Supplementary information

Table SI. AMPAR and ChTx diffusion coefficients. Median values of AMPAR and ChTx diffusion coefficients in synaptic and extrasynaptic locations (***: Mann-Whitney test, $p<0.0001$).

Table SII. Explored area of AMPAR and ChTx. Median explored areas (μm^2) of AMPAR and ChTx in synaptic and extrasynaptic compartments (**: unpaired two-tailed t-test, $p<0.005$).

Movies S1 and S2. Movies of AMPAR diffusion in hippocampal neurons of n-cof$^{flx/flx}$ (movie S1) and n-cof$^{flx/flx,CaMKII\text{-}cre}$ mice (movie S2). QD-AMPAR are shown in red, FM-labelled synapses are shown in green and QD-trajectories are shown in white.

Table SIII. Latrunculin A and jasplakinolide dependent AMPAR diffusion coefficients. Median values of AMPAR diffusion coefficients in synaptic and extrasynaptic locations after treatment with latrunculin A and jasplakinolide (Mann-Whitney test, **: $p<0.01$, *: $p<0.05$)

Supplementary Materials and methods

Biochemistry

F/G-actin ratio of total protein lysates: Freshly dissected tissue was lysed in ice-cold 1xPHEM extraction buffer (in mM: 60 PIPES, 20 HEPES, 10 EGTA, 2 $MgCl_2$, 1%

TritonX-100, pH 7.0) by using a tight fitting Douncer. F-actin was pelleted by a 10 min centrifugation at 10,000 rpm in an Eppendorf centrifuge. Supernatant and pellet were brought up to equivalent volumes in 1xSDS sample buffer and equal amounts of both fractions were separated by SDS-PAGE. Actin was quantified by western blot using an anti-actin antibody (MP Biochemicals, clone C4). *Preparation and fractionation of synaptosomes* was carried out on dissected cortices and hippocampi as previously described (Lopes et al., 1999; Phillips et al., 2001). For details, see below. Following antibodies were used: Synaptophysin, NSF, and Chapsyin-110/PSD-93, ADF (Sigma), PSD-95 and stargazin/TARP γ8 (Millipore), GRIP/ABP, SAP102, Shank 1 (Synaptic Systems), phospho-ADF/cofilin (Cell Signaling). *Preparation of synaptosomes and fractionation* into soluble extrasynaptic, presynaptic matrix, and PSD was carried out on dissected cortices and hippocampi as previously described (Lopes et al., 1999; Phillips et al., 2001). Briefly, the cortex or hippocampus from one mouse of each genotype was lysed at the same time and synaptosomes were enriched on a floatation gradient containing 45% Percoll in Krebs-Ringer buffer. The synaptosomes were re-suspended in HEPES-Krebs buffer and lysed by addition of an equal volume of 2xPHEM extraction buffer. After incubation on ice for 15 min, F-actin was pelleted by a 10 min centrifugation at 10,000 rpm in an Eppendorf centrifuge. The supernatant and the pellet were brought up to equivalent volumes in 1xSDS sample buffer and equal amounts of the two fractions were separated by SDS-PAGE.

Genotyping of mice

Genotyping of mice was performed by PCR using the following primers:

(CGCTGGACCAGAGCACGCGGCATC), (CTGGAAGGGTTGTTACAACCCTGG) and (CATGAAGGTTCGCAAGTCCTCAAC) for detecting the n-cof^{flx} allele; (GTGGCAGATGGCGCGGCAACACCATT)/(GCCTGCATTACCGGTCGATGCA ACGA) for the CaMKII-cre transgene; (TCTGAGTGGCAAAGGACCTTAGG) and (CGCTGAACTTGTGGCCGTTTACG) for the *Thy1*-GFP-M transgene. Animal care and experimental testing were conducted at the EMBL Monterotondo according to the approved license and the institutional guidelines that are in compliance with international laws and policies (European Community Council Directive 86 609, Official Journal L358, December 18, 1986). When not specified in the respective materials and methods section, age of mice used for the experiments was between 8-16 weeks.

Electrophysiology

Mice were sacrificed by cervical dislocation. For input-output curves, paired pulse facilitation, post-tetanic stimulation, short-term depression and long-term depression brains were removed from the skull and the hippocampi were quickly dissected out in ice-cold oxygenated artificial cerebrospinal fluid (ACSF) containing the following (in mM): 120 NaCl, 2.5 KCl, 2.5 $CaCl_2$, 1.2 $MgCl_2$, 26.2 $NaHCO_3$, 1.0 NaH_2PO_4, 11.0 glucose, bubbled with a mixture of 95% O_2 / 5% CO_2, pH 7.4. Transverse hippocampal slices (400 μM) were cut with a manual tissue chopper (Stoelting, Wood Dale, IL) and maintained in a humidified holding chamber at room temperature for at least one hour for recovery. Slices were then individually transferred to an interface recording chamber (Fine Science Tools, Foster City, CA), where they were continuously perfused with ASCF maintained at 29°C and at a flowing rate of 1.5-2

ml/min. Extracellular field excitatory postsynaptic potentials (fEPSPs) were recorded in *stratum radiatum* of area CA1 of the hippocampus with glass electrodes filled with 3M NaCl coupled to the input stage of a Warner IE-210 amplifier (Handen, CT). Stimuli were delivered (0.1 ms pulse duration) to the commissural/Schaffer collateral afferents with a concentric bipolar stainless steel electrode at a stimulus intensity that elicited a fEPSP amplitude that was ~ 50% of maximum, and at a frequency of 0.033 Hz (LTD experiments), 0.1 Hz (for input-output and PPF experiments) or 0.2 Hz (for STD and PTP experiments). We set the basal stimulation intensity for our experiments at ~ 50% of the intensity that evoked maximal fEPSP amplitude. Input-output curves were built by measuring the fiber volley and fEPSP of the responses evoked by stimulating afferent fibers with current intensities ranging from 10 to 300 μA. Paired pulse facilitation (PPF) was induced by applying pairs of stimuli at the following interstimulus intervals (in ms): 10, 25, 50, 75, 100, 150, 200, 250 and 300. Post-tetanic potentiation (PTP) was evoked using a single train of 100 Hz for one second in the presence of APV (50 μM) in the perfusion medium in order to avoid the induction of NMDA-dependent forms of synaptic plasticity. Short-term depression (STD) was monitored during stimulation at 10 Hz for two full minutes in the presence of APV. For long-term depression (LTD), a low-frequency stimulation (LFS) consisting of 900 pairs of stimuli (distance 50 ms) at 1 Hz was used. The presynaptic fiber volley was measured as the amplitude to the negative peak that appears after the stimulus artifact. Synaptic activity was measured as the maximal slope of the rising phase of the fEPSP.

For long-term potentiation and for recording of AMPA and NMDA receptor mediated currents, mice were sacrificed by cervical dislocation and their brains were rapidly removed and dissected in chilled solution (4°C) containing (in mM): 87 NaCl, 2 KCl,

0.5 $CaCl_2$, 7 $MgCl_2$, 26 $NaHCO_3$, 1.25 NaH_2PO_4, 25 glucose, 75 sucrose, bubbled with a mixture of 95% O_2/5% CO_2. 370 µm thick, horizontal hippocampal slices were cut with a vibratome (VT1000S; Leica, Nussloch, Germany), preincubated for 30 min at 37°C and then transferred to a recording solution containing (in mM): 125 NaCl, 2.5 KCl, 2 $CaCl_2$, 1.3 $MgSO_4$, 26 $NaHCO_3$, 1.25 NaH_2PO_4, 10 glucose, 2 sodium pyruvate, 3 myo inositol, 0.44 ascorbic acid, bubbled with a mixture of 95% O_2/5% CO_2. Patch pipettes had resistances of 4-8 MΩ when filled with a solution containing (in mM): 117.5 $CsMeSO_4$, 2.5 CsCl, 8 NaCl, 10 HEPES, 10 TEA, 0.2 EGTA, 4 Na_2ATP, 0.6 Na_2GTP, 5 QX-314; pH was adjusted to 7.2 with CsOH. Slices were transferred to a recording chamber, which was continuously perfused at a rate of 1.5-2 ml/min with recording solution at room temperature. CA1 hippocampal neurons were visualized with DIC-infrared optics using a 60 (numerical aperture 1) water immersion objective on an upright microscope (Eclipe E600-FN; Nikon, Düsseldorf, Germany). Electrophysiological responses were recorded with an EPC 10 patch-clamp amplifier (HEKA Elektronik, Lambrecht, Germany) and PatchMaster and FitMaster software (HEKA).

For stimulation of Schaffer collaterals, monopolar glass electrodes filled with recording solution, were placed in *stratum radiatum* at the CA1 region. The stimulus was adjusted to evoke a measurable (100-200 pA), monosynaptic EPSC. AMPAR EPSCs were measured at a holding potential of -70 mV, and NMDAR EPSCS were measured at +40 mV and at 150 ms after the stimulus, at which point the AMPAR EPSC has completely decayed.

For LTP experiments, pipettes were filled with 3 M NaCl and fEPSPs were measured at a stimulus intensity that elicited an amplitude that was ~50% of maximum. Stimulation was given at a frequency of 0.033 Hz. LTP was elicited with one or four

one second 100 Hz trains, given with a 30 s pause between trains. Measurements that showed on average a potentiation less than 10% were rejected from further analysis. Data are presented as means ± S.E.M.

Behavior analysis

Mice were housed in groups of four or five in individually ventilated cages at constant temperature (22±1 °C) and humidity (50±5 %) with food and water *ad libitum*. Experiments were performed during the light period. *Morris water maze task:* Experiment was performed as described previously (Lu et al., 1997). Before starting the learning test a visible platform test was performed to exclude motor and visual acuity impairment. The platform was marked with a flag and tracking length and latency to reach the platform were recorded. Position, tracking length, swim speed and latency of finding the platform were recorded automatically by using the software Viewer (Biobserve GmbH, Bonn, Germany). *Contextual and auditory cue conditioned fear:* Experiment 1 was performed, as already described (Lu et al., 1997), in a conditioning chamber equipped with a tone stimulus, a house light and a stainless-steel rod floor through which a footshock is administered, all controlled by Freezeframe software (Coulbourn Instruments, Allentown, PA, USA). During training, mice were placed individually and allowed to explore the chamber for 120 s. A continuous tone (3600 Hz, 95 dB), which served as a conditioned stimulus (CS) was then presented for 30 s, ending concurrently with a 2 s foot shock (I = 0.75 mA), which served as the unconditioned stimulus (US). Mice were removed from the chamber 30 s later and returned to their home cage. 24 hours later, mice were re-exposed to the same chamber for 6 min for contextual memory. Then, the auditory CS test (6 min) was performed 12 hr later. During the latter test, the context of the

chamber was altered by covering the floor and walls with colored plastic, changed noise background and olfactory characteristics. During the first 3 min of the test the CS was absent (pre-CS stage), then it was turned on for 3 min. Fear responses during the contextual and CS tests were assessed by scoring the subjects' freezing response using FreezeView software (Coulbourn Instruments, Allentown, PA, USA). Freezing was defined as absence of any movement, except breathing. Sometimes slight head movements, and occasional tail rattling were observed. The animals were usually in a crouching position. Experiment 2: contextual and cued memory testing were performed 30 min after training (performed as described for experiment 1). After 6 min of re-exposing mice to the chamber (context test), mice received a pair of 60 sec tone (cue 1 and cue 2) with an ITI of 2 min. Mice were returned to their home cages 60s after cue 2. *Spontaneous alternation performances* using a Y-maze was performed as described before (Hughes, 2004). Briefly, during the 5 min testing period, total number of visited arms, the number of spontaneous alternation performance (SAP) as well as the relative numbers of alternate arm returns (AAR) or same arm returns (SAR) were scored for measuring spatial working memory performance. *Spatial working memory in a modified Y-maze:* was performed following a previously described experimental setup (Fuchs et al., 2007). Mice were maintained on a restricted feeding schedule at 85% of their free-feeding weight and were habituated to the Y-maze, and to drinking sweetened, condensed milk, over several days before testing. During testing, mice were placed in the start arm of the Y-maze and rewards (0.1 ml condensed milk) were positioned at the terminals of both non-starting arms. Each trial consisted of a sample run and a choice run. On the sample run, the mice were forced either left or right by the presence of a barrier, according to a pseudorandom sequence (with equal numbers of left and right turns per

session, and with not more than two consecutive turns in the same direction). Subsequently, the barrier was removed and, with a time interval of 15 sec, the mice were again placed in the starting arm for the choice run. The animals were rewarded for choosing the previously unvisited arm. Mice were run one trial at a time with an inter-trial interval of approximately 10 min. Each daily session consisted of 8 trials. *Hole-board analysis:* Experiment was performed essentially as described before (Van der Zee et al., 1992). The hole-board is an enclosed square arena (69.85cm x 69.85cm) made of black plexiglass containing four rows of four equidistant holes (2.5 cm diam.; 2 cm deep) in the floor plate. Food-restricted mice (85% of normal body weight) were required to locate pieces of sweet pellets (fruit loops) in 4 of 16 holes. Two days prior to training, mice are familiarized with fruit loops in their home cage and the quantity of food is restricted. Subsequently, mice were habituated to the maze by allowing them to explore and freely eat in a baited maze for 5 min in 2 separate sessions for 2 days. On session 1, mice were put in group (per cage) in the center of the arena by allowing them to search in a fully baited maze (floor and all holes). On the second habituation session, animals were placed individually inside the maze, which this time contains food pellets in the holes only (all 16). From day 2, on sessions 3 and 4, mice were put in the starting box and only when the mouse entered the arena with its four paws, the door slid down and the 3 minutes session started. Acquisition training started on the third day. Mice are subjected to a morning and afternoon session. Each session consisted of 5 trials (3 minutes each) after which animals are rewarded with a few fruit loops in their home cage. Normal food was available only at the end of the afternoon session. A total of 6 training sessions were given over a 3-day period. Animals are removed from the maze as soon as all pellets were consumed or at the end of the 3 minutes trial. After each animal, the floor and

the holes are cleaned with 70 % alcohol to remove possible odor cues. Number of working memory errors (visits to non-baited holes) was assessed for each session. *Conditioned place preference*: Experiment was performed essentially as described before (Hiroi et al., 1997). The experimental chamber consists of two large compartments with doors and distinguishable by visual (*stripes* and *circles*) and tactile cues separated by a central compartment for transition. Mice were pre-exposed, during the habituation phase, to the apparatus with free access for 20 min to all 3 compartments where mice were injected, morning and afternoon, a saline solution (0.01 ml/g body weight of 0.9% NaCl). During the conditioning phase, animals were confined, alternating the vehicle and drug pairings, to one compartment of the shuttle box with the door closed for 20 min. Twice a day for 3 consecutive days, mice were injected with either saline and placed in the saline-paired compartment (*circles)* or cocaine hydrochloride (Sigma Aldrich) (dissolved in saline solution (10 mg/kg at a volume of 0.01 ml/g body weight) and placed in the cocaine-paired compartment (*stripes)*. Post-conditioning test was conducted 24 h after the last conditioning session. The animals in a drug-free state were placed in the shuttle box with doors opened for 20 min. The time spent and distance travelled in each compartment was recorded.

Lateral Diffusion

Single particle tracking: To track the movement of receptors, quantum dots (QDs) emitting at 605 nm conjugated with goat F(ab')2 anti-rabbit or anti-mouse IgG (Invitrogen) were coupled to primary antibody directed against extracellular epitopes of AMPAR subunit GluR2 (mouse monoclonal, clone, BD Pharmingen). QDs (30 nM) were incubated with the antibody (5 nM, 30 min) and subsequently 15 min with

casein to block unspecific binding. Primary hippocampal cultures (21-24 days in vitro, DIV) were incubated for 10 min at 37°C with the pre-coupled QDs (0.06 nM) and rinsed. ChTx (Sigma Aldrich, Lyon, France) was biotinylated with the EZ-Link Sulfo-NHS-Biotin kit (Pierce, Perbio Science France, Brebières, France), using a low ratio of ChTx:biotin (1:2). Incubation of neurons with biotinylated ChTX was performed as described before (Renner et al., 2009a). Briefly, neurons were incubated for 10 min with biotinylated ChTx (2 μM), washed, and incubated for 1 min with streptavidin-coated QDs emitting at 605 nm (0.2– 0.3 nM; Invitrogen, Cergy Pontoise, France). Synapses were labeled with FM4-64 (15 s in a solution with FM and KCl 40 mM). All incubation steps and washes were performed at 37°C. Cells were imaged within 30 min after QD staining. Single particle imaging was performed as described by Bannai and colleagues (Bannai et al., 2006). Tracking and quantitative analysis was performed as previously described (Dahan et al., 2003; Ehrensperger et al., 2007).

Single molecule imaging of AMPAR: Cy5 fluorophore was coupled to anti-GluR2 antibody following the manufacturer's instructions (Amersham Biosciences, Buckinghamshire, UK). Coverslips were first incubated at 37°C for 5 min with 2 mM rhodamine 123 (Rh123; Molecular Probes, Invitrogen, Cergy Pontoise, France) and then for 10 min at room temperature with Cy5-antiGluR2 (0.1 μM). For image acquisition, an inverted microscope (Olympus IX70; Olympus France, Paris, France) with a 100X oil-immersion objective (NA = 1.4) was used. Samples were illuminated for 15 ms at 633 nm by a He-Ne laser for Cy5 detection (JDS Uniphase, Milpitas, USA). An appropriate filter set (DCLP650, HQ575/50; Chroma Technology, Roper Scientific, Evry, France) was used for detection with a CCD camera (Micromax; Princeton Instruments, Evry, France). Rh123 was imaged using a 532 nm YAG laser

(Coherent, Orsay, France) using another filter set (DCLP498, Chroma Technology; A515, Omega Optical, Brattelboro, USA). Given the resolution, trajectories with $D<10^{-2}\mu m^2/s$ were classified as immobile. *Drug treatment*: Actin filaments were depolymerized with latrunculin A (3 µM; Sigma Aldrich, Lyon, France) solubilised in DMSO (Sigma Aldrich, Lyon, France). Cells were pre-incubated for 10 min with latrunculin or the control solution (0.002% DMSO), and then incubated with Cy5-antiGluR2 diluted in latrunculin- or DMSO-containing MEMr. Recordings were performed in presence of the drugs. Actin stabilization was done by pre-incubating the neurons with jasplakinolide (Molecular Probes, The Netherlands) for 30 min at 100 nM.

Autoradiography

Freshly dissected unfixed brains were frozen on dry ice and kept at -80 °C. 16 µm coronal sections were cut by using a cryostat microtome, thaw mounted on to slides (SuperFrost Plus, Menzel-Gläser) and stored at -80 °C until further processing.

[^{3}H]-AMPA binding assay: Procedure was performed as previously described (Le Jeune et al., 1996)). In brief, coronal sections sections of two to four month old mice were equilibrated to -20 °C o/n and thereafter to 4 °C for 1 h. Subsequently, sections were incubated for 1 h in 50 mM Tris-acetic acid (pH 7.2) followed up by 45 min incubation in 50 mM Tris-acetic acid (pH 7.2) in the presence of 50 nM [^{3}H]-AMPA (PerkinElmer) and 100 mM potassium thiocyanate. Slices were washed 3 times in 100 mM potassium thiocyanate containing 50 mM Tris-acetic acid (pH 7.2) for a total rinse time of 15 s, rapidly dipped in cold distilled H_2O and kept at RT o/n for drying. After 6 weeks of exposure time at 4 °C, [^{3}H]-sensitive Hyperfilm (BioMax MS, Kodak) were analysed. Non-specific binding was determined by adding 1 mM

unlabeled L-glutamate to the incubation medium. Under these conditions, specifically bound [^{3}H]-AMPA represented at least 95 % of totally bound ligand in the various brain regions enriched with [^{3}H]-AMPA sites. *[^{3}H]-MK801 binding assay*: Experiment was performed according to a previously performed method (Glazewski et al., 1995; Le Jeune et al., 1996). In brief, sections were pre-incubated for 10 min at 4 °C in 50 mM Tris-HCl (pH 7.4) containing 2.5 mM $CaCl_2$. For labelling with [^{3}H]-MK801 (3nM; specific activity 22.0 Ci/mmol, PerkinElmer) sections were incubated for 60 min at RT in 50 mM Tris-HCl (pH 7.4) containing 5 µM glycine, 5 µM glutamate and 5 µM spermidine. Subsequent, sections were rinsed three times with ice-cold Tris-HCl over a period of 15 sec total rinse time, twice dipped into ice-cold H_2O. Finally sections were twice rapidly dipped into acetone containing 2.5% glutaraldehyde and dried o/n at RT.

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Table SI: AMPAR and ChTx diffusion coefficients.

		Diffusion coefficient ($\mu m^2/s$)			
		synaptic		extrasynaptic	
		n-cof$^{flx/flx}$	n-cof$^{flx/flx,CaMKII-cre}$	n-cof$^{flx/flx}$	n-cof$^{flx/flx,CaMKII-cre}$
AMPAR (GluR2)	**n**	158	219	341	425
	Median	0.024	ns	0.015	0.160	***	0.043
ChTx	**n**	722	601	6636	2522
	Median	0.023	ns	0.022	0.207	ns	0.199

Table SII: Explored area of AMPAR and ChTx.

		Median explored area (μm^2)			
		synaptic		extrasynaptic	
		n-cof$^{flx/flx}$	n-cof$^{flx/flx,CaMKII-cre}$	n-cof$^{flx/flx}$	n-cof$^{flx/flx,CaMKII-cre}$
AMPAR (GluR2)	**n**	163	224	348	433
	Median	0.00895 — ns —	0.00585	0.1257 — ** —	0.0244
ChTx	**n**	726	605	6640	2542
	Median	0.00845 — ns —	0.00777	0.1828 — ns —	0.1609

Table SIII: Latrunculin A and jasplakinolide dependent AMPAR diffusion coefficients.

		Diffusion coefficient ($\mu m^2/s$)					
		synaptic			extrasynaptic		
		control	latrunculin A	jasplakinolide	control	latrunculin A	jasplakinolide
AMPAR (GluR2)	**n**	118	71	80	276	787	175
	Median	0.047	0.070	0.030	0.078	0.129	0.049
		*			*		
		ns			**		