

Stress at Learning Facilitates Memory Formation by Regulating AMPA Receptor Trafficking Through a Glucocorticoid Action

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Stress and glucocorticoids (GCs) can facilitate memory formation. However, the molecular mechanisms mediating their effects are largely unknown. α -Amino-3-hydroxy-5-methyl-4-isoxazolepropionic acid (AMPA) receptor (AMPA) trafficking has been implicated in the changes in synaptic strength at central glutamatergic synapses associated with memory formation. In cell cultures, corticosterone has been shown to condition the synaptic trafficking of the AMPAR GluA2 subunit. In this study, we investigated the involvement of GluA2 trafficking in the facilitation of learning by stress. Using the water maze spatial task involving different stress levels, mice trained under more stressful conditions (water at 22°C) showed better learning and memory, and higher post-training corticosterone levels, than mice trained under lower stress (water at 30°C). Strikingly, this facilitated learning by stress was accompanied by enhanced synaptic expression of GluA2 AMPARs that was not observed in mice trained under less stressful conditions. Interfering with GC actions by injecting the GC synthesis inhibitor, metyrapone, blocked both the memory facilitation and the enhanced GluA2 trafficking induced by stressful learning. Intracerebroventricular infusion of the peptide, pep2m, that blocks GluA2 synaptic trafficking by interfering with the interaction between N-ethylmaleimide-sensitive factor and GluA2, impaired immediate performance at learning as well as long-term memory retrieval, supporting a causal role for GluA2 trafficking in stress-induced facilitation of spatial learning and memory. Evidence for the involvement of the neural cell adhesion molecule N-cadherin in interaction with GluA2 is also provided. These findings underscore a new mechanism whereby stress can improve memory function.

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INTRODUCTION

Stress experienced within the training context can potentiate memory formation by enhanced levels of glucocorticoid (GC) hormones (de Kloet *et al*, 1999; Joels *et al*, 2006; Roozendaal *et al*, 2006; Sandi and Pinelo-Nava, 2007). GCs (corticosterone in rodents), released by the adrenal glands into the bloodstream, readily gain access to the brain where, through binding to specific (mineralocorticoid—MR and glucocorticoid-GR) receptors, they can affect brain function (De Kloet *et al*, 1998).

The molecular pathways underlying the facilitating role of stress and GRs on memory formation are largely unknown. Recent work on stress-induced memory facilitation has linked the activation of the mitogen-activated protein kinase (MAPK) signaling pathway to the effects of GCs (Revest *et al*, 2005), and phosphorylation and trafficking of the GluA1 AMPA (α -amino-3-hydroxy-5-methyl-4-isoxazolepropionic acid) glutamate receptors (AMPARs) to the

effects of norepinephrine (Hu *et al*, 2007). AMPARs are responsible for the majority of the fast excitatory transmission in the brain. They are heterotetramers comprised of a combinatorial assembly of four subunits, GluA1, GluA2, GluA3, and GluA4 (latest nomenclature NC_IUPHAR (Collingridge *et al*, 2009)). The majority of AMPARs in the central nervous system are composed of GluA2-containing heteromers (Adesnik and Nicoll, 2007; Lu *et al*, 2009; Malinow and Malenka, 2002; Shepherd and Huganir, 2007). GluA2 has a critical role in controlling various AMPAR properties, including Ca^{2+} permeability, subunit assembly of AMPARs (Derkach *et al*, 2007; Sprengel, 2006), and AMPAR synaptic targeting (Lu *et al*, 2009). In the hippocampus, the GluA2 subunit mainly occurs in principal pyramidal neurons (Tokuoka and Goda, 2008) and excitatory synaptic currents appear to be primarily mediated by GluA2-containing AMPARs (Plant *et al*, 2006).

Recently, corticosterone was shown to trigger time-dependent increases in GluA2-AMPA surface mobility and synaptic surface content in rat hippocampal cultures (Groc *et al*, 2008). Moreover, within minutes, corticosterone was able to potentiate the increase of synaptic surface GluA2 content induced by a chemical long-term potentiation stimulus (Groc *et al*, 2008). However, it is not known whether corticosterone can exert similar effects in living

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animals submitted to stressful learning protocols. This is of particular relevance as synaptic trafficking of AMPARs is induced by learning and long-term potentiation (LTP) (Whitlock *et al*, 2006; Williams *et al*, 2007), but it is not known whether the stress/GR component contributes to this effect. In this study, we investigate the contribution of GluA2-AMPA trafficking to stress-induced and GC-mediated facilitation of spatial memory. Using the water maze spatial task involving different stress levels, we find that mice trained under more stressful conditions (water at 22°C) show better learning and memory, as well as increased post-training corticosterone levels and synaptic expression of GluA2 AMPARs, than mice trained under lower stress (water at 30°C). The requirement of a GC action was evaluated by injecting either an inhibitor of GC synthesis (metyrapone) or an antagonist of the GC. The causal involvement of GluA2 trafficking in the behavioral effects of stress was evaluated by injecting mice with a synthetic peptide pep2m, which interferes with N-ethylmaleimide-sensitive factor (NSF)-mediated GluA2 trafficking.

MATERIALS AND METHODS

Animals

Adult male C57B6J mice (8 weeks on day of arrival; Charles River Laboratories) were used. Animal experiments were approved through a license issued by the Cantonal Veterinary Authorities (Vaud, Switzerland). Animals were housed in groups of two to four and kept on a 12-h light/dark cycle (lights on at 0700 hours) in our in-house animal care facility with access to food and water *ad libitum*. They were left to habituate to the new environment for at least 2 weeks before behavioral assessment. All experiments were conducted between 0800 and 1400 hours to avoid the influence of circadian hormonal fluctuations.

Morris Water Maze Training

The Morris water maze is a hippocampus-dependent task (Morris *et al*, 1982, 1990). The apparatus consisted of a large white circular pool (140 cm in diameter) filled with opaque colored water. Mice were trained to locate a hidden platform located in the middle of one of the virtual quadrants with the help of extra-maze visual cues. The hidden platform (10 × 10 cm) was submerged 0.5 cm beneath the water surface and placed in a fixed location in the middle of one of the virtual quadrants. Both pool and platform were made of white polyvinyl plastic and offered no intra-maze cues to guide escape behavior. Different groups of mice were trained at either 22 or 30°C water temperature.

The general experimental procedure was organized as follows. Mice received a pretraining session (day 1) followed by up to 3 days (days 2–4) of spatial training (but note that as we focused on early learning mechanisms, most experiments ended immediately after training on day 2). The goal of the pretraining session was to habituate mice to the apparatus and water (temperature either 22 or 30°C). First, they were given a free swim trial (without platform) for 2 min. At the end of this trial, the platform was rapidly placed in the pool and the mouse allowed to stay on it for

15 s. After a 10 min intertrial interval, mice were given a 60 s training trial. Spatial training was given over the subsequent 2–4 days. Each spatial training session consisted of six trials (intertrial interval 6 min) that for data analyses purposes were collapsed in blocks (B) of two trials. Each trial started with the mouse facing the wall at one of three possible start positions. If the mouse did not find the platform within 60 s, it was guided toward it. Each mouse remained on the platform for 15 s before being taken out and placed in a holding cage warmed by a red heating bulb. Twenty-four hours after the last training session (ie, on either day 3 or day 5, depending on the specific experiment), memory was assessed through a 60 s probe test held without the platform. As stated above, in most of the biochemical experiments, mice were killed immediately after trial 6 (B3) on day 2. However, some experiments were conducted to study time dynamics of training-induced receptor trafficking regulation with animals then being killed either immediately after trial 2 (B1) on day 2 or 45 min after the end of the last training trial (B3+45 min) on day 2.

One experiment involved 'yoked' controls (Y), that received the same experimental procedures as mice trained at 22°C, but with no platform in the pool (ie, yoked mice were exposed to the pool, over days 1 and 2, for the same amount of time per trial as mice exposed to the hidden platform version of the water maze), then killed immediately after B3 on day 2.

A cued version of water maze addressed to evaluate the motivation of mice trained at 30°C to swim to the escape platform. In all trials, a 10 cm flag attached to the base of the platform marked the platform location. Mice were released from the same release positions as mice trained in a hidden version of the water maze.

Video tracking software (Ethovision, Noldus, Wageningen, Netherlands) was used for automatic recording of a variety of parameters (escape latencies, distances swum, cumulative distance, and velocities). As there were occasional difference in swim speed between animals trained at the two water temperatures (see Supplementary Figure S1), the cumulative distance to the platform (distance to the platform location summed over all videotracking samples of a given trial), rather than the latency, was used for the statistical analyses. For the probe tests, the percent time spent in each quadrant of the pool was measured and time spent in the 'target' (ie, where the platform was located during training) quadrant was calculated. In addition, for the probe test on day 3, cumulative distance to the virtual platform was also calculated.

Corticosterone Analysis

Trunk blood was taken at decapitation. Plasma corticosterone was quantified using Correlate-EIA Corticosterone Enzyme Immunoassay Kit (Assay Design, MI) according to the manufacturer's instructions.

Synaptoneurosome Preparation

Hippocampal tissue was obtained from mice submitted to different experimental procedures and from naive untrained littermate controls (Ctrl). Following dissection, the hippocampus was immediately placed in ice-cold dissection buffer

(212.7 mM sucrose/2.6 mM KCl/1.23 mM NaH₂PO₄/26 mM NaHCO₃/10 mM dextrose/1.0 mM MgCl₂/0.5 mM CaCl₂/0.1 mM Kynurenic acid and saturated with 95% O₂/5% CO₂). Synaptoneurosomes (SNS) were prepared according to the method of Hollingsworth *et al* (1985), and as described by (Quinlan *et al*, 1999). See Supplementary Materials and Methods for a detailed description.

Cell-Surface Biotinylation

To label cell-surface proteins on SNS, we followed a protocol previously described (Williams *et al*, 2007). Briefly, a fixed amount of SNS (120 µg in 50 µl of PBS) was incubated with a membrane-impermeable biotin moiety (200 µl of 1.5 mg/ml sulfo-NHS-SS-biotin; Pierce Biotechnology, IL) for 30 min at 4°C. Unreacted biotin was then quenched by addition of an equal volume of 100 mM glycine in PBS. The synaptoneurosomes were again precipitated by centrifugation at 3600 g for 10 min at 4°C and then washed in 100 mM glycine in PBS. Following centrifugation, labeled SNS were solubilized in homogenization buffer containing 1% SDS and protease and phosphatase inhibitor cocktail (Roche, Switzerland). The labeled solubilized synaptoneurosomes fraction was then incubated with 20 µl of neutravidin conjugated to agarose beads for 2 h at 4°C to precipitate the biotin bound proteins. The agarose beads were precipitated by sequential centrifugation (10 000 g) followed by two washing steps in solubilization buffer. The final precipitated beads were resuspended in 2 × sample buffer and boiled for 10 min at 95°C to release the biotin–neutravidin complex. After precipitation of the agarose beads, the supernatant was taken and frozen at –20°C.

Quantitative Western Blotting

Protein content in whole and SNS hippocampal samples was quantified using the DC protein assay (Biorad Laboratories AG, Switzerland). Equal protein samples were prepared at a concentration of 0.5 µg/ml in 33 mM NaCl, 70 mM Tris-HCl, 1 mM EDTA, 2% (w/v) SDS, 0.01% (w/v) bromophenol blue, 10% glycerol, pH 6.8. Proteins were resolved on 10% polyacrylamide gels, and transferred to nitrocellulose membranes. For analysis of cell-surface proteins, a fixed proportion of protein derived from a standard amount of SNS (120 µg) was used. Membranes were incubated with specific primary and secondary antibodies (see Supplementary Materials and Methods for specific western blot conditions) and the immunocomplexes were visualized using a chemiluminescence peroxidase substrate (Super-Signal West Dura, Pierce Biotechnology) and immunoreactivity detected using the Biorad ChemiDoc XRS system. Densitometry analysis on the bands was calculated using Biorad Quantity One (4.2.3) software (Biorad Laboratories). Absorbance for each of the synaptic protein antibodies was normalized to within-lane actin absorbance. Average densitometric data are reported for each experimental group as percentage of control values.

Surgery and Cannulation

Mice were anesthetized with an intraperitoneal (i.p.) injection of a 10 mg/kg/ml xylazine and 70 mg/kg/ml

ketamine mix, and then placed in a stereotaxic frame. A 26-gauge guide cannula (Plastic One Inc., VA) was placed into the right lateral ventricle (0.4 mm posterior to the bregma, 1.0 mm lateral to the midline, and 2.0 mm from the surface of the skull using a flat skull position). The guide cannula was secured to the skull using dental acrylic bound to glue. A dummy cannula (Plastic One Inc.) was inserted into the guide cannula to maintain cannula patency before insertion of the infusion cannula during drug administration. Mice were allowed to recover for at least 1 week before injections were performed. Injections were performed using a 33-gauge stainless steel internal cannula (Plastic One Inc.). The internal cannula was connected to a 10 µl Hamilton syringe (Hamilton, Switzerland) by a PE20 tube. Peptides diluted in artificial sterile CSF were administered at a constant rate of 0.25 µl/min, and the injection cannula was removed 1 min following the termination of the injection to avoid spillage from the guide cannula.

Drug Administration

The corticosterone synthesis inhibitor metyrapone (75 mg/kg) was injected i.p. 1 h before pretraining (day 1) and to training on day 2; control animals were injected with an equal volume (200 µl) of saline vehicle. The GR receptor antagonist RU-38486 (40 mg/kg; Sigma Aldrich, Switzerland) and corticosterone (10 mg/kg; soluble corticosterone 2-hydroxypropyl-β-cyclodextrin complex (Sigma Aldrich)) were also injected i.p. For more details, see Supplementary Material and Methods. The peptide pep2m (Primm Srl, Milan, Italy) that interferes with the interaction between the C terminus of the GluA2 and NSF was administered intracerebroventricularly (i.c.v.) (for details on the procedures followed for surgery and cannulation, see Supplementary Material and Methods). A scrambled version of the pep2m peptide (scr-pep2m, Primm Srl) served as control. Both peptides were fused to an 11-amino-acid sequence derived from the TAT protein of the human immunodeficiency virus that facilitates intracellular delivery of peptides and were synthesized by Primm Srl. We injected 1 µl of either pep2m or scr-pep2m (450 nM dissolved in artificial CSF) by i.c.v. injection 30 min before training on day 2.

Statistics

Results are expressed as mean ± SEM. Before evaluating group differences, data normality was checked using Levene's and Bartlett's tests for equal variances. When normality was confirmed, data were analyzed with either Student's *t*-tests (with Welch's correction where necessary; one-tailed for comparisons testing the *a priori* hypothesis that 22°C-trained mice would show stronger memory than 30°C-trained mice in the probe test of the water maze) or ANOVA (one-way or repeated measures) followed by the Bonferroni *post hoc* test, as appropriate. When normality was rejected, data were analyzed using the nonparametric Kruskal–Wallis test followed by Dunn's multiple comparison *post hoc* test. Significance was set at $P \leq 0.05$.

RESULTS

Cold Water Facilitates Spatial Learning and Memory and Increases Plasma Corticosterone

We exploited previous observations in rats indicating that the strength of memory developed in a spatial water maze task changes with variations in the water temperature that lead to different degrees of corticosterone activation (Akirav *et al*, 2004; Sandi *et al*, 1997), and trained mice using an adapted protocol. Our training schedule involved one pretraining day (day 1), followed by three training days (days 2–4) consisting of six trials per day, with data analyzed represented in blocks (B) of two trials. Mice trained in the water maze at 22°C ($n=8$) showed better overall learning over the three training days (Figure 1a; $F_{(1,18)}=16.46$, $p=0.001$) and stronger long-term memory in a probe test conducted 24 h after the final trial (Figure 1b and c; $p<0.05$) than mice trained at 30°C ($n=12$).

From data on Figure 1a, it became apparent that performance of mice trained at 22°C was already significantly better than in 30°C-trained mice on the first training day (day 2: $F_{(1,18)}=10.89$, $p=0.004$). We, therefore, set to study whether GluA2 would be involved in this rapid phenomenon and started by characterizing behavioral differences induced by the different training conditions in these early training days. We first verified that these behavioral differences were also reflected in indexes of better memory on the following day, as mice trained at 22°C

showed a shorter cumulative distance to the platform on the first trial conducted on day 3 (Figure 1d; $p<0.05$). We should mention that, as showed above, we found that training at the two water temperatures applied in our study led to differences on the probe trial performed on day 5, which indicates that differences in stress at learning has a long-term effect on memory formation (Figure 1b and c). As we focused our study on early effects of stress at training on learning acquisition, and given that probe trial effects (in terms of a quadrant preference) after a single training day are only rarely found, the parameter cumulative distance to the platform was instead the one evaluated to test for memory effects on day 3.

We then measured plasma corticosterone levels immediately after training mice on day 2 and confirmed that training at 22°C is more stressful than training at 30°C (Figure 1e; $p=0.008$). We also found that mice trained at 30°C under cued platform conditions were significantly more accurate to reach the platform than mice trained at the same water temperature with a hidden platform, which excluded a lack of motivation in 30°C-trained mice to escape from the water (Figure 1f; $F_{(1,8)}=8.47$, $p=0.02$).

Stress Facilitates Hippocampal GluA2 Synaptic Trafficking

We then aimed to evaluate the effect of stressful learning on AMPAR trafficking by focusing on the first training day

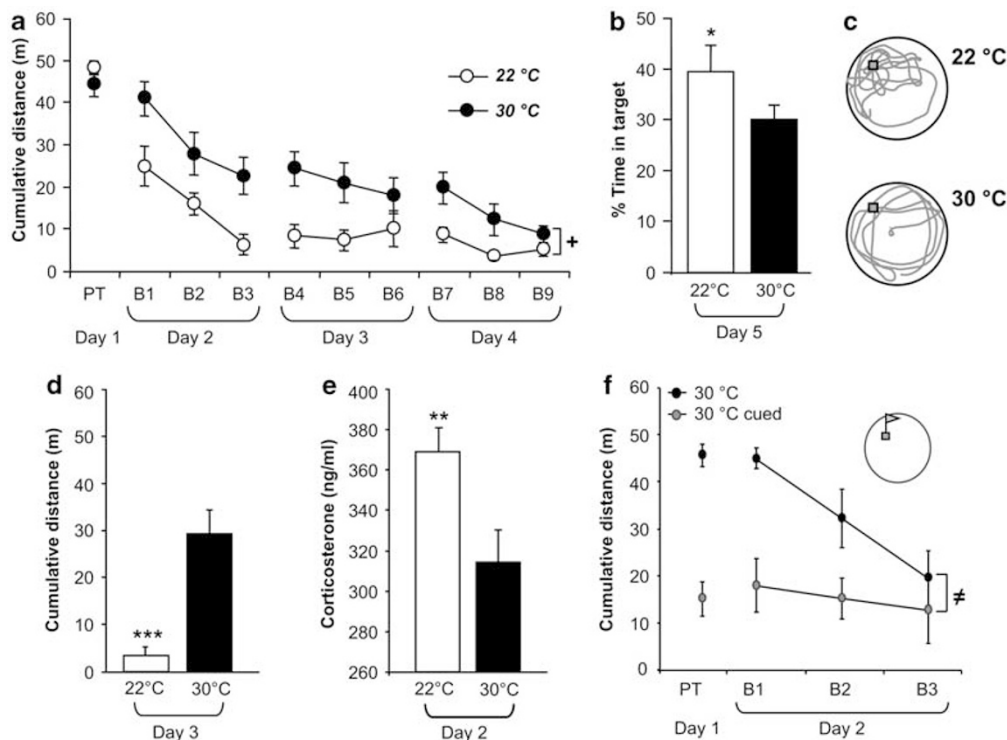


Figure 1 Stress at training facilitates spatial learning and memory, and increases plasma corticosterone. (a) Mice trained at 22°C learned better on days 2–4 and (b) spent significantly more time in the target quadrant in a probe test conducted on day 5 (c) as illustrated in representative representations of swim paths ($n=8-12$). (d) Indication that 22°C-trained mice also show improved memory after training on days 1 and 2 is given by their lower cumulative distance to the platform than 30°C-trained mice on the first trial of day 3. (e) Post-training plasma corticosterone levels on day 2 were significantly higher in 22°C ($n=24$) than in 30°C-trained mice ($n=21$). (f) Mice trained at 30°C in a cued version of the water maze have a significantly shorter cumulative distance to the platform than mice trained in the standard hidden version of the water maze. Data represent mean \pm SEM. * $p<0.05$ (repeated measures ANOVA); * $p<0.05$, ** $p<0.01$, and *** $p<0.001$ vs 30°C. $\neq p<0.05$, 30° vs 30°C cued.

(ie, day 2). Thus, the hippocampus was dissected out immediately after training to prepare an SNS biochemical fraction (enriched for synaptic proteins and validated to detect trafficking of hippocampal AMPARs after learning and *in vivo* LTP (Whitlock *et al*, 2006; Williams *et al*, 2007)). Conditions were established to be in the linear range of the immunoblot assays (Supplementary Figure S2) and their quantitative analyses performed without experimenter having knowledge of the experimental condition.

Mice trained at 22°C exhibited higher levels of GluA2 in the SNS fraction compared with control mice (Figure 2a; Kruskal–Wallis $p=0.002$; Ctrl vs 22°C, $p<0.01$), whereas GluA2 expression levels in 30°C-trained mice were not significantly different from controls. The increase was, to some extent, specific to this subunit, as the GluA1 (Figure 2b) and the NR1 subunit of the NMDA receptor (Figure 2d) remained unchanged. However, expression of GluA3 was also increased in 22°C-trained mice ($p<0.01$),

but a mild upregulation in 30°C-trained mice did not reach significance (Figure 2c). These changes were likely to be hippocampus-specific as we found no learning-induced change in GluA1, GluA2, GluA3, or NR1 subunits in an SNS fraction prepared from the cerebellum of trained mice (data not shown). Moreover, stress alone is not sufficient to enhance synaptic GluA2 expression, as yoked controls, that were exposed to the water maze at 22°C for the same time as trained animals but without a platform (22°C-Y), and displayed elevated plasma corticosterone levels (naive: 27.43 ± 4.49 ng/ml; 22°C-Y: 331.33 ± 17.85 ng/ml), showed no changes in synaptic GluA2 expression (Figure 2e).

To ascertain if the observed changes in GluA2 expression in SNS are expressed at the cell surface, we used a biotin-based method (Williams *et al*, 2007) that produces a fraction that is highly enriched for cell-surface membrane synaptic proteins. Briefly, this method is based on the recovery of surface membrane-associated proteins from the

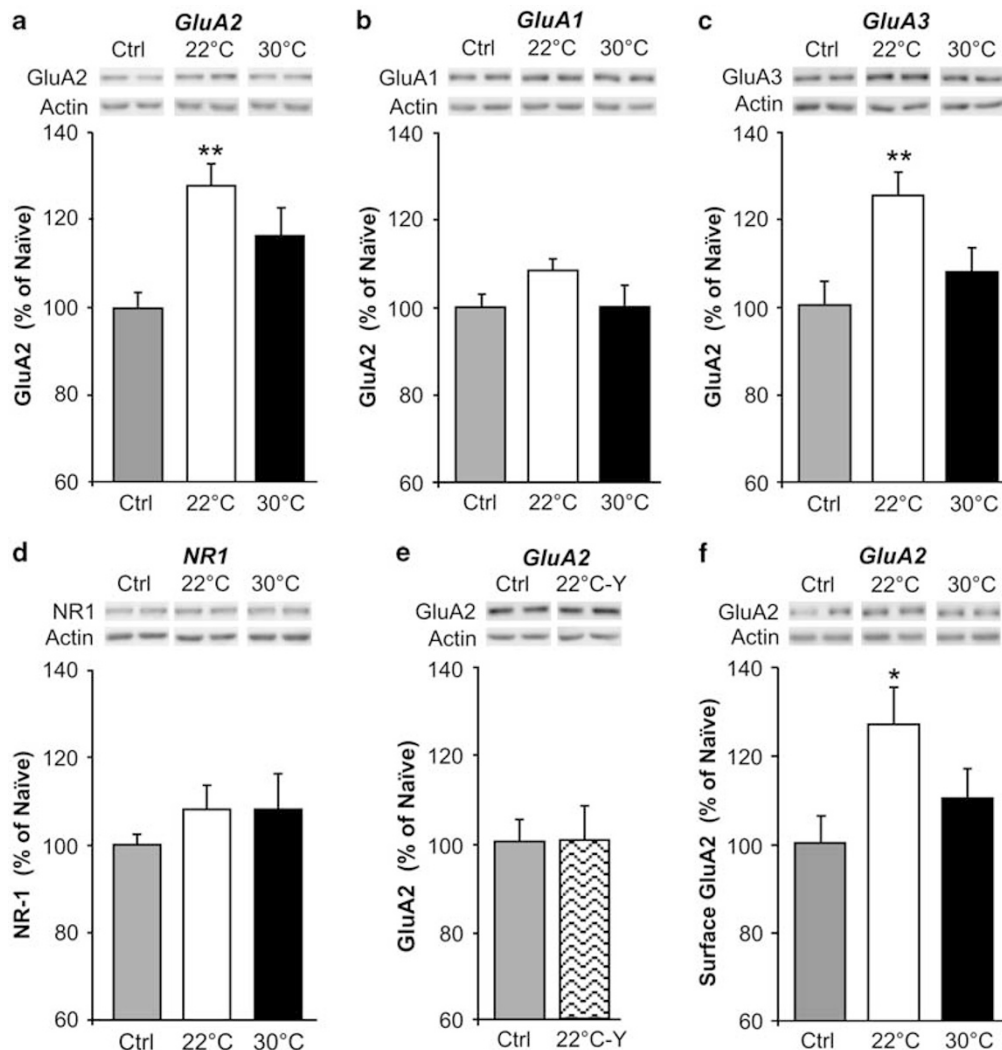


Figure 2 Stress facilitates synaptic GluA2 trafficking. (a–d) Results from immunoblot analyses of synaptic GluA2, GluA1, GluA3, and NR1 receptors, including representative blots and corresponding actin, from naive controls (Ctrl) and from mice trained at 22 or 30°C ($n=18$ –27 per group). Training at 22°C resulted in a significant increase in synaptic GluA2 and GluA3 expression. (e) Synaptic GluA2 trafficking was not altered in 22°C-yoked controls (22°C-Y, $n=8$). (f) The expression of GluA2 on the synaptoneurosomal cell surface is increased in 22°C-trained compared with Ctrl mice ($n=6$, per group). Data represent mean \pm SEM. * $p<0.05$, ** $p<0.01$ vs Ctrl.

SNS fraction by biotinylation and subsequent precipitation with a neutravidin-agarose conjugate. In control experiments, western blot analyses confirmed the expression of all AMPAR subunits on this SNS surface extract (data not shown). In samples from trained mice, we verified that immediately after training on day 2, expression levels of SNS cell-surface GluA2 are significantly higher in 22°C-trained mice than in control animals (Figure 2f; $p < 0.05$, $n = 6$ per group), whereas mice trained at 30°C do not differ from controls.

In a crude homogenized hippocampal fraction, expression of GluA2 was unchanged (Supplementary Figure S3b, a lack of changes in the total hippocampal fraction for other glutamate receptor subunits is shown in Supplementary Figure S3a-d) indicating that the observed modulations are related to receptor trafficking and questioning the involvement of transcriptional effects.

Blockade of Stressful Learning-Elicited Corticosterone Release Prevents Stress-Induced Memory Facilitation and GluA2 Trafficking

To test whether enhanced plasma corticosterone levels observed in 22°C-trained mice would contribute to the memory facilitating effect and GluA2 trafficking, a new experiment was performed in which mice were treated with the corticosterone synthesis inhibitor metyrapone (75 mg/kg, i.p. injection) 1 h before being trained at 22°C on both the pretraining day 1 and training day 2. Then, these mice were divided in two sets (each one including vehicle- and metyrapone-treated groups): one set to evaluate their long-term memory after 24 h (day 3), and another set in which the hippocampus was dissected out immediately after training (on day 2). Metyrapone had no effect on learning acquisition on day 2 (Figure 3a; day 2; $F_{(1,34)} = 1.61$, $p = 0.21$)

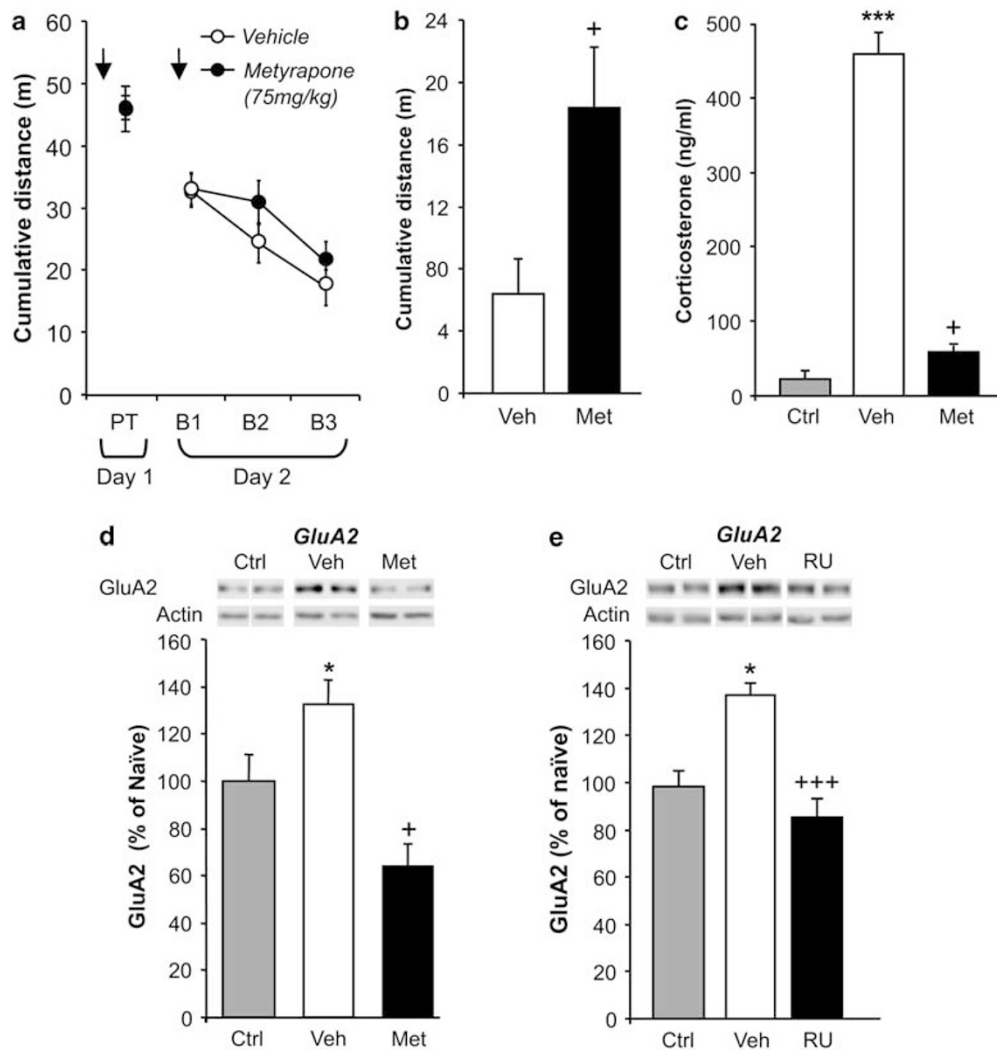


Figure 3 Effects of metyrapone and RU486 on learning and memory and synaptic AMPAR trafficking during stressful learning. (a) Mice trained at 22°C that were treated with metyrapone pretraining (Met, 75 mg/kg, i.p.; $n = 18$) were not impaired at spatial learning on day 2, but (b) were impaired in memory retrieval on day 3, as compared to vehicle-injected mice (Veh; $n = 18$). (c) Post-training corticosterone release was prevented by metyrapone treatment. (d) Metyrapone also interfered with the post-training trafficking of synaptic GluA2 (Ctrl, $n = 9$; Veh, $n = 12$; Met, $n = 8$). (e) RU 38486 antagonized the training-induced increase in synaptic GluA2 (Ctrl, $n = 9$; Veh, $n = 6$; Met, $n = 7$). Arrows indicate injection timing. Data represent mean \pm SEM. * $p < 0.05$, *** $p < 0.001$ vs Veh, * $p < 0.05$ and *** $p < 0.001$ vs Ctrl.

but impaired memory consolidation as indicated by reduced retrieval on day 3 (Figure 3b; $p=0.02$). Interestingly, a separate experiment indicated a lack of effect of metyrapone injections in animals trained at 30°C water (data not shown).

In the second set, animals were killed immediately after training (on day 2). We verified that metyrapone was effective in preventing training-induced corticosterone release (Figure 3c; $p<0.05$). Strikingly, metyrapone also prevented the modulation of GluA2 synaptic trafficking induced by 22°C water training ($p<0.001$), with SNS GluA2 levels in the vehicle-treated group being higher than those found in both untrained controls ($p<0.05$) and trained and metyrapone-injected mice ($P<0.01$) (Figure 3d). By contrast, metyrapone had no effect on synaptic GluA1 expression (data not shown). To investigate the involvement of GR receptors—which mediate corticosterone actions and were shown to be essential for consolidation of long-term spatial memory (Oitzl *et al*, 2001)—we injected the GR antagonist RU38486 before training and found that it inhibited the increase in GluA2 observed in 22°C-trained mice (Figure 3e; RU vs Ctrl $p<0.05$). Corticosterone injections (10 mg/kg; i.p.) did not modify SNS GluA2 levels when given to untrained mice on two consecutive days (a protocol followed to mimic hormone increases induced by training on days 1 and 2), which is reminiscent of the lack of GluA2 modulation in 22°C yoked controls that show enhanced plasma corticosterone levels without a concomitant spatial learning (Figure 2f) (Supplementary Figure S4).

Taken together, these data support a key role for corticosterone in stressful learning-induced facilitation of spatial memory formation and concurrent modulation of GluA2 trafficking.

Intracerebroventricular Infusion of a Peptide that Blocks GluA2 Trafficking Prevents the Stress-Induced Memory Facilitation

A key question arising from these findings is whether the enhanced GluA2 trafficking contributes to the memory

facilitating effect resulting from stressful learning. To tackle this question, mice were given an i.c.v injection of either a synthetic peptide (TAT)-pep2m, which interferes with NSF-mediated GluA2 trafficking (Nishimune *et al*, 1998; Yao *et al*, 2008), or a scrambled control peptide (scr-pep2m) 30 min before training at 22°C on day 2. Both the learning rate on day 2 (Figure 4a; $F_{(1,31)}=6.67$, $p=0.02$) and the expression of long-term memory (Figure 4b; day 3, $p\leq 0.05$) were impaired in pep2m-treated mice compared with scr-pep2m-treated mice. Post-training corticosterone levels were similarly increased in both groups (Figure 4c). These findings implicate increased GluA2 trafficking in the learning-enhancing effects of stressful learning.

Stressful Learning Facilitates Hippocampal N-Cadherin Trafficking With Time Dynamics Similar to GluA2

Through a reciprocal interaction with the cell adhesion molecule, N-cadherin, GluA2 has been shown to promote the formation and growth of dendritic spines (Saglietti *et al*, 2007). As enhanced spine density has been linked with memory consolidation (Diamond *et al*, 2006), we measured N-cadherin expression under our experimental conditions. Strikingly, we found the same pattern of results as for GluA2: a significant increase in synaptic N-cadherin in the 22°C ($p<0.05$) but not in the 30°C-trained group (Figure 5a). This change was only observed in the SNS fraction—total N-cadherin levels were unchanged (Supplementary Figure S5). Specificity for N-cadherin modulation was supported by the absence of post-training changes in synaptic levels of the neural cell adhesion molecule (NCAM)-180 (Figure 5b; lack of changes in total values can be observed in Supplementary Figure S5).

Next, a time course analysis was performed to determine when post-training changes in GluA1, GluA2, GluA3, and N-cadherin occur on day 2 with regard to the training phase (Figure 6a–d, respectively). None of the molecules presented changes among the different experimental groups when samples were taken after two training trials (B1). For GluA1, while no differences were observed either immediately

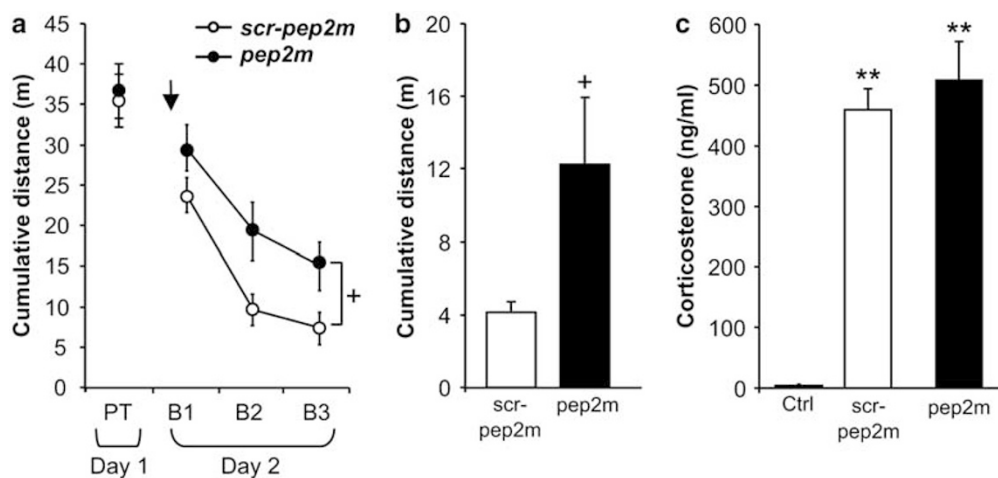


Figure 4 Requirement of GluA2 in stress facilitation of learning and memory. Mice pre-treated with (TAT)-pep2m (450 nM, i.c.v., $n=17$) showed (a) impaired spatial learning on day 2 as compared with performance of control scr-pep2m (Scr)-treated mice (450 nM, i.c.v., $n=16$) (b) impaired spatial memory on day 3 compared with Scr-treated mice. (c) Pep2m had no effect on post-training plasma corticosterone induction ($n=5-6$ per group). Arrow indicates injection time. Data represent mean \pm SEM. $^+p\leq 0.05$ vs scr-pep2m, $^{**}p<0.01$ vs Ctrl.

following training on day 2 (B3), mice trained at 22°C displayed significantly higher levels for this subunit than naive controls 45 min after B3 (Figure 5a; $F_{(2, 27)} = 4.41$,

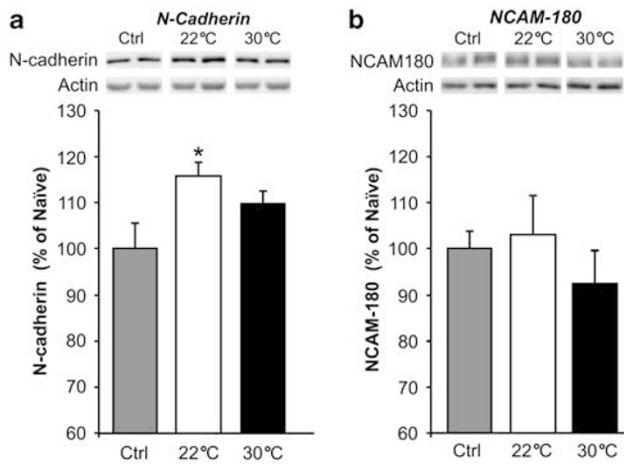


Figure 5 Regulation of N-cadherin by stressful learning. (a) Synaptic trafficking of the GluA2 binding partner N-cadherin was increased by stressful learning (22°C training), whereas, (b) synaptic levels of the neural cell adhesion molecule (NCAM)-180 remained unaltered. Data represent mean \pm SEM. * $p < 0.05$ vs Ctrl.

$p = 0.02$; 22°C vs naive, $p < 0.05$). As already noted, changes in GluA2, GluA3, and N-cadherin expression in 22°C-trained mice were observed immediately after training on day 2 (B3; depicted data from experiments reported before). At 45 min after training, GluA2 (Figure 6b; B3 + 45 min; $F_{(2,23)} = 7.28$, $p = 0.004$; 22°C vs naive, $p < 0.05$) and GluA3 (Figure 6c; B3 + 45 min; $F_{(2,23)} = 9.07$, $p = 0.001$; 22°C vs naive, $p < 0.01$) remained upregulated with regard to controls. Importantly, at this time point, expression levels of GluA2 ($p < 0.01$), GluA3 ($p < 0.01$) and N-cadherin ($p < 0.01$) were significantly higher than those of 30°C-trained mice.

DISCUSSION

In this study, we show that mice trained under more stressful conditions (water at 22°C) exhibit better learning and memory in the spatial water maze task, as well as higher post-training plasma corticosterone levels, than mice trained under lower stress (water at 30°C). This facilitation of learning by stress is coupled to increased synaptic expression of the GluA2-AMPA subunit in the hippocampus as observed immediately after learning. This effect is

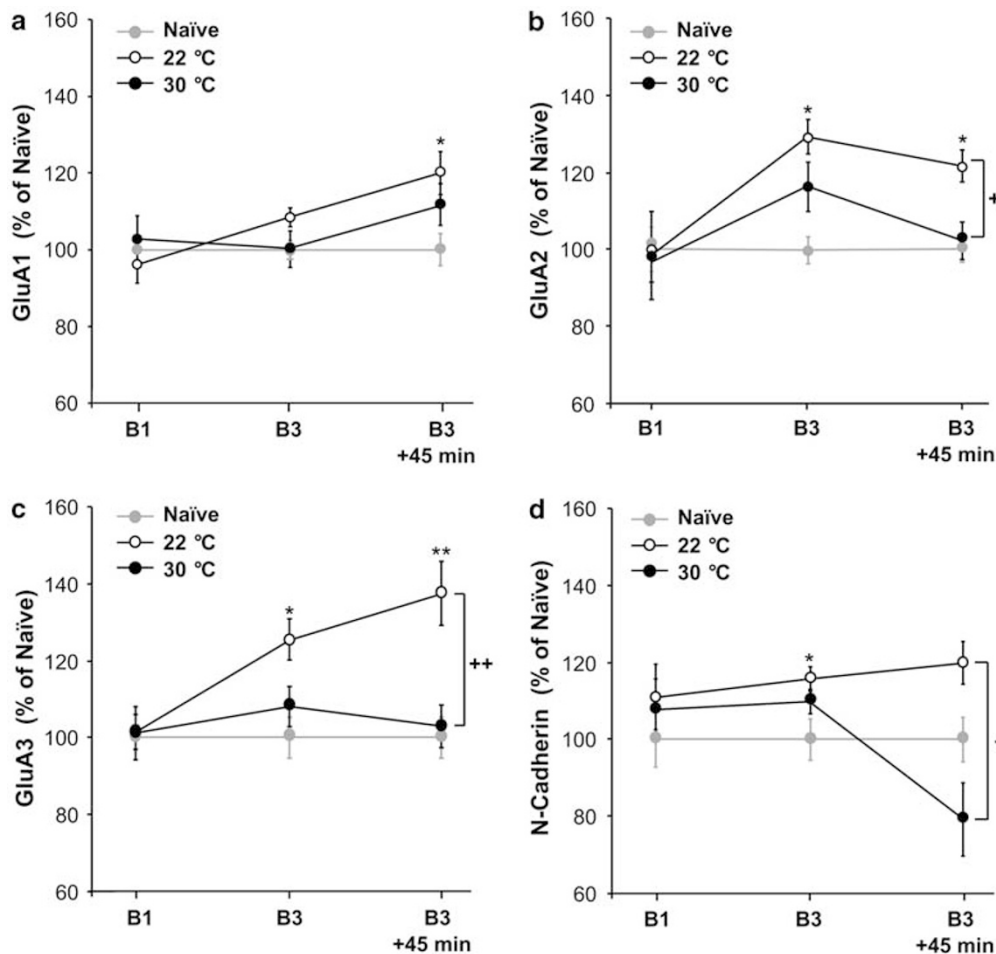


Figure 6 Time dynamics of changes in synaptic protein expression in animals trained under different stress levels. Mice were trained in the water maze at either 22 or 30°C on days 1 and 2, and samples taken at different time points on day 2; ie, after two training trials (B1), after six training trials (B3), and 45 min after B3. Results are represented for synaptic expression of (a) GluA1, (b) GluA2, (c) GluA3, and (d) N-cadherin. Data represent mean \pm SEM. * $p < 0.05$, ** $p < 0.01$ vs Ctrl, + $p < 0.05$, ++ $p < 0.01$ vs 30°C.

subunit-specific as indicated by a lack of significant changes observed immediately after stressful training in the GluA1 or NR1 subunits, while GluA3 was also elevated. The changes in GluA2 synaptic expression are not matched by parallel changes in the total hippocampal fraction, which highlights a role for receptor trafficking mechanisms rather than GluA2 transcription on the observed effects. That the observed increased synaptic GluA2 expression is corticosterone-dependent and necessary for stress enhancement of memory is indicated by the concomitant inhibition of enhanced memory and GluA2 trafficking by treatment with the corticosterone synthesis inhibitor metyrapone before mice were trained in 22°C water. Intracerebroventricular infusion of a peptide that interferes with NSF–GluA2 interaction and, consequently, with GluA2 synaptic trafficking (Garry *et al*, 2003; Yao *et al*, 2008), impairs immediate performance at learning having as well an impact on subsequent memory retrieval, supporting a key role for GluA2 trafficking in stress-induced facilitation of spatial learning and memory. Furthermore, we find a similar stressful learning-induced synaptic trafficking for the cell adhesion molecule N-cadherin, with which GluA2 has been shown to reciprocally interact for the formation and growth of dendritic spines (Saglietti *et al*, 2007; Tai *et al*, 2008), suggesting a potential interaction between these two molecules in mediating the behavioral effects of stress.

We also show that the increase in GluA2 levels observed in the SNS fraction immediately after stressful learning was concomitant with a parallel increase of this subunit's cell-surface expression. This observation agrees with a recently emphasized role for GluA2 in synaptic targeting (Lu *et al*, 2009) and emphasizes its modulation by stressful learning.

Although we did not find evidence for rapid GluA1 trafficking either during or immediately after stressful learning, we should be cautious not to exclude this subunit from the phenomenon under study. Our trafficking assessments for GluA1 were only performed in SNS preparations, while measurements of cell-surface expression were only performed for the GluA2 subunit, which was the main focus of our study on the basis of its recent implication in GR actions (Groc *et al*, 2008). Importantly, we found increased incorporation of GluA1 in the SNS preparations when samples were taken 45 min after training mice in 22°C water, indicating the involvement of this subunit in later plastic mechanisms triggered by stressful learning. *In vitro*, corticosterone was also found to enhance GluA1 synaptic delivery 2 h after corticosterone treatment (Groc *et al*, 2008). Previous *in vitro* studies have indicated a leading role for the GluA1 subunit in activity-dependent synaptic delivery of GluA1/A2 heteromers that are eventually replaced by the cycling of GluA2/A3 heteromers (Hayashi *et al*, 2000; Malinow and Malenka, 2002; Passafaro *et al*, 2003; Shi *et al*, 2001). *In vivo*, hippocampal LTP has also been shown to involve a rapid synaptic delivery of the GluA1, GluA2, and GluA3 subunits (Williams *et al*, 2007), with expression levels for the two former subunits being upregulated up to 48 h following LTP induction (Kennard *et al*, 2009). In behaving animals, training in an inhibitory avoidance task led to increased synaptic delivery in both GluA1 and GluA2 in the rat hippocampus (Whitlock *et al*, 2006). Moreover, Hu *et al* (2007) showed that emotional

stress, as well as enhanced norepinephrine levels, induces phosphorylation of GluA1 at sites critical for its synaptic delivery. This phosphorylation was shown to be necessary and sufficient to lower the threshold for GluA1 synaptic incorporation during LTP. Accordingly, it is tempting to hypothesize that the changes in GluA1 synaptic incorporation found in our study, 45 min after stressful training, were preceded by increases in GluA1 phosphorylation. Future studies should directly address these slight discrepancies in the timing of incorporation of the different AMPAR subunits under different learning and stressful learning conditions. As GC interactions with noradrenergic mechanisms are essential for some forms of memory modulation by stress (Roosendaal *et al*, 2008), and the GluA1 subunit has been implicated in the emotional enhancement of learning induced by norepinephrine (Hu *et al*, 2007), it will also be important to investigate whether the GC-dependent trafficking mechanisms described in this study are dependent or not of concurrent noradrenergic actions.

Collectively, our results indicate that, under our experimental conditions, enhanced GluA2 trafficking is dependent on the co-occurrence of both information processing elicited by spatial learning and training-induced high corticosterone levels, while enhanced corticosterone, on its own, is not sufficient to enhance synaptic GluA2 expression in the hippocampus: (1) Increased synaptic trafficking is significant in mice trained at 22°C, but not in those trained at 30°C, the former showing higher corticosterone levels than the latter; (2) Preventing corticosterone release in 22°C-trained mice through pretraining injections of metyrapone interferes with both stress-induced facilitation of memory and synaptic incorporation of GluA2. (3) Yoked-controls exposed to the water maze at 22°C without a concomitant training task do not vary their synaptic GluA2 expression despite displaying high plasma corticosterone levels. (4) Similarly, no changes are found in untrained animals that were injected with corticosterone. This is reminiscent of a synergistic effect in the increase of synaptic surface GluA2 content observed in hippocampal cultures within minutes after concurrent application of corticosterone and a chemical LTP stimulus (Groc *et al*, 2008). However, our results are at odds with this hippocampal culture study in that they also find that a brief application of corticosterone is sufficient to increase GluA2 surface content (Groc *et al*, 2008). Possible explanations for this discrepancy could relate to differences between *in vivo* and *in vitro* mechanisms, or differences in mobility dynamics between native intact (our study) and semiconductor quantum-dotted (Groc *et al*, 2008) receptors.

In our study, it is tempting to speculate that the increased GluA2 synaptic expression observed in mice trained under stressful conditions is involved in their learning and memory enhancement. Given that pretraining i.c.v. injection of the pep2m peptide, which interferes with NSF-mediated GluA2 trafficking (Nishimune *et al*, 1998; Yao *et al*, 2008), impairs performance already at learning, our results, indeed, support a role for GluA2 trafficking on spatial learning. Interference with the NSF–GluA2 interaction has previously been shown to impair synaptic transmission (Luscher *et al*, 1999; Luthi *et al*, 1999; Song *et al*, 1998) and LTP (Yao *et al*, 2008). Recently, the pep2m peptide has also been shown to interfere with the

maintenance of LTP and increased surface GluA2 expression associated with the application of PKMzeta protein to hippocampal cultures (Yao *et al*, 2008). Therefore, our data strongly implicate the NSF–GluA2 interaction in the facilitating effects induced by experiencing a certain degree of stress concomitant to learning. This is in agreement with impaired spatial learning described in mice with a forebrain-specific reduction in GluA2 (Shimshek *et al*, 2006). The fact that, immediately after training, only 22°C-, but not 30°C-, trained mice show significantly increased synaptic GluA2 expression when compared with controls supports a role for these receptors during the learning phase. However, we should note that receptor levels from mice trained at 30°C laid in between the 22°C and the control groups, which indicates that a mild nonsignificant effect also occurred in this mice while they weakly learned the task. Importantly, the two groups showed significant differences in synaptic GluA2 expression when analyzed at a later time point during the consolidation phase, with 22°C-trained mice showing significantly higher levels than 30°C-trained mice at 45 min post-training. Together with the fact that pretraining injection of pep2m peptide also had an effect on memory evaluated 24 h afterwards, this data suggest that, in addition to their role in learning acquisition, increased synaptic GluA2 expression is involved in the facilitating effects of stress on memory formation. These data are in agreement with the fact that metyrapone injections blocked both GluA2 synaptic incorporation and the stress-induced long-term memory enhancement, while it did not affect task performance in mice trained at 30°C.

How could increased synaptic expression of GluA2 be related to the improvement of memory processes by stress? Increasing evidence indicates key roles for GluA2 in the regulation of synaptic strength, spine shape, and synaptogenesis, all processes that are thought to mediate at least some forms of memory formation (Bailey and Kandel, 2008; Morris, 2006). Synaptic strength is determined to a large extent by synaptic trafficking of AMPARs, including GluA2 (Malinow and Malenka, 2002). Mice with a forebrain-specific reduction in GluA2 show alterations in synaptic structure (Medvedev *et al*, 2008), which suggests that GluA2 is important in regulating synaptic morphology. Overexpression of GluA2 has been shown to enhance AMPAR clustering and to increase spine size, and these changes occur together with enhanced presynaptic release (Passafaro *et al*, 2003). Interestingly, a positive correlation between the abundance of postsynaptic GluA2 and presynaptic release probability at single synapses follows enhanced network activity in cultured hippocampal neurons (Tokuoka and Goda, 2008). Synaptic adhesion molecules have been suggested as potential candidates to promote this correlation, given their ability to link the presynaptic and postsynaptic terminals in an activity-dependent manner (Tokuoka and Goda, 2008).

Our results showing a converging pattern of regulation for N-cadherin and GluA2 by stressful learning are in line with previously described interactions between these two molecules (Saglietti *et al*, 2007). Strong evidence in cultured hippocampal neurons and in GluA2 knockout mice has implicated GluA2 in the formation, shape, and maintenance of dendritic spines (Medvedev *et al*, 2008; Passafaro *et al*,

2003; Saglietti *et al*, 2007), with the GluA2 N-terminal domain (NTD) having a critical role (Passafaro *et al*, 2003; Saglietti *et al*, 2007). In parallel, N-cadherin, whose expression is particularly concentrated at synapses, is essential for proper development of dendritic spines (Takahashi *et al*, 2003). The NTD of GluA2 can interact directly with N-cadherin, in *cis* or in *trans* and, in hippocampal neurons, N-cadherin and GluA2 (through its NTD) can form a synaptic complex that stimulates presynaptic development and function and promotes dendritic spine formation (Saglietti *et al*, 2007). From these findings, Saglietti *et al* (2007) has proposed a model whereby accumulation of GluA2-containing AMPARs at the synapse would promote recruitment of N-cadherin and associated molecules, thereby stabilizing and enlarging the synapse. Therefore, this model strongly supports the view that these mechanisms might underlie enhanced learning and memory in mice trained in the water maze at 22°C in our study. In future studies, it will be important to determine the potential involvement of other cell adhesion molecules. Recently, a role for the neurexin-1 β /neuroligin-1 complex in the recruitment of GluA2 was described. As binding between these two cell adhesion molecules is known to have an important role in synapse initiation, these data point to a new role of AMPARs in synaptogenesis (Heine *et al*, 2008).

It should be noted that this study focuses on the facilitating effects of stress triggered by the learning task and, therefore, the mechanisms reported here are not proposed to explain all type of stress and memory interactions. In fact, the available literature indicates that the relationship between stress intensity and spatial learning follows an inverted-U shape manner (Sandi and Pinelo-Nava, 2007), which suggests that the 22°C-water condition is acting as a moderate stressor. The question thus arises as to which mechanisms will be involved when animals are trained under high stress that leads to learning impairment. Importantly, using a computational model, we have recently proposed that the facilitating actions of stress alter the trade between animals' exploration *vs* exploitation of the current knowledge (translated in changes in task performance accuracy), while high stress levels might impair learning by increasing animals' impulsivity (manifested as changes in future reward discounting) (Luksys *et al*, 2009).

In conclusion, this study provides strong evidence that the effects of GCs on GluA2 trafficking are critically involved in stress induced facilitation of memory formation. These findings can have important implications for the understanding of the intriguing pathways whereby stress and memory interact.

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DISCLOSURE

The authors declare no conflict of interest.

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