Neuron, Volume *83*

Supplemental Information

Efficient, Complete Deletion of Synaptic Proteins using CRISPR

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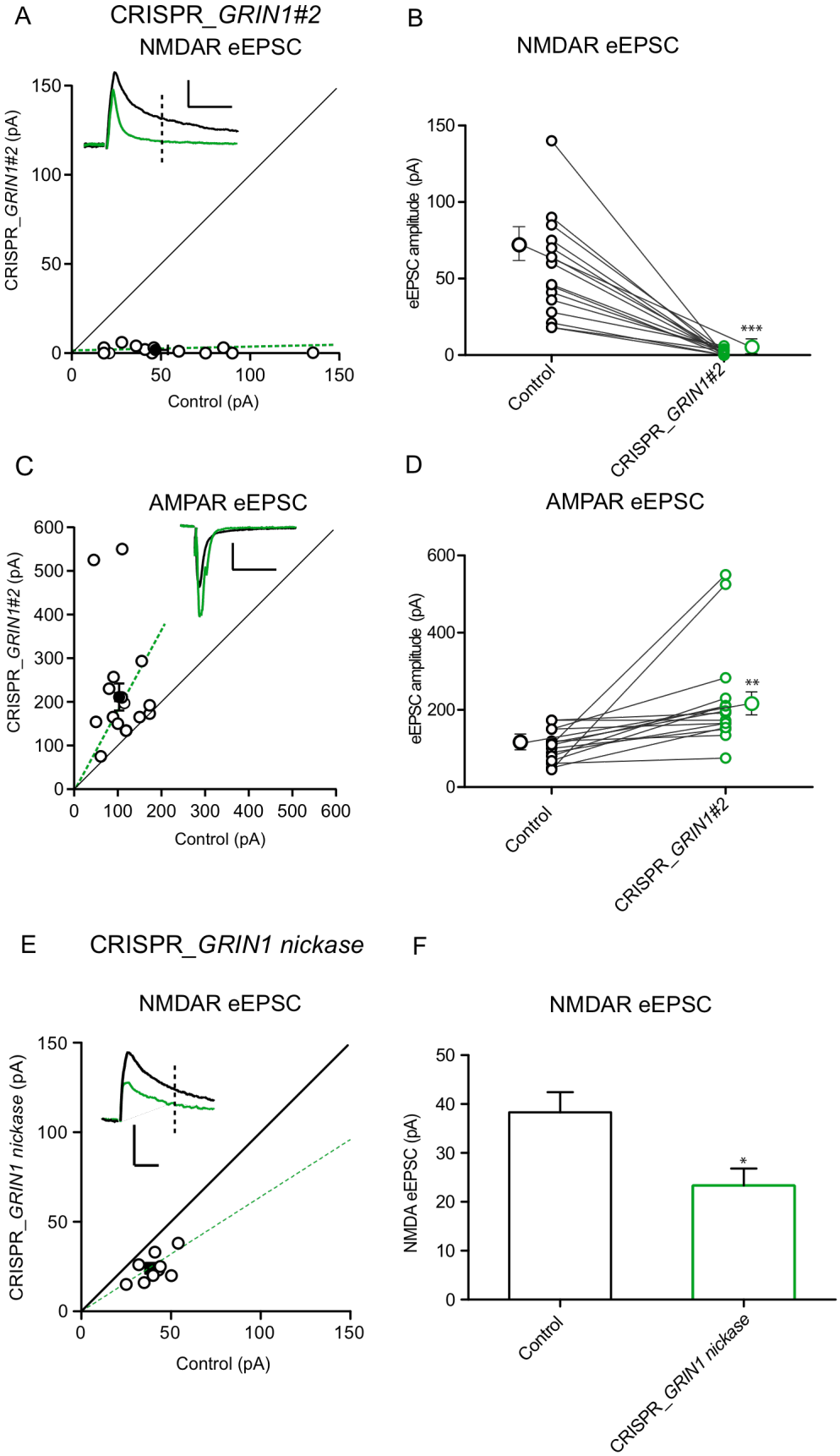
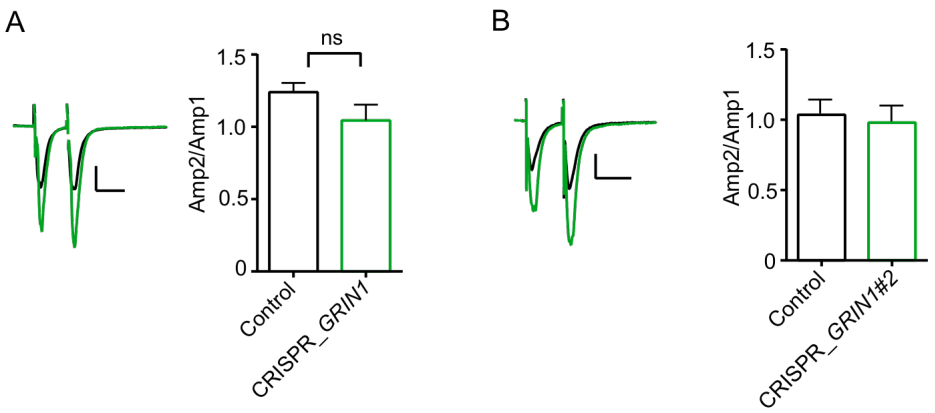
Inventory of Supplementary Materials:



Incontro et al. Figure S1

Figure S1: CRISPR *GRIN1* KO with a different target at an intronic/exonic site and CRISPR *GRIN1* nickase effect on NMDAR eEPSCs (related to Figure 1).

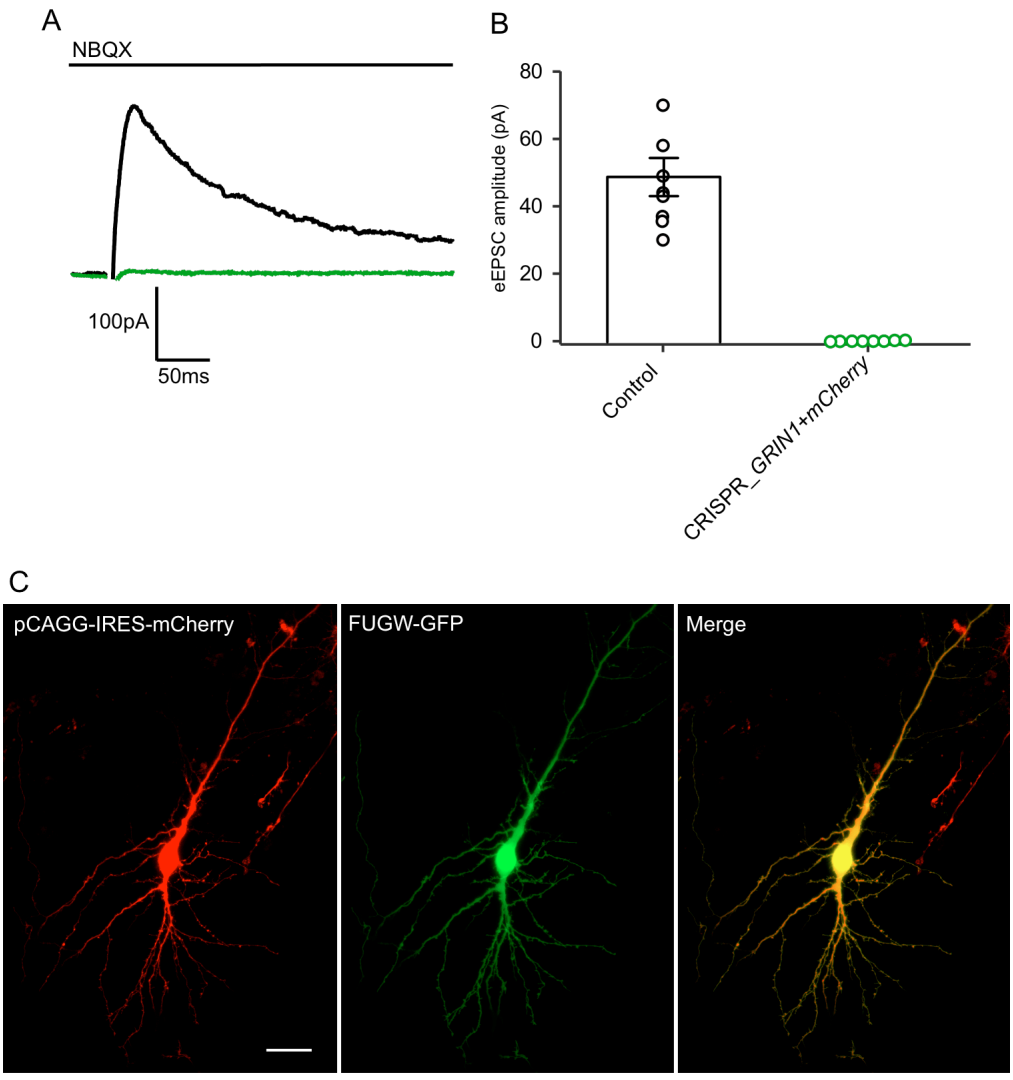
(A) CRISPR *GRIN1*#2 NMDAR EPSC scatter plot for single pairs (open circles) and mean ± SEM (filled circle). Scale bar 50 pA and 50 ms. (B) Paired average of single pairs from control and transfected cells. Mean ± SEM for Control and CRISPR_*GRIN1* are 49.8 ± 6.5 pA, n=19 and 1.8 ± 0.5 pA, n=19, respectively. *** $p<0.0001$ Wilcoxon signed rank test. (C) AMPAR EPSCs scatter plot and bar graph for single pairs (open circles) and mean ± SEM (filled circle). Scale bar 50 pA and 50 ms. (D) Paired average of single pairs from control and transfected cells. Control 104.2 ± 8.6 pA, n=19; CRISPR_*GRIN1* 215.4 ± 28 pA, n=19. ** p=0.0011 Wilcoxon signed rank test. (E) CRISPR_*GRIN1* nickase NMDAR EPSC scatter plot for single pairs (open circles) and mean ± SEM (filled circle). Scale bar 50 pA and 50 ms. (F) Graph bars represent mean values of NMDAR EPSC amplitudes for control and CRISPR_*GRIN1* nickase. Mean ± SEM are Control 38.2 ± 3.6 n=9; CRISPR_*GRIN1 nickase* 22 ± 2.9 n=9. * P=0.03 Wilcoxon signed rank test.



Incontro et al. Figure S2

Figure S2: CRISPR/Cas9 deletion of *GRIN1* doesn't affect Paired Pulse Ratio of AMPAR eEPSCs (related to Figure 2).

(A) Paired Pulse Ratio traces and bar graph for control and transfected cells, for the first *GRIN1* target shown in Figure 2. Mean values of AMPA second to first amplitude are 1.24 ± 0.06 n=7 and 1.045 ± 0.1 n=7 respectively. P=0.43 Student's unpaired t-test. (B) Paired average of single pairs from control and transfected cells, for the second *GRIN1* target corresponding to Figure S1. AMPA second to first amplitude mean ± SEM for control and rescue are 1.01 ± 0.15 n=8 and 0.98 ± 0.12 n=8, respectively. P= 0.84 Student's unpaired t-test.



Incontro et al. Figure S3

Figure S3: CRISPR/Cas9 deletion of *GRIN1* co-transfected with p-CAGG-IRES-mCherry and FUGW-GFP plasmids (related to Figure 3).

(A) Sample traces of NMDAR evoked EPSCs, from a transfected CRISPR/Cas9 cell co-transfected with *GRIN1*#1, FUGW-GFP and p-CAGG-IRES-mCherry (green trace) and a neighboring control cell (black trace) in the presence of NBQX (10 μM). (B) Bar graph showing the averaged eEPSC amplitudes of Control, 43.7 ± 4.3 pA, n=9 and CRISPR_*GRIN1* + mCherry, 0 pA, n=9. (C) Representative confocal stacks from CRISPR/Cas9 cells. Scale bar 20 μm.

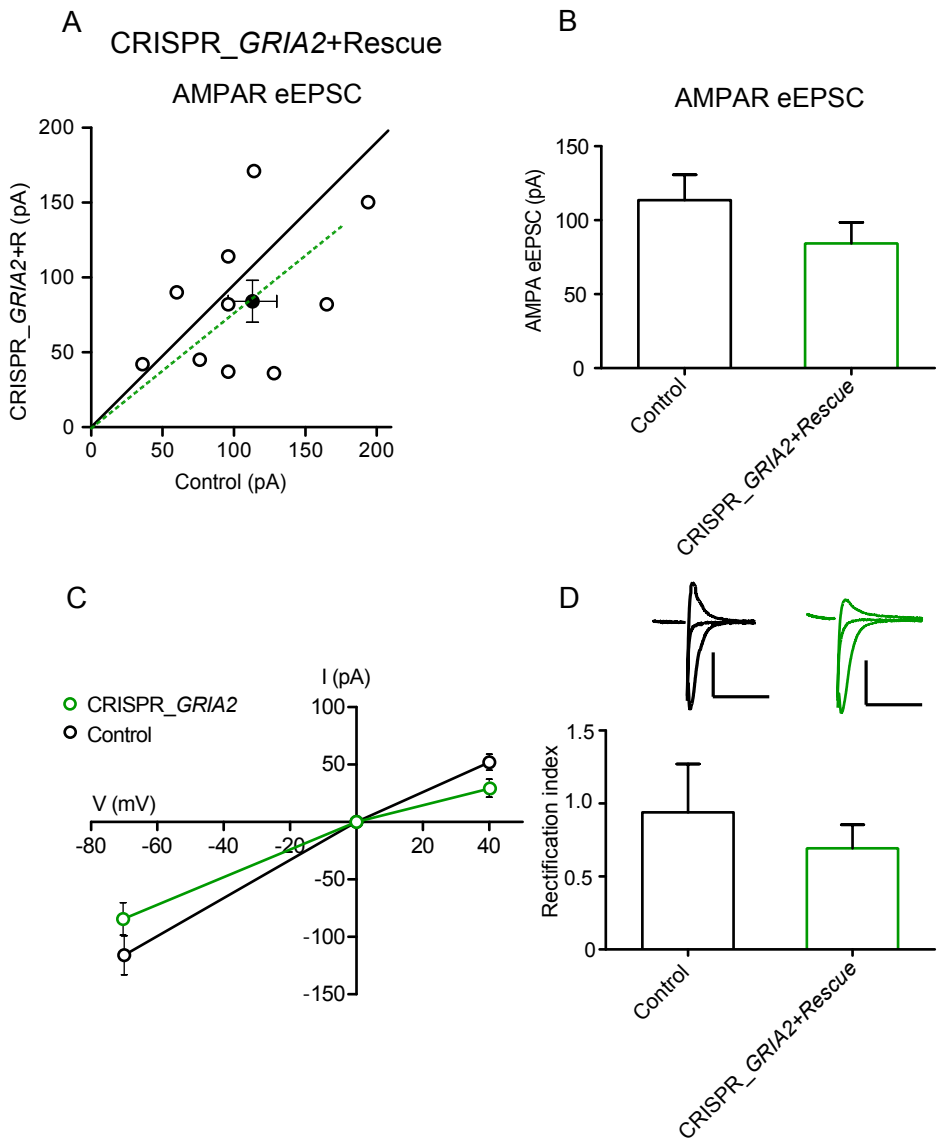


Figure S4: *GRIA2* Rescue with GluA2 cDNA resistant to Cas9 (related to Figure 4).

(A) AMPAR EPSC amplitudes of control and neighboring neurons transfected with CRISPR_ *GRIA2* + Rescue. Scatter plot for single pairs (open circles) and mean ± SEM (filled circle). Scale bar 50 pA and 10 ms. (B) Graph bars represent mean values of AMPAR EPSC amplitudes for control and CRISPR_*GRIA2* + Rescue. Mean ± SEM are Control 113.5 ± 17.1 n=11; CRISPR_*GRIA2* + Rescue 84.2 ± 14.2 n=11. P=0.1 Wilcoxon signed rank test. (C) Graph representing I/V plots for Control and CRISPR_*GRIA2* + Rescue cells: the inward rectification of *GRIA2* KO cells is largely rescued by GluA2 expression. (D) Mean ± SEM of rectification index values for Control (0.88 ± 0.1) and CRISPR_*GRIA2* cells (0.66 ± 0.04). P=0.05 Student's unpaired t-test.

Supplemental experimental procedures

Methods

Molecular biology

The first step in the design of gRNAs involved identification of the best sequence to target. We targeted the 5' end of the protein-coding region because a frameshift is likely to result in a complete loss of function. When possible, we also selected guide RNAs (gRNAs) encompassing an exon-intron junction (*GRIN1*#2, *GRIA2*#1), which should enable rescue using a cDNA. This particular design also has the potential to affect splice sites and thereby produce a null mutation with both in-frame and out-of-frame insertions or deletions. When this was not possible (*GRIN1*#1, *GRIA2*#2), we designed gRNAs targeting a domain of the protein essential for its folding, activity or stability since the frequency of in-frame insertions or deletions occurring with double strand DNA repair can vary substantially among loci (Koike-Yusa et al., 2014). These considerations should help to reduce the likelihood of residual, functional protein due to in-frame repair.

To screen the *GRIN1* and *GRIA2* gRNA sequences for potential off-target effects, we used the Cas9 design target tool (http://crispr.mit.edu). The human codon-optimized Cas9 and chimeric gRNA expression plasmid (pX330) as well as the lentiviral backbone plasmid (lentiCRISPR) both developed by the Zhang lab (Cong et al., 2013; Ran et al., 2013b) were obtained from Addgene. To generate (gRNA) plasmids, a pair of annealed oligos (20bp) was ligated into the single gRNA scaffold of pX330 or lentiCRISPR. The primers used to design the specific gRNA targets were: *GRIN1*#1 forward (5' to 3') CACC G aaccaggccaataagcgaca; *GRIN1*#1reverse (5' to 3') AAAC tgtcgcttattggcctggtt C; *GRIN1*#2 forward (5' to 3') CACC G actaggatagcgtagacctg; *GRIN1*#2 reverse (5' to 3') AAAC caggtctacgctatcctagt C; *GRIA2*#1 forward (5' to 3') CACC G ctaacagcatacagataggt; *GRIA2*#1 reverse (5' to 3') AAAC acctatctgtatgctgttag C; *GRIA2*#2 forward (5' to 3') CACC G ctaacagcatacagataggt; *GRIA2*#2 reverse (5' to 3') AAAC acctatctgtatgctgttag C. To generate gRNA plasmids, using the double nickase pX335, two pairs (one for each DNA strand) of annealed oligos (20bp) were ligated into the single gRNA scaffold of each pX335. The primers used to design the nickase targets were: *GRIN1*#1 forward and reverse (same as above) for one DNA strand; *GRIN1*#3 forward (5' to 3') CACC G agccgtgtcgcttattggcc; *GRIN1*#3 reverse (5' to 3') AAAC ggccaataagcgacacggct C, for the other DNA strand. In the primer sequences the lowercase character represents the gRNA. To rescue the GluN1 deletion, a GluN1-1a cDNA in pCAGGS-IRES-mCherry was used and co- transfected with the CRISPR/Cas9 construct. To rescue the GluA2 deletion, a GluA2 cDNA resistant to Cas9 was used. For the rescue control experiment, a pCAGGS-IRES-mCherry plasmid was co-transfected with CRISPR_*GRIN1*. We co-coated all the bullets with GFP- expressing vector FUGW plasmid (Addgene) in order to identify transfected cells.

Antibodies

The GluN1 mouse monoclonal antibody was obtained from BD Transduction Laboratories, the β-Actin mouse monoclonal and the GluA2 mouse monoclonal antibody from Millipore. The synaptophysin rabbit polyclonal antibody was obtained from Zymed.

Cell Culture and Lentivirus Production

HEK293T cells were maintained in DMEH-21 medium with 10% fetal bovine serum (FBS) in 5% CO_2 at 37^{o}C. Lentivirus was produced by transfecting HEK293T cells with lentiCRISPR, psPAX2 and pVSVG using Fugene HD (Roche) according to the manufacturer's instructions.

Neuronal culture transduction and western blot

Dissociated cultures of postnatal rat hippocampal neurons were transduced with freshly made viral supernatant at DIV4. For western blot analysis, neurons (DIV18) were lysed in 150 mM NaCl, 50 mM Tris-HCl, pH 8.0, 1% TX-100, and protease inhibitors (Roche) including 1 mM EGTA and 1 mM PMSF. After sedimentation at 14,000g to remove nuclei and cell debris, 5 mg protein was separated by electrophoresis through polyacrylamide, transferred to nitrocellulose, and the membranes immunoblotted for GluN1, GluA2, β-Actin and synaptophysin and the appropriate secondary antibodies conjugated to IRDye800 (Rockland). The membrane was imaged with a LICOR system (Odyssey). For indels analysis, neurons (DIV10) were lysed in 10 mM Tris pH8.0, 100 mM NaCl, 10 mM EDTA pH 8.0, 0.5% SDS, and proteinase K. After overnight incubation at 55^{o}C, genomic DNA was isolated by phenol/chloroform extraction, and used as a template for PCR amplification of the region around the Cas9 cleavage site. PCR products were cloned into pBSK II (+) and individual clones were analyzed by sequencing.

Neuronal transfection

Sparse biolistic transfections of organotypic slice cultures were performed as previously described (Lu et al. 2009; Schnell et al., 2002). Briefly, 50 μg of each plasmid DNA was coated on 1 μm diameter gold particles in 0.5 mM spermidine, precipitated with 0.1 mM $CaCl_2$, and washed four times in pure ethanol. The gold particles were coated onto PVC tubing, dried using ultra-pure N_2 gas, and stored at 4^{o}C in desiccant. DNA-coated gold particles were delivered with a Helios Gene Gun (BioRad). Construct expression was confirmed by GFP and/or mCherry fluorescence.

Confocal imaging

CA1 pyramidal neurons in organotypic hippocampal slice cultures made from P6 rat pups were biolistically transfected with FUGW-GFP, p-CAGG-IRES-mCherry and CRISPR_*GRIN1*#1 constructs ~18-20 hr after plating. Confocal imaging was performed on live tissue in HEPES buffered aCSF (125 mM NaCl; 5 mM KCl; 10 mM D-Glucose; 10 mM HEPES; 2 mM MgSO4; 2 mM CaCl2; pH 7.3) 14 days after transfection using a Nikon Spectral C1si confocal microscope with a NIR Apo 40x W objective. Z-stacks were made of 30 μm sections using EZ-C1 software (Nikon).

Electrophysiology in slice cultures

Cultured slices were prepared and transfected as previously described (Schnell et al. 2002). Experiments were done at different days (5 to 15) after biolistic transfection with CRISPR/Cas9 constructs. Slices were maintained in aCSF supplemented with 5-20 μM 2-chloroadenosine to dampen epileptiform activity, and $GABA_A$ receptors were blocked with picrotoxin (0.1 mM) and bicuculline (0.01 mM), in a solution saturated with 95% O_2 / 5% CO_2. CA1 pyramidal cells were visualized by infrared differential interference contrast microscopy. The internal solution contained (in mM) $CsMeSO_4$ 135, NaCl 8, HEPEs 10, Na_3GTP 0.3, MgATP 4, EGTA 0.3, QX- 314 5, and spermine 0.1. Cells were recorded with 3 to 5 MΩ borosilicate glass pipettes, following stimulation of Schaffer collaterals with monopolar glass electrodes filled with ACSF placed in stratum radiatum at the CA1 region. All paired recordings involved simultaneous whole-cell recordings from one GFP-positive neuron and neighboring GFP-negative neuron. GFP positive neurons were identified by epifluorescence microscopy. Series resistance was monitored and not compensated, and cells in which series resistance was above 30 MΩ or varied by 25% during recording session were discarded. Synaptic responses were collected with a Multiclamp 700A-amplifier (Axon Instruments, Foster City, CA), filtered at 2 kHz, digitized at 10 Hz. The stimulus was adjusted to evoke a measurable, monosynaptic EPSC in the control cell. AMPAR-mediated responses were isolated by voltage-clamping the cell at -70 mV, whereas NMDA responses were recorded at +40 mV and amplitudes measured at 150 ms after stimulation to avoid contamination by AMPAR current. In Fig.1, NBQX (50 μM) was added to the ACSF followed by a switch to ACSF containing NBQX (50 μM) and D-AP-5 (100 μM). Rectification indices were calculated as the ratio of the slopes of the two lines connecting average EPSC values at -70 and 0 mV, and, 0 and +40 mV, respectively, in presence of 100 μM D-AP-5 to block NMDAR mediated EPSCs.

Statistical analysis

Significance of evoked dual whole-cell recordings of transfected neurons compared to controls was determined using the Wilcoxon signed-rank sum test. Paired-pulse ratios and rectification values were analyzed with a Student's t test.

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