Supporting Information

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SI Materials and Methods

All animal work was approved by animal use committees at Center for Neuroscience and Cell Biology and Centro de Biología Molecular Severo Ochoa.

Materials. Rat ghrelin, the growth hormone secretagogue type 1a (GHS-R1a) antagonist [D-Lys^3]-GHRP-6, tetrodotoxin (TTX), 2-amino-5-phosphonopentanoic acid (AP5), picrotoxin, and H-89 were purchased from Tocris Bioscience. The GHS-R1a agonist MK-0677 and the phosphatidylinositol 3-kinase inhibitor LY294002 were purchased from Axon Medchem and bisindolylmaleimide IV was purchased from Sigma-Aldrich. The anti-actin (A5441), anti-tubulin (T7816), and anti-CaMKIIα (C265) antibodies were purchased from Sigma-Aldrich; the anti-Akt (9272S), anti–P-Ser473(Akt) (9271), anti-protein kinase C (PKC) substrates (2261S), and anti–PSD-95 (3450) antibodies were obtained from Cell Signaling; the anti–GHS-R1a (AB15159) (for Western blot), anti-GluA1 (AB1504), anti–P-Ser845 (GluA1) (AB5849), anti–P-Ser239/240 (stargazin) (AB15435), anti-Vglut1 (AB5905) and anti–P-Thr286/287 (α/βCaMKII) (06-881) antibodies were from Millipore; the anti–P-Ser831 (GluA1) (2041) was from Tocris Bioscience; and the anti-synaptophysin (ab32594) and the anti-MAP2 (ab5392) antibodies were from Abcam. The anti-GFP (598) antibody was purchased from MBL International, the anti–GHS-R1a (GHSR11-A) antibody (for immunocytochemistry) was from Alpha Diagnostic International, and the anti-transferrin receptor antibody (136800) was from Invitrogen. The antibody for the N-terminal of GluA1 was a kind gift from Andrew Irving (University of Dundee, Dundee, Scotland). All other reagents were purchased from Sigma-Aldrich or from Merck unless specified otherwise.

Neuronal Culture. Primary cultures of rat hippocampal neurons were prepared from the hippocampi of E18-E19 Wistar rat embryos, after treatment with trypsin [0.06% (wt/vol), 15 min, 37 °C] (GIBCO, Invitrogen) in Ca^{2+}- and Mg^{2+}-free Hanks' balanced salt solution [5.36 mM KCl, 0.44 mM monopotassium phosphate (KH_2PO_4), 137 mM NaCl, 4.16 mM sodium bicarbonate ($NaHCO_3$), 0.34 mM disodium phosphate (Na_2HPO_4)·$2H_2O$, 5 mM glucose, 1 mM sodium pyruvate, 10 mM Hepes, and 0.001% (wt/vol) phenol red]. Hippocampal cells were washed with Hanks' balanced salt solution six times. The cells were mechanically dissociated and then plated in six-well plates (8.9×10^4 cells per cm^2) coated with poly-D-lysine (0.1 mg/mL) for biochemical purposes or at a final density of 3×10^5 cells per dish on poly-D-lysine-coated coverslips in 60-mm culture dishes for imaging purposes. The cells were plated in neuronal plating medium [minimum essential medium (MEM); GIBCO, Invitrogen) supplemented with 10% (vol/vol) horse serum (GIBCO, Invitrogen), 0.6% (wt/vol) glucose, and 1 mM pyruvic acid]. Once neurons attached to the substrate, after 2–4 h, in case of high-density cultures, the neuronal plating medium was replaced by neuronal culture medium containing neurobasal medium (GIBCO, Invitrogen,) supplemented with B27 supplement (1:50 dilution; GIBCO, Invitrogen,), 25 μM glutamate, 0.5 mM glutamine, and 0.12 mg/mL gentamycin (GIBCO, Invitrogen). The coverslips were flipped over an astroglial feeder layer in 60-mm culture dishes containing neuronal culture medium. These neurons grew face down over the feeder layer but were kept separate from the glia by wax dots on the neuronal side of the coverslips. To prevent overgrowth of glia, neuron cultures were treated with 5 μM cytosine arabinoside after 3 d. Cultures were maintained in a humidified incubator of 5% (vol/vol) CO_2/95% (vol/vol) air at 37 °C, feeding the cells once per week by replacing one-third of the medium per well or dish, using neuronal culture medium without glutamate. Cultures were used after 7, 15, 16, 19, or 21 d.

Hippocampal Slice Preparation and Culture and Sindbis Virus Expression. Hippocampal slices were prepared from young Wistar rats of either sex (postnatal day 5–6) as previously described (1). Briefly, after dissection of the hippocampi in ice-cold gassed [5% (vol/vol) CO_2/95% (vol/vol) O_2] dissection solution (in millimolars: 10 glucose, 4 KCl, 24 $NaHCO_3$, 234 sucrose, 0.5 $MgCl_2$·$6H_2O$, 0.7 $CaCl_2$·$2H_2O$, 0.03 phenol red, at pH 7.4), 400-μm transverse slices were prepared using a tissue slicer. Slices were transferred to slice culture inserts (Millipore) and cultured in culture medium [(MEM; GIBCO, Invitrogen) supplemented with 20% (vol/vol) horse serum (GIBCO, Invitrogen), 1 mM glutamine, 1 mM $CaCl_2$, 2 magnesium sulfate ($MgSO_4$), 1 mg/L insulin, 0.0012% (wt/vol) ascorbic acid, 30 mM Hepes, 13 mM glucose, 5.2 mM $NaHCO_3$, at pH 7.25, and final osmolarity of 320 mOsm/L]. Cultures were maintained in a humidified incubator of 5% (vol/vol) CO_2/95% (vol/vol) air at 35.5 °C, and the culture medium was replaced every 2–3 d. The recombinant GluA1-GFP was delivered into slices using Sindbis virus after 1–2 in culture, as previously described (2). Recombinant protein expression was typically for 2 d.

Cell-Surface Protein Biotinylation in Acute Hippocampal Slices. Hippocampal transverse slices (350 μm) obtained from C57BL/6 mice 8–10 wk old were prepared using a tissue slicer. Slices (five per condition) were incubated for 2 h at 37 °C in artificial cerebrospinal fluid (ACSF) [in millimolars: 124 NaCl, 3 KCl, 2 $CaCl_2$, 25 $NaHCO_3$, 1.1 monosodium phosphate (NaH_2PO_4), 2 $MgSO_4$, 10 D-glucose, pH 7.4 (control condition)], or 1 μM MK-0677 in ACSF, under continuous gasification with 5% (vol/vol) CO_2/95% (vol/vol) O_2. Slices were transferred to ACSF solution containing 0.3 mg/mL NHS-SS-biotin (Pierce, Thermo Fisher Scientific) and kept on ice with agitation for 45 min. Slices were then incubated in ACSF with 1 μM lysine to block all reactive NHS-SS-biotin in excess. Slices were then collected in 500 μL of lysis buffer [1% (vol/vol) Triton X-100, 0.1% (vol/vol) SDS, 1 mM EDTA, 50 mM NaCl, 20 mM Tris, pH 7.5, supplemented with 1 mM DTT, 0.1 mM phenylmethylsulfonyl fluoride (PMSF), 1 μg/mL chymostatin, 1 μg/mL leupeptin, 1 μg/mL antipain, 1 μg/mL pepstatin] and sonicated on ice for 30 s. To discard extra debris, homogenates were centrifuged for 5 min at 4 °C at 16,200 × *g* and supernatants collected. Protein concentration was quantified by the bicinchoninic acid (BCA) method (Pierce, Thermo Fisher Scientific), and the same amount of protein (500 μg) was used in all experimental conditions. To precipitate biotinylated proteins, 80 μL of Ultra-link immobilized neutravidin plus beads (Pierce, Thermo Fisher Scientific) were added to samples for 2 h at 4 °C, under constant agitation. Nonbiotinylated proteins were removed by centrifugation at 2,500 × *g* for 2 min, and beads were washed three times with lysis buffer. Biotinylated proteins were eluted with denaturing buffer at 95 °C for 5 min. Samples were processed for Western blotting analysis. To ensure that only cell-surface proteins were labeled with biotin, Western blot against actin was performed for all biotinylated samples (data not shown).

Biochemistry. Protein extracts were prepared in lysis buffer [10 mM Hepes (pH 7.4); 150 mM NaCl; 10 mM EDTA; 1% (vol/vol) Triton X-100 supplemented with 1 mM DTT; 0.1 mM PMSF; 1 μg/mL chymostatin, 1 μg/mL leupeptin, 1 μg/mL antipain, and 1 μg/mL pepstatin (CLAP); and a mixture of phosphatase inhibitors (1×, Roche)]. After centrifugation at 16,100 × *g* for 10 min at 4 °C, protein in the supernatant was quantified using the BCA assay kit (Pierce, Thermo Fisher Scientific), and the samples were denatured with 5× concentrated denaturating buffer [62.5 mM Tris·HCl (pH 6.8), 10% (vol/vol) Glycerol, 2% (vol/vol) SDS, 0.01% (wt/vol) bromophenol blue, and 5% (vol/vol) β-mercaptoethanol (added fresh)], and boiled for 5 min. Protein extracts were resolved by SDS/PAGE in 7.5% (vol/vol) or 12% (vol/vol) polyacrylamide gels. For Western blot analysis, proteins were transferred onto a PVDF membrane (Millipore) by electroblotting (40 V, overnight at 4 °C). The membranes were blocked for 1 h at room temperature in Tris-buffered saline (137 mM NaCl, 20 mM Tris·HCl, pH 7.6) containing 0.1% (vol/vol) Tween-20 (TBS-T), and 5% (wt/vol) low-fat milk or BSA. Membranes were probed during 1 h, at room temperature, or overnight, at 4 °C, with the primary antibodies diluted in TBS-T containing 5% or 0.5% (wt/vol) low-fat milk or 5% (wt/vol) BSA. Following several washes, membranes were incubated for 1 h with alkaline phosphatase-conjugated secondary antibodies (anti-mouse or anti-rabbit, depending on the primary antibody host species) at room temperature, washed again, and incubated with enhanced chemifluorescence (ECF) substrate (GE Healthcare) for 5 min at room temperature. Membranes were scanned with the Storm 860 scanner (GE Healthcare), and quantified using the ImageQuant software under linear exposure conditions. When necessary, the membranes were stripped (0.2 M NaOH for 5 min) and reprobed.

Subcellular Fractionation. The procedure for purification of synaptossomes was adapted from ref. 3. Two hundred milligrams of hippocampi, dissected from adult Wistar rats, were collected and homogenized in a motor-driven glass Teflon homogenizer [30 stokes, 900 rpm (Heidolph, mechanical overhead stirrer RZR 1), at 4 °C] in Hepes-buffered sucrose (HBS) buffer [0.32 M sucrose, 4 mM Hepes (pH 7.4)] supplemented with protease and phosphatase inhibitors [0.2 mM PMSF, 1 μg/mL CLAP, 0.1 mM sodium orthovanadate (Na_3VO_4), and 50 mM NaF]. The hippocampal homogenate was collected and centrifuged at 900 × *g* for 15 min, at 4 °C, to obtain the nonnuclear fraction (S1). The resultant supernatant was centrifuged at 18,000 × *g* for 15 min, at 4 °C, to yield the crude synaptossomal pellet (P2). P2 was resuspended in HBS (nonsupplemented with Na_3VO_4 and supplemented also with the protease inhibitor 1 mM DTT) and centrifuged at 18,000 × *g* for 15 min, at 4 °C, to yield the washed crude synaptossomal fraction. Protein concentrations were determined using the BCA assay kit (Pierce, Thermo Fisher Scientific), and samples were denatured with 5×-concentrated denaturating buffer, warmed for 5 min at 95 °C, and separated by SDS/PAGE using an equal amount of protein for each fraction.

Neuron Transfection. GFP-tagged GHS-R1a (GHS-R1a-GFP) (4) was recombinantly expressed in primary cultures of hippocampal neurons at 9 d in vitro (DIV) using the calcium phosphate transfection protocol adapted from ref. 5. The GHS-R1a-GFP plasmid (4 μg per coverslip) was diluted in Tris·EDTA transfection buffer (10 mM Tris·HCl and 2.5 mM EDTA, pH 7.3). Briefly, a $CaCl_2$ solution (2.5 M in 10 mM Hepes) was then added, drop-wise, to the plasmid DNA-containing solution to give a final concentration of 250 mM $CaCl_2$. This was then added to an equivalent volume of Hepes-buffered transfection solution (274 mM NaCl, 10 mM KCl, 1.4 mM Na_2HPO_4, 11 mM dextrose, 42 mM Hepes, pH 7). The mixture was vortexed gently for 2–3 s, and the precipitate was allowed to develop at room temperature for 30 min, protected from light, and vortexed every 5 min. The precipitated DNA was added drop-wise to the coverslips, and the cultures were incubated with the precipitate for 1–3 h in the presence of kynurenic acid (2 mM). Each coverslip was transferred to a fresh well of the 12-well plate containing 1 mL of conditioned culture medium with kynurenic acid (2 mM), slightly acidified with HCl (~5 mM final concentration), and the plate was returned to a 37 °C, 5% (vol/vol) CO_2/95% (vol/vol) air incubator for 10–15 min. Coverslips were then transferred to the original dish containing the conditioned medium. The cells were then returned to a 37 °C, 5% (vol/vol) CO_2/95% (vol/vol) air incubator to allow expression of the transfected construct. Protein expression was typically for 7 d.

Immunocytochemistry. For labeling surface GluA1-containing AMPA receptors (GluA1-AMPARs), live neurons were incubated for 10 min at room temperature using an antibody against an extracellular epitope in the GluA1 N terminus diluted in conditioned neuronal culture medium or extracellular solution (ECS) [used for chemical long-term potentiation (cLTP)]. Neurons were then fixed for 15 min in 4% (wt/vol) sucrose and 4% (wt/vol) paraformaldehyde in PBS (PBS: 137 mM NaCl, 2.7 mM KCl, 1.8 mM KH_2PO_4 and 10 mM $Na_2HPO_4{\cdot}2H_2O$, pH 7.4) at room temperature, and permeabilized with PBS + 0.25% (vol/vol) Triton X-100 for 5 min, at 4 °C. Neurons were then incubated in 10% (wt/vol) BSA in PBS for 30 min at 37 °C to block nonspecific staining, and incubated in appropriate primary antibody diluted in 3% (wt/vol) BSA in PBS (2 h, 37 °C or overnight, 4 °C). After washing 6 times in PBS, cells were incubated with the secondary antibody diluted in 3% (wt/vol) BSA in PBS (45 min, 37 °C). The coverslips were mounted using fluorescent mounting medium from DAKO.

cLTP Protocol. cLTP was induced as previously described (6). Nineteen-day in vitro hippocampal cultures were thoroughly washed with ECS containing (in millimolars): 150 NaCl, 2 $CaCl_2$, 5 KCl, 10 Hepes, 30 Glucose, 0.001 TTX, 0.01 strychnine, 0.03 picrotoxin, pH 7.4. After washing, neurons were stimulated with or without glycine (300 μM) at room temperature for 3 min in ECS and then incubated for 20–25 min in ECS (no added glycine) in a 37 °C, 5% (vol/vol) CO_2/95% (vol/vol) air incubator. Surface GluA1-AMPARs were labeled, fixed, and probed as described above.

Electrophysiology. For recordings of evoked synaptic responses, a cut was made between CA3 and CA1 to minimize recurrent activity. The recording chamber was perfused with external solution (in millimolars: 119 NaCl, 2.5 KCl, 1 NaH_2PO_4, 11 glucose, 26 $NaHCO_3$, 4 $MgCl_2$, 4 $CaCl_2$, and 0.004 2-chloroadenosine, at pH 7.4), and was gassed with 5% (vol/vol) CO_2/95% (vol/vol) O_2. Some recordings included AP5 (100 μM) or picrotoxin (100 μM) to block NMDA or $GABA_A$ receptors, respectively. For voltage-clamp recordings, patch-recording pipettes (3–6 MΩ) were filled with internal solution (in millimolars: 115 cesium methanesulfonate, 20 CsCl, 10 Hepes, 2.5 $MgCl_2$, 4 Na_2ATP, 0.4 Na_3GTP, 10 sodium phosphocreatine and 0.6 EGTA, at pH 7.25). For current-clamp recordings, the internal solution contained (in millimolars) 115 K gluconate, 20 KCl, 10 Hepes, 2 $MgCl_2$, 4 Na_2-ATP, 0.3 Na_3-GTP, at pH 7.25. Synaptic responses were evoked with bipolar electrodes using single-voltage pulses (200 μs, up to 20 V). The stimulating electrodes were placed over the Schaffer collateral fibers between 300 and 500 μm from the CA1-recorded cells. Synaptic responses were averaged over 50 trials. For recordings of spontaneous activity, no cut was made between CA3 and CA1, 100 μM picrotoxin was added to the external solution, 2-chloroadenosine was omitted, and $MgCl_2$ and $CaCl_2$ concentrations were changed to 1.2 and 2 mM, respectively. Whole-cell recordings were carried out with a Multiclamp 700A amplifier (Molecular Devices).

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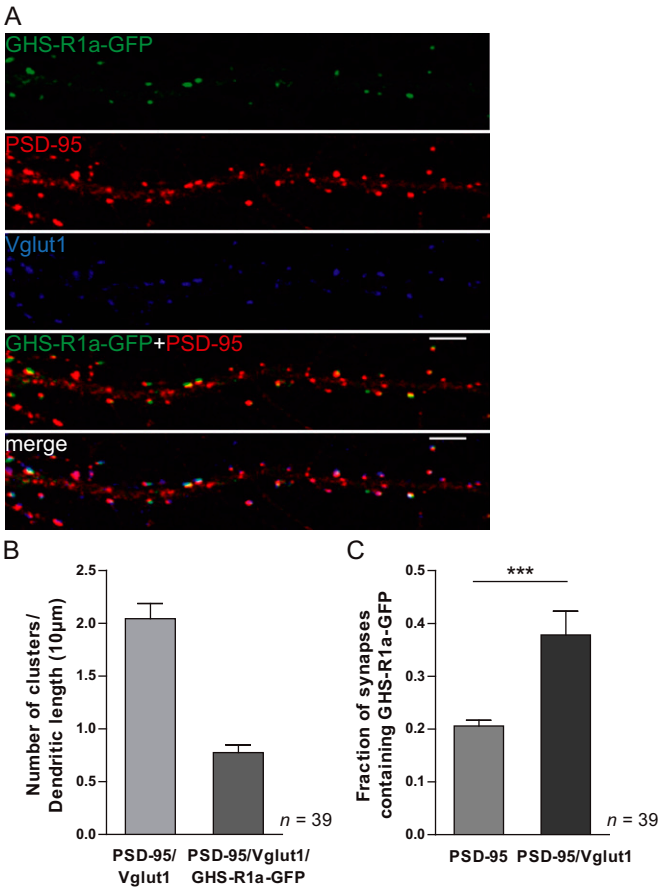

Fig. S1. GHS-R1a localizes preferentially to the vicinity of functional synapses. (*A*) Representative immunofluorescence images of hippocampal neurons in culture transfected with a GFP-tagged GHS-R1a construct. Neurons were stained for GFP, PSD-95, and Vglut1. (Scale bars: 5 μm.) (*B*) Quantification of the number of dendritic clusters per dendritic length that are positive for both PSD-95 and Vglut1 (functional synapses) and of the number of functional glutamatergic synapses that contain GHS-R1a-GFP (PSD-95/Vglut1/GHS-R1a-GFP). *n* represents the total number of analyzed cells in three independent experiments. Error bars represent SEM. (*C*) GHS-R1a-GFP is preferentially localized to the vicinity of functional excitatory synapses. The fraction of synapses containing GHS-R1a-GFP was calculated by evaluating the presence of GHS-R1a-GFP at regions of overlap between PSD-95 and Vglut1 clusters, or at PSD-95 clusters. *n* represents the total number of analyzed cells in three independent experiments. Error bars represent SEM. The statistical significance was calculated using the Mann–Whitney test (***$P < 0.001$).

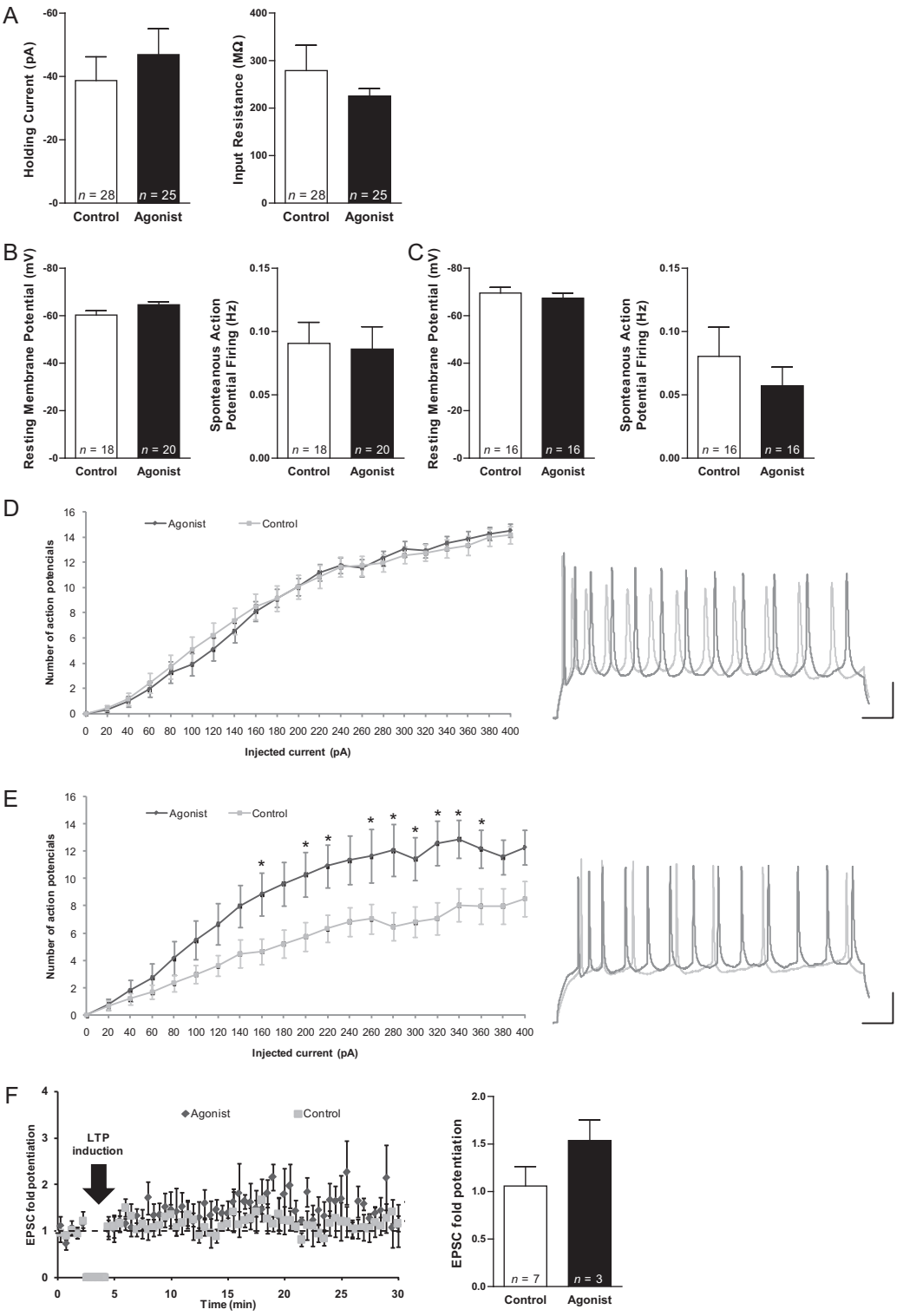

Fig. S2. MK-0677 treatment did not change neuronal passive membrane properties or spontaneous activity, and did not have an effect on the nonpotentiated (unpaired) pathway. (*A*) Effects of ghrelin receptor agonist MK-0677 treatment on passive membrane properties of CA1 hippocampal neurons. Application of MK-0677 to cultured hippocampal slices did not alter input resistance (*Right*) or holding current (*Left*), compared with untreated control neurons. (*B* and *C*) Effect of MK-0677 treatment on resting membrane potential (*Left*) and spontaneous action potential firing (*Right*) of CA1 (*B*) and CA3 (*C*) neurons. (*D* and *E*, *Left*) Effect of MK-0677 treatment on the number of action potentials fired after current injection [frequency-intensity (f-I) curves] of CA1 (*D*) and CA3 (*E*) neurons. Representative traces are shown (*D* and *E*, *Right*). (Scale bars: vertical 20 mV; horizontal, 100 ms.) (*F*, *Left*) Time course of normalized AMPAR-mediated synaptic responses for the unpaired pathway, before and after LTP induction. For simplicity, each time point in the plot corresponds to the average of six consecutive stimulations (sampling rate: 0.2 Hz). Organotypic slice cultures were incubated in culture medium, or in medium containing the ghrelin receptor agonist MK-0677 (1 μM, 20 h). (*F*, *Right*) Plot shows quantification of average synaptic potentiation from the unpaired pathway 20–30 min after LTP induction. (*A*–*F*) Error bars represent SEM. The statistical significance was calculated using the Mann–Whitney test (*$P < 0.05$). *n* represents the number of cells.

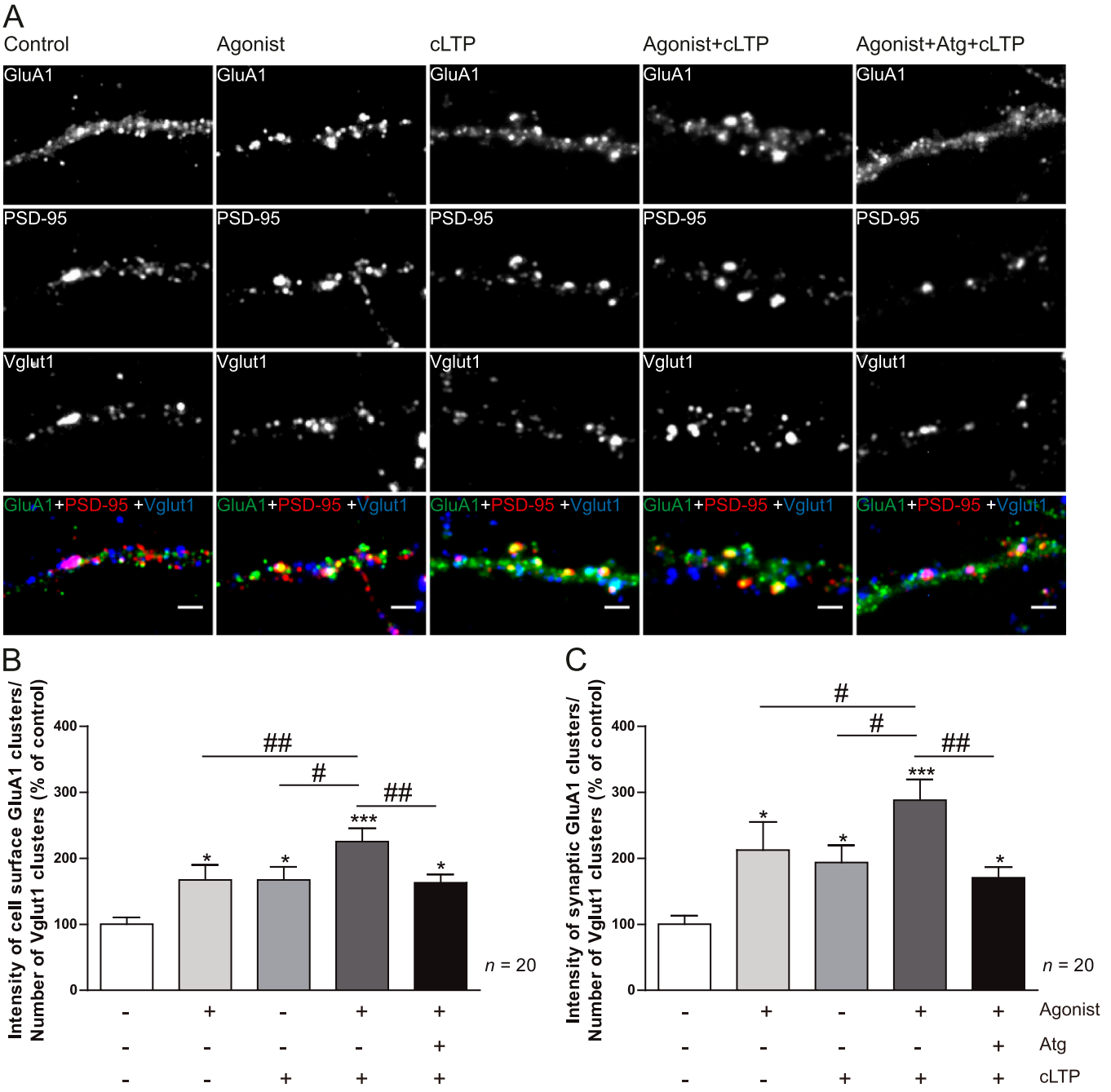

Fig. S3. Ghrelin receptor activation increases the chemical LTP-induced delivery of GluA1-AMPAR to synapses. (*A*) Nineteen-day in vitro hippocampal neurons in culture were submitted to the following stimuli: ghrelin receptor agonist MK-0677 1 μM for 20 h, cLTP (300 μM glycine for 3 min in the absence of Mg^{2+}), cLTP in neurons treated with the ghrelin receptor agonist MK-0677, or simultaneously with the ghrelin receptor agonist and antagonist [D-Lys^3]-GHRP-6 (Atg; 100 μM). Neurons were kept at 37 °C for 20 min (without glycine) and were live stained for GluA1 using an antibody against an extracellular epitope in the GluA1 N terminus. After fixation, neurons were stained for PSD-95, Vglut1, and MAP2. Synaptic GluA1 was defined as the GluA1 signal overlapping with Vglut1. (Scale bars: 2 μm.) Neurons were analyzed for the total GluA1 cell-surface fluorescence intensity (*B*) and for the GluA1 synaptic cluster fluorescence intensity (*C*) per number of Vglut1 clusters per dendritic length. (*B* and *C*) Results are expressed as the percentage of control cells, and are averaged from two independent experiments. Error bars represent SEM. The statistical significance was calculated using the Kruskal–Wallis test ($P < 0.0001$) followed by Dunn's multiple comparison test ($^{*}P < 0.05$ and $^{***}P < 0.001$). Comparisons between pairs were performed using the Mann–Whitney test ($^{\#}P < 0.05$ and $^{\#\#}P < 0.01$). *n* represents the number of cells.

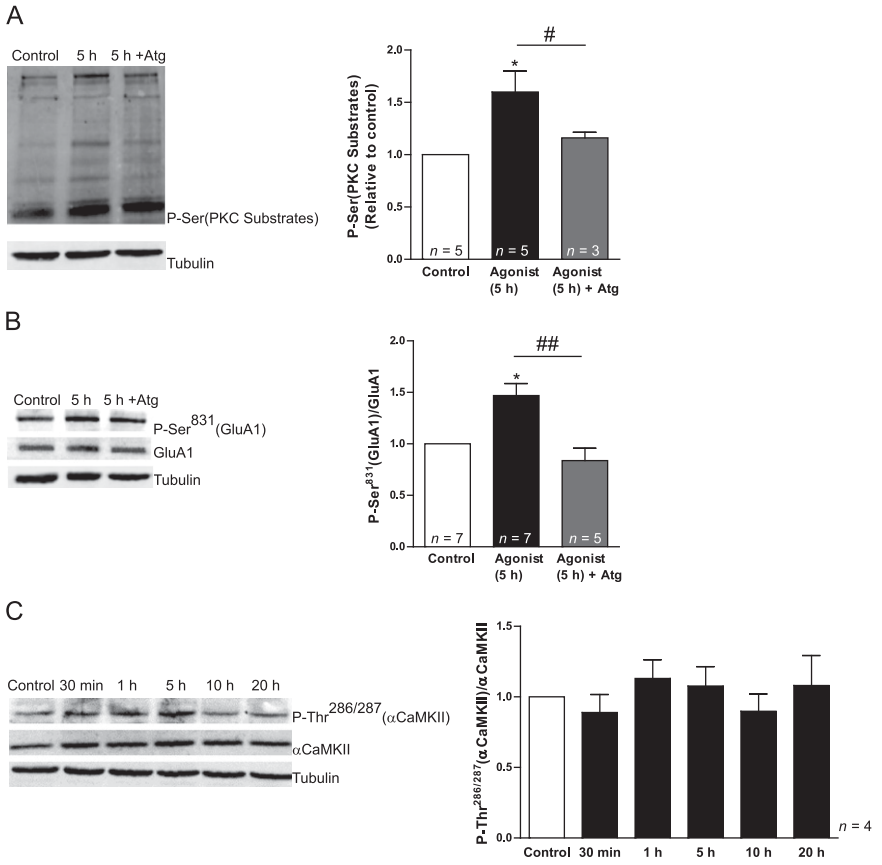

Fig. S4. Ghrelin receptor activation in organotypic hippocampal slices activates PKC signaling pathway and triggers changes in GluA1 phosphorylation. (*A–C, Left*) Western blot analysis of protein extracts from hippocampal slices incubated with culture medium or with medium containing the ghrelin receptor agonist MK-0677 (1 μM) for the indicated periods of time, or with MK-0677 in the presence of the ghrelin receptor antagonist [D-Lys^3]-GHRP-6 (Atg; 100 μM). The primary antibodies detected phosphorylation of endogenous proteins at phospho-Ser PKC substrate motifs (*A*), phosphorylation of GluA1 at Ser^{831} (*B*) and phosphorylation of αCaMKII at Thr$^{286/287}$ (*C*). Total GluA1 and total αCaMKII were also detected and tubulin was used as a loading control. (*A–C, Right*) The graphs represent the quantification of band intensities relative to control extract. Error bars represent SEM. The statistical significance was calculated using one-way analysis of variance ($P = 0.0240$ and $P = 0.0042$) followed by Dunnett's multiple comparison test ($^*P < 0.05$). Comparisons between pairs were performed using the Student *t* test ($^{\#}P < 0.05$ and $^{\#\#}P < 0.01$). *n* represents the number of independent experiments.