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Supporting Online Material for

CKAMP44: A Brain-Specific Protein Attenuating Short-Term Synaptic Plasticity in the Dentate Gyrus

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Supporting Online Material

Materials and methods

Identification of proteins in the immunoprecipitated AMPA receptor complex

Forebrains from GluA1 knockouts (S*1*) and wildtype littermates were dissected and separately homogenized in isotonic buffer, containing 0.32 M sucrose, 10 mM HEPES, pH 7.4 and the complete set of protease inhibitors. After centrifugation at 1,000 g for 10 min, the supernatant was spun at 20,000 g for 20 min. The pellet was resuspended in Triton X-100 extraction buffer (25 mM HEPES, pH 7.4, 150mM NaCl, 1% TritonX-100 plus protease inhibitors), adjusted to 5 mg protein per ml buffer, and incubated at 4°C for 45 min. The lysate was spun at 20,000 g for 10 min, and 10 µg rabbit anti-AMPA receptor 1 cross-linked to protein A agarose beads (Protein A-Agarose Fast Flow) with dimethyl pimelimidate (10mM), and were added to 2 ml of supernatant. After incubation for 4 hrs at 4°C, the beads were spun down at 500 g for 1 min and washed 4 times with Triton X-100 extraction buffer. Bound proteins were eluted from the beads by incubation with 0.1% TFA. After centrifugation the supernatant was dried in a SpeedVac and the solubilized protein complex was digested with trypsin. Peptides from wildtype sample were tagged with iTRAQ reagent 114, and from GluA1 knockout mice with iTRAQ reagent 117. The tagged samples were pooled, separated by nanoC18 liquid chromatography, deposited onto the MALDI metal target with Probot, and analyzed by the 4700 Proteomics Analyzer MALDI (S*2*). The mass spectra were searched with the NCBI database. To remove redundancy in the NCBI database, protein sequences that shared >90% similarity across 85% of the sequence length were grouped as a single protein cluster. The fold difference in protein amount between wild-type and GluA1 knockout is given by the average ratio of peak areas of iTRAQ signature ion 114 divided by those of iTRAQ signature ion 117 across all peptides matched to a protein.

The Mass spec data of the AMPAR IPs revealed peptide fragments of the hypothetical murine protein LOC72555 as putative AMPAR interacting protein. By RT-PCR from total brain we found that the Riken cDNA clone predicted the wrong N-terminus. According to our analysis, the protein translation is initiated at the Met codon in position 41 of LOC72555.

RNA analysis

A mouse tissue Northern blot (Mouse Multiple Tissue Northern Blot, Clontech, Palo Alto, CA) was prehybridized in Quickhyb hybridization buffer (Stratagene, Heidelberg, Germany) for 1 hr at 68°C. The CKAMP44 cDNA probe (960 bp, spanning exons 1- 5) was labeled with ^{32}P-CTP using a kit (Rediprime II, Amersham Biosciences, Freiburg, Germany), separated from free ^{32}P-CTP via a spin column (Bio-Spin, Bio-Rad, Munich, Germany) and added to hybridization buffer. After 2 hrs of incubation at 68°C, the membrane was washed 3 x with 2 x SSC, 0.1 % SDS at 50 °C for 15 min, followed by 2 washes with 0.1 x SSC, 0.1% SDS, at 40°C for 30 min. Sealed blots were exposed to X-ray film for 1-5 days at -80°C.

For tissue specific reverse transcriptase-PCR analysis, total RNA from different tissues of an adult C57/BL6 mouse was reverse transcribed into cDNA and used as template for PCR amplification. Specific primer pairs were used to amplify the cDNA

fragments of CKAMP44 (VM-A172: 5'-TCC ATA TGA ACA GCA GCC TCC AG3', VM-A173: 5'-TTC TGT CCA TTC TGC TGT GCA G-3') or ß-actin (ACTBrealFor: 5'-CTG GAA CGG TGA AGG CGA CA-3', ACTBrealRev: 5'-GGT GAG GGA CTT CCT GTA ACC ACT-3').

In situ hybridization was performed on horizontal brain sections from P28 adult C57/BL6 mice. Brains were removed and frozen on dry ice (S*2*). Sections of 14 µm were cut with a cryostat and slide-mounted, fixed for 5 minutes in 4% paraformaldehyde, rinsed in PBS and dehydrated in a series of ascending ethanol concentrations. Sections were stored in 100% ethanol at 4°C until use. *In situ* hybridization with an ^{35}S-labeled antisense 45-mer oligodeoxyribonucleotide probe (c24.is3, 5'-GCA TGA CCC AGG AAA AGC ATG ACT CCT TAT GAG TAG GTC TGT GGT-3') to the 3' UTR of CKAMP44 were performed as previously described (S*3*).

DNA constructs

The full-length coding cDNA for CKAMP44 was obtained by RT-PCR from brain RNA of adult BALB/c mice and subcloned into pcDNA3.1/hygro(+), yielding the CKAMP44 expression plasmid pcDNA3.1/hygro-CKAMP44. This plasmid was modified to obtain pcDNA3.1/hygro-FLAG-CKAMP44 for expression of FLAG-CKAMP44. The coding sequence for the tandem FLAG tag (sense 5'-GGT GAT TAT AAA GAT CAT GAT ATC GAT TAC AAG GAT GAC GAT GAC AAG CAC-3', corresponding peptide: GDYKDHDIDYKDDDDKH) was inserted by PCR into the CKAMP44 cDNA between codons 29 (CAC, histidine) and 30 (GGG, glycine).

To express short-hairpin RNAs (shRNAs) under the control of the polymerase-III H1 gene promotor, DNA-encoded short-hairpin constructs were annealed from synthetic oligonucleotides and cloned as BglII/HindIII fragments into pSuper (S*4*). Short-hairpin oligonucleotides were designed using iRNAi 2 software (http://mekentosj.com/irnai). shRNA1 (plasmid pSuperMV5): 5'-GAT CCC CCC AGA GCA CCT GTA CCA ACT TCA AGA GAG TTG GTA CAG GTG CTC TGG TTT TTG GAA A-3' and 5'-AGC TTT TCC AAA AAC CAG AGC ACC TGT ACC AAC TCT CTT GAA GTT GGT ACA GGT GCT CTG GGG G-3'; ShRNA2 (plasmid pSuper137): 5'-GAT CCC CTC GTG GTC CAC GTG CAG CAT TCA AGA GAT GCT GCA CGT GGA CCA CGA TTT TTG GAA A-3' and 5'-AGC TTT TCC AAA AAT CGT GGT CCA CGT GCA GCA TCT CTT GAA TGC TGC ACG TGG ACC ACG AGGG-3'; shRNA scrambled (plasmid pSuperMV9): 5'-GAT CCC CCC AGA GCA CGT GCA CTA ACT TCA AGA GAG TTA GTG CAC GTG CTC TGG TTT TTG GAA A-3' and 5'-AGC TTT TCC AAA AAC CAG AGC ACG TGC ACT AAC TCT CTT GAA GTT AGT GCA CGT GCT CTG GGG G-3'.

Plasmids for neuronal transfections or production of recombinant AAV (rAAV) particles. All plasmids for expression of recombinant proteins or shRNAs were modified from the AAV vector pAAV-6P-SEWB (S*5*). For expression of CKAMP44 or FLAG-CKAMP44, the human Synapsin I promotor and the coding cDNA followed by the bovine growth hormone polyadenylation site were inserted as XbaI-BpiI fragment into pAAV-6P-SEWB to build AAV vectors with bicistronic expression units. Plasmids were: pAAV-6P-SEWB, expression of GFP; pAAV-Syn-CKAMP44-GFP, co-expression of CKAMP44 and GFP; pAAV-Syn-FLAG-CKAMP44-GFP, co-expression of FLAG-CKAMP44 and GFP. For expression of shRNAs, the transcriptional control units from pSuper containing the H1 promotor followed by the DNA-encoded short hairpin constructs were inserted as XbaI-XhoI fragments into pAAV-6P-SEWB to build AAV vectors with bicistronic

expression units. Plasmids: pAAV-MV5-GFP, co-expression of shRNA-5 and GFP; pAAV-137-GFP, co-expression of shRNA-137 and GFP; pAAV-MV9-GFP, coexpression of shRNA-5m and GFP.

Production and injection of rAAV particles

For rAAV particle production, HEK293 cells were co-transfected with an AAV vector carrying the transcription units of interest and helper plasmids in equimolar ratios by calcium phosphate-mediated plasmid transfection. Cells were lysed 72 hrs after transfection by three freeze-thaw cycles, and the cell debris was removed by centrifugation. The supernatant containing the viral particles was treated with benzonase, and viral particles were purified by iodixanol density centrifugation (S*6, S7*) in a Ti70 rotor at 60.000 rpm. Iodixanol was removed, and the virus was concentrated in PBS buffer in an Amicon Ultra 15 centrifugation filter. The remaining 500 µl solution containing the viral particles was filtered through a Milex GV 0.22 µm pore size. The rAAV particles were stored at -20°C and thawed prior to infection. For P0 injections we used AAV1/2-serotyped viral particles (S*8*). The rAAV particles were injected into newborn C57/BL6 mice, cryo-anaesthesized on ice for 5 minutes. Using a microinjection pump with a Hamilton 710 syringe, 1 µl virus solution was injected bilaterally into hippocampi, 1 mm rostral and 1 mm lateral from lambda at a depth of 1.5 mm measured from the skin. In addition, 1 µl virus was injected into the lateral brain ventricles, 2 mm rostral and 0.7 mm lateral from lambda at a depth of 2 mm measured from the skin. Animals were sacrificed for electrophysiological measurements 15-30 days after injection. All virus injections were performed according to the guidelines for animal treatment (Regierungspräsidium Karlsruhe, license 359185.81/G56/05).

Antibodies and immunoblot analysis

For generation of anti-CKAMP44 antibodies (ab-CKAMP44-66), two rabbits were immunized with a mix of two peptides (pep-66: KTWDPSDQSLRRQAYG, pep-67: CTQPYFITNSKTEVTV) according to the Double XP program performed by Eurogentec. To obtain ab-CKAMP44-66, the antiserum was affinity-purified against pep-66 and stored at a concentration of 0.1 mg/ml in PBS containing 0.01% Thimerosal. Commercial rabbit antibodies were anti-GluR-1, anti-GluR-2/3, anti-NR2B, anti-synapsin 1, anti-GluR-2/3, anti-NR1, anti-stargazin/Cacng2, anti-FLAG, anti-GFP, IgG and mouse antibodies: anti-GluR-2, anti-FLAG (M2), anti-synaptophysin, anti-PSD-95, anti-GFP.

For immunoblot analysis, protein samples were boiled in SDS sample buffer (S*9*). Denatured proteins were separated by SDS-PAGE, transferred onto PVDF membranes and probed with antibodies. For statistical analysis, antibody signals were quantified with Scion Image software, and values were normalized to the corresponding ß-actin signals. Sample sizes were $n \geq 6$, and statistical analysis was performed with Student's t-test.

Heterologous expression in HEK293 cells

Human embryonic kidney cells (HEK293) were grown in DMEM plus 10% fetal bovine serum. Cells were transfected by lipofection and cultured for 48-72 hrs after transfection. For protein extracts, cells were washed with PBS and harvested in lysis buffer (50 mM

Tris, pH 7.5, 150 mM NaCl, 50 mM KCl, 1% Triton X-100, 0.5% NP-40 containing protease inhibitors). After centrifugation for 10 min at 20,000 g, the supernatant was used for immunoblot analysis.

Immunoprecipitation of CKAMP44 from mouse forebrain homogenates

Crude membrane fractions were prepared from adult C57/BL6 mice and AMPAR subunit knockout mice. Forebrains were dissected, homogenized in buffer A (0.32 M sucrose, 10 mM HEPES, pH 7.4 plus Complete protease inhibitors), and spun at 1,000 g for 10 min. Crude membranes were pelleted at 20,000 g for 20 min. The pellet was resuspended in buffer B (50 mM Tris, pH 7.4, 150 mM NaCl, 50 mM KCl, 1% Triton X-100, 0.5 % NP-40 plus Complete protease inhibitors), incubated at 4°C for 45 min and subjected to centrifugation for 10 min at 20,000 g. The supernatant was used for immunoprecipitations. Affinity-purified anti-CKAMP44 was prebound on protein A agarose beads and added to the supernatant. After incubation for 4 hrs at 4°C, the antibody-protein A agarose beads were spun at 500 g for 1 min and washed 4 x with buffer C (50 mM Tris, pH 7.4, 150 mM NaCl, 50 mM KCl, 0.5 % Triton X-100, 0.25 % NP-40). Bound proteins were eluted by incubation with excess antigen peptide pep-66.

Subcellular fractionation

Subcellular fractions were prepared from forebrain homogenates of 3 month-old C57/BL6 mice as described (S*10*) with some modifications. Forebrains were homogenized in 0.32 M sucrose buffer, pH 7.5 and centrifuged at 1,000 g for 10 min. The supernatant was centrifuged at 18,000 g for 30 min to obtain cytoplasm and crude membrane fractions. Cytoplasm was centrifuged at 120,000 g for 2 hrs to recover the microsome fraction as a pellet. Crude membrane was layered on top of a 0.85/1.2 M sucrose density gradient and ultracentrifuged at 120,000 g for 2 hrs. The synaptosome fraction was collected at the interface of 0.85/1.2 M sucrose. Synaptosomes were hypotonically shocked and subjected to a second round of sucrose density gradient ultracentrifugation. The synaptic membrane fraction collected from the interface of 0.85/1.2 M sucrose gradient was mixed with Triton X-100 to a final concentration of 1 %, incubated for 15 min, and layered on top of a 1.2/1.5/2.0 M sucrose density gradient. After 2 hrs of ultracentrifugation, the PSD fraction was collected at the interface of 1.5/2.0 M sucrose. 10 µg of each fraction were separated by SDS-PAGE and blotted on a PVDF membrane.

Hippocampal cultures and immunocytochemistry

Rat hippocampal neurons were prepared (S*10*) with some modifications. Hippocampal primary cultures were prepared from E18 Wistar rat embryonic tissue by dissociation with trypsin, plated on poly-L-lysine/laminin-coated 12 mm glass coverslips at a density of 50,000 cells per coverslip and cultured in Neurobasal media with B27 supplement. Hippocampal neurons were transfected or infected with rAAV at days in vitro (DIV) 5-6. After DIV 19-21, cells were processed for immunostaining or harvested for immunoblot detection. For immunocytochemical surface staining cultures were incubated with primary antibodies under non-permeabilizing conditions. Coverslips were mounted on slides with Mowiol.

Confocal microscopy and image acquisition

For immunocytochemistry of primary hippocampal neurons, acquisition of images was performed on a laser-scanning confocal microscope using a Plan-Apochromat 63x objective and LSM 510 Meta software. Optical sections were taken at 512x512 pixel resolution and averaged four times. All images presented are projection images from individual Z-stacks.

Electrophysiology of *Xenopus laevis* oocytes

Stage V–VI *Xenopus laevis* oocytes were prepared as previously described (S*11*). Oocytes were injected up to 24 hr after isolation with the indicated cRNA and assayed 3 days later. Plasmids used: GluA1(flip; R at the R/G editing site), GluA2(flip, Q; and G at the R/G editing site), GluA3(flip, G at the R/G editing site), GluK2(VCQ), GluN1(1a) and CKAMP44 (the CKAMP44a splice variant was used for recombinant expression throughout this study) in pGEMHE, and GluN2B in pCDNA3. As a control for non-specific effects on protein synthesis or surface expression, we co-injected cRNA for GABA transporter 1 (GAT1). Two electrode voltage-clamp recordings were carried out at room temperature using GeneClamp500 connected to digidata1322A and pCLAMP8.2. Electrodes were filled with 3 M KCl and had a resistance of 0.5–1MΩ. Oocytes were continuously perfused with recording solution (in mM, 10 HEPES, pH 7.4, 90 NaCl, 1 KCl, and 1.5 $CaCl_2$). For GluA1 and GluA2 recordings, oocytes were clamped at -70 mV and glutamate (1 mM) was applied together with cyclothiazide (co-application) (0.1 mM; RBI) to reduce receptor desensitization. For GluK2, clamped oocytes (-70 mV) were incubated for 5 min with concanavaline A (1 mg/ml) prior to glutamate (1 mM) application. Oocytes expressing GluN1/N2B were clamped at +30 mV (to avoid Mg^{+2} block) and currents were induced by 0.1 mM glutamate and glycine. Data were analyzed using pCLAMP 8.2 and ORIGIN 8 software.

Surface protein biotinylation assay

Surface protein biotinylation assay was done as described previously (S*11*). Briefly, 15 oocytes from each sample were incubated with 0.5mg/ml EZ-link Sulfo-NHS-SS-Biotin in ND for 30 min at 17^{o}C with gentle shaking. Oocytes were washed 5 times with ND, homogenized through a p200 pipette tip in buffer H containing 100mM NaCl, 20mM Tris-Cl, pH 7.4, 1% Triton X-100 and protease inhibitor cocktail in a volume of 40 □l per oocytes, and centrifuged at 14,000 x g for 5 min. A fraction (50 □l) from the cleared lysate was reserved for whole-cell protein sample (total/T) and the remaining lysate was incubated with 50 □l of 50% streptavidin-Sepharose slurry for 3 hr at 4^{o}C. Beads were washed 5 times in buffer H, and biotinylated proteins were eluted by 5 min boiling in SDS-PAGE sample buffer. Total (T) and biotinylated surface (S) fractions were separated on SDS-PAGE (8%), blotted, and immune-reacted with rabbit polyclonal antibodies to GluA1 and to Actin (serving as a control for cell integrity during biotinylation). Bands were visualized and quantified by the chemiluminescence protocol with the imaging system Chemi Doc XRS.

CKAMP44 KO mice

The CKAMP44 KO mice were generated using gene targeting. In 129 SvEvBrd ES cells the CKAMP44 promoter region together with exon1 were replaced by the PGK-bgeo/puro selection cassette. ES cell-derived chimeric mice were back-crossed with C57Bl6 mice and analyzed as F2 and F3 animals. Homozygous CKAMP44 KO mice are viable and show no signs of major anomalies.

Electrophysiology

Brains were removed from deeply anaesthetized (isoflurane) P15-P30 mice. Transverse hippocampal 250 µm slices were cut in 4°C solution containing (in mM): 125 NaCl, 25 $NaHCO_3$, 1.25 NaH_2PO_4, 2.5 KCl, 2 $CaCl_2$, 1 $MgCl_2$, 25 glucose; bubbled with 95%O_2/5%CO_2 (pH 7.4). Whole-cell recordings were performed at 34°C using pipettes pulled from borosilicate glass capillaries with a resistance of 3-5 MΩ when filled with the following solution (in mM): 120 Cs-gluconate, 10 CsCl, 8 NaCl, 10 HEPES, 10 phosphocreatine-Na, 0.3 Na_3GTP, 2 MgATP, 0.2 EGTA (pH 7.3, adjusted with NaOH). Liquid junction potentials were not corrected. Series resistance and input resistance were continuously monitored, by measuring peak and steady-state currents in response to small hyperpolarizing pulses. Slices were continuously perfused with ACSF containing (in mM): 25 NaCl, 25 $NaHCO_3$, 1.25 NaH_2PO_4, 2.5 KCl, 2 $CaCl_2$, 1 $MgCl_2$, 25 glucose, 0.01 SR 95531 hydrobromide; bubbled with 95%O_2/5%CO_2 (pH 7.4). 10µM glycine was added for recording NMDAR-mediated currents. Fast application of glutamate onto outside-out and nucleated patches was performed as described (S*12*) using theta glass tubing mounted on a piezo translator. Application pipettes were tested by perfusing solutions with different salt concentrations through the two barrels onto open patch pipettes and recording current changes with 1 and 500 ms moves of the application pipette. Only application pipettes with current change 20-80% rise times below 100 µs and with a reasonable symmetrical on- and offset were used. The application solution contained (in mM): 135 NaCl, 10 HEPES, 5.4 KCl, 1.8 $CaCl_2$, 1 $MgCl_2$, 5 glucose (pH 7.2). Recovery from desensitization, dose response, and equilibrium desensitization experiments were performed by applying glutamate onto nucleated patches since they usually showed less current rundown and inter-pulse variability than outside-out patches. Synaptic EPSCs were evoked by Schaffer collateral/commissural fiber stimulation (0.1 Hz) at a holding potential of -70 mV (for AMPAR-mediated currents) or +40 mV (for NMDAR-mediated currents). NMDAR-mediated current amplitudes were measured 20 ms after the start of the stimulus artifact. Paired-pulse responses were elicited by two equal in strength stimulations of Schaffer collateral/commissural fibers in the stratum radiatum with 20, 50, 100, 200, 1000, and 3000 ms interstimulus intervals. For recording PPR of AMPAR- or NMDAR-mediated EPSCs, we blocked NMDARs and AMPARs with 10 µM CNQX and 50 µM D-APV, respectively. NMDAR-mediated EPSCs for PPR experiments were recorded at a holding potential of -30mV. mEPSCs were recorded at a holding potential of -70 mV with 1 µM tetrodotoxin to block spontaneous activity, and 50 µM D-APV to block NMDARs. CA1 pyramidal cells were visually identified using an upright microscope equipped with infrared-differential interference contrast and standard epifluorescence. Stimulus delivery and data acquisition were performed using Pulse software. Signals were filtered at 3 kHz, sampled at 10 kHz, and off-line analysis was performed using Igor Pro. AMPAR-current decay and desensitization were fitted with one

or two exponentials, and in case of double exponential fits, the weighted tau (τ_w) was calculated as $\tau_w = (\tau_f \times a_f) + (\tau_s \times a_s)$, where a_f and a_s are the relative amplitudes of the fast (τ_f) and slow (τ_s) exponential components. EPSCs were fitted with two exponentials if the chi-square error was smaller with a double than single exponential fit.

Data are presented as mean ± standard deviation (SD) or standard error of the mean (SEM) and as median ± interquartil range (IQR).See Table S2-6 for values. Statistical differences between groups were examined by Student's t-test or ANOVA, followed by Bonferroni test when the values showed a normal distribution, or by Mann-Whitney Rank Sum Test and Kruskall-Wallis One Way ANOVA, followed by Dunn's method of multiple comparisons for non-Gaussian distributed values. P values < 0.05 were considered statistically significant (* = $p < 0.05$, * = $p < 0.01$, *** = $p < 0.001$)

Supporting Text

We combined immunopurification (IP) and mass spectrometry to identify proteins interacting with endogenous AMPARs in the mature mice forebrain. The tryptic peptides of IP samples from wild type and GluR-A knock out mice were tagged with iTRAQ reagents 114 and 117, respectively, pooled together, separated by nano-liquid chromatography, detected and quantified by mass spectrometry.

As anticipated the AMPA receptors were absent in the GluR-A knock sample; therefore peptide corresponding to the GluA1 as well as the GluA2 subunit were identified with high iTRAQ ratios. In contrast, proteins that bind non-specifically should be present equally in samples of wild type and knock out mice. Consequently, iTRAQ ratios for non specific proteins such as mitochondrial proteins, cytoskeletal proteins and myelin basic proteins scored at values close to 1. A high iTRAQ ratio was detected for the Gamma-2 subunit of voltage-gated Ca^{2+} channel (TARPγ2), which is known to be a specific and strong AMPA receptor interacting protein. The depletion of TARPγ2 in anti-GluA1-IP samples of GluR-A knock out mice demonstrated the informative value of high iTRAQ ratio between wild type and GluR-A knockout samples for identifying proteins associated with the AMPA receptor.

A hypothetical protein (LOC72555) showed up with a similarly high iTRAQ ratio as TARPγ2 suggesting the protein of unknown function to be associated with AMPA receptors. We named this CKAMP44.

Supplementary Table 1. **Quantitative proteomics of AMPA receptor 1 complex detected a novel interacting protein (CKAMP44)**

Accession number	Protein name	iTRAQ average ratio 114/117	Peptide count
38511947	AMPA receptor 1	65,67	19
496140	AMPA receptor 2	61,92	18
148697713	Tarp γ2	9,21	2
149267279	**PREDICTED: hypothetical protein LOC72555 isoform 2 (named CKAMP44)**	**8,40**	**6**
168984593	AMPA receptor 3	7,91	5
164419753	AMPA receptor 4	7,57	2
13562102	NG5 protein	6,25	1
3218528	pancortin-3	4,66	1
148688184	coiled-coil domain containing 18, isoform CRA_b	4,32	1
148699231	Tarp γ8	4,24	2
6978593	calcium/calmodulin-dependent protein kinase II alpha	3,40	2
21450321	Na+/K+ -ATPase alpha 3 subunit	1,93	3
74139650	unnamed protein product (ATP synthase, H+ transporting mitochondrial F1 complex, beta subunit)	1,90	4
902008	adenine nucleotide translocase-1	1,87	2
34740335	tubulin, alpha 1B	1,80	2
74177597	unnamed protein product (pyruvate dehydrogenase (lipoamide) beta)	1,72	5
69885032	myelin basic protein isoform 1	1,70	2
74137438	unnamed protein product (ATPase, Na+/K+ transporting, beta 1 polypeptide)	1,64	2
13242237	heat shock protein 8	1,59	10
74139457	unnamed protein product (ATP synthase, H+ transporting, mitochondrial F1complex, alpha subunit, isoform 1)	1,48	4
148666415	mCG141792 (Immunoglobulin domain variable region (v) subfamily)	1,48	1
121044	Ig gamma-3 chain C region	1,45	1
29436595	protein-L-isoaspartate (D-aspartate) O-methyltransferase	1,44	4
86262157	hypothetical protein LOC239796	1,37	8
146325018	Dihydrolipoamide acetyltransferase component of pyruvate dehydrogenase complex	1,29	3
211827181	Acsf3 protein	1,29	1
14917005	Stress-70 protein, mitochondrial	1,25	3
6679261	pyruvate dehydrogenase E1 alpha 1	1,19	2
12833728	unnamed protein product (microtubule-associated	1,16	1

	protein 1 light chain 3 beta)		
6680522	Shaw-related voltage-gated potassium channel protein	1,12	2
113680120	complement component 1, q subcomponent, C chain	1,07	2
115202	Complement C1q subcomponent subunit B	1,06	3
26336661	unnamed protein product (solute carrier family 8 (sodium/calcium exchanger))	0,98	1
16716569	protease, serine, 1	0,85	1
20150118	Native Crystal Structure Of The Recombinant Monoclonal Wild Type Anti-Testosterone Fab Fragment	0,75	2
15826279	Chain L, Crossreactive Binding Of A Circularized Peptide To An Anti- Tgfalpha Antibody	0,69	1
17390852	Igh-1a protein	0,55	4

Supplementary Table 2. **Electrophysiological values of *Xenopus laevis* oocytes recordings.** * = p<0.05, ** = p<0.01, *** = p<0.001

***Xenopus laevis* oocytes** (mean ± SD)				
	Control (GAT1) (3ng cRNA)	n	CKAMP44 (3ng cRNA)	n
GluA1 (3ng cRNA) Amplitude (nA)	1380 ± 140	10	88 ± 41***	10
GluA2 (2ng cRNA) Amplitude (nA)	2100 ± 315	7	420 ± 60***	7
GluA3 (3ng cRNA) Amplitude (nA)	2500 ± 180	5	81 ± 20***	5
GluK2 (3ng cRNA) Amplitude (nA)	1570 ± 220	15	1460 ± 157	15
GluN1/2B (3ng cRNA) Amplitude (nA)	1370 ± 164	10	1343 ± 206	10

GluA1 (3ng cRNA) Amplitude (nA)	Control (GAT1)		CKAMP44	
+ 3 ng cRNA	1220 ± 61	10	122 ± 61***	10
+ 1 ng cRNA	1300 ± 100	10	430 ± 120***	10
+ 0.3 ng cRNA			740 ± 120**	10
+ 0.1 ng cRNA			1140 ± 122	10
+ 0.03 ng cRNA			1260 ± 73	10

GluA1 (3ng cRNA)	Control (GAT1) (1ng cRNA)		CKAMP44 (1 ng cRNA)	
Glutamate (+0.1mM CTZ) EC50 (□M)	15.1 ± 0.5	5	3.6 ± 0.1***	5
CTZ (+ 1mM Glutamate) EC50 (□M)	35 ± 3	5	225 ± 27***	5

	Control (GAT1) (2ng cRNA)		CKAMP44 (2 ng cRNA)	
GluA1 (2ng cRNA) Amplitude (nA)	840 ± 100	7	126 ± 30***	7
GluA1(L497Y) (4ng cRNA) Amplitude (nA)	1170 ± 155	15	994 ± 140	15

Supplementary Table 3. **Electrophysiological values of outside-out patch recordings.**

Outside-out patches CA1						
	Control	n	CKAMP44 over-expression	n	CKAMP44-KO	n
Amplitude (pA) (median ± IQR)	254 [135-332]	25	289 [122-424]	18	172 [84-289]	16
20-80% rise time (ms) (mean ± SD)	0.42 ± 0.15	25	0.41 ± 0.17	18	0.44 ± 0.15	16
Deactivation τ_w (ms) (median ± IQR)	2.2 [1.74-2.81]	25	3.86 [3.7-4.55]*	18	2.19 [1.77-2.76]	16
Desensitization τ_w (ms) (median ± IQR)	8.81 [6.3–15.1]	17	6.47 [5.81-7.2]	10	8.39 [6.34-10.3]	15
Deactivation τ_w (ms) (median ± IQR) with CTZ pre-application	6.7 [5.7–8.1]	13	26.3 [20.3–34.7]*	13	6.5 [4.5–10.9]	16

Supplementary Table 4. **Electrophysiological values of nucleated patch recordings.**

Nucleated Patches CA1						
	Control	n	CKAMP44 over-expression	n	CKAMP44-KO	n
% SS/peak current (500 ms glut) (median ± IQR)	0.73 [0.62–0.81]	23	0.07 [0.04 –0.1]*	14	1.37 [0.89–1.73]*	20
τ_{deact} (ms) (median ± IQR)	3.8 [3.2–4.4]	25	5.17* [4.65–6.13]	14	3.69 [3.3-4.05]	20
τ_{des} (ms) (median ± IQR)	12.7 [10.5–15.5]	22	7.5 [7.1–8.4]*	13	19.5 [13.1–22.4]*	20
% desens. 10 ms after 1st pulse (mean ± SD)	40.5 ± 9.5	26	11.6 ± 5***	13	48.4 ± 7.2**	19
Recovery from desensitization τ_w (ms) (median ± IQR)	118 [88–195]	25	536 [463–556]*	13	62 [49–72]*	18
Glutamate EC50 (µM) (mean ± SD)	433 ± 196	20	159 ± 49***	10	519 ± 187	13
Hill coefficient EC50 (mean ± SD)	1.5 ± 0.3	20	1.4 ± 0.2	10	1.5 ± 0.1	13
Glutamate IC50 (µM) (mean ± SD)	3.1 ± 1.1	5	1.2 ± 0.3**	5		
Hill coefficient IC50 (mean ± SD)	-1.7 ± 1.3	5	2.9 ± 1.0	5		
% SS/peak current (500 ms glut + CTZ) (mean ± SD)	50 ± 14	5	10 ± 4***	6		
Deactivation after 500 ms glut + CTZ (ms)	10 ± 0.9	5	35 ± 9.2***	6		
% SS/peak current (20 s glut + CTZ) (mean ± SD)	72 ± 13	5	25 ± 12***	5		

CKAMP knockdown experiments, CA1							
Recovery from desensitization τ_w (ms) $p < 0.05$ for shRNA1 and shRNA2 versus control and scrambled shRNA							
control	n	shRNA1	n	shRNA2	n	Scrambled shRNA	n
114 ms [102-150]	22	69 ms [52.2-75.9]	9	75 ms [61-105]	17	139 ms [110-180]	14

% steady state/peak current (500 ms glutamate) $p < 0.05$ for shRNA1 and shRNA2 versus control and scrambled shRNA							
control	n	shRNA1	n	shRNA2	n	Scrambled shRNA	n
0.92 % [0.7-1.33]	22	2.68 % [1.5-3.5	9	1.65 % [1.2-2.3]	17	0.78 % [0.65-1.33]	14

Supplementary Table 5. **Electrophysiological values of CA1 synaptic recordings.**

Synaptic recordings (EPSCs and mEPSCs) CA1						
	Control	n	CKAMP44 over-expression	n	CKAMP44-KO	n
-70/+40 ratio ("AMPA/NMDA") (mean ± SD)	2.24 ± 0.77	12	2.55 ± 0.92	11	2.36 ± 0.71	10
AMPA decay (ms) (mean ± SD)	5.14 ± 0.99	22	5.81 ± 1.22	14	5.22 ± 1.18	10
mEPSC IEI (ms) (mean ± SD)	1106 ± 579	19	810 ± 328	15	1311 ± 358	10
mEPSC amplitude (pA) (mean ± SD)	-19.1 ± 4.2	19	-21.6 ± 3.2	15	-18.2 ± 2.2	
mEPSC 20-80% rise time (ms) (mean ± SD)	0.81 ± 0.16	19	0.83 ± 0.15	15	0.77 ± 0.1	10
mEPSC τ_w decay (ms) (mean ± SD)	4.89 ± 1.02	19	4.96 ± 0.8	15	4.68 ± 0.39	10
mEPSC τ_w decay + CTZ (ms) (mean ± SD	14.2 ± 1.9	12	25.8 ± 4.4	12	15.1 ± 2.3	12

AMPA EPSC PPR CA1 (mean ± SEM)						
20 ms IEI	1.53 ± 0.05	31	1.11 ± 0.1***	14	1.65 ± 0.06	15
50 ms IEI	1.5 ± 0.04	31	1.13 ± 0.07***	14	1.59 ± 0.05	15
100 ms IEI	1.35 ± 0.03	31	1.05 ± 0.04***	13	1.41 ± 0.04	15
200 ms IEI	1.2 ± 0.03	30	1.00 ± 0.06**	13	1.2 ± 0.03	15
1000 ms IEI	1.01 ± 0.02	27	0.93 ± 0.03*	13	1.02 ± 0.02	15
3000 ms IEI	1.00 ± 0.01	24	1.00 ± 0.01	11	0.95 ± 0.02	15
NMDA EPSC PPR CA1 (mean ± SEM)						
50 ms IEI	1.37 ± 0.08	7	1.47 ± 0.17	6		
100 ms IEI	1.53 ± 0.2	6	1.54 ± 0.18	6		
200 ms IEI	1.20 ± 0.06	6	1.16 ± 0.08	6		
1000 ms IEI	1.01 ± 0.02	6	0.97 ± 0.04	6		
3000 ms IEI	0.97 ± 0.02	5	0.99 ± 0.04	5		
AMPA EPSC train (10 Hz, n = 5 EPSCs, and "recovery EPSCs" after 300, 1000 and 3000 ms) (mean ± SEM)						
Peak amplitude 2. EPSC(% of 1st)	130 ± 3	23	104 ± 4***	12	138 ± 5	15
Peak amplitude 3. EPSC (% of 1st)	135 ± 4	23	96 ± 5***	12	140 ± 5	15
Peak amplitude 4. EPSC (% of 1st)	134 ± 4	23	87 ± 6***	12	146 ± 6	15
Peak amplitude 5. EPSC (% of 1st)	140 ± 5	23	84 ± 5***	12	144 ± 7	15
Peak amplitude 300 ms after train (% of 1st)	113 ± 3	23	79 ± 3***	12	123 ± 4	15
Peak amplitude 1000 ms after train (% of 1st)	110 ± 3	19	103 ± 4	10	106 ± 3	12
Peak amplitude 3000 ms after train (% of 1st)	112 ± 3	16	108 ± 3	9	117 ± 8	6

Supplementary Table 6. **Electrophysiological values of DG synaptic recordings.**

AMPA EPSC PPR DG med perforant path (mean ± SEM)						
	Control	n	CKAMP44 over-expression	n	CKAMP44-KO	n
20 ms IEI	0.89 ± 0.05	13	1.07 ± 0.40	16	0.76 ± 0.05	13
50 ms IEI	0.93 ± 0.04	13	1.09 ± 0.03	16	0.87 ± 0.05	13
100 ms IEI	0.89 ± 0.03	13	1.00 ± 0.03	16	0.80 ± 0.04	13
200 ms IEI	0.82 ± 0.04	11	0.90 ± 0.02	15	0.80 ± 0.03	13
1000 ms IEI	0.92 ± 0.03	11	0.88 ± 0.02	14	0.95 ± 0.03	13
3000 ms IEI	0.96 ± 0.03	10	0.95 ± 0.02	14	1.00 ± 0.03	12
AMPA EPSC PPR DG lat perforant path (mean ± SEM)						
20 ms IEI	1.19 ± 0.06	11	1.57 ± 0.10	16	1.14 ± 0.05	13
50 ms IEI	1.27 ± 0.06	11	1.51 ± 0.05	15	1.23 ± 0.07	13

100 ms IEI	1.09 ± 0.04	11	1.33 ± 0.05	15	1.06 ± 0.04	12
200 ms IEI	0.96 ± 0.03	11	1.06 ± 0.03	15	0.89 ± 0.03	12
1000 ms IEI	0.97 ± 0.03	11	0.92 ± 0.03	10	0.93 ± 0.03	10
3000 ms IEI	0.94 ± 0.03	10	0.98 ± 0.02	9	0.96 ± 0.03	10
NMDA EPSC PPR DG (mean ± SEM)						
50 ms IEI	2.01 ± 0.14	11			2.10 ± 0.14	9
100 ms IEI	1.62 ± 0.07	11			1.71 ± 0.07	9
200 ms IEI	1.41 ± 0.08	11			1.29 ± 0.06	9
1000 ms IEI	0.91 ± 0.05	11			0.94 ± 0.03	9
3000 ms IEI	0.95 ± 0.02	11			0.98 ± 0.03	9

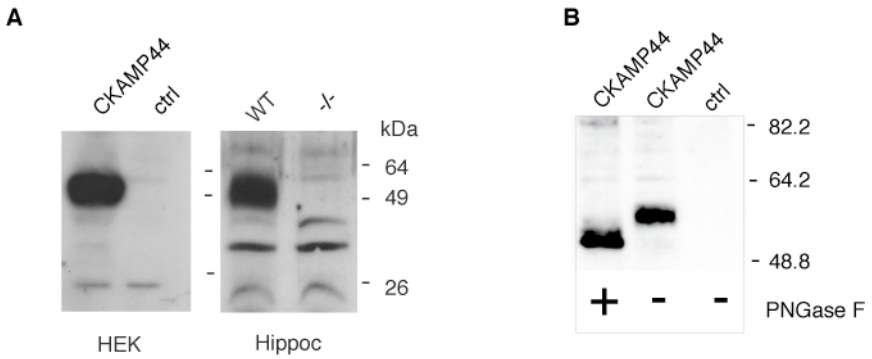

Fig. S1. Specificity of the CKAMP44 antibody and glycosylation of CKAMP44 **(A)** Immunoblot of CKAMP44 in recombinantly expressing HEK293 cells. Our CKAMP44 antibody specifically detects recombinantly expressed CKAMP44. Untransfected HEK293 cells served as control for the CKAMP44 antibody. Specificity of the antibody was shown by absence of the CKAMP44 signal in KO mice. **(B)** Due to glycosylation (and putative Cys-knot structure) the electrophoretic mobility of CKAMP44 was higher than expected. Treatment with PNGase F (bottom) reduces the molecular weight of CKAMP44.

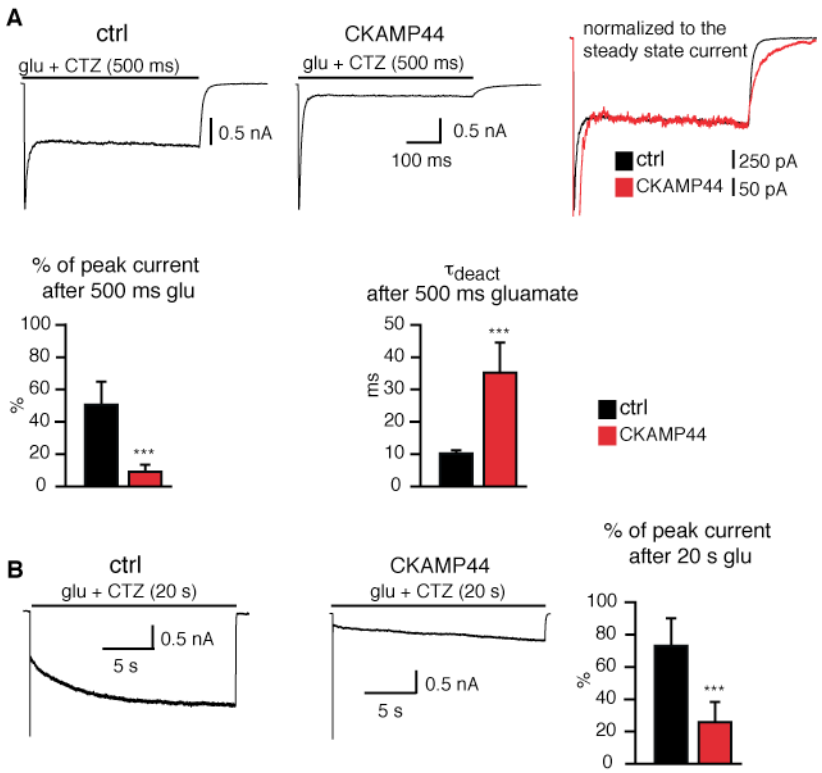

Fig. S2. CKAMP44 reduces the cyclothiazide effect on AMPAR-mediated currents in nucleated patches. **(A)** Glutamate-evoked currents in nucleated patches with co-application of CTZ were studied, since experiments in oocytes had shown that co-expression of CKAMP44 reduces AMPAR steady-state current amplitude in presence of CTZ. Steady-state/peak current ratios (500-ms glutamate pulse) in the presence of CTZ are smaller in nucleated patches of cells over-expressing CKAMP44 compared to control cells. τ_{deact} at the end of the 500-ms glutamate pulse is slower in CKAMP44 over-expressing cells. **(B)** A short peak current precedes a slowly increasing current during a 20-s glutamate + CTZ pulse. The current (calculated as percentage of the peak) at the end of the 20-s pulse is smaller in nucleated patches of cells over-expressing CKAMP44, compared to control patches.

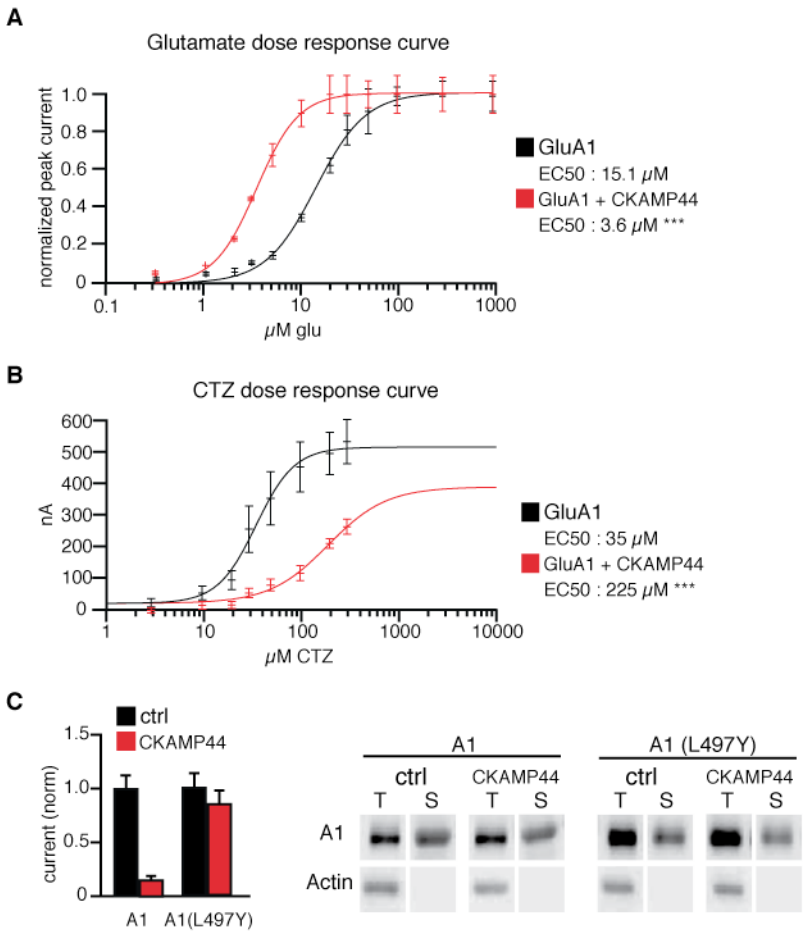

Fig. S3. CKAMP44 modulates AMPAR affinity to glutamate and CTZ in oocytes but has no influence on non-desensitizing AMPARs. **(A)** CKAMP44 increases glutamate affinity of GluA1 expressed in *Xenopus laevis* oocytes. Steady-state GluA1-mediated currents were recorded with co-application of CTZ. **(B)** CKAMP44 decreases CTZ affinity of GluA1 expressed in oocytes. Steady-state GluA1-mediated currents were recorded in the presence of different CTZ concentrations. **(C)** CKAMP44 co-expression does not alter the steady-state current of the non-desensitizing L497Y GluA1 mutant. The ratio of surface bound (S) to total (T) GluA1 is not changed by co-expression of CKAMP44, as quantified by Western blot analysis of total and biotinylated protein.

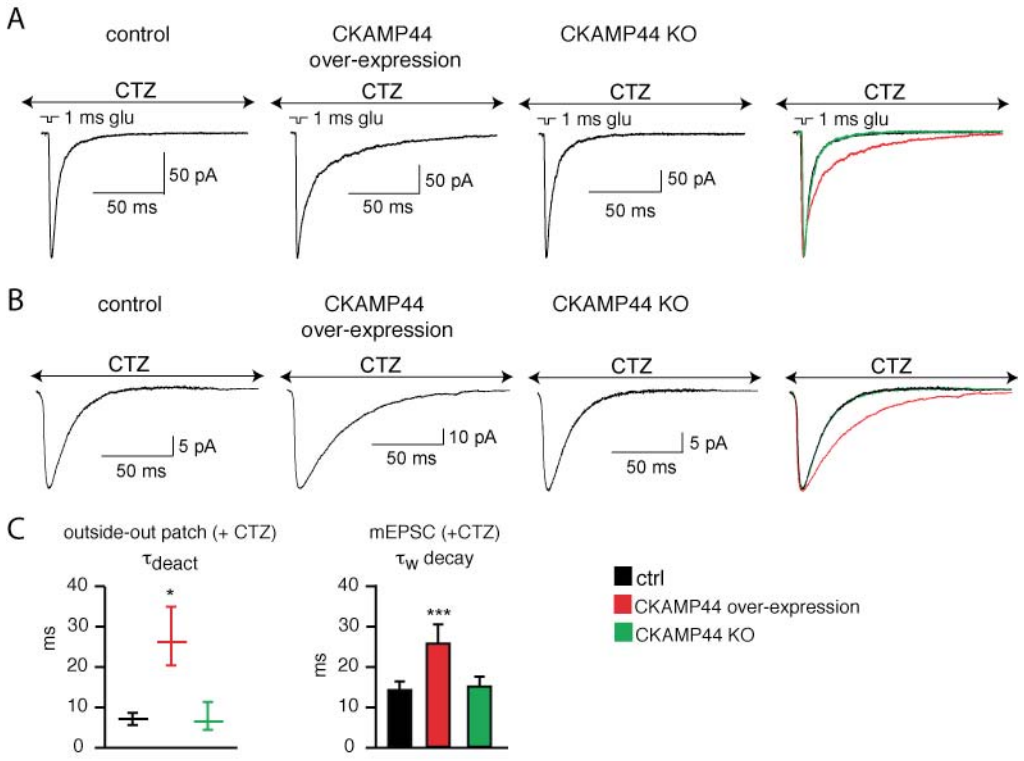

Fig. S4. CKAMP44 modulates kinetic properties of AMPARs in the presence of CTZ. **(A)** Sample traces of AMPAR-mediated currents in outside-out patches of control, CKAMP44 over-expressing and KO cells. **(B)** Sample traces of AMPAR-mediated currents in outside-out patches of control, CKAMP44 over-expressing and KO cells.
(C) CKAMP44 over-expression increases τ_{deact} of AMPARs-mediated currents in CA1 outside-out patches (median ± IQR). CKAMP44 over-expression increases mEPSC decay time constant in CA1 neurons.

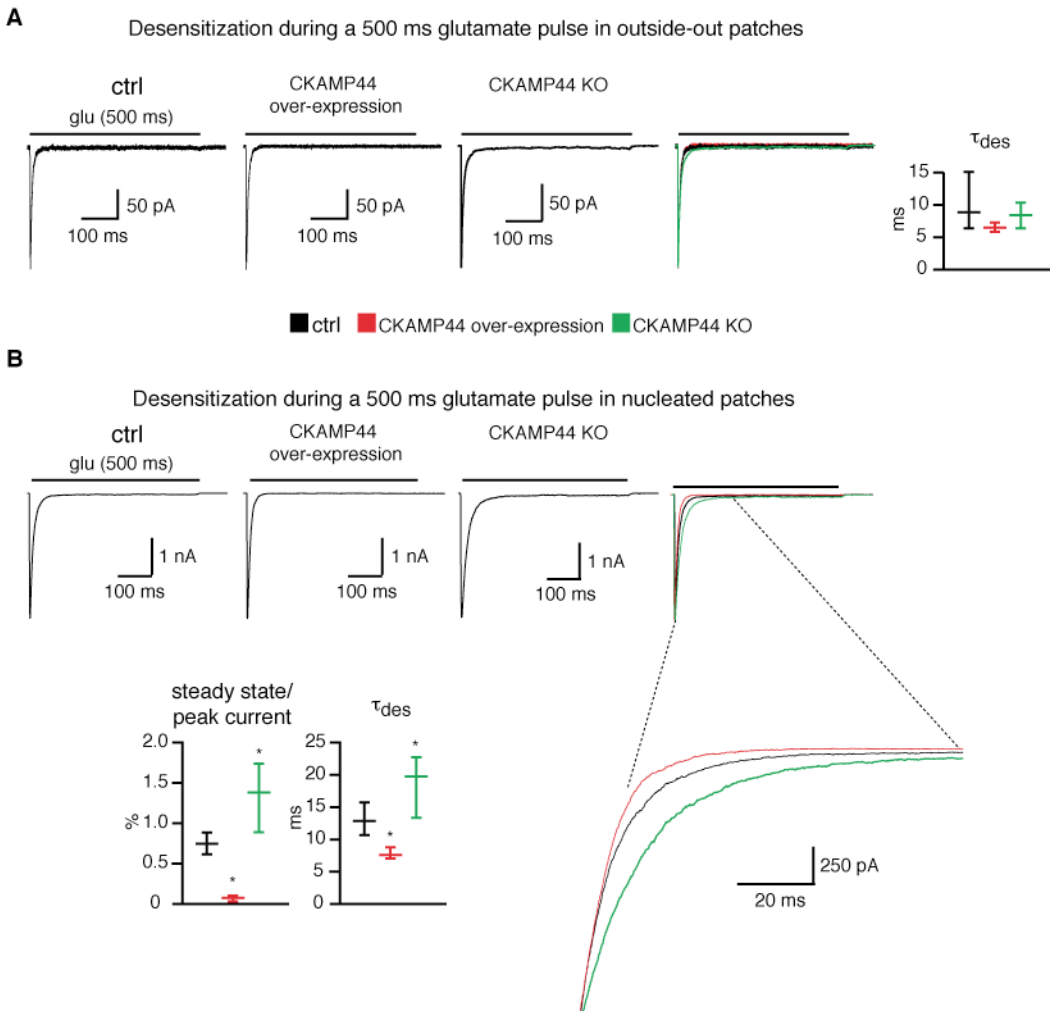

Fig. S5. CKAMP44 increases AMPAR desensitization. **(A)** There is a trend to a smaller desensitization time constant (τ_{des}) of AMPAR-mediated currents during 500-ms glutamate pulses in outside-out patches of cells over-expressing CKAMP44, compared to patches of control and CKAMP44 KO cells. **(B)** CKAMP44 over-expression reduces and CKAMP44 KO increases steady-state currents (calculated as the percentage of peak current amplitude) of AMPAR-mediated currents evoked by 500-ms glutamate pulses compared to nucleated patches of control cells. The τ_{des} of AMPAR-mediated current desensitization is smaller in nucleated patches of CKAMP44 over-expressing cells and bigger in nucleated patches of CKAMP44 KO cells, when compared to patches of control cells. The extended view of the first 100 ms of the desensitizing current illustrates differences in τ_w and in steady state current.

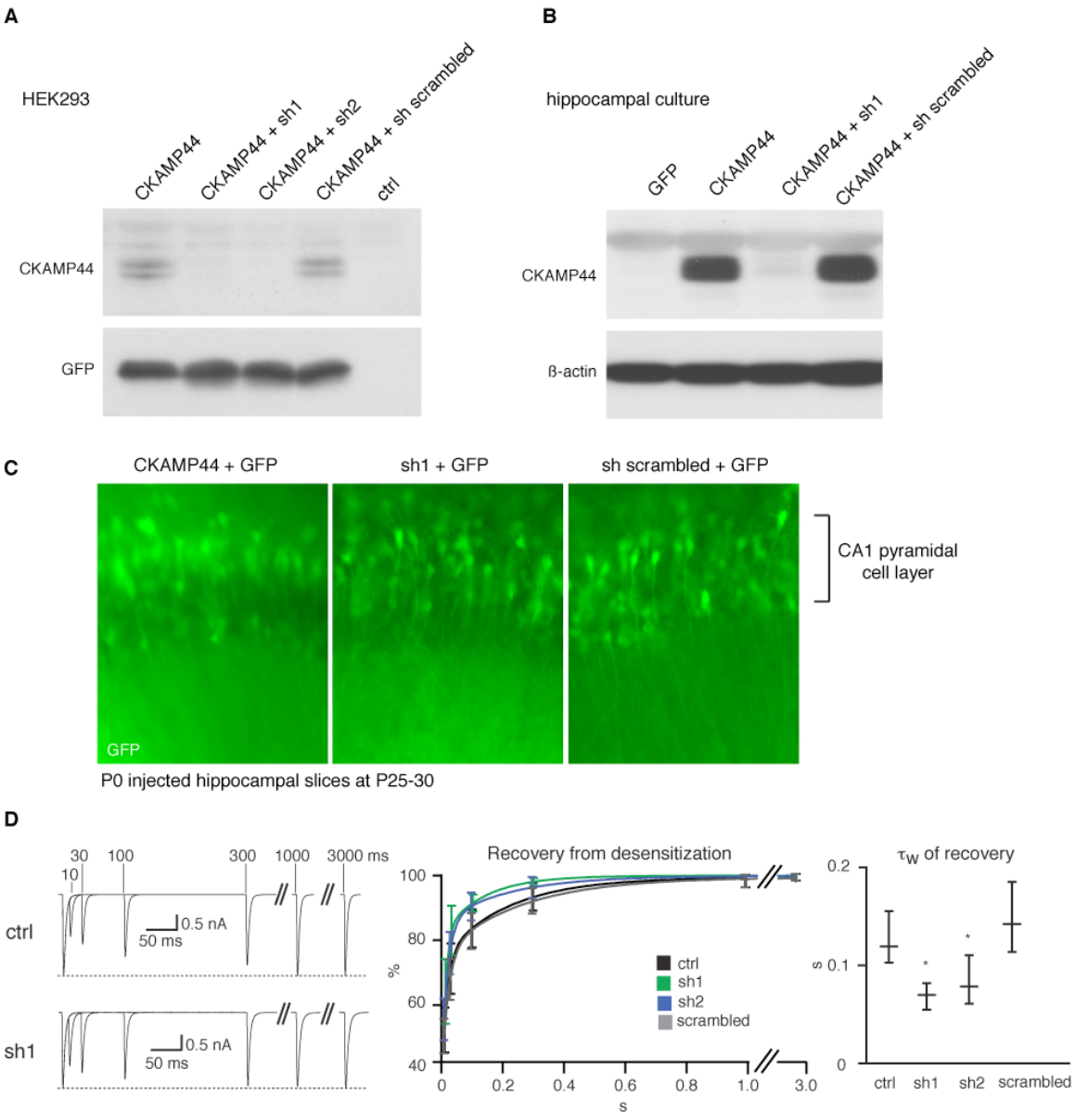

Fig. S6. To exclude that the observed changes in desensitization in nucleated patches of CA1 cells from CKAMP44 KO mice result from compensatory changes during embryonic development, we used viral delivery into newborns of two short hairpin RNA constructs (shRNA1 and shRNA2) to knock down CKAMP44 postnatally (see also Table S4). **(A)** Immunoblots of lysates from transfected HEK293 cells expressing CKAMP44 and GFP plus indicated shRNAs. Lysates from untransfected HEK293 cells are shown in the control lane (ctrl) on the right. **(B)** Immunoblots of lysates from rAAV-infected hippocampal cultures expressing GFP or FLAG-CKAMP44, co-infected with rAAVs expressing shRNAs as indicated. **(C)** **r**AAV-mediated expression of CKAMP44 or shRNAs together with the reporter protein GFP in CA1 neurons of mice infected at P0. For electrophysiological recordings, the infected cells. **(D)** CKAMP44 knockdown with

two different shRNAs (sh1 and sh2) leads to a faster recovery from desensitization (two 1-ms pulses of 1 mM glutamate with different inter-event intervals) compared to control cells or cells infected with rAAV expressing a scrambled version of the shRNA. Example traces of control and shRNA1 (sh1) expressing cells are shown.

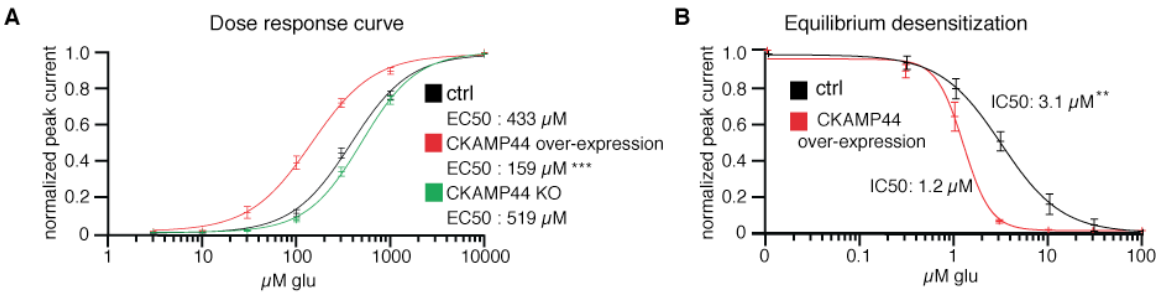

Fig. S7. CKAMP44 over-expression decreases glutamate EC50 and IC50 **(A)** AMPARs of cells over-expressing CKAMP44 exhibit increased glutamate affinity, with a left shift in the dose-response curve, compared to AMPARs of control cells. CKAMP44 KO does not significantly change the EC50 **(B)** AMPARs of cells over-expressing CKAMP44 show a lower IC50 of glutamate equilibrium desensitization than control cells. Glutamate equilibrium desensitization was quantified by changes in peak current amplitudes evoked with 1-mM glutamate pulses during a continuous exposure to increasing glutamate concentrations (0.3, 1, 3, 10, 30 and 100 μM).

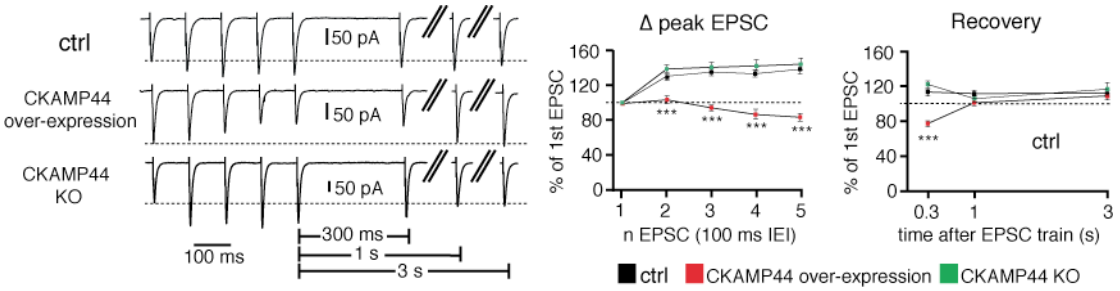

Fig. S8. CKAMP44 modulates short-term plasticity of AMPAR-mediated currents during a train of 5 EPSCs evoked at a frequency of 10 Hz. Over-expression of CKAMP44 caused a decrease in EPSC amplitude of EPSC2-5, whereas EPSC amplitudes increased in control and CKAMP44 KO cells. EPSC amplitudes 300 ms after the train (but not anymore after 1 and 3 s) were still significantly smaller in CKAMP44 over-expressing than control and CKAMP44 KO cells (see also Table S5).

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