared from 20- to 34-d-old *Cav1*$^{-/-}$ mice (made in B6129SF2/J and backcrossed for five generations with WT C57BL/6J (Jackson Laboratory). Mice were deeply anesthetized and killed by decapitation; hippocampi were rapidly removed and incubated in cold sucrose cutting solution (234 mM sucrose, 2.5 mM KCl, 1.25 mM NaH_2PO_4, 10.0 mM $MgSO_4$, 0.5 mM $CaCl_2$, 26 mM $NaHCO_3$, and 11 mM glucose) aerated with 95% O_2/5% CO_2. Coronal slices (400 μm thick for field recordings and 200 μm thick for patch-clamp) were cut with a vibrating microslicer (DTK-1000; Dosaka-EM). After cutting slices were incubated for 2–8 h at room temperature in 75% recording solution (125 mM NaCl, 2.5 mM KCl, 2 mM $CaCl_2$, 1 mM $MgCl_2$, 26 mM NaH_2PO_4, and 10 mM glucose) and 25% cutting solution. For recording, slices were transferred to a submersion recording chamber and perfused (2 mL/min) with recording solution saturated with 95% O_2/5% CO_2 at 32 °C for at least 50 min before stimulation. In some experiments, the CA3 region was rapidly removed from the slices immediately after cutting; no significant difference was observed in recordings obtained from intact slices.

Electrophysiology. For extracellular field recordings, slices were maintained at 32 °C and perfused with external solution at 2 mL/min. Recording pipettes (1–2 MΩ) filled with external solution were placed in the *stratum radiatum* of area CA1. Field EPSPs (fEPSPs) were evoked by square pulses (10–100 μA, 100 μs) to Schaffer collateral/commissural afferents with a concentric bipolar tungsten stimulating electrode. Baseline presynaptic stimulation was delivered once every 30 s at a stimulation intensity yielding 40–60% of the maximal response. The maximal slope on the fEPSP rising phase was used to measure the magnitude and stability of synaptic responses. Baseline slope was calculated from the mean values recorded for 20 min before drug application or synaptic conditioning. LTD was induced either by single or multiple applications of 100 μM DHPG for 5 min, or a single application for 20 min or by low frequency synaptic stimulation (LFS) at 1 Hz for 20 min. Data were acquired with a Multiclamp 700B amplifier (Molecular Devices) and analyzed by Igor Pro software (WaveMetrics), respectively.

For whole-cell voltage-clamp recordings from CA1 pyramidal neurons, slices were maintained at 32 °C and perfused with external solution (2.5–3 mL/min) containing picrotoxin (100 μM, to block inhibitory synaptic transmission), and neurons were visualized by infrared–differential interference contrast (IR–DIC) microscopy. Recording pipettes (3–5 MΩ) were filled with intracellular solution (145 mM Cs gluconate, 8 mM NaCl, 2 mM Mg-ATP, 10 mM Hepes, 1 mM EGTA, 2 mM ATP-Mg, 0.3 mM GTP-Na, 0.1 mM spermine, 5 mM lidocaine N-ethyl bromide) at pH 7.2 (290–310 mOsm). To measure the resting membrane potential, Cs gluconate in the internal solution was replaced by K gluconate. EPSCs were evoked as for fEPSPs with square pulses (10–100 μA, 100 μs) delivered once every 10 s with a concentric bipolar tungsten electrode placed in the stratum radiatum along the Schaffer collaterals 50–200 μm from the recorded neurons. Input resistance (R_{in}) and effective time constant of charging of dendritic membrane were measured under voltage-clamp with −10-mV steps 80 ms in duration from a holding potential of −60 mV (Table 1). Throughout the intracellular recordings of hyperpolarization in response to DHPG application (1, 2), series resistance (R_s) was monitored every 10 s by measuring the peak current in response to a 2-mV, 20-ms hyperpolarizing step. R_s ranged from 10 to 20 MΩ; experiments showing >20% drift in R_s were excluded from further analysis. The rectification index (RI) was defined as the chord conductance of EPSCs recorded at +40 mV divided by that at −60 mV, both in the presence of D-APV (50 μM) ($RI = (I_{+40}/40)/(I_{-60}/(-60))$). The EPSP reversal potential was near 0 mV. The ratio of NMDA to AMPA responses was calculated as the peak amplitude of the D-APV–sensitive component to the D-APV–insensitive component of the EPSCs recorded at +40 mV. Whole-cell, patch-clamp recordings were performed with a DAGAN 3900A amplifier (DAGAN) and pCLAMP software (Molecular Devices). EPSCs were filtered at 2 kHz and digitized at 10 kHz. Data were analyzed with Origin software (OriginLab).

Biochemical Analysis of Surface Receptors. Hippocampal slices (400-μm-thick) were incubated at room temperature in external recording solution aerated with 95% O_2/5% CO_2 for 1–2 h before labeling surface proteins with biotin. Pools of 2–3 slices per condition were placed on ice, rinsed with ice-cold ACSF-Hepes (124 mM NaCl, 5 mM KCl, 1.25 mM NaH_2PO_4, 26 mM $NaHCO_3$, 2 mM $CaCl_2$, 2 mM $MgCl_2$, 10 mM dextrose, and 20 mM Hepes at pH 7.4), and incubated with 1 mg/mL sulfo-NHS-LC-biotin (Pierce) in ACSF-Hepes for 30 min. Slices were washed three times with Tris-buffered saline, homogenized in lysis buffer (50 mM Tris·HCl, 150 mM NaCl, 2 mM EDTA, 50 mM NaH_2PO_4, 1% Triton X-100, 0.1% SDS, and 0.5% Na-deoxycholate at pH 7.4) with complete protease inhibitors (Sigma Aldrich), and 1 mM Na_3VO_4 and briefly sonicated. After removing insoluble material by centrifugation, the protein content in the soluble extract was measured with the Bio-Rad protein assay reagent (Bio-Rad Laboratories). Equal amounts of protein per condition were incubated with 80 μL of Neutravidin beads (Pierce) for 2 h at 4 °C. Beads were washed three times with lysis buffer and one time with phosphate buffer, and bound proteins were eluted in sample buffer (with 100 mM DTT) at 70 °C for 20 min. Total and biotinylated proteins were fractionated by SDS/PAGE, transferred to membranes, probed with anti-mGluR5 (LabVision) or anti-actin antibodies (LabVision), and visualized by enhanced chemiluminescence. Band densities were measured with the NIH Image/Image J software.

Synaptoneurosomes (SNs). SNs were prepared according to ref. 3. Briefly, mouse brain cortices were rinsed in ice-cold gradient buffer (5 mM Tris·HCl, 0.25 mM DTT, 1 mM EDTA, and 0.32 M sucrose at pH 7.4) and homogenized. Homogenates were spun at 1,000 × *g* for 10 min, and the supernatants overlaid onto gradients were made with 2 mL each of 3, 10, 15, and 23% Percoll.

Gradients were centrifuged at 31,000 × *g* for 5 min; the SN fraction at the interface between 15 and 23% Percoll was collected, diluted in 1 mM EDTA with 0.32 M sucrose, spun at 20,000 × *g* for 30 min and dissolved in buffer containing 125 mM NaCl, 2.5 mM KCl, and 2 mM $CaCl_2$.

MEK, ERK1/2 Phosphorylation. Acute hippocampal slices (400-μm-thick) were incubated at room temperature in normal external recording solution bubbled with 95% O_2/5% CO_2 for 120 min before drug treatment. To assay MEK and ERK1/2 phosphorylation, slices were incubated with 100 μM DHPG for 5 min and rapidly rinsed; treated and untreated (control) slices were snap-frozen in dry ice and stored at −80 °C. After thawing on ice, the tissue was homogenized in lysis buffer (20 mM Tris·Cl, 37 mM NaCl, 2 mM EDTA, 1% Triton X-100, and 10% glycerol at pH 7.4) with complete protease and phosphatase inhibitors (mixtures I and II; Sigma Aldrich). After removing insoluble material by centrifugation, the total protein content was measured, and equal amounts of protein (30 μg) for each condition were resolved by SDS/PAGE and transferred to membranes. Membranes were probed with the following antibodies: anti–p-MEK (Ser217/221), anti-MEK, anti–p-ERK1/2 (Thr202/Tyr204), and anti-ERK1/2 (Cell Signaling Technology). Detection and quantification of band densities were carried out as described above. ERK1 and ERK2 bands could be distinguished by their differing mobilities.

Statistics. Data are expressed as mean ± SE; Student's *t* tests and ANOVA were used as appropriate. Significance was taken as $P < 0.05$.

1. Congar P, Leinekugel X, Ben-Ari Y, Crépel V (1997) A long-lasting calcium-activated nonselective cationic current is generated by synaptic stimulation or exogenous activation of group I metabotropic glutamate receptors in CA1 pyramidal neurons. *J Neurosci* 17:5366–5379.
2. Partridge LD, Valenzuela CF (1999) Ca^{2+} store-dependent potentiation of Ca^{2+}-activated non-selective cation channels in rat hippocampal neurones in vitro. *J Physiol* 521:617–627.
3. Dunkley PR, et al. (1988) A rapid Percoll gradient procedure for isolation of synaptosomes directly from an S1 fraction: Homogeneity and morphology of subcellular fractions. *Brain Res* 441:59–71.

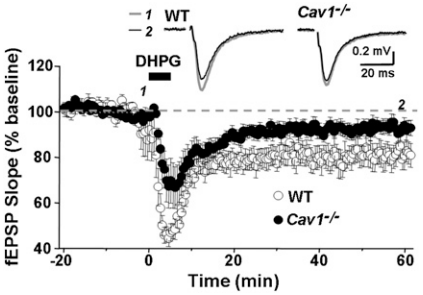

Fig. S1. DHPG-induced LTD at Sch-CA1 synapses is attenuated in *Cav1*$^{-/-}$ mice at postnatal ages P10–11. The fEPSPs in the CA1 region were evoked by stimulation of Schaffer collateral axons; LTD was induced by bath application of (*RS*)-3,5-DHPG (100 μM, 5 min). The fEPSP slope (mean ± SEM) is plotted as percent of pre-DHPG baseline: WT, 76 ± 4% $n = 7$; *Cav1*$^{-/-}$, 92 ± 3% $n = 8$; $P < 0.05$ WT vs. *Cav1*$^{-/-}$, $P < 0.05$ *Cav1*$^{-/-}$ *vs.* baseline. (*Inset*) The fEPSP traces (average of 3–5 traces) from a representative experiment taken at indicated times (*1*, pre-DHPG baseline, thick gray trace; *2*, 50–60 min after DHPG for *Cav1*$^{-/-}$, thin black trace).

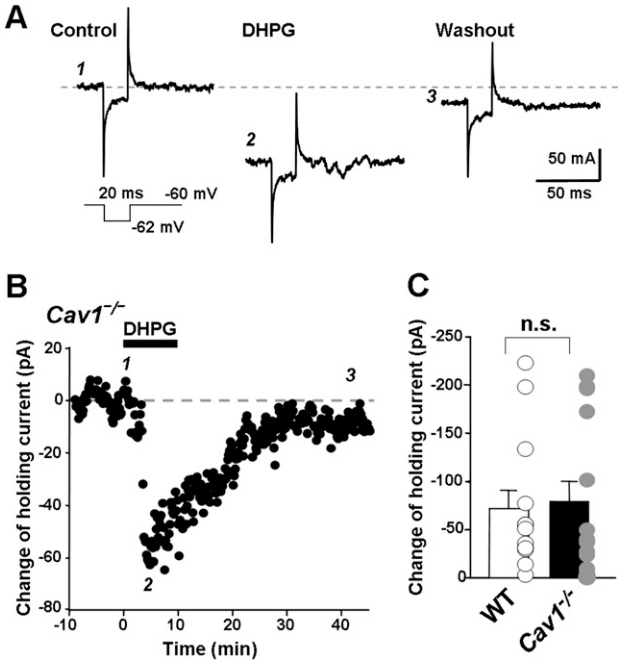

Fig. S2. The DHPG-induced inward current is normal in CA1 neurons of *Cav1*$^{-/-}$ mice. (*A*) DHPG (100 μM, 10 min) increased holding current in CA1 neurons at −60 mV. The current recovered after washout. A 2-mV test pulse showed that input conductance did not change detectably. (*B*) Representative experiment showing change in holding current in response to DHPG in *Cav1*$^{-/-}$ neurons. Values are plotted every 10 s with traces shown in *A* taken at indicated times: *1*, baseline (dotted line); *2*, DHPG response; *3*, washout. (*C*) Summary graph of maximum change in DHPG-induced inward current in WT and *Cav1*$^{-/-}$ mice ($n = 13$ for each, $P > 0.05$).

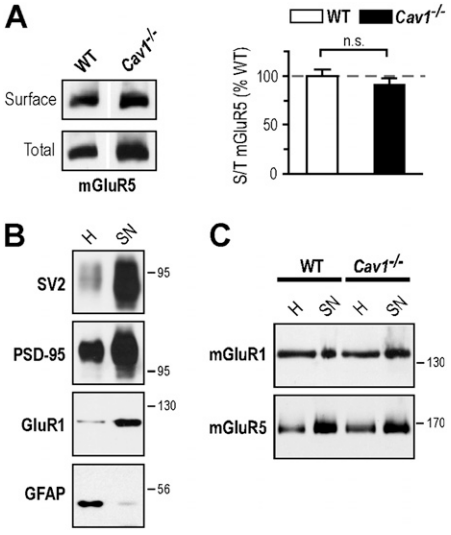

Fig. S3. Total and surface Group I mGluR expression is not significantly altered in *Cav1*$^{-/-}$ mice. (*A*) Expression of mGluR5 in hippocampal slices. Surface proteins isolated by biotinylation and total proteins were probed with anti-mGluR5 and quantified in immunoblots. Representative blot (*Left*) and quantification (*Right*): Surface expression was calculated as the ratio of biotinylated to total mGluR5 normalized to the WT ratio (*Cav1*$^{-/-}$ 91 ± 7% of WT; $n = 7$ for WT, 8 for *Cav1*$^{-/-}$, $P > 0.05$). (*B*) A single blot showing synaptic protein enrichment in WT neocortical synaptoneurosomes. Equal amounts of synaptoneurosomes (SN) and total homogenate (H) were probed with antibodies to synaptic vesicle associated protein 2 (SV2), PSD-95, the AMPAR subunit GluR1, and glial fibrillary acidic protein (GFAP). (SN, % total homogenate H): SV2, 400%; PSD-95, 200%; GluR1, 700%; GFAP, 6%. (*C*) Representative immunoblots from homogenates and synaptoneurosomes probed with anti-mGluR1 and anti-mGluR5 antibodies. Abundance in synaptoneurosomes (relative to total homogenate) of mGluR1 or mGluR5 was similar in WT and *Cav1*$^{-/-}$ mice. Total abundance in homogenates (relative to α-tubulin) of mGluR1a (WT, 0.6 ± 0.2; *Cav1*$^{-/-}$, 0.62 ± 0.3; $n = 3$, $P > 0.05$) or mGluR5 (WT, 2.1 ± 0.8; *Cav1*$^{-/-}$, 2.1 ± 0.4; $n = 3$, $P > 0.05$) was also similar in WT *vs.* *Cav1*$^{-/-}$ mice.

Table S1. Passive properties of CA1 pyramidal neurons

Mouse strain	Resting potential, mV (*n*)	Input resistance, MΩ (*n*)	Time constant, ms (*n*)
WT	−61 ± 1.3 (8)	284 ± 29 (17)	2.3 ± 0.13 (17)
Cav1$^{-/-}$	−59 ± 1.7 (9)	310 ± 30 (15)	2.6 ± 0.23 (15)