Supporting Information

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SI Materials and Methods

Hippocampal Neuronal Culture. Whole hippocampi were dissected from embryonic day 18 (E18) Sprague–Dawley rats, dissociated in calcium- and magnesium-free Hanks' Buffered Salt Solution containing 0.125% trypsin for 15 min, triturated in Dulbecco's modified Eagle's medium (DMEM) (Invitrogen)/10% fetal bovine serum (FBS), and plated at 0.4 million cells per well in 6-well plates or 0.1 million cells per well per 24-well plates for immunostaining. Cells were grown at 37 °C, 5% CO_2, and 95% humidity, first in 10% FBS/DMEM, followed 1 d later by serum-free medium and Neurobasal plus B27 (B27NB; Invitrogen). Cultures were grown in serum-free medium for 10–12 d before the start of the experiments, and the medium was changed every 3 d. Fresh medium was applied 24 h before each experiment. These cultures yielded virtually all neurons.

Fluorescent Immunostaining for Surface GluR1. Rat hippocampal cultures were treated with AR-A014418 (generously donated by AstraZeneca Pharmaceuticals; 2 μM, 10 μM) for 1 or 48 h at 37 °C. After addition of α-amino-3-hydroxyl-5-methyl-4-isoxazole-propionate (AMPA) (100 μM; Tocris) for 15 min, surface immunostaining was performed. After treatment, cells were blocked with 0.5% BSA in B27NB medium for 10 min at 37 °C, followed by treatment with rabbit anti-GluR1 N-terminal antibody (1:10; Oncogene Research) in 0.1% BSA in B27NB for 10 min. Cells were washed with neurobasal medium and fixed with 4% paraformaldehyde in PBS for 1 h on ice. After being washed with PBS three times, cells were incubated with Cy3 goat anti-rabbit antibody (1:30; Jackson ImmunoResearch) in 5% goat serum in PBS for 1 h in the dark at 37 °C, washed with B27NB three times, and mounted with a Prolong Antifade kit (Molecular Probes). *Z*-stack images were acquired by 510 Meta confocal microscope (Carl Zeiss MicroImaging) under exactly the same conditions. To acquire high-quality images for quantification, we took only five confocal images randomly from each coverslip. The cell body and longest dendrite from every neuron in the image treated in each group was circled for quantification. Fluorescent intensities of GluR1 (red) of the cell body and the individual longest dendrite in each image were determined using 510 Meta software (Carl Zeiss MicroImaging).

Peptide Design and Synthesis. The peptides TAT-KCLpCDK, TAT-KLC, and TAT-Con were designed and synthesized to block the phosphorylation of KLC2 by glycogen synthase kinase 3 (GSK-3) kinase [TAT-KLCpCDK (33 aa): YGRKKRRQRR-RLSDSRTL*S* (GSK-3β site) SSSMDLSRRS (p) *S* (CDK5 site) LVG; TAT-KLC (33 aa): YGRKKRRQRRR-LSDSRTL*S*SSSMDLSRRS*S*LVG; and TAT-Con (33 aa): YGRKKRRQRRR-LSDSRTLASSSM-DLSRRSALVG; phosphoglycogen synthase peptide-2: YRR-AAVPPSPSLSRHSSPHQS(p)EDEEE]. The TAT sequence, an HIV sequence able to deliver peptides into the brain, passes the blood–brain barrier (Celtek Bioscience). TAT-KCLpCDK peptide is phosphorylated at its CDK-5 site to provide the docking position for GSK-3 kinase. The TAT-Con sequence containing the same sequence with mutated GSK-3β and CDK5 motifs (serine to alanine) was also synthesized to serve as a control.

Surface Biotinylation and Western Blot Analysis of GluR1 and GluR2. Hippocampal neurons (12 DIV) were treated with the GSK-3 inhibitor AR-A014418 (10 μM) or SB-216763 (1 μM) for 1 h. Next, either AMPA (100 μM), N-methyl-D-aspartate (NMDA) (50 μM)/glycine (10 μM), or insulin (30 nM) were added to the medium for an additional 30 min. To study the effect of TAT peptide on internalization, peptide treatment concentrations were maintained at 80 μM for 1 h followed by AMPA (100 μM) stimulation for an additional 30 min. For the cAMP-induced insertion, neurons (12 DIV) were treated with AR-A014418 (10 μM) for 1 h followed by Sp-cAMP (50 μM) treatment for an additional 30 min. After treatment, ice-cold PBS with calcium and magnesium (pH 7.4; Invitrogen) was added to the cultures to prevent receptor internalization. After three washes with ice-cold PBS, cells were incubated in sulfo-NHS-LC-biotin (Pierce; 0.25 mg/mL in cold PBS) for 30 min. Surface biotinylation was stopped by removing the above solution and incubating with 10 mM ice-cold glycine in PBS (PBS) for 20 min. Cells were then washed three times with cold PBS and lysed with RIPA (radioimmunoprecipitation assay) buffer (20 mM Hepes, pH 7.4, 100 mM NaCl, 1 mM EGTA, 1 mM Na orthovanadate, 50 mM NaF, 1% Nonidet P-40, 1% deoxycholate, 0.1% SDS plus protease inhibitor mixture (Sigma) and phosphatase inhibitor mixtures I & II (Sigma). Equal amounts of biotinylated proteins were precipitated with 100 μL of ImmunoPure immobilized streptavidin (Pierce). Biotinylated proteins were separated on a 10% SDS/PAGE gel and transferred to a nitrocellulose membrane. The membranes were probed with a polyclonal anti-GluR1 antibody (1:200; Chemicon) and anti-GluR2 antibody (1:200; Chemicon) followed by peroxidase-conjugated goat anti-rabbit or goat anti-mouse IgG (1:2,000; Vector). To assess β-catenin levels, protein cell homogenates were separated by 4–20% gels and probed by rabbit anti-β-catenin antibody (Abcam). Immunoreactive bands were visualized by electrochemiluminescence (ECL; GE Healthcare). ECL signal intensities were quantified by a Kodak Image program (Eastman Kodak).

Surface Receptor Cross-Linking with Bis[Sulfosuccinimidyl] Suberate to Distinguish Surface and Intracellular AMPA Receptors in Vivo. Male c57/B6 mice (7–8 wk old, starting weight 25–30 g; Harlan) were housed three to four per cage in a 12-h light/dark cycle with free access to water and food. After a 1-wk accommodation period, the mice were treated with the GSK inhibitor AR-A014418 dissolved in dimethyl sulfoxide (DMSO). Control animals were injected with the same amount of DMSO. TAT-peptide-treated mice were implanted with a minipump, and either TAT-KLCpCDK or TAT-Con was infused for 12–13 d (see the detailed methods below). All mice were killed by decapitation during the morning hours and immediately processed for cross-linking studies (n = 6 per group). Bis[sulfosuccinimidyl] suberate (BS3) selectively cross-links cell-surface receptors, forming high molecular weight aggregates, but does not modify intracellular receptors. Thus, surface and intracellular receptor pools can be distinguished based on molecular weight using SDS/PAGE and Western blotting. Our control experiments validated the use of the high molecular weight band as a measure of surface-expressed proteins and confirmed that BS3 does not access intracellular antigens, as would be predicted from its chemical structure.

After injection with A014418, mice were decapitated. Brains were rapidly removed, prefrontal cortex and hippocampal tissues were isolated, and bilateral pieces of prefrontal cortex or hippocampal tissue from each rat were chopped into 400-mm slices using a McIllwain tissue chopper (The Vibratome Company). Slices were added to Eppendorf tubes containing ice-cold artificial cerebrospinal fluid (ACSF) spiked with 2 mM BS3 (Pierce). Incubation with gentle agitation proceeded for 30 min at 4 °C. Cross-linking was terminated by quenching the reaction with 100 mM glycine (10 min, 4 °C). The slices were pelleted by brief centrifugation (1,000 × g, 2 min), and the supernatant was discarded. Pellets were resuspended in ice-cold lysis buffer containing protease

and phosphatase inhibitors [25 mM Hepes, pH 7.4, 500 mM NaCl, 2 mM EDTA, 1 mM DTT, 1 mM phenylmethyl sulfonyl fluoride, 20 mM NaF, 1 mM sodium orthovanadate, 10 mM sodium pyrophosphate, 1 mM microcystin-LF, 1 mM okadaic acid, 1× protease inhibitor mixture (Calbiochem), and 0.1% Nonidet P-40 (vol/vol)] and homogenized rapidly by sonicating for 5 s. Total protein concentration of lysates was determined. Samples were aliquoted (15 aliquots per rat) and stored at –80 °C for future analysis. After Western blot analysis with anti-GluR1 antibody, data were analyzed by paired Student's *t* test.

Immunoprecipitation. Hippocampal neurons or rat hippocampal tissue were lysed or sonicated with RIPA buffer [20 mM Hepes, pH 7.4, 100 mM NaCl, 1 mM EGTA, 1 mM Na orthovanadate, 50 mM NaF, 1% Nonidet P-40, 1% deoxycholate, 0.1% SDS plus protease inhibitor mixture (Sigma), and phosphatase inhibitor mixtures I & II (Sigma)]. After setting on ice for 30 min, the cell lysates were centrifuged for 10 min at 140,000 × g. The supernatant was collected and transferred to another tube. The protein sample (40–120 μg) was preincubated with 50 μL of protein G Sepharose slurry (GE Healthcare) or 25 μL of protein A/G agarose beads (Santa Cruz Biotechnology) and rotated at 4 °C for 1 h. After mixing, the sample was centrifuged and the supernatant (unbound fraction) was collected. The precleared protein was incubated with either 4–8 μg of mouse anti-KLC2 antibody (raised against the C terminus of KLC2; Abcam), 4–8 μg of rabbit anti-GluR1 IgG (Chemicon), 4–8 μg of mouse anti-Bcl-2 IgG (Abcam), or normal rabbit IgG (Santa Cruz Biotechnology) overnight at 4 °C before adding 50 μL of protein G Sepharose slurry for 4 h or 25 μL of protein A/G agarose beads for 2 h at 4 °C. The beads were then washed three times in ice-cold lysis buffer and resuspended in an adjusted volume of protein sample buffer. Fractions were then boiled for 10 min, separated by 4–15% gradient SDS/PAGE, and transblotted to nitrocellulose membranes for immunodetection with anti-GluR1 antibody (Chemicon), anti-phosphoserine antibody (1:1,000; Abcam), or anti-KLC antibody (1:200; Abcam). The total protein samples were subjected to Western blot analysis with anti-TauS396 (a GSK-3 site) antibody (1:200; Abcam).

Electrophysiological Recording. Hippocampal slices (400-μm thickness) were prepared from Sprague–Dawley rats aged 16–23 postnatal d, and placed in an incubation chamber at 34 °C for 1.5 h before recording. A single slice was transferred to a recording chamber and perfused at room temperature with ACSF containing 125 mM NaCl, 2.5 mM KCl, 1 mM $MgCl_2$, 2 mM $CaCl_2$, 1.25 mM KH_2PO_4, 26 mM $NaHCO_3$, and 20 mM glucose and bubbled with 95% O_2/5% CO_2. Whole-cell recordings of CA1 neurons were performed using the "blind" method with a MultiClamp 700A amplifier (Axon Instruments) at holding potential –60 mV. The recording pipettes (~6 MΩ) were filled with intracellular solution containing 122.5 mM Cs-gluconate, 17.5 mM $CsCl_2$, 2 mM $MgCl_2$, 10 mM Hepes, 0.5 mM EGTA, 4 mM K-ATP, and 5 mM QX-314, with pH adjusted to 7.2 by CsOH (290–300 mOsm) with or without TAT-KLCpCDK (40 μM) or TAT-Con peptide (40 μM). Series and input resistance were monitored throughout each experiment and cells were excluded from data analysis if a greater than 20% change in the series or input resistance occurred during the course of the experiment. Excitatory postsynaptic currents (EPSCs) were evoked every 30 s by stimulation (0.05-ms duration) of the Schaffer collateral-commissural pathway with a bipolar tungsten electrode in the presence of bicuculline methiodide (10 μM) (to block $GABA_A$ receptor-mediated inhibitory synaptic currents). Peptides tested were dissolved (40 μM) in the intracellular solution. After break-in, 10 min of baseline EPSCs were recorded. After obtaining a stable EPSC baseline, long-term potentiation (LTP) was induced by paired protocol, 200 pulses at 2 Hz while holding at –5 mV, or long-term depression (LTD) was induced by 300 pulses at 1 Hz while holding at –45 mV. The stimulation intensity of induction was the same as that used during baseline recording. After LTP or LTD induction, the holding potential was switched back to –60 mV and EPSC recordings at the baseline stimulus rate were recorded for 1 h thereafter.

GSK-3β Kinase Assay. Recombinant rat GSK-3β activity was assayed in a total volume of 50 μL of kinase buffer containing 8 mM Mops (pH 7.4), 10 mM $MgCl_2$, 1 mM DTT, 250 μM ATP, 2 μCi of [γ-^{32}P] ATP, and 100 μM phosphoglycogen synthase peptide-2 (YRRAAVPPSPSLSRHSSPH QSEDEEE), TAT-KLC peptide, TAT-KLCpCDK peptide, or TAT-Con control peptide, and incubated at 30 °C for 30 min. Reactions were terminated by putting the samples on ice and spotting them on the paper. Filter papers were washed three times in 60–100 mL 0.75% phosphoric acid for 15 min, once more in acetone for 5 min, dried, and then counted in a liquid scintillation counter.

Behavioral Tests. All animal treatments, procedures, and care were approved by the National Institute of Mental Health Animal Care and Use Committee and followed the *Guide for the Care and Use of Laboratory Animals* (ISBN 0-309-05377-3) (1). Male Swiss CD1 mice (8–9 wk old, starting weight 35–45 g; Harlan) were housed individually in a 12-h light/dark cycle and had free access to water and food.

Surgery to implant minipumps. Instruments used to perform stereotaxic surgeries were equipped with a gas anesthesia mask (David Kopf Instruments) for delivery of isoflurane at a flow rate of 5 liters per minute (lpm) for anesthetic induction and 2 lpm for maintenance. TAT-KLCpCDK and TAT-Con peptides were dissolved in saline (Alzet) at a concentration of 20 mg/mL. Osmotic minipumps (model 1002; flow rate, 0.25 l/h; Alzet/Durect) were filled with TAT-KLCpCDK or TAT-Con (20 mg/mL, 120 μg/d). The minipump with TAT-KLCpCDK, TAT-Con, or vehicle was attached to a brain infusion kit (BIK model I; Alzet/Durect) with polyethylene tubing. BIK cannulas were guided to the third ventricle and pumps were implanted s.c. between the scapulae. Animals recovered for 7 d before undergoing behavioral tests. Mice underwent the tail suspension test on day 8 and the AMPH-induced hyperactivity test on day 12.

Tail suspension test. A 15-cm length of tape 1.9-cm wide (TimeMed Labeling Systems) was positioned with ≈2 mm of tail protruding. The long length of the tape used, and the short distance from the tape to the end of the tail, prevented the mice from being able to balance themselves in preparation for any climbing behavior. Mice were suspended by their tails for 6 min during videotaped sessions. In a series of pilot experiments using this procedure, we observed that no mice climbed their tails. Immobility time was quantified by a naive observer for a full 6 min; data were further confirmed by reading the immobility time with Topscan (Clever Sys. Inc.).

Forced swim test. Mice were placed in a cylinder of water between 23 and 25 °C. Mice were videotaped during a 6-min session, which was later analyzed for activity during the final 4 min. Mobility was defined as any movement beyond what was necessary to maintain the head above water. At the end of the trial session, mice were taken out of the water, dried with a paper towel, and placed back in their home cages. A blinded observer scored the videotapes.

AMPH-induced activity. A small arena (40 × 40 cm) was used to study AMPH-induced activity. Twelve days after implantation of minipumps containing TAT-KLCpCDK, TAT-Con peptide, or vehicle, mice were placed in the center of the open field for 60 min for baseline recording. Animals were then injected with AMPH. Immediately after AMPH injection (3 mg/kg, i.p. injection), mice were again placed in the center of the arena and their behavior was recorded for an additional 60 min. Total distance traveled was analyzed using Topscan.

Statistics. Data for biochemical experiments were combined from two or three independent experiments. Data were analyzed either by Student's *t* test or one-way analysis of variance (ANOVA).

Data for AMPH-induced hyperactivity were analyzed using a repeated-measures ANOVA with AMPH (before and after) as a repeated-measures factor and TAT-KLCpCDK or TAT-Con as a main factor. Statistical significance was defined as $P < 0.05$.

1. Institute of Laboratory Animal Research, Commission on Life Sciences, National Research Council (1996) *Guide for the Care and Use of Laboratory Animals* (National Academy Press, Washington, DC), p 140.

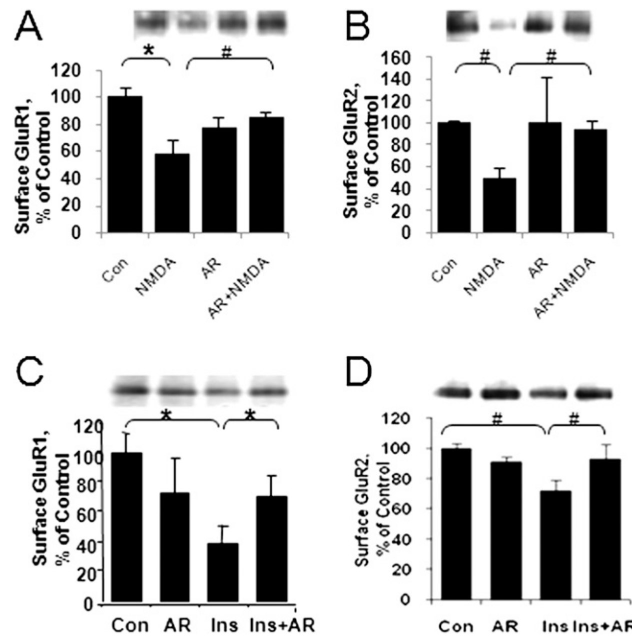

Fig. S1. The GSK-3 inhibitor AR-A014418 significantly blocked NMDA- and insulin-induced internalization of GluR1 and GluR2 receptors in hippocampal neurons. Hippocampal neurons (12 DIV) were treated with AR-A014418 (10 μM) for 1 h and NMDA (50 μM)/glycine (10 μM) or insulin (30 nM) for 30 min. A biotinylation assay was performed to determine surface GluR1 and GluR2 levels. (*A*) AR-A014418 significantly inhibited NMDA-induced GluR1 receptor internalization (one-way ANOVA, Bonferroni's multiple comparison test, $N = 2$, $n = 29$, $*P = 0.0069$; Student's t test, unpaired, two-tailed, $n = 7$–8 per group, $\#P = 0.0459$). (*B*) AR-A014418 significantly blocked NMDA-induced GluR2 receptor internalization (Student's t test, unpaired, two-tailed, $n = 7$–8 per group; control versus NMDA, $\#P = 0.0005$; NMDA versus NMDA + AR, $\#P = 0.0067$). (*C*) AR-A014418 significantly blocked insulin-induced GluR1 receptor internalization (one-way ANOVA, Bonferroni's multiple comparison test, $N = 2$, $n = 27$, $*P < 0.01$). (*D*) AR-A014418 significantly blocked insulin-induced GluR2 receptor internalization (Student's t test, unpaired, two-tailed, $n = 5$–9 per group; control versus insulin, $\#P = 0.0217$; insulin versus insulin + AR, $\#P = 0.0438$).