Metabotropic Glutamate Receptor 5/Homer Interactions Underlie Stress Effects on Fear

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Background: Glutamatergic transmission is one of the main components of the stress response; nevertheless, its role in the emotional stress sequelae is not known. Here, we investigated whether interactions between group I metabotropic glutamate receptors (metabotropic glutamate receptor 1 and metabotropic glutamate receptor 5 [mGluR5]) and Homer proteins mediate the delayed and persistent enhancement of fear induced by acute stress.

Methods: Antagonists and inverse agonists of metabotropic glutamate receptor 1 and mGluR5 were injected into the hippocampus after immobilization stress and before contextual fear conditioning. Metabotropic glutamate receptor 5 was displaced from constitutive Homer scaffolds by viral transfection of Homer1a or injection of Tat decoy peptides. The effects of these manipulations on stress-enhanced fear were determined.

Results: We show that stress induces interactions between hippocampal mGluR5 and Homer1a; causes a sustained, ligand-independent mGluR5 activity; and enhances contextual fear. Consistent with this mechanism, enhancement of fear was abolished by delayed poststress application of inverse agonists, but not antagonists, of mGluR5. The effect of stress was mimicked by virally transfected Homer1a or injection of Tat-metabotropic glutamate receptor C-tail decoy peptides into the hippocampus.

Conclusions: Constitutive activation of mGluR5 is identified as a principal hippocampal mechanism underlying the delayed stress effects on emotion and memory. Inverse agonists, but not antagonists, of mGluR5 are therefore proposed as a preventive treatment option for acute and posttraumatic stress disorders.

Key Words: Conditioned fear, constitutive activity, Homer, metabotropic glutamate receptor 5, posttraumatic stress disorder, stress

Preventive strategies for stress-mediated disorders, such as acute and posttraumatic stress disorder (PTSD), face several unique problems. Notably, the unpredictability of stressor occurrence requires retroactive interference to prevent later enhancement of fear. Optimally, treatments would reduce the persistent emotional effects of stress while leaving memory processes intact. This is particularly difficult to achieve, however, given that both stress (1) and episodic memory (2) are mediated by the hippocampus. Addressing these issues requires the identification of mechanisms specifically contributing to the stress component of memory modulation.

Sensitization to stress and subsequent enhancement of fear conditioning to environmental contexts has been highlighted as a possible cause and aggravating factor of PTSD in susceptible individuals (3). These endophenotypes of PTSD can be successfully modeled in rodents exposed to acute immobilization (4) causing enhanced contextual fear conditioning. The actions of stress include sustained increase of hippocampal excitability (5) and enhanced fear conditioning, both of which initially require corticotropin-releasing factor (4) and corticosterone (6,7). Stress hormones, however, are insufficient for persistent enhancement of aversive memories (8).

Another important component of the stress response is the glutamatergic system. Stress triggers transient glutamate release (9) and activation of *N*-methyl-D-aspartate (10) receptors. Glutamate also primes hippocampal excitability by activating type I metabotropic receptors (metabotropic glutamate receptor 1 [mGluR1] and metabotropic glutamate receptor 5 [mGluR5]) and thus causes a sustained reduction of the activation threshold for forthcoming hippocampal inputs (11). Here we show, using pharmacological interference with hippocampal metabotropic glutamate receptor (mGluR) type I or their interaction with Homer scaffolds, that mGluR5 mediates the delayed, stress-induced enhancement of contextual fear. This effect involves increased binding of Homer1a and reduced binding of Homer1b/c to mGluR5. Accordingly, the effect of stress was mimicked, in an mGluR5-dependent manner, by viral overexpression of Homer1a in the hippocampus.

Methods and Materials

Animals

Nine-week-old male BALB/c mice (Charles River, Hollister, California) were individually housed after 9 weeks of age and maintained in an enclosed animal cubicle provided with its own ventilation system (15 air exchanges/hour), at a 12/12 dark light cycle (7:00 AM–7:00 PM), 40% to 50% humidity, and 20°C ± 2°C (12). All studies were approved by the Animal Care and Use Committee of Northwestern University in compliance with National Institutes of Health standards.

Immobilization Stress

Immobilization of mice was performed by taping their forelimbs for 1 hour (4,13). Mice were lightly anesthesized with isoflurane and placed on their back on a plastic surface. Their forelimbs and hind-

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limbs were fixed to the surface using autoclave tape. Control mice were left in their home cages.

Fear Conditioning

Fear conditioning was conducted 6 hours after the end of immobilization, unless indicated otherwise. Training consisted of a 3-min exposure of mice to the conditioning box (context), followed by a 30-sec tone (75 dB sound pressure level 10 kHz 200 msec pulsed), terminating with a foot shock (2 sec, .7 mA, constant current), as described previously (12,14). The contextual memory tests were performed 24 hours later by re-exposing the mice for 3 min to the conditioning context. The tone-dependent memory tests were performed in a novel context by presenting three 30-sec tones separated by 30-sec intertrial intervals. Freezing, defined as a lack of movement besides heart rate and respiration, was recorded every 5th (tone) or 10th (context) second by two trained observers (one unaware of experimental conditions) for 3 min. The number of scores indicating freezing was calculated as a mean from both observers and expressed as a percentage of the total number of observations (4,12).

Cannulation and Injections

Cannulation into the dorsal hippocampus was performed as described (4). Briefly, mice were anesthetized with an intraperitoneal injection of 1.2% Avertin (2,2,2-tri-bromethanol dissolved in .7% isoamyl alcohol (Sigma-Aldrich, St Louis, Missouri); .4 mL per mouse). Double-guided cannulae (internal, 28 gauge; guide, 26 gauge) were inserted into the dorsal hippocampi (anteroposterior: −1.5 mm, mediolateral: ± 1 mm, dorsoventral: −2 mm from skull). The cannulae were secured to the skull with dental acrylic (3M Inc., St. Paul, Minnesota). Mice were allowed to recover for at least 3 days. Microinjections were performed under light isoflurane anesthesia over a 15-sec period (.25 μL per site, 1 μL/min).

Drugs

MPEP hydrochloride (Tocris, Ellisville, Missouri) was dissolved in 10% dimethyl sulfoxide (DMSO) in artificial cerebrospinal fluid (aCSF) (5 mg/2 mL) for systemic injections, and 20% dimethyl sulfoxide in aCSF for intrahippocampal injections. MTEP hydrochloride (Tocris) was dissolved in 20% DMSO in aCSF. MCPG (Tocris) was dissolved to 100 mmol/L in sodium hydroxide, diluted with aCSF, and stabilized at pH = 7.4. CPCCOEt ethyl ester (Tocris) and BAY 36-7620 (Tocris) were dissolved in aCSF. The doses of individual compounds are indicated in each experiment.

Viral Vectors

Recombinant adeno-associated viral (rAAV) vector constructs, as previously described (15), were used to express Homer1a protein fused with Venus fluorescent protein (H1aV), a green fluorescent protein (GFP) variant (rAAV-H1aV) within the dorsal hippocampus. A GFP expressing rAAV vector was used as a control (rAAV-GFP). The titer of each virus was 2×10^{11} plaque-forming units per milliliter.

Bilateral infusions, each .25 μL (.5 μL/mouse) of either rAAV-H1aV or rAAV-GFP were injected into the dorsal hippocampus via a previously implanted cannula. Contextual fear conditioning was conducted 5 or 10 days after injection.

Peptides

We used a Tat-mGluR C-tail decoy peptide and control as previously described (16,17). The decoy peptide, Tat-mGluR5 (YGRKKRRQRRRALTPPSPFR; active peptide), comprised the enabled/vasodilator-stimulated phosphoprotein homology 1 (EVH1) binding domain for Homer (PPSPFR) fused to the arginine-enriched cell membrane transduction domain of the human immunodeficiency virus 1 (YGRKKRRQRRRALT) to gain cell permeability (18). As a comparison, we used a peptide rendered incapable of binding with Homer due to a dual point mutation (PLSPRR; scramble peptide). We additionally used a fluorescent, fluorescein isothiocyanate (FITC)-tagged active peptide and a FITC-tagged cell impermeable peptide. The latter consisted of the active EVH1 binding domain fused to the 14 amino acid sequence adjacent to the cell membrane transduction domain of the Tat peptide (17) (FITC-KALGISYGRKKALTPPSPFR; Tat_{38-48}). Peptides were dissolved in aCSF for injection at the final concentrations immediately before use. Intrahippocampal injections were conducted 1 hour before contextual fear conditioning or 1 hour before hippocampal dissection for co-immunoprecipitation and immunoblotting.

Protein Extraction

Individual dorsal hippocampi (the rostral 2.5 mm septal pole) were collected 3 or 6 hours after immobilization stress and from naive mice. For viral and peptide experiments, mice were injected with vehicle, active or scrambled peptide 1 hour before individual dorsal hippocampi were dissected, frozen in liquid nitrogen, and stored at −80°C. Cytoplasmic, membrane, cytoskeletal, and nuclear fractions were prepared using the ProteoExtract kit for subcellular proteome extraction (EMD Biosciences, Gibbstown, New Jersey) and validated as described (12).

Co-immunoprecipitation and Immunoblot

Dorsohippocampal membrane fractions (2–5 samples from each group) were pooled to obtain 250 μg/group for each co-immunoprecipitation. Immunoprecipitation was performed using the Catch and Release kit (Millipore, Billerica, Massachusetts) for Homer1a (12) and MAGmol Microbeads (Miltenyi Biotec, Bergisch Gladbach, Germany) with a preclearing step for Homer1b/c (19). Washing and elution were performed as described in the respective user's manuals.

Briefly, 250 μg of membrane protein for each treatment group and 4 μg of mGluR5 antibody (Millipore) were used for each immunoprecipitation and rabbit immunoglobulin G (Sigma-Aldrich) served as a negative control. Eluates and input samples were reduced in loading buffer with dithiothreitol and boiled for 5 min, then subjected to sodium dodecyl sulfate polyacrylamide gel electrophoresis (25 μg/well) and blotted to polyvinylidene difluoride membranes (Millipore). Using the SnapID system (Millipore), membranes were saturated with I-block (Tropix, Foster City, California), incubated with primary (Anti-Homer1b/c 1:150, Anti-β-actin 1:150, Santa Cruz Antibodies; Pan-Anti-Homer 1:350, Synaptic Systems Goettingen, Germany; mGluR5 1:500, Millipore) and corresponding secondary antibodies (Goat Anti-Rabbit 1:3500, Tropix). For detection, we used alkaline phosphatase chemiluminescence. Interassay variability between blots was determined by a standard control sample for each individual fraction obtained from pooled hippocampi of three naive or vehicle-injected mice in the stress and peptide experiments, respectively.

Perfusion and Immunohistochemistry

Mice were anesthetized with an intraperitoneal injection of 240 mg/kg of Avertin and transcardially perfused with ice-cold 4% paraformaldehyde in phosphate buffer (pH 7.4, 150 mL/mouse). Brains were cut on a cryostat at 50 μm. Sections from fluorescent peptide experiments were mounted on slides for microscopy and counterstained with 4′-6-diamidino-2-phenylindole (Vector Laboratories, Burlingame, California). Brains from viral vector expressing mice were processed with immunohistochemistry as described previously (12) with specific antibodies for Homer1a (1:2000, Santa Cruz

Antibodies) or anti-GFP (1:100,000, Millipore) and Anti-Goat or Anti-Mouse secondary antibodies, respectively (both 1:200; Vector Laboratories). Digital images for light and immunofluorescent microscopy were captured with a cooled color charge-coupled device camera (RTKE Diagnostic Instruments, Sterling Heights, Michigan) and SPOT software (Diagnostic Instruments) for Macintosh.

Neuronal Cultures and Immunofluorescent Labeling

The hippocampi from E18 mice were isolated and dissociated with trypsin (Invitrogen, Carlsbad, California) as described previously (20). Cells were plated on coverslips coated with poly-D-lysine (.1 mg/mL; Sigma-Aldrich, St. Louis, Missouri) in 24-well culture plates at a density of 60,000 to 80,000 cells and grown in NeuroBasal medium (Invitrogen; 2 mmol/L GlutaMax, .5% gentamicin, and 2% B27). One half of the medium was replaced with identical medium every 4 days. After 2 weeks of culturing, 5 μmol/L of FITC-tagged active or Tat_{38-48} peptide was applied for 1 hour before fixing with 4% formaldehyde in phosphate-buffered saline. Light microscopy determined distribution of the peptides.

Statistics

Unpaired Student *t* test was used for comparisons of two groups. One-, two- or three-way analysis of variance (ANOVA) was used for multiple group comparisons where appropriate. Scheffe's test was used for post hoc analyses. The α level $p < .05$ was used for all analyses.

Results

Acute Stress Induces Enhanced and Persistent Fear

In BALB/c mice, a strain highly sensitive to the neuroendocrine and behavioral sequels of stress (21), 1 hour of immobilization stress before fear conditioning (Figure 1A) resulted in a significant increase of contextual fear 1 day later, as revealed by a context-specific freezing response (Figure 1B; see also Figure S1 in Supplement 1). Fear conditioning to the tone did not significantly differ between stressed and nonstressed mice [$t(14) = .138$, $p = .894$; Figure S1 in Supplement 1]. The stress effect emerged 1 hour after the end of immobilization, was maximal between 3 and 6 hours poststress, and was not observed when the stress-training interval was extended to 24 hours [$F(5,42) = 7.35$. $p < .01$]. The 6-hour poststress time window thus opened an opportunity to retroactively interfere with the effects of stress on contextual fear.

The enhancement of contextual fear was persistent, as revealed by a significant increase of freezing after 1, 7, and 28 days [repeated measure ANOVA, $F(1,7) = 12.38$, $p < .01$, no stress] (Figure 1C). This effect was completely and lastingly abolished by systemic (intraperitoneal) administration of the mGluR5 inverse agonist MPEP (5 mg/kg, injection volumes 40–50 μL/mouse) [three-way ANOVA, stress × drug interaction, $F(1,20) = 7.392$, $p < .05$, stress × drug × day, $F(2,40) = 2.489$, $p = .104$; Figure S2 in Supplement 1) injected 1 hour after the end of stress. Notably, MPEP did not alter freezing of nonstressed mice, suggesting that, at these time points, MPEP did not affect fear conditioning or subsequent memory retrieval. The latter observation is most likely due to the rapid clearance of MPEP within 15 min postinjection in mice (22).

Inverse Agonists of mGluR5 Selectively Prevent Induction of Stress-Enhanced Fear

The hippocampus is a well-established site of the lasting effects of stress and mGluR on synaptic plasticity and memory (11,23). Although glutamate is the primary endogenous ligand of type I mGluRs, these receptors can also be activated via ligand-independent dissociation from their constitutive "long" Homer scaffold proteins (Homer1–3) (24,25). Whereas inverse agonists (drugs that act at the same receptor as agonists, yet produce an opposite effect), such as MPEP and MTEP, reverse both types of activity, competitive receptor antagonists (drugs that block the actions of agonists without exerting their own activity), such as MCPG, specifically block glutamate-dependent effects (24). Here, we compared the ability of the potent mGluR5-specific inverse agonists MPEP and MTEP and the competitive antagonist MCPG to reverse the long-lasting activation of mGluR5 after stress. MPEP (50 nmol/.5 μL/mouse, selected from pilot studies) or vehicle (20% DMSO, .5 μL/mouse) was injected via previously implanted cannula into the dorsal hippocampus (Figure S3 in Supplement 1). A time course study revealed that a single intrahippocampal injection of MPEP (50 nmol/mouse) was sufficient to prevent stress-enhanced fear even after a 5-hour delay [stress × drug interaction, $F(1,48) = 18.173$, $p < .001$; Figure 2A]. Importantly, neither stress nor MPEP altered locomotor activity or shock response during training (Figure S4A in Supplement 1). Similarly,

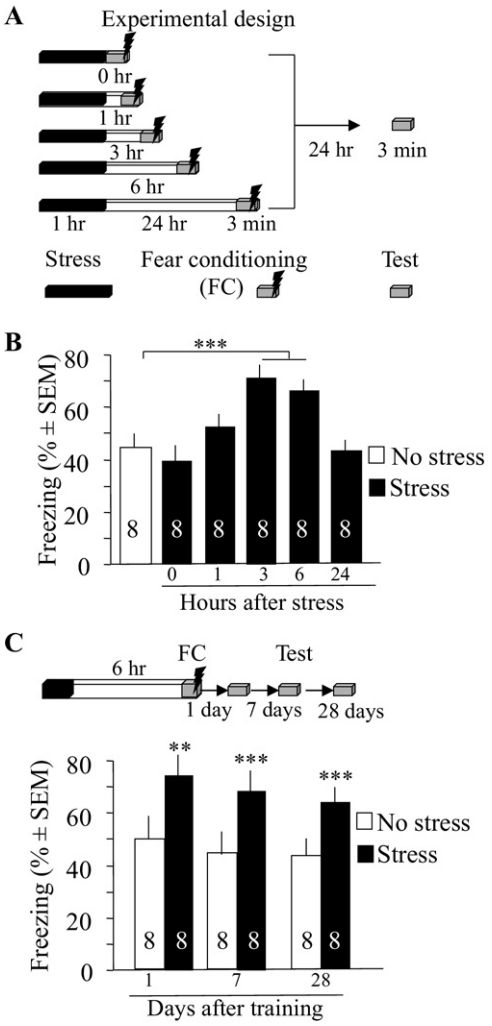

Figure 1. Stress-enhanced fear conditioning requires ongoing activity of metabotropic glutamate receptor 5. **(A)** Design of the stress/conditioning phase of the experiment indicating the time window between stress and fear conditioning (FC). **(B)** FC performed 3 and 6 hours after stress resulted in a significant enhancement of fear tested 24 hours later. **(C)** The enhancing effect of stress on fear lasted for at least a month. $^{**}p < .01$, $^{***}p < .001$ vs. nonstress/vehicle. The lightning bolt sign indicates footshock. The number of mice per group is marked on the bars for experiment.

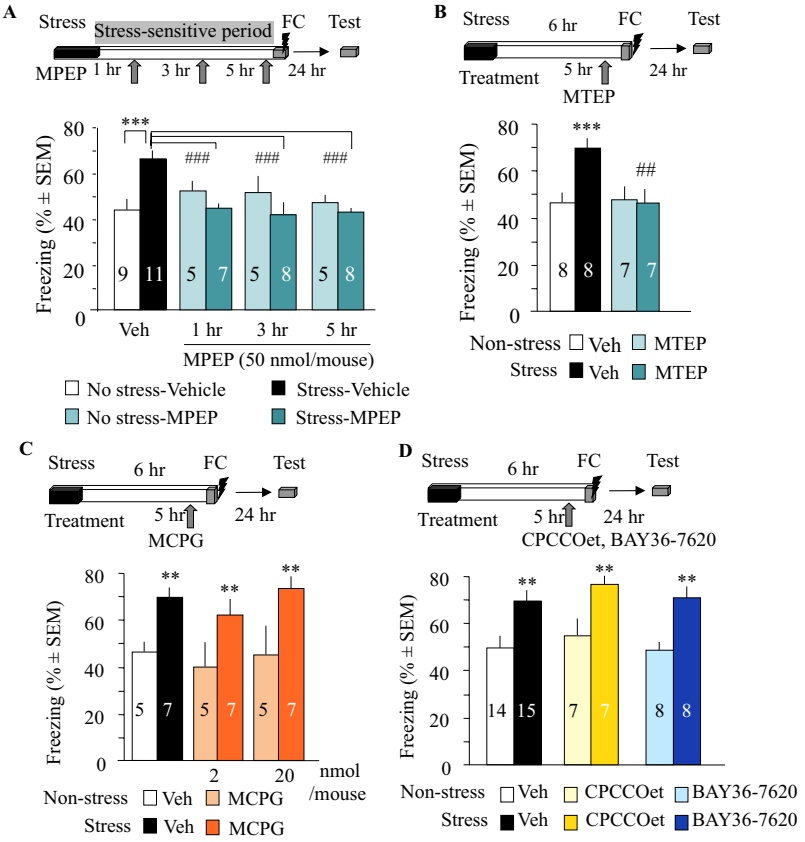

Figure 2. Stress-enhanced fear conditioning (FC) requires ligand-independent but not ligand-dependent activation of metabotropic glutamate receptor 5 or metabotropic glutamate receptor 1 in the hippocampus. **(A)** Intrahippocampal injection of 50 nmol/mouse MPEP up to 5 hours poststress reduced enhancement of fear. **(B)** The potent metabotropic glutamate receptor 5 inverse agonist MTEP injected intrahippocampally 5 hours after stress prevented stress enhancement contextual fear. **(C)** Intrahippocampal injection of the metabotropic glutamate receptor competitive antagonist MCPG 5 hours after stress did not prevent the enhancement of fear. **(D)** Intrahippocampal injection of the metabotropic glutamate receptor 1 antagonist CPCCOEt (100 nmol/mouse) or inverse agonist BAY 36-7620 (100 nmol/mouse) 5 hours poststress did not reverse stress-enhanced fear. $^{**}p < .01$, $^{***}p < .001$ vs. nonstress/vehicle; $^{\#\#}p < .01$, $^{\#\#\#}p < .001$ vs. stress/vehicle. Veh, vehicle.

intrahippocampal injection of MTEP (15 nmol/.5 μL/mouse) or vehicle (20% DMSO, .5 μL/mouse) administered 5 hours after stress prevented the enhancement of fear by prior stress [stress × drug interaction, $F(1,26) = 5.996$, $p < .05$; Figure 2B]. MTEP did not alter freezing of nonstressed mice ($p = .538$), demonstrating that the drug did not interfere with fear conditioning or memory retrieval.

The type I mGluR antagonist MCPG injected intrahippocampally (2 or 20 nmol/mouse) 5 hours poststress did not prevent stress-enhanced fear [stress effect, $F(1,34) = 19.93$, $p < .001$; stress × drug interaction, $F(2,34) = .12$, $p = .89$; Figure 2C], suggesting that this effect was mediated by a ligand-independent activation of mGluR5. The efficacy of the employed dose of MCPG to block mGluR-mediated effects was verified by the established ability of this drug to impair memory retrieval [test × drug interaction: $F(1,13) = 9.3$, $p < .01$; Figure S5 in Supplement 1).

Given the similarity between the two subtypes of type I mGluRs, we also investigated the role of mGluR1 in stress-enhanced fear conditioning. Intrahippocampal injection of the mGluR1 inverse agonist BAY 36-7620 (100 nmol/mouse) or antagonist CPCCOEt (100 nmol/mouse) 5 hours after stress was ineffective [stress effect,

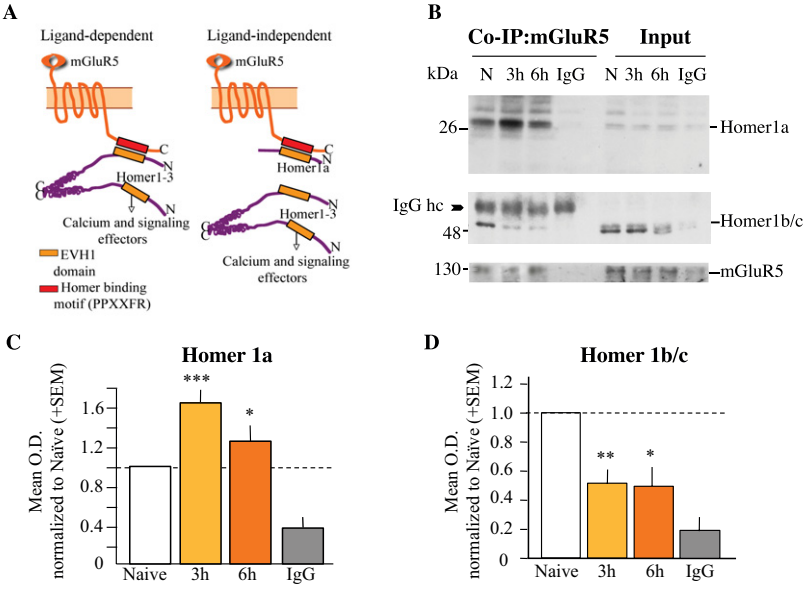

Figure 3. Metabotropic glutamate receptor 5 (mGluR5)/Homer1a interactions are induced by stress. **(A)** A model of mGluR5/Homer interactions showing ligand-dependent and independent mGluR5 activity and displacement of mGluR5 from Homer scaffolds by Homer1a. This may indirectly alter the effect of Homer1 to 3 on calcium homeostasis mediated by ryanodine and inositol triphosphate channels. **(B)** A representative immunoblot showing hippocampal mGluR5/Homer1a and mGluR5/Homer1b/c interactions 3 and 6 hours after stress. Left: co-immunoprecipitation, Right: input; input/immunoglobulin G is the input sample used for immunoglobulin G co-immunoprecipitation. The co-immunoprecipitation experiment was replicated with six pull-downs of independent sample pools, each prepared from two to five individual membrane fractions/group ($n = 12$–15 hippocampi/group). **(C)** Quantification of the immunoblot signals showing mGluR5/Homer1a interactions significantly increased after stress, whereas **(D)** mGluR5/Homer1b/c interactions exhibited a significant decrease. $^{*}p < .05$, $^{**}p < .01$, $^{***}p < .001$ versus naive. C, C-terminus; Co-IP, co-immunoprecipitation; EVH1, enabled/vasolidator-stimulated phosphoprotein homology 1; IgG hc, immunoglobulin G heavy chain; mGluR5, metabotropic glutamate receptor 5; N, naïve; O.D., optical density.

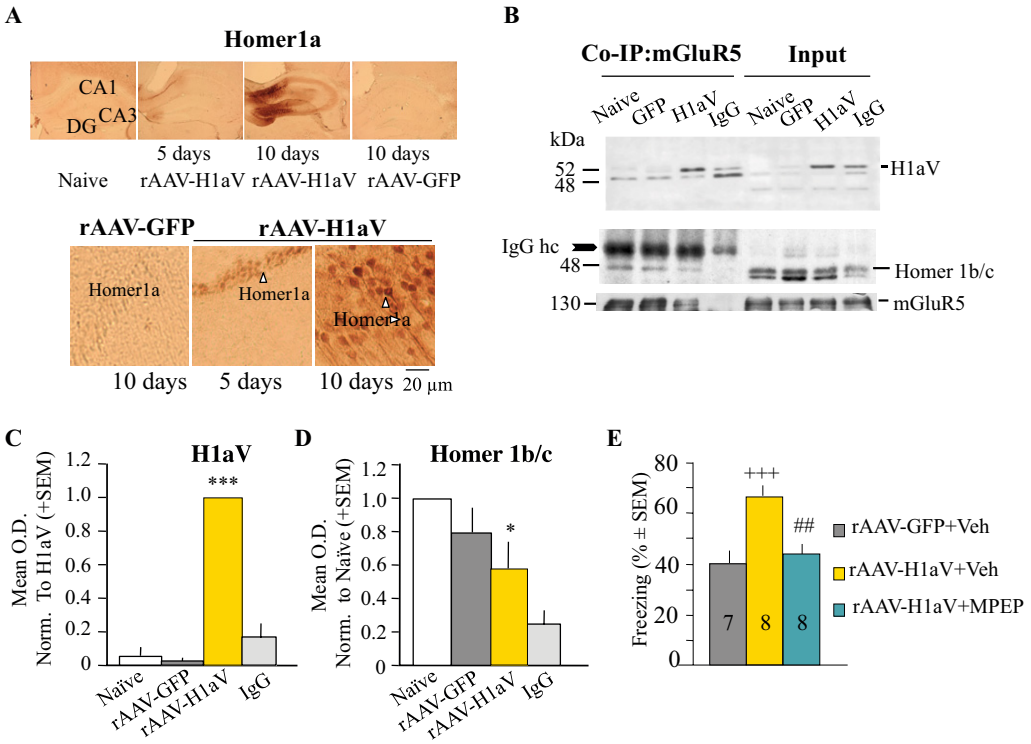

Figure 4. Overexpression of Homer1a mimics the effects of stress. **(A)** Mice injected with recombinant adeno-associated viral (rAAV)-Homer1a protein fused with Venus fluorescent protein (H1aV) or rAAV- green fluorescent protein (GFP) show robust protein expression in the dorsal hippocampus 10 days later. Homer1a antibody revealed strong signals at 10, but not 5, days after rAAV-H1aV injection. Both viral vectors showed high levels of expression as detected by GFP antibody. H1aV levels and distribution in cornus ammonis 1 dorsohippocampal neurons 5 and 10 days after viral injection. White arrows indicate Homer1a-positive neurons. **(B)** H1aV interacts with metabotropic glutamate receptor 5 (mGluR5) as revealed by robust co-immunoprecipitation of both proteins 10 days after injection of rAAV-H1aV, whereas mGluR5/Homer1b/c interactions decrease. Input/immunoglobulin G is the input sample used for immunoglobulin G co-immunoprecipitation; the co-immunoprecipitation experiment was replicated with three pull-downs of independent sample pools, each prepared from two to four individual membrane fractions/group ($n = 10–13$ hippocampi/group). **(C)** Quantification of the immunoblot signals showing significant increase of mGluR5/H1aV interaction when compared with all other groups. The data were normalized to H1aV because there was no detectable signal in the naive control. **(D)** Quantification of the immunoblot signals showing significant decrease of mGluR5/Homer1b/c signals 10 days after hippocampal injection of rAAV-H1aV. **(E)** Overexpression of H1aV leads to enhanced contextual fear conditioning. Injection of MPEP 1 hour before training reverses this enhancement. *$p < .05$, ***$p < .001$ vs. naive, +++ $p < .001$ vs. rAAV-GFP, ## $p < .01$ vs. rAAV-H1aV/vehicle. CA1, cornus ammonis 1; CA3, cornus ammonis 3; Co-IP, co-immunoprecipitation; DG, dentate gyrus; GFP, green fluorescent protein; H1aV, Homer1a protein fused with Venus fluorescent protein; IgG hc, immunoglobulin G heavy chain; mGluR5, metabotropic glutamate receptor 5; O.D., optical density; rAAV, recombinant adeno-associated viral; Veh, vehicle.

$F(1,53) = 25.67$, $p < .001$; stress × drug interaction, $F(2,53) = .04$, $p = .97$; Figure 2D]. Hippocampal mGluR1 is thus not required for the delayed enhancement of fear by stress.

Viral Overexpression of Homer1a Mimics the Effects of Stress on Fear

Interactions with different isoforms of Homer proteins are key determinants of ligand-dependent (Homer1–3) (16,25) and ligand-independent (Homer1a) (24) activity of mGluR5. Endogenously, the dominant negative short Homer1a triggers ligand-independent mGluR activity (26,27) by displacing the long Homer1 to 3 isoforms from the C-terminus of mGluR5 (24) (Figure 3A). Stress did not significantly alter the hippocampal levels of mGluR5, Homer1b/c or Homer1a, as determined 6 hours after stress (Figure S6A,B in Supplement 1) using a pan-anti-Homer antibody reacting with these isoforms (Figure S7A,B in Supplement 1). However, the binding of mGluR5 to Homer1a was induced 3 and 6 hours after stress, as shown by their significantly stronger co-immunoprecipitation signals when compared with the naive group [stress effect, $F(3,12) = 39.061$, $p < .001$; Figure 3B,C]. At the same time, interactions between mGluR5 and Homer1b/c were significantly decreased [stress effect, $F(3,12) = 39.061$, $p < .01$; Figure 3B,D]. Stress-induced mGluR5/Homer1a interaction is therefore a candidate mechanism for the long-lasting constitutive activity of mGluR5 and enhancement of fear.

Whether Homer1a is sufficient to enhance contextual fear conditioning without prior stress was examined by hippocampally expressing the H1aV fusion protein using an rAAV-H1aV viral vector. This protein acts as an active form of Homer1a (15). Mice injected with rAAV-H1aV or rAAV-GFP showed low levels of these proteins 5 days and a robust increase 10 days after viral injection into dorsal hippocampus (Figure 4A; see also Figure S6C in Supplement 1), as revealed by an anti-Homer1a antibody recognizing the overexpressed protein. On day 5, H1aV was present in the soma but not dendrites or axons, correlating with the lack of behavioral effect at this time point [$t(15) = .37$, $p = .72$]. However, 10 days after injection, H1aV reached detectable levels throughout the entire neuropil of cornus ammonis 1 (CA1) and dentate gyrus neurons (Figure 4A) and exhibited strong interaction with mGluR5 [$F(3,12) = 29.6$, $p < .001$; Figure 4B,C]. At the same time, H1aV injections resulted in a significant decrease of mGluR5/Homer1b/c interactions [$t(4) = 3.641$, $p < .05$; Figure 4D], whereas rAAV-GFP was ineffective [$t(4) = 2.056$, $p = .109$; Figure 4D] when compared with the naive group. Further-

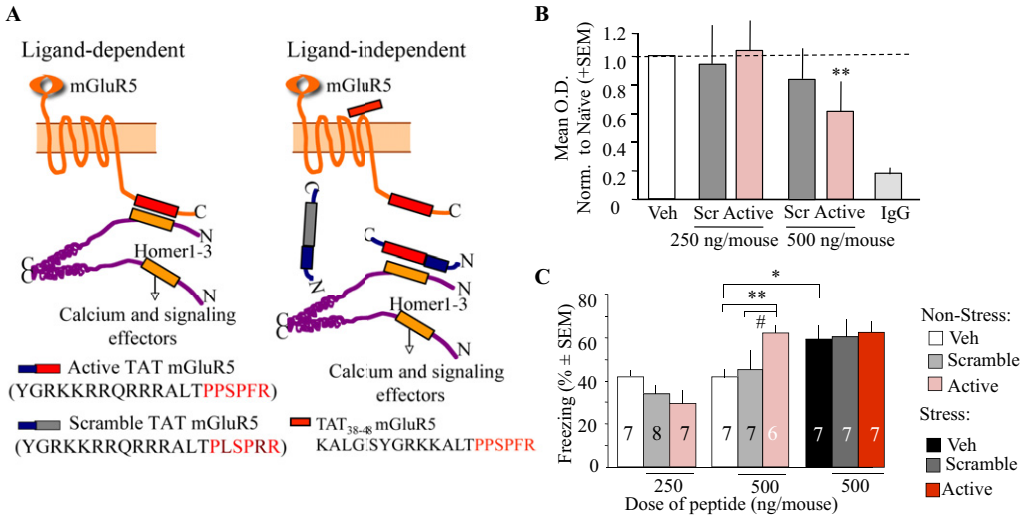

Figure 5. Uncoupling metabotropic glutamate receptor 5 (mGluR5) from long Homer isoforms mimics the effects of stress on fear. **(A)** A model of endogenous mGluR5/Homer interactions showing displacement of mGluR5 from Homer scaffolds by Tat decoy peptides. **(B)** Quantification of co-immunoprectpitation signals after intrahippocampal injection of active peptide causing reduction of mGluR5/Homer1b/c interactions at a dose of 500, but not 250, ng/mouse. The scramble peptide was ineffective. The co-immunoprecipitation experiment was replicated with four pull-downs of independent sample pools, each prepared from two to three individual membrane fractions/group (n = 10–12 hippocampi/group). **(C)** Intrahippocampal injection of active peptide at a dose of 500 ng/mouse 1 hour before training enhanced fear conditioning of nonstressed mice when compared with the scramble- or vehicle-injected control mice. Prior stress prevented further enhancement of contextual fear by Tat peptides. *$p < .05$, **$p < .01$ vs. vehicle, #$p < .05$ vs. scramble. C, C-terminus; IgG, immunoglobulin G; mGluR5, metabotropic glutamate receptor 5; N, N-terminus; O.D., optical density; Scr, scramble; Veh, vehicle.

more, after contextual fear conditioning, these mice (n = 8) showed significantly enhanced freezing to the context when compared with mice expressing rAAV-GFP [$t(13) = 3.89$, $p < .01$; n = 7, Figure 4E]. Viral overexpression of H1aV did not alter baseline activity or shock reactivity during training (Figure S4B in Supplement 1). Injection of MPEP 1 hour before contextual fear conditioning abolished the enhancement by Homer1a overexpression [$F(2,20) = 10.99$, $p < .001$; $t(14) = 4.15$, $p < .001$; H1aV/vehicle vs. H1aV/MPEP; n = 8, Figure 4E], demonstrating that Homer1a enhances contextual fear conditioning via mGluR5.

Displacement of mGluR5 from Homer Scaffolds Mimics the Effects of Stress on Fear

We next determined whether displacement of constitutive Homer (Homer1b/c) from the EVH1 binding domain of mGluR5 is the key underlying mechanism of stress-enhanced contextual fear conditioning. We used decoy peptides that contain the Homer binding site of mGluR5 and specifically disrupt mGluR5/Homer interactions (Figure 5A). On their own, these peptides are cell impermeable; thus, we used the protein transduction domain of human immunodeficiency virus 1 (18) to generate the active Tat-mGluR C-tail decoy peptide (active) and an inactive nonbinding control peptide with a dual point mutation in the EVH1 domain (scramble) (16,17). We first confirmed the in vivo efficacy of the active peptide by co-immunoprecipitation of mGluR5 and Homer1b/c. Injection of 500, but not 250, ng/mouse of active peptide reduced the interaction of mGluR5 with Homer1b/c as determined 1 hour later [$F(5,22) = 5.84$, $p < .01$; Figure 5B]. The effective dose of the active peptide injected 1 hour before training significantly enhanced fear conditioning in nonstressed mice [dose, $F(1,41) = 9.61$, $p < .01$; dose × peptide interaction $F(2,41) = 7.45$, $p < .01$; post hoc analyses: $t(11) = 4.626$, $p < .01$ vs. vehicle; $t(11) = 1.994$, $p < .05$ vs. scramble; Figure 5C]. Injection of Tat peptides 5 hour after stress did not further enhance contextual fear conditioning [$F(2,19) = .052$, $p = .949$; Figure 5C], suggesting that stress occluded the peptide effects.

We further examined the cell permeability and intracellular action of the active peptide by synthesizing fluorescent-tagged active peptide with the addition of an FITC tag (active) and a cell impermeable peptide with identical binding domain but an adjacent sequence from the Tat protein (Tat_{38-48}) (17,18). The cell permeable, but not impermeable, peptide entered cell bodies in dissociated cell cultures and CA1 neurons in vivo (Figure 6A; see also Figure S8 in Supplement 1). Active Tat significantly impaired mGluR5/Homer1b/c interactions, whereas the impermeable Tat_{38-48} was ineffective [$F(3,12) = 8.53$, $p < .01$; Figure 6B,C]. Further, only the cell permeable active peptide enhanced contextual fear conditioning when injected into the dorsal hippocampus 1 hour before training [$F(2,20) = 9.37$, $p < .001$; $t(13) = 3.51$, $p < .01$ active vs. vehicle; $t(13) = 3.74$, $p < .01$ active vs. Tat_{38-48}; Figure 6D) and this effect was completely reversed by MPEP injected 15 min later [$t(13) = 3.422$, $p < .01$ active-MPEP vs. active-vehicle; Figure 6E). None of the applied peptides affected locomotor activity or reactivity to the shock, demonstrating that the enhancing effects of the active peptide was not due to sensorimotor alterations (Figure S4C,D in Supplement 1). These findings support the view that uncoupling mGluR5 from long forms of Homer is sufficient to emulate stress and enhance contextual fear conditioning.

Discussion

Overall, we demonstrated that ongoing activity of mGluR5 caused emergent and persistent enhancement of contextual fear conditioning in the aftermath of stress. This effect required displacement of mGluR5 from its constitutive Homer scaffolds by Homer1a, resulting in sustained agonist-independent activation of mGluR5 lasting for several hours. The behavioral effects of mGluR5/Homer1a may be thus be due to ligand-independent mGluR5 activity, uncoupling of mGluR5 from signaling via constitutive Homers, or both mechanisms.

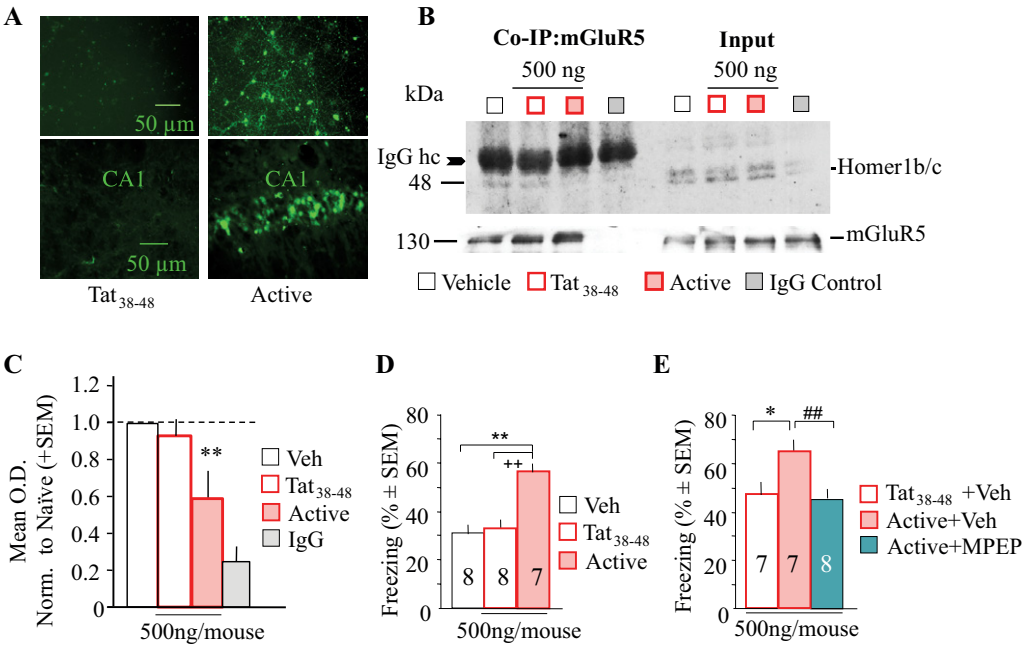

Figure 6. Tat peptides reduce metabotropic glutamate receptor 5 (mGluR5) interactions and enhance fear in an mGluR5-dependent manner. **(A)** Fluorescein isothiocyanate (FITC)-tagged active peptide, contrary to the FITC-tagged Tat_{38-48} peptide, readily entered into cultured neurons (upper panels) and in cornus ammonis 1 pyramidal cells in vivo (lower panels). **(B)** A representative co-immunoprecipitation image showing reduced mGluR5/Homer1b/c interactions after injection of active Tat. Input/immunoglobulin G is the input sample used for immunoglobulin G co-immunoprecipitation. The co-immunoprecipitation experiment was replicated with three pull-downs of independent sample pools, each prepared from two to three individual membrane fractions/group (n = 6–9 hippocampi/group.) **(C)** Quantification of the immunoblot signals showing a significant effect of active Tat but not impermeable Tat_{38-48} on mGluR5/Homer1b/c interactions. **(D)** Intrahippocampal injection of 500 ng/mouse of FITC-tagged active peptide 1 hour before training enhanced fear when compared with the cell impermeable Tat_{38-48} peptide or vehicle. **(E)** Enhancement of fear by active Tat peptide was completely abolished by MPEP (50 nmol/mouse) injected 15 min later. *$p < .05$, **$p < .01$ vs. vehicle, ++ $p < .01$ vs. Tat_{38-48}, ## $p < .01$ vs. active + vehicle. CA1, cornus ammonis 1; Co-IP, co-immunoprecipitation; IgG hc, immunoglobulin G heavy chain; mGluR5, metabotropic glutamate receptor 5; O.D., optical density; Veh, vehicle.

Several studies indicate that mGluR5 receptors are involved in fear conditioning (28–30), although pretraining hippocampal manipulations have shown a role of mGluR1, but not mGluR5, in contextual fear (31). By injecting MPEP and MTEP at least 1 hour before training, we avoided direct drug effects on fear conditioning. The latest time point was selected because in mice, the half-life of these drugs is 15 min (22). Thus, the drugs selectively prevented the effects of stress.

Given that the stress-sensitive time window did not extend over 24 hours and that enhancement of fear did not generalize to a novel context or tone, mGluR5/Homer1a interactions most likely specifically strengthened the acquisition/consolidation of the contextual fear memory, rather than anxiety. This model predicts that stress-enhanced memory, triggering high levels of freezing upon retrieval, would not require mGluR5/Homer1a interactions once memory formation has been completed.

Contrary to the inverse agonists MPEP and MTEP, the competitive antagonist MCPG failed to reverse the stress effects on fear. Although higher doses of MCPG might have been required to fully exclude an effect of a more extensive, stress-induced release of glutamate, its levels typically return to baseline shortly (30 min) after stress (9). Under conditions of lower extracellular glutamate levels, MCPG was effective (as indicated by its significant antagonistic effects on memory retrieval) but was still not able to reverse the stress effects on fear. Thus, based on the significant efficacy of inverse agonists but not antagonist, we suggested that the mGluR5 involvement was ligand-independent.

Ligand-dependent mGluR activity requires Homer scaffolds for the initiation and propagation of protein kinase signaling (16,17,27). Alternatively, ligand-independent activation of mGluR5, as observed here, is triggered when Homer1a displaces the long Homer isoforms such as Homer1b/c, Homer2b, or Homer3 (24,25) from the EVH1 binding site on mGluR5. Homer1a thus disinhibits ryanodine and inositol triphosphate channels and thereby stimulates intracellular calcium release, activates big K+ channels (24), and decreases cell excitability (32). By preventing nonspecific increases in excitability and baseline neuronal firing rates and thus increasing signal-to-noise ratio (33), Homer1a may contribute to the stress-induced facilitation observed in our study.

Homer1a messenger RNA is typically induced within minutes or hours of various environmental manipulations (26,27). However, attempts to find a parallel increase of protein levels in the hippocampus have not been successful (34–36), probably due to rapid proteosomal degration of Homer1a protein (37). Similarly, interactions between mGluR5 and Homer1a observed after stress were not accompanied by a significant increase of Homer1a levels. There are several alternative mechanisms for the observed interactions. Stress may trigger relocalization and synaptic accumulation of Homer1a (38). Alternatively, given that Homer1a and Homer1b/c do not have identical interacting partners (39), stress may induce a reorganization of their complexes, including displacement of Homer1b/c from mGluR5 and binding of Homer1a. Either of these mechanisms would increase the probability of mGluR5/Homer1a complex formation and cause ongoing and ligand-independent mGluR5 activation in the absence of increased Homer1a protein levels.

Enhancement of fear conditioning by rAAV-H1aV or Tat active peptides in the absence of prior stress indicated that displacement

of long Homer proteins from mGluR5 was required for this behavioral effect. The effect of Tat active peptides was occluded by prior stress, suggesting the fear-enhancing action of stress and Tat peptides engaged similar molecular mechanisms. It is important to note that the employed Tat active peptides disrupt mGluR5/Homer interactions without detectably interfering with phosphatidylinositol and dynamic calcium responses to agonists or causing visible changes in the distribution of mGluR5, Homer1b/c, and Shank (17).

Stress has opposing effects on different types of learning mediated by the hippocampus (40). Acute stressors reliably enhance formation of aversive memory (4,6–8) but impair spatial learning (41) and working memory (42). Homer1a overexpression in the hippocampus impairs spatial working memory (15). Conversely, our findings showed that Homer1a overexpression in the hippocampus enhances contextual fear, in line with the impairment found in Homer1a knockout mice (43). Therefore, Homer1a putatively mediates the disparate effects of stress on memory, probably by engaging different binding partners. Using the mGluR5/Homer1a mechanism, the hippocampus may provide the key brain regions of the fear circuit, such as the amygdala and prefrontal cortex (44), with aversively biased, to the detriment of neutral contextual information (45), and thus facilitate the formation of a memory triggering exaggerated fear. Poststress administration of mGluR5 inverse agonists or interventions that destabilize interactions between mGluR5 and Homer1a are therefore promising strategies for reducing fear and preventing acute stress disorder and PTSD in high-risk individuals. Notably, such approaches would not significantly interfere with memory processes and are thus unlikely to produce detrimental mnemonic side effects.

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Supplementary material cited in this article is available online.

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