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

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Abstract

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 (mGluR1 and mGluR5) and Homer proteins mediate the delayed and persistent enhancement of fear induced by acute stress.

Methods—Antagonists and inverse agonists of mGluR1 and mGluR5 were injected into the hippocampus after immobilization stress and before contextual fear conditioning. mGluR5 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 post-stress application of inverse agonists, but not antagonists, of mGluR5. The effect of stress was mimicked by virally transfected of Homer1a or injection of TAT-mGluR 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.

Keywords

Metabotropic glutamate receptor 5; Homer; constitutive activity; stress; conditioned fear; post-traumatic stress disorder

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Preventive strategies for stress-mediated disorders, such as acute and post-traumatic 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 NMDA (10) receptors. Glutamate also primes hippocampal excitability by activating type I metabotropic receptors (mGluR1 and mGluR5), and thus causes a sustained reduction of the activation threshold for forthcoming hippocampal inputs (11). Here we show, using pharmacological interference with hippocampal 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, USA) were individually housed after 9 weeks of age and maintained in an enclosed animal cubicle provided with its own ventilation system (15 air exchanges/hr), at a 12/12 dark light cycle (7 am–7 pm), 40–50% humidity, and $20 \pm 2^\circ\text{C}$ (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 hr (4,13). Mice were lightly anesthetized with isoflurane and placed on their back on a plastic surface. Their fore and hind 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 SPL 10kHz 200ms pulsed), terminating with a foot shock (2 sec, 0.7 mA, constant current), as described previously (12,14). The contextual memory tests were performed 24 hr 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) sec 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 0.7% isoamyl alcohol; 0.4 ml per mouse). Double-guided cannulae (internal, 28 gauge; guide, 26 gauge) were inserted into the dorsal hippocampi (AP: -1.5 mm ML: $\pm$ 1 mm, DV: -2 mm from skull). The cannulae were secured to the skull with dental acrylic (3M Inc.). Mice were allowed to recover for at least 3 days. Microinjections were performed under light isoflurane anesthesia over a 15 sec period (0.25 μ l per site, 1 μ l/min).

Drugs

2-Methyl-6-(phenylethynyl)pyridine hydrochloride (MPEP, Tocris) was dissolved in 10% DMSO in aCSF (5 mg/2 ml) for systemic injections, and 20% DMSO in aCSF for intrahippocampal injections. 3-((2-Methyl-1,3-thiazol-4-yl)ethynyl)pyridine hydrochloride (MTEP, Tocris) was dissolved in 20% DMSO in aCSF. (RS)- α -Methyl-4-carboxyphenylglycine (MCPG, Tocris) was dissolved to 100mmol in NaOH, diluted with aCSF and stabilized at pH = 7.4. 7-(Hydroxyimino)cyclopropa[b]chromen-1 α -carboxylate ethyl ester (CPCCOet, Tocris) (3aS,6aS)-Hexahydro-5-methylene-6a-(2-naphthalenyl)methyl-1H-cyclopenta[c]furan-1-one (BAY 36-7620, Tocris) was dissolved in aCSF. The doses of individual compounds are indicated in each experiment.

Viral Vectors

Recombinant adeno-associated viral vector (rAAV) constructs, as previously described (15), were used to express Homer1a protein fused with Venus fluorescent protein, 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} PFU/ml.

Bilateral infusions, each 0.25 μ l (0.5 μ l/mouse) of either rAAV-H1aV or rAAV-GFP were injected into the dorsal hippocampus via 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 (YGRKKRRQRRRALTPSPFR; Active peptide) comprised of the EVH1 binding domain for Homer (PPSPFR) fused to the arginine-enriched cell membrane transduction domain of the HIV-1 virus (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 utilized a fluorescent, 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) (FITCKALGISYGRKKALTPSPFR; TAT₃₈₋₄₈). Peptides were dissolved in aCSF for injection at the final concentrations immediately prior to use. Intrahippocampal injections were conducted 1 hour prior to contextual fear conditioning or 1 hour prior to hippocampal dissection for co-immunoprecipitation and immunoblotting.

Protein extraction

Individual dorsal hippocampi (the rostral 2.5 mm septal pole) were collected three or six hours after immobilization stress and from naïve mice. For viral and peptide experiments, mice were injected with vehicle, active or scrambled peptide one hour before individual dorsal hippocampi were dissected, frozen in liquid nitrogen and stored at -8°C . Cytoplasmic, membrane, cytoskeletal and nuclear fractions were prepared by using the ProteoExtract kit for subcellular proteome extraction (EMD Biosciences) and validated as described (12).

Coimmunoprecipitation and Immunoblot

Dorsohippocampal membrane fractions (2–5 samples from each group) were pooled to obtain $250\mu\text{g}/\text{group}$ for each co-immunoprecipitation. Immunoprecipitation was performed using the Catch and Release kit (Upstate) for Homer1a (12) and MAGmol Microbeads (Milteny Biotec) with a pre-clearing step for Homer1b/c (19). Washing and elution were performed as described in the respective user's manuals.

Briefly, $250\mu\text{g}$ of membrane protein for each treatment group and $4\mu\text{g}$ of mGluR5 antibody (Millipore) was used for each immunoprecipitation, and rabbit IgG (Sigma) served as a negative control. Eluates and input samples were reduced in loading buffer with DTT and boiled for 5 minutes, then subjected to SDS polyacrylamide gel electrophoresis ($25\mu\text{g}/\text{well}$) and blotted to PVDF membranes (Millipore). Using the SnapID system (Millipore), membranes were saturated with I-block (Tropix), incubated with primary (Anti-Homer1b/c 1:150, Anti- β -Actin 1:150, Santa Cruz Antibodies; Pan-Anti-Homer 1:350; Synaptic Systems; mGluR5 1:500, Millipore) and corresponding secondary antibodies (Goat Anti-Rabbit 1:3500, Tropix). For detection, we used alkaline phosphatase chemiluminescence. Inter-assay variability between blots was determined by a standard control sample for each individual fraction obtained from pooled hippocampi of three naïve or vehicle injected mice in the stress and peptide experiments, respectively.

Perfusion and immunohistochemistry

Mice were anesthetized with an intraperitoneal injection of $240\text{ mg}/\text{kg}$ of Avertin and transcardially perfused with ice-cold 4% paraformaldehyde in phosphate buffer (pH 7.4, $150\text{ ml}/\text{mouse}$). Brains were cut on a cryostat at $50\mu\text{m}$. Sections from fluorescent peptide experiments were mounted on slides for microscopy and counterstained with 4',6-diamidino-2-phenylindole (DAPI, Vector). 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 CCD camera (RTKE Diagnostic Instruments) and SPOT software for Macintosh.

Neuronal cultures and immunofluorescent labeling

The hippocampus from E18 mice was isolated and dissociated with trypsin (Invitrogen, Carlsbad, CA) as described previously (20). Cells were plated on coverslips coated with poly-D-lysine ($0.1\text{mg}/\text{ml}$; Sigma-Aldrich, St. Louis, MO) in 24-well culture plates at a density of $60\,000$ – $80\,000$ cells and grown in NeuroBasal medium (Invitrogen; 2 mM GlutaMax, 0.5% gentamicin and 2% B27). One-half of the medium was replaced with identical medium every 4 days. After two weeks of culturing, $5\mu\text{M}$ of FITC-tagged Active or TAT_{38–48} peptide was applied for 1 h before fixing with 4% formaldehyde in PBS. Light microscopy determined distribution of the peptides.

Statistics

Unpaired Student's *t* test was used for comparisons of two groups. One-, two- or three-way ANOVA was used for multiple group comparisons where appropriate. Scheffe's test was used for post-hoc analyses. $P < 0.05$ was used as the α level 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), one hour of immobilization stress prior to fear conditioning (Figure 1A) resulted in a significant increase of contextual fear one day later, as revealed by a context-specific freezing response (Figure 1B, see also Supplement: Figure S1). Fear conditioning to the tone did not significantly differ between stressed and non-stressed mice [$t(14) = 0.138$, $P = 0.894$, Supplement: Figure S1]. The stress effect emerged 1 hour after the end of immobilization, was maximal between 3–6 hours post-stress and was not observed when the stress-training interval was extended to 24 hours [$F(5,42) = 7.35$, $P < 0.01$]. The 6-hour post-stress 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, $F(1,7) = 12.38$, $P < 0.01$, no stress] (Figure 1C). This effect was completely and lastingly abolished by systemic (intraperitoneal) administration of the mGluR5 inverse agonist MPEP (5mg/kg, injection volumes 40–50 μ l/mouse) [three-way ANOVA, Stress $\times$ Drug interaction, $F(1,20) = 7.392$, $P < 0.05$, Stress $\times$ Drug $\times$ Day, $F(2,40) = 2.489$, $P = 0.104$; Supplement: Figure S2] injected 1 hr after the end of stress. Notably, MPEP did not alter freezing of non-stressed 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 post-injection 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/0.5 μ l/mouse, selected from pilot studies) or vehicle (20% DMSO, 0.5 μ l/mouse) was injected *via* previously implanted cannula into the dorsal hippocampus (Supplement: Figure S3). 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-hr delay [Stress $\times$ Drug interaction, $F(1,48) = 18.173$, $P < 0.001$; Figure 2A]. Importantly, neither stress nor MPEP altered locomotor activity or shock response during training (Supplement: Figure S4A). Similarly, intrahippocampal injection of MTEP (15 nmol/0.5 μ l/mouse) or vehicle (20% DMSO, 0.5 μ l/mouse) administered 5 hrs after stress prevented the enhancement of fear by prior stress [Stress $\times$ Drug interaction, $F(1,26) = 5.996$, $P < 0.05$; Figure 2B]. MTEP did not alter freezing of non-stressed mice ($P = 0.538$), demonstrating that the drug did not interfere with fear conditioning or memory retrieval.

The type I mGluR antagonist MCPG injected intrahippocampally (2 nmol or 20 nmol/mouse) 5 hours post-stress did not prevent stress-enhanced fear [Stress effect, $F(1,34) = 19.93$, $P < 0.001$; Stress $\times$ Drug interaction, $F(2,34) = 0.12$, $P = 0.89$; 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 $\times$ Drug interaction: $F(1,13) = 9.3$, $P < 0.01$, Supplement: Figure S5].

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 BAY36-7620 (100 nmol/mouse) or antagonist CPCCOet (100 nmol/mouse) 5 hours after stress was ineffective [Stress effect, $F(1,53) = 25.67$, $P < 0.001$; Stress $\times$ Drug interaction, $F(2,53) = 0.04$, $P = 0.97$; Figure 2D]. Hippocampal mGluR1 is thus not required for the delayed enhancement of fear by stress.

Viral over-expression 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 -independent (Homer1a) (24) activity of mGluR5. Endogenously, the dominant negative “short” Homer1a triggers ligand-independent mGluR activity (26,27) by displacing the “long” Homer1-3 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 hrs after stress (Supplement: Figure S6A,B) using a pan-anti-Homer antibody reacting with these isoforms (Supplement: Figure S7A,B). However, the binding of mGluR5 to Homer1a was induced three and six hours after stress, as shown by their significantly stronger co-immunoprecipitation signals when compared to the naïve group [Stress effect, $F(3,12) = 39.061$, $P < 0.001$; Figure 3B,C]. At the same time, interactions between mGluR5 and Homer1b/c were significantly decreased [Stress effect, $F(3,12) = 39.061$, $P < 0.01$; 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 Homer1a-Venus (H1aV) fusion protein using a 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 Supplement: Figure S6C), as revealed by an anti-Homer1a antibody recognizing the over-expressed 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 [$t(15) = 0.37$, $P = 0.72$]. However, 10 days after injection H1aV reached detectable levels throughout the entire neuropil of CA1 and dentate gyrus neurons (Figure 4A) and exhibited strong interaction with mGluR5 [$F(3,12) = 29.6$, $P < 0.001$; Figure 4B,C]. At the same time, H1aV injections resulted in a significant decrease of mGluR5/Homer1b/c interactions [$t(4) = 3.641$, $P < 0.05$; Figure 4D] whereas rAAV-GFP was ineffective [$t(4) = 2.056$, $P = 0.109$; Figure 4D] when compared to the naïve group. Furthermore, after contextual fear conditioning, these mice ($N = 8$) showed significantly enhanced freezing to the context when compared to mice expressing rAAV-GFP [$t(13) = 3.89$, $P < 0.01$; $N = 7$, Figure 4E]. Viral overexpression of H1aV did not alter baseline activity or shock reactivity during training (Supplement: Figure S4B). Injection of MPEP 1 hour prior to contextual fear conditioning abolished the enhancement by Homer1a overexpression [$F(2,20) = 10.99$, $P < 0.001$; $t(14) = 4.15$, $P < 0.001$ H1aV/veh 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 utilized 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 HIV-1 (18) to generate the active TAT-mGluR C-tail decoy peptide (active) and an inactive non-binding 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 hr later [$F(5,22) = 5.84$, $P < 0.01$; Figure 5B]. The effective dose of the active peptide injected 1 hr before training, significantly enhanced fear conditioning in non-stressed mice [Dose, $F(1,41) = 9.61$, $P < 0.01$; Dose $\times$ Peptide interaction $F(2,41) = 7.45$, $P < 0.01$; post-hoc analyses: $t(11) = 4.626$, $P < 0.01$ vs vehicle; $t(11) = 1.994$, $P < 0.05$ vs scramble; Figure 5C]. Injection of TAT peptides 5 hr after stress, did not further enhance contextual fear conditioning [$F(2,19) = 0.052$, $P = 0.949$; 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 a FITC tag (Active), and a cell impermeable peptide with identical binding domain, but an adjacent sequence from the TAT protein (TAT₃₈₋₄₈) (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 Supplement: Figure S8). Active TAT significantly impaired mGluR5/Homer1b/c interactions whereas the impermeable TAT₃₈₋₄₈ was ineffective [$F(3,12) = 8.53$, $P < 0.01$; Figure 6B,C]. Further, only the cell permeable active peptide enhanced contextual fear conditioning when injected into the dorsal hippocampus 1 hr before training [$F(2,20) = 9.37$, $P < 0.001$; $t(13) = 3.51$, $P < 0.01$ Active vs vehicle; $t(13) = 3.74$, $P < 0.01$ Active vs TAT₃₈₋₄₈; Figure 6D] and this effect was completely reversed by MPEP injected 15 min later [$t(13) = 3.422$, $P < 0.01$ Active-MPEP vs Active-Veh; 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 (Supplement: Figure S4C,D). 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.

Several studies indicate that mGluR5 receptors are involved in fear conditioning (28,29,30), although pre-training hippocampal manipulations have shown a role of mGluR1 but not mGluR5 in contextual fear (31). By injecting MPEP and MTEP at least 1 hr 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 hrs, 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 Ca^{2+} release, activates big K^+ (BK) 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 mRNA is typically induced within minutes or hours of various environmental manipulations (26,27). Attempts to find a parallel increase of protein levels in the hippocampus have not been successful however (34,35), (36), probably due to rapid proteosomal degradation 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 re-localization 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 Ca^{2+} 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 (4,6,7,8), 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. Post-stress administration of mGluR5 inverse agonists or interventions that de-stabilize 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.

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

Acknowledgments

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References

1. Sala M, Perez J, Soloff P, Ucelli di Nemi S, Caverzasi E, Soares JC, et al. Stress and hippocampal abnormalities in psychiatric disorders. *Eur Neuropsychopharmacol* 2004;14:393–405. [PubMed: 15336301]
2. Eichenbaum H. A cortical-hippocampal system for declarative memory. *Nat Rev Neurosci* 2000;1:41–50. [PubMed: 11252767]
3. Grillon C. Startle reactivity and anxiety disorders: aversive conditioning, context, and neurobiology. *Biol Psychiatry* 2002;52:958–975. [PubMed: 12437937]
4. Radulovic J, Ruhmann A, Liepold T, Spiess J. Modulation of learning and anxiety by corticotropin-releasing factor (CRF) and stress: differential roles of CRF receptors 1 and 2. *J Neurosci* 1999;19:5016–5025. [PubMed: 10366634]
5. Blank T, Nijholt I, Grammatopoulos DK, Randevo HS, Hillhouse EW, Spiess J. Corticotropin-releasing factor receptors couple to multiple G-proteins to activate diverse intracellular signaling pathways in mouse hippocampus: role in neuronal excitability and associative learning. *J Neurosci* 2003;23:700–707. [PubMed: 12533630]
6. Cordero MI, Venero C, Krut ND, Sandi C. Prior exposure to a single stress session facilitates subsequent contextual fear conditioning in rats Evidence for a role of corticosterone. *Horm Behav* 2003;44:338–345. [PubMed: 14613728]
7. Revest JM, Di Blasi F, Kitchener P, Rouge-Pont F, Desmedt A, Turiault M, et al. The MAPK pathway and Egr-1 mediate stress-related behavioral effects of glucocorticoids. *Nat Neurosci* 2005;8:664–672. [PubMed: 15834420]
8. Beylin AV, Shors TJ. Glucocorticoids are necessary for enhancing the acquisition of associative memories after acute stressful experience. *Horm Behav* 2003;43:124–131. [PubMed: 12614642]
9. Lowy MT, Gault L, Yamamoto BK. Adrenalectomy attenuates stress-induced elevations in extracellular glutamate concentrations in the hippocampus. *J Neurochem* 1993;61:1957–1960. [PubMed: 7901339]
10. Shors TJ, Mathew PR. NMDA receptor antagonism in the lateral/basolateral but not central nucleus of the amygdala prevents the induction of facilitated learning in response to stress. *Learn Mem* 1998;5:220–230. [PubMed: 10454366]
11. Cohen AS, Abraham WC. Facilitation of long-term potentiation by prior activation of metabotropic glutamate receptors. *J Neurophysiol* 1996;76:953–962. [PubMed: 8871210]
12. Schrick C, Fischer A, Srivastava DP, Tronson NC, Penzes P, Radulovic J. N-cadherin regulates cytoskeletally associated IQGAP1/ERK signaling and memory formation. *Neuron* 2007;55:786–798. [PubMed: 17785185]

13. Smith MA, Makino S, Kvetnansky R, Post RM. Effects of stress on neurotrophic factor expression in the rat brain. *Ann N Y Acad Sci* 1995;771:234–239. [PubMed: 8597402]
14. Radulovic J, Kammermeier J, Spiess J. Relationship between fos production and classical fear conditioning: effects of novelty, latent inhibition, and unconditioned stimulus preexposure. *J Neurosci* 1998;18:7452–7461. [PubMed: 9736664]
15. Celikel T, Marx V, Freudenberg F, Zivkovic A, Resnik E, Hasan MT, et al. Select overexpression of homer1a in dorsal hippocampus impairs spatial working memory. *Front Neurosci* 2007;1:97–110. [PubMed: 18982121]
16. Ronesi JA, Huber KM. Homer interactions are necessary for metabotropic glutamate receptor-induced long-term depression and translational activation. *J Neurosci* 2008;28:543–547. [PubMed: 18184796]
17. Mao L, Yang L, Tang Q, Samdani S, Zhang G, Wang JQ. The scaffold protein Homer1b/c links metabotropic glutamate receptor 5 to extracellular signal-regulated protein kinase cascades in neurons. *J Neurosci* 2005;25:2741–2752. [PubMed: 15758184]
18. Schwarze SR, Ho A, Vocero-Akbani A, Dowdy SF. In vivo protein transduction: delivery of a biologically active protein into the mouse. *Science* 1999;285:1569–1572. [PubMed: 10477521]
19. Fischer A, Sananbenesi F, Schrick C, Spiess J, Radulovic J. Cyclin-dependent kinase 5 is required for associative learning. *J Neurosci* 2002;22:3700–3707. [PubMed: 11978846]
20. Gao C, Sun X, Wolf ME. Activation of D1 dopamine receptors increases surface expression of AMPA receptors and facilitates their synaptic incorporation in cultured hippocampal neurons. *J Neurochem* 2006;98:1664–1677. [PubMed: 16800848]
21. Shanks N, Anisman H. Stressor-provoked behavioral changes in six strains of mice. *Behav Neurosci* 1988;102:894–905. [PubMed: 3214540]
22. Anderson JJ, Bradbury MJ, Giracello DR, Chapman DF, Holtz G, Roppe J, et al. In vivo receptor occupancy of mGlu5 receptor antagonists using the novel radioligand [3H]3-methoxy-5-(pyridin-2-ylethynyl)pyridine. *Eur J Pharmacol* 2003;473:35–40. [PubMed: 12877935]
23. Bangasser DA, Shors TJ. The hippocampus is necessary for enhancements and impairments of learning following stress. *Nat Neurosci* 2007;10:1401–1403. [PubMed: 17906620]
24. Ango F, Prezeau L, Muller T, Tu JC, Xiao B, Worley PF, et al. Agonist-independent activation of metabotropic glutamate receptors by the intracellular protein Homer. *Nature* 2001;411:962–965. [PubMed: 11418862]
25. Kammermeier PJ. Endogenous homer proteins regulate metabotropic glutamate receptor signaling in neurons. *J Neurosci* 2008;28:8560–8567. [PubMed: 18716215]
26. Kato A, Ozawa F, Saitoh Y, Hirai K, Inokuchi K. vesl, a gene encoding VASP/Ena family related protein, is upregulated during seizure, long-term potentiation and synaptogenesis. *FEBS Lett* 1997;412:183–189. [PubMed: 9257717]
27. de Bartolomeis A, Iasevoli F. The Homer family and the signal transduction system at glutamatergic postsynaptic density: potential role in behavior and pharmacotherapy. *Psychopharmacol Bull* 2003;37:51–83. [PubMed: 14608240]
28. Gravius A, Pietraszek M, Schmidt WJ, Danysz W. Functional interaction of NMDA and group I metabotropic glutamate receptors in negatively reinforced learning in rats. *Psychopharmacology (Berl)* 2006;185:58–65. [PubMed: 16402194]
29. Schulz B, Fendt M, Gasparini F, Lingenhohl K, Kuhn R, Koch M. The metabotropic glutamate receptor antagonist 2-methyl-6-(phenylethynyl)-pyridine (MPEP) blocks fear conditioning in rats. *Neuropharmacology* 2001;41:1–7. [PubMed: 11445180]
30. Rodrigues SM, Bauer EP, Farb CR, Schafe GE, LeDoux JE. The group I metabotropic glutamate receptor mGluR5 is required for fear memory formation and long-term potentiation in the lateral amygdala. *J Neurosci* 2002;22:5219–5229. [PubMed: 12077217]
31. Maciejak P, Taracha E, Lehner M, Szyndler J, Bidzinski A, Skorzevska A, et al. Hippocampal mGluR1 and consolidation of contextual fear conditioning. *Brain Res Bull* 2003;62:39–45. [PubMed: 14596890]
32. Sakagami Y, Yamamoto K, Sugiura S, Inokuchi K, Hayashi T, Kato N. Essential roles of Homer-1a in homeostatic regulation of pyramidal cell excitability: a possible link to clinical benefits of electroconvulsive shock. *Eur J Neurosci* 2005;21:3229–3239. [PubMed: 16026461]

33. Matthews EA, Disterhoft JF. Blocking the BK channel impedes acquisition of trace eyeblink conditioning. *Learn Mem* 2009;16:106–109. [PubMed: 19181615]
34. Igaz LM, Bekinschtein P, Izquierdo I, Medina JH. One-trial aversive learning induces late changes in hippocampal CaMKIIalpha, Homer 1a, Syntaxin 1a and ERK2 protein levels. *Brain Res Mol Brain Res* 2004;132:1–12. [PubMed: 15548423]
35. Zhang GC, Mao LM, Liu XY, Parelkar NK, Arora A, Yang L, et al. In vivo regulation of Homer1a expression in the striatum by cocaine. *Mol Pharmacol* 2007;71:1148–1158. [PubMed: 17234898]
36. Kato A, Ozawa F, Saitoh Y, Fukazawa Y, Sugiyama H, Inokuchi K. Novel members of the Vesl/Homer family of PDZ proteins that bind metabotropic glutamate receptors. *J Biol Chem* 1998;273:23969–23975. [PubMed: 9727012]
37. Ageta H, Kato A, Hatakeyama S, Nakayama K, Isojima Y, Sugiyama H. Regulation of the level of Vesl-1S/Homer-1a proteins by ubiquitin-proteasome proteolytic systems. *J Biol Chem* 2001;276:15893–15897. [PubMed: 11278836]
38. Kato A, Fukazawa Y, Ozawa F, Inokuchi K, Sugiyama H. Activation of ERK cascade promotes accumulation of Vesl-1S/Homer-1a immunoreactivity at synapses. *Brain Res Mol Brain Res* 2003;118:33–44. [PubMed: 14559352]
39. Calo L, Bruno V, Spinsanti P, Molinari G, Korkhov V, Esposito Z, et al. Interactions between ephrin-B and metabotropic glutamate 1 receptors in brain tissue and cultured neurons. *J Neurosci* 2005;25:2245–2254. [PubMed: 15745950]
40. Sandi C, Pinelo-Nava MT. Stress and memory: behavioral effects and neurobiological mechanisms. *Neural Plast* 2007;2007:78970. [PubMed: 18060012]
41. Diamond DM, Park CR, Heman KL, Rose GM. Exposing rats to a predator impairs spatial working memory in the radial arm water maze. *Hippocampus* 1999;9:542–552. [PubMed: 10560925]
42. Woodson JC, Macintosh D, Fleshner M, Diamond DM. Emotion-induced amnesia in rats: working memory-specific impairment, corticosterone-memory correlation, and fear versus arousal effects on memory. *Learn Mem* 2003;10:326–336. [PubMed: 14557605]
43. Inoue N, Nakao H, Migishima R, Hino T, Matsui M, Hayashi F, et al. Requirement of the immediate early gene vesl-1S/homer-1a for fear memory formation. *Mol Brain* 2009;2:7. [PubMed: 19265511]
44. Rodrigues SM, LeDoux JE, Sapolsky RM. The influence of stress hormones on fear circuitry. *Annu Rev Neurosci* 2009;32:289–313. [PubMed: 19400714]
45. Gray, J.; McNaughton, N. *The neuropsychology of anxiety*. 2nd ed.. Oxford: Oxford University Press; 2000.

Figure 1.

Stress-enhanced fear conditioning requires ongoing activity of mGluR5. (A) Design of the stress/conditioning phase of the experiment indicating the time window between stress and fear conditioning. (B) Fear conditioning performed 3 and 6 hrs after stress resulted in a significant enhancement of fear tested 24 hr later. (C) The enhancing effect of stress on fear lasted for at least a month. * $P < 0.05$, *** $P < 0.001$ vs non-stress/vehicle; ### $P < 0.001$ vs stress/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 requires ligand-independent but not ligand-dependent activation of mGluR5 or mGluR1 in the hippocampus. (A) Intrahippocampal injection of 50 nmol/mouse MPEP up to 5 hr post-stress reduced enhancement of fear. (B) The potent mGluR5 inverse agonist MTEP injected intrahippocampally 5 hr after stress prevented stress enhanced contextual fear. (C) Intrahippocampal injection of the mGluR competitive antagonist MCPG 5 hr after stress did not prevent the enhancement of fear. (D) Intrahippocampal injection of the mGluR1 antagonist CPCCOet (100 nmol/mouse), or inverse agonist BAY36-7620 (100 nmol/mouse) 5 hr post-stress did not reverse stress-enhanced fear.

** $P < 0.01$, *** $P < 0.001$ vs non-stress/ vehicle; # $P < 0.05$, ## $P < 0.01$, ### $P < 0.001$ vs stress/vehicle.

Figure 3. 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 Homer1-3 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 hrs after stress. Left: coimmunoprecipitation (CoIP), Right: input; Input/IgG is the input sample used for IgG co-immunoprecipitation; N: naïve. The co-immunoprecipitation experiment was replicated with 6 pull-downs of independent sample pools, each prepared from 2–5 individual membrane fractions/group (a total of 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. * $P < 0.05$, ** $P < 0.01$, *** $P < 0.001$ vs naïve.

Figure 4.

Overexpression of Homer1a mimics the effects of stress. (A) Mice injected with rAAV-H1aV or and rAAV-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 CA1 dorsohippocampal neurons 5 and 10 days after viral injection. White arrows indicate Homer1a-positive neurons. (B) H1aV interacts with mGluR5 as revealed by robust co-immunoprecipitation of both proteins 10 days after injection of rAAV-H1aV, whereas mGluR5/Homer1b/c interactions decrease. Input/IgG is the input sample used for IgG co-immunoprecipitation; The co-immunoprecipitation experiment was replicated with 3 pull-downs of independent sample pools, each prepared from 2–4 individual membrane fractions/group (a total of N=10–13 hippocampi/group). (C) Quantification of the immunoblot signals showing significant increase of mGluR5/H1aV interaction when compared to all other groups. The data were normalized to H1aV because there was no detectable signal in the naïve 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 hr prior to training reverses this enhancement. * $P < 0.05$, *** $P < 0.001$ vs naïve, +++ $P < 0.001$ vs rAAV-GFP, ## $P < 0.01$ vs rAAV-H1aV/Vehicle.

Figure 5.

Uncoupling 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 coimmunoprecipitation 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 4 pull-downs of independent sample pools, each prepared from 2–3 individual membrane fractions/group (a total of N=10–12 hippocampi/group). **(C)** Intrahippocampal injection of active peptide at a dose of 500 ng/mouse 1 hr before training enhanced fear conditioning of non-stressed mice when compared to the scramble- or vehicle-injected controls. Prior stress prevented further enhancement of contextual fear by TAT peptides. * $P < 0.05$, ** $P < 0.01$ vs vehicle, # $P < 0.05$ vs Scramble.

Figure 6.

TAT peptides reduce mGluR5 interactions and enhance fear in an mGluR5-dependent manner. (A) FITC-tagged active peptide, contrary to the FITC-tagged TAT₃₈₋₄₈ peptide, readily entered into cultured neurons (upper panels) and in CA1 pyramidal cells *in vivo* (lower panels). (B) A representative co-immunoprecipitation image showing reduced mGluR5/Homer1b/c interactions after injection of active TAT. Input/IgG is the input sample used for IgG co-immunoprecipitation. The co-immunoprecipitation experiment was replicated with 3 pull-downs of independent sample pools, each prepared from 2–3 individual membrane fractions/group (a total of N=6–9 hippocampi/group.) (C) Quantification of the immunoblot signals showing a significant effect of active TAT but not impermeable TAT₃₈₋₄₈ on mGluR5/Homer1b/c interactions. (D) Intrahippocampal injection of 500 ng/mouse of FITC-tagged active peptide 1hr before training enhanced fear when compared to the cell impermeable TAT₃₈₋₄₈ peptide or vehicle. (E) Enhancement of fear by active TAT peptide was completely abolished by MPEP (50 nmol/mouse) injected 15 min later. * $P < 0.05$, ** $P < 0.01$ vs vehicle, ++ $P < 0.01$ vs TAT₃₈₋₄₈, ## $P < 0.01$ vs Active + vehicle.