

Emotion Enhances Learning via Norepinephrine Regulation of AMPA-Receptor Trafficking

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SUMMARY

Emotion enhances our ability to form vivid memories of even trivial events. Norepinephrine (NE), a neuromodulator released during emotional arousal, plays a central role in the emotional regulation of memory. However, the underlying molecular mechanism remains elusive. Toward this aim, we have examined the role of NE in contextual memory formation and in the synaptic delivery of GluR1-containing α -amino-3-hydroxy-5-methyl-4-isoxazolepropionic acid (AMPA)-type glutamate receptors during long-term potentiation (LTP), a candidate synaptic mechanism for learning. We found that NE, as well as emotional stress, induces phosphorylation of GluR1 at sites critical for its synaptic delivery. Phosphorylation at these sites is necessary and sufficient to lower the threshold for GluR1 synaptic incorporation during LTP. In behavioral experiments, NE can lower the threshold for memory formation in wild-type mice but not in mice carrying mutations in the GluR1 phosphorylation sites. Our results indicate that NE-driven phosphorylation of GluR1 facilitates the synaptic delivery of GluR1-containing AMPARs, lowering the threshold for LTP, thereby providing a molecular mechanism for how emotion enhances learning and memory.

INTRODUCTION

A memory system, such as the hippocampal formation, needs to efficiently distinguish more significant from the less relevant experiences and give priority to the transformation of the former into long-term memory. One factor that is believed to guide the hippocampus in this challeng-

ing task is the emotional load of an experience: heightened states of emotion can facilitate learning and memory formation (Christianson, 1992; McGaugh, 2000; Richter-Levin and Akirav, 2003). An extreme example of such regulation occurs in posttraumatic stress disorder (PTSD), which is characterized by persistent vivid memories of traumatic events (Ehlers and Clark, 2000). Thus, understanding how emotion regulates memory is an important challenge in memory research.

The stress hormone norepinephrine (NE) plays a central role in the emotional regulation of memory via the brain β -adrenergic receptors (β -ARs) (Cahill et al., 1994; Frankland et al., 2004; McGaugh, 2000). During emotional arousal, NE is released by neurons originating from the locus ceruleus and lateral brain stem tegmentum. These neurons project widely to many brain regions, including the hippocampus and the amygdala, key regions involved in the formation of emotional memory (Fanselow and Gale, 2003; Kandel et al., 2000; LeDoux, 2000; Maren, 2001; Silva et al., 1998). NE activates adenylyl cyclase and cAMP-dependent protein kinase (PKA) (Hall, 2004; Kandel et al., 2000; Vanhose and Winder, 2003) and calcium/calmodulin-dependent protein kinase II (CaMKII) (Wang et al., 2004) via β -adrenergic receptors. In parallel to their powerful effects on memory formation, NE and β -adrenergic stimulation show profound effects on the induction of long-term potentiation (LTP), an important form of synaptic plasticity that is widely believed to be a cellular substrate for aspects of memory (Gelinas and Nguyen, 2005; Katsuki et al., 1997; Sarvey et al., 1989; Thomas et al., 1996; Watabe et al., 2000; Winder et al., 1999). Although several candidate molecules, including calcium-activated potassium channel (Haas and Konnerth, 1983; Madison and Nicoll, 1982), voltage-dependent calcium channel (Gray and Johnston, 1987) and *N*-methyl-D-aspartate (NMDA) receptor (Huang et al., 1993; Raman et al., 1996), have been suggested to be the downstream targets of NE, direct functional evidence for the involvement of these molecules in NE-regulated LTP is missing. Most importantly, there is no evidence in behaving

animals showing a role for these candidate molecules in NE-facilitated learning. Therefore, the molecular mechanism linking NE signaling to LTP facilitation and memory enhancement remains to be elucidated.

There is considerable evidence that synaptic insertion of GluR1 subunit-containing AMPA receptors (GluR1 receptors) contributes to the synaptic strengthening observed during LTP induction (Barry and Ziff, 2002; Bredt and Nicoll, 2003; Malenka and Bear, 2004; Malinow and Malenka, 2002; Sheng and Hyoung Lee, 2003; Song and Huganir, 2002) as well as in experience-driven plasticity (Clem and Barth, 2006; Rumpel et al., 2005; Takahashi et al., 2003). AMPA receptors are ionotropic glutamate receptors that mediate the majority of the fast excitatory transmission in the brain. They are heterotetramers, comprised of a combinatorial assembly of four subunits, GluR1–GluR4 (Hollmann and Heinemann, 1994; Wisden and Seeburg, 1993). The GluR1 subunit plays a central role in hippocampal LTP (Hayashi et al., 2000; Lee et al., 2003; Shi et al., 1999; Zamanillo et al., 1999) and is regulated by protein phosphorylation at several major sites on its intracellular carboxy-terminal domain (Boehm et al., 2006; Roche et al., 1996). The functional role of phosphorylation of Ser831 and Ser845 sites, in particular, has been extensively characterized. Ser831 is phosphorylated by CaMKII and protein kinase C (PKC), whereas Ser845 is phosphorylated by PKA (Barria et al., 1997; Mammen et al., 1997; Roche et al., 1996). Phosphorylation of these sites regulates both the channel properties (Banke et al., 2000; Barria et al., 1997; Derkach et al., 1999; Roche et al., 1996) and the synaptic incorporation of the receptor (Ehlers, 2000; Esteban et al., 2003; Qin et al., 2005). Genetically modified mice with knockin mutations that block phosphorylation at Ser845 and Ser831 sites of GluR1 show subtle disturbances in synaptic plasticity and learning (Lee et al., 2003). These previous results suggest that these phosphorylation sites may play modulatory rather than essential roles in plasticity and memory formation.

We set out to test the hypothesis that NE-driven phosphorylation of GluR1 facilitates AMPA-receptor trafficking to synapses and LTP induction, and as a consequence, lowers the threshold for formation of memory. We show here that (1) NE signaling induces phosphorylation of the Ser845 and Ser831 sites of GluR1 both in vitro and in vivo, and emotional stress mimics this modulation; (2) NE facilitation of CA1 LTP follows a similar time course as the GluR1 phosphorylation; (3) the synaptic delivery of GluR1 is facilitated by NE through the phosphorylation at Ser845 and Ser831 sites; and (4) phosphomutant mice with knockin mutations on the GluR1 phosphorylation sites have similar defects in NE-facilitated LTP and NE-enhanced contextual memory task: weak stimuli, which lead to NE-facilitated synaptic plasticity and memory in wild-type mice do not show facilitation in phosphomutant mice, and strong stimuli lead to plasticity and learning that are little affected by NE or phosphomutation. These results establish a central role of NE-regulated AMPA receptor trafficking in the emotional regulation of learning and memory.

RESULTS

NE Induces Phosphorylation of GluR1 at Sites Critical for Its Synaptic Delivery

The GluR1 subunit of the AMPAR contains two phosphorylation sites, Ser845 (PKA site) and Ser831 (PKC/CaMKII site), which regulate GluR1 trafficking and function in vitro and in vivo (Banke et al., 2000; Barria et al., 1997; Derkach et al., 1999; Esteban et al., 2003; Kameyama et al., 1998; Lee et al., 2000, 2003). To test the effect of NE on GluR1 phosphorylation on these two sites, we incubated organotypic hippocampal slices in NE-containing medium (10 μ M, 10 min) and assayed for the change in GluR1 phosphorylation. NE treatment caused a dramatic increase in the phosphorylation of GluR1 at Ser845 ($224 \pm 18\%$ of control, $p < 0.001$) and a smaller but significant increase of phosphorylation at Ser831 ($129 \pm 11\%$ of control, $p < 0.05$) (Figure 1A). Phosphorylation of both sites was relatively transient: 30 min after NE washout, the magnitude of phosphorylation was reduced to $155 \pm 29\%$ ($p < 0.05$) for Ser845 and $114 \pm 13\%$ ($p = 0.05$) for Ser831. By 90 min after NE washout, the level of phosphorylation was no longer significantly different from basal levels (Figure 1A). Similar results were obtained when NE was applied to acute hippocampal slices made from adult mice (data not shown). Ser818, another phosphorylation site on GluR1 recently implicated in AMPAR trafficking (Boehm et al., 2006) does not show NE-induced phosphorylation change ($108 \pm 4\%$ of control, $p = 0.9$).

To further characterize the signaling events that lead to NE-induced GluR1 phosphorylation, we applied NE on organotypic hippocampal slice pretreated with different pharmacological reagents (Figure 1B). Consistent with a previous study that showed β -AR activation couples to PKA phosphorylation of GluR1 (Vanhoose and Winder, 2003), NE-induced GluR1 Ser845 phosphorylation was blocked by the β -AR antagonist propranolol (10 μ M) but not by the α -AR antagonist phentolamine (50 μ M) (Figure 1B). As expected for a PKA phosphorylation site, Ser845 phosphorylation was sensitive to PKA inhibitor KT5720 (4 μ M) but not CaMKII inhibitor KN-93 (20 μ M) (Figure 1B). Similar analysis on Ser831 site revealed that the NE-induced Ser831 phosphorylation depends on β -AR signaling and CaMKII activity (Figure 1B). These results suggest that NE signals through β -ARs and then through PKA and CaMKII in a parallel fashion, leading to phosphorylation of GluR1 at Ser845 and Ser831 sites respectively.

In Vivo Phosphorylation of GluR1 Induced by Epinephrine and Emotional Stress

We next examined whether similar modulation of GluR1 by NE occurs in animals in vivo. Mice were injected systemically with either saline or epinephrine, a drug that can elevate NE level in the central nervous system (CNS) (McGaugh et al., 1996; Liang et al., 1986), and hippocampal lysates were analyzed (Figure 2Aa). Compared with saline injection, epinephrine (0.5 mg/kg, a dose that was

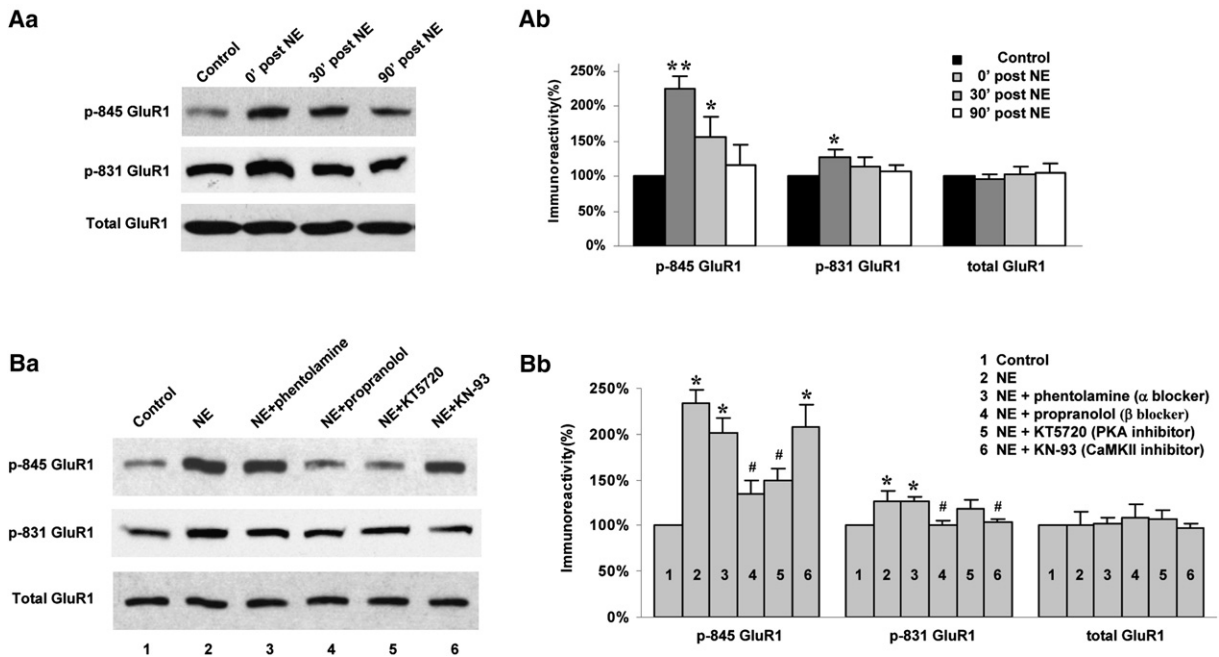


Figure 1. NE Induces Transient Phosphorylation of GluR1 at Ser831 and Ser845 In Vitro

(A) In (Aa) are representative immunoblots showing the amount of phospho-Ser845-GluR1 (top panel), phospho-Ser831-GluR1 (middle panel), and total GluR1 (bottom panel) from the organotypic hippocampal slice cultures. From left to right are samples without NE treatment (control) and samples taken at 0, 30, and 90 min after NE withdrawal, respectively. In (Ab) are immunoreactivity normalized to control slices. ** indicates $p < 0.001$; * indicates $p < 0.05$, Student's t test. Error bars indicate standard error of the mean (SEM).

(B) In (Ba) are representative immunoblots showing phospho-GluR1 and total GluR1 of hippocampal slices treated with NE in presence of different drugs. In (Bb) are immunoreactivity normalized to control slices. (* indicates $p < 0.05$ compared with control slices; # indicates $p < 0.05$ compared with NE treated slices, student's t test.) Error bars indicate SEM.

previously shown to enhance contextual fear memory in mice [Frankland et al., 2004]) caused increased phosphorylation of GluR1 at the Ser845 ($172 \pm 28\%$ of control, $p < 0.05$) site (Figure 2A). There is also a tendency of increase at the Ser831 ($131 \pm 16\%$ of control, $p = 0.08$) site (Figure 2A).

Next, to test if natural emotional stress can produce similar effects on GluR1 phosphorylation, we employed a fox urine assay (Figure 2Ba). Fox urine exposure causes innate fear (Figure 2Bd) and augments NE release in the brain [Hayley et al., 2001]. Hippocampi from mice exposed to fox urine showed elevated Ser845 ($200 \pm 34\%$, $p < 0.05$) and Ser831 phosphorylation ($126 \pm 10\%$, $p < 0.05$) of GluR1 (Figure 2B). The increase in phosphorylation was largely abolished in mice preinjected with the β -AR blocker propranolol ($112 \pm 17\%$, $p = 0.45$ for Ser845; $103 \pm 13\%$, $p = 0.58$ for Ser831), indicating that NE and β -ARs likely mediate the effect of emotional stress on GluR1 phosphorylation (Figure 2B).

NE Facilitates LTP Induction in a Time-Dependent Fashion

A predominant component of hippocampal activity in behaving animals occurs at the θ frequency (5–12 Hz). It has been shown that NE and β -adrenergic stimulation lower the threshold for induction of LTP in the θ frequency range

in CA1 hippocampal neurons [Gelinias and Nguyen, 2005; Katsuki et al., 1997; Sarvey et al., 1989; Thomas et al., 1996; Watabe et al., 2000; Winder et al., 1999]. This effect is not due to NE-caused decrease of synaptic inhibition, which depends on α -adrenergic receptors [Madison and Nicoll, 1988; Thomas et al., 1996]. We speculated that phosphorylation of GluR1 is a critical molecular event downstream of NE mediating its facilitatory effect on LTP. If so, considering the transient nature of NE-induced GluR1 phosphorylation, one would predict NE modulation on CA1 LTP follows a similar time course.

To test the time course of NE facilitation of LTP, we recorded evoked field excitatory postsynaptic potentials (fEPSP) in the stratum radiatum of acute rat hippocampal slices and applied NE at different time points prior to LTP induction (Figure 3A). A low-frequency tetanic stimulus (10 Hz, 900 pulses) delivered immediately after a brief bath application of NE (10 μ M, 10 min) produced significant LTP ($156 \pm 8\%$, $n = 6$, $p < 0.001$; Figure 3B), consistent with previous results [Gelinias and Nguyen, 2005; Katsuki et al., 1997; Sarvey et al., 1989; Watabe et al., 2000; Winder et al., 1999]. NE application, or the tetanic stimulus alone, produced no persistent change in synaptic strength (Figure 3B). When 10 Hz stimulation was delivered 30 min after washout of NE, this led to reduced LTP ($124 \pm 8\%$, $n = 10$, $p < 0.01$; Figure 3B).

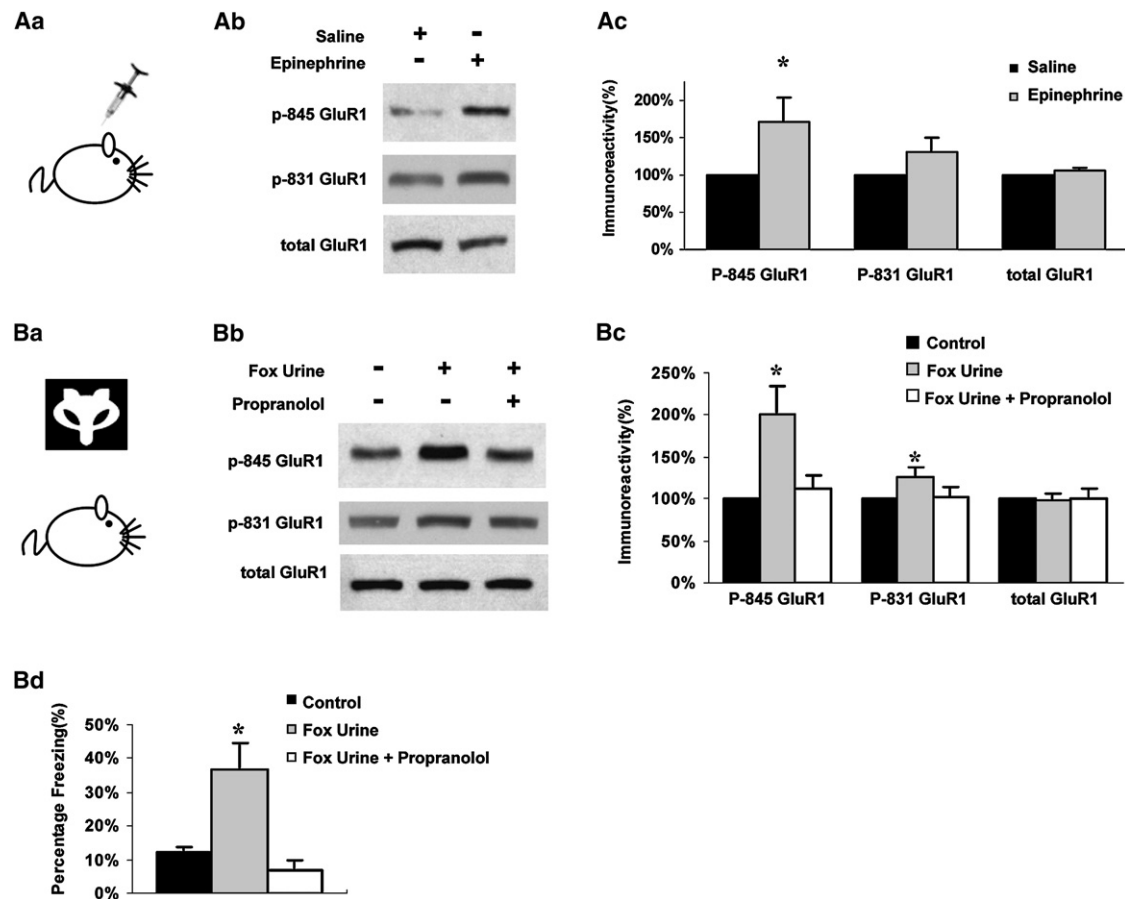


Figure 2. In Vivo Phosphorylation of GluR1 Induced by Epinephrine and Emotional Stress

(A) Systemic injection of epinephrine induces GluR1 phosphorylation in the hippocampus. In (Aa) is the schematic of the experiment. Saline or epinephrine (0.5mg/kg, intraperitoneally [IP]) was injected into mice within 15 min before the dissection of hippocampi for western analysis. In (Ab) are immunoblots showing the amount of p-845 GluR1, p-831 GluR1, and total GluR1 from the hippocampi of saline or epinephrine-injected mice. In (Ac) is quantification of the results in (Ab). Error bars indicate SEM.

(B) Fear-induced GluR1 phosphorylation in the hippocampus depends on NE signaling. In (Ba) is the schematic of the experiment. Mice (preinjected with saline or propranolol (20 mg/kg, IP) were exposed to fox urine for 5 min before the dissection of hippocampi for western analysis. In (Bb) are immunoblots showing the amount of phospho-GluR1 and total GluR1 from the hippocampi of experimental mice. In (Bc) is quantification of the results in (Bb). Error bars indicate SEM. In (Bd) is correlated freezing behavior of mice used in (Ba)–(Bc). Mice were monitored, and percentage freezing was calculated during the 5 min exposure period.

When the 10 Hz stimulation was applied 90 min after NE washout, no LTP was produced ($104 \pm 5\%$, $n = 7$, $p = 0.56$; Figure 3B). Therefore, the time course of the facilitatory effect of NE on CA1 LTP induction strongly correlates with the time course of GluR1 phosphorylation decay after NE removal, suggesting a potential connection of these two phenomena (Figures 3C and 3D).

Synaptic Delivery of GluR1 Receptors Is facilitated by NE

To establish more directly the effect of GluR1 phosphorylation on synaptic plasticity, we examined GluR1 trafficking to synapses, a mechanism underlying the expression of LTP (Barry and Ziff, 2002; Bredt and Nicoll, 2003; Malenka and Bear, 2004; Malinow and Malenka, 2002; Sheng and Hyung Lee, 2003; Song and Haganir, 2002). We wished

to determine if the NE-driven phosphorylation of GluR1 facilitates the delivery of GluR receptors into synapses. To test this possibility, we employed the “electrophysiological tagging” method to monitor the incorporation of recombinant GluR1 into synapses (Hayashi et al., 2000; Shi et al., 2001). Briefly, expression of recombinant GluR1 in organotypic hippocampal slice cultures leads to an increased proportion of homomeric channels, which display rectification (Boulter et al., 1990): little outward current at positive membrane potentials compared to endogenous AMPA receptors (Hestrin, 1992). Thus, synaptic insertion of GluR1-GFP can be detected as an increased rectification of AMPA receptor-mediated synaptic responses (Hayashi et al., 2000; Shi et al., 2001).

At the basal condition, neurons expressing GluR1-GFP displayed no increase in rectification compared to

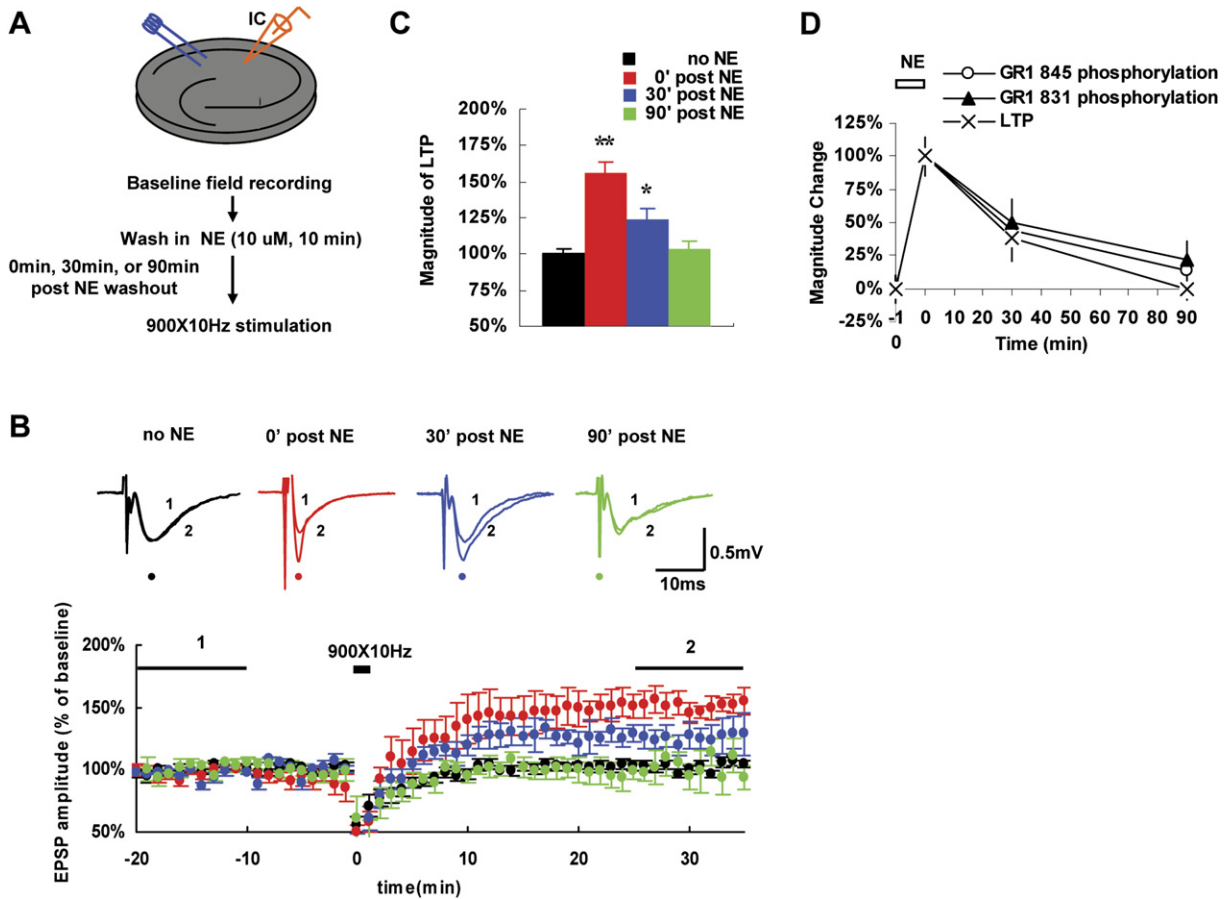


Figure 3. NE Facilitates LTP Induction in a Time-Dependent Manner

(A) Schematic of the experimental setup and protocol for the NE-LTP experiment.
 (B) Representative average field EPSP response obtained before (Ba) and after (Bb) LTP-inducing stimulus in rat hippocampal CA1 neurons. Lower plot, normalized field EPSP amplitude. Baseline is measured between -20 to -10 min. Nine hundred pulses of 10 Hz stimulus were delivered at time 0. NE was applied for 10 min and washed out at 0 (red), 30 (blue), or 90 min (green) prior the onset of the 10 Hz stimulation. ** indicates $p < 0.001$; * indicates $p < 0.01$, Student's t test. Error bars indicate SEM.
 (C) Summary of the data in (B). Magnitude of LTP is defined by the ratio of the average fEPSP between ~ 25 and 35 min (Cb) and average fEPSP between approximately -20 to -10 min (Ca). Error bars indicate SEM.
 (D) The time course of GluR1 phosphorylation correlates with the LTP magnitude change after NE treatment. White bar indicates the time when NE was applied. Data are normalized to the maximum change (the -10 min time point is set to 0, and the maximum change is set to 100%). Error bars indicate SEM.

noninfected cells ($102 \pm 7\%$, $p = 0.23$), as shown previously (Hayashi et al., 2000; Shi et al., 2001). Application of NE or the 10 Hz stimulation alone also caused no change of rectification in infected neurons (Figures 4B and 4C). In contrast, if NE (10 μ M, 10 min) was applied just prior to the 10 Hz (900 pulses) stimulation (Figures 4A), cells expressing GluR1-GFP showed increased synaptic rectification compared to neighboring noninfected cells (Figure 4D), indicating GluR1 was driven into synapses. This result indicates that NE facilitates the synaptic delivery of GluR1 under a low-frequency electrical stimulus, which by itself does not induce GluR1 synaptic incorporation.

Next we used electrophysiological tagging in combination with GluR1 mutagenesis to determine the role of Ser845 and Ser831 phosphorylation in NE-facilitated

synaptic incorporation of GluR1. We first mimicked constitutive phosphorylation of Ser831 and Ser845 by substituting these residues with negatively charged aspartates (GluR1DD) (Figure 4Ga). In neurons expressing GluR1DD, a 10 Hz stimulation led to transmission that was potentiated and displayed increased rectification, indicating the receptor was delivered into synapses (Figure 4G). Therefore, expression of GluR1DD mimics the effect of NE. As a control, neurons expressing GluR1DD but not receiving the 10 Hz stimulation did not show significant change in transmission or rectification (Figure 4Gc). Conversely, we made GluR1 with serine to alanine mutations at positions 831 and 845 (GluR1AA), which prevents phosphorylation at these sites (Figure 4Ha). In cells expressing GluR1AA, NE application with a 10 Hz stimulation failed to

induce synaptic delivery of the recombinant receptor (Figure 4H). Indeed, transmission in infected cells was depressed compared to nearby noninfected cells after NE and a 10 Hz stimulation (Figure 4Hc). This would be expected if GluR1AA acts in a dominant-negative manner, reducing, at least in part, endogenous GluR1 synaptic incorporation, while the neighboring (noninfected) cells undergo normal GluR1 delivery in response to NE and the 10 Hz. Thus GluR1AA blocks the effect of NE. Together these results indicate that phosphorylation at Ser831 and Ser845 of GluR1 is both sufficient and necessary to mediate the facilitation effect of NE on the synaptic delivery of GluR1-containing AMPARs.

NE-Facilitated LTP Is Defective in GluR1 Double-Phosphomutant Mice

To further address the role of NE-induced GluR1 phosphorylation in LTP and memory, we used GluR1AA knockin mice, in which GluR1 Ser845 and Ser831 are mutated to alanine to prevent phosphorylation at these sites (Lee et al., 2003). Acute hippocampal slices prepared from either the wild-type or the phosphomutant mice were tested for LTP-induction protocols using a range of frequencies (namely 100 Hz, 10 Hz, 1 Hz), in presence or absence of NE (Figure 5). Wild-type slices undergo either LTP, no change in EPSP, or LTD (long-term depression), in response to 100 Hz, 10 Hz or 1 Hz stimulation respectively (Figures 5A, 5C, 5E, and 5H). This frequency-response relationship of hippocampal CA1 synapses is important for the ability of these synapses to function as information storage devices (Bach et al., 1995; Bienenstock et al., 1982; Kirkwood et al., 1996). Consistent with previous reports (Katsuki et al., 1997), NE caused a leftward shift of the frequency-response curve, essentially lowering the threshold frequency for LTP in wild-type slices (Figures 5A, 5C, and 5H).

The phosphomutant slices, however, showed deficits in NE modulation of the frequency-response curve, with a notable defect in the 10 Hz-induced synaptic plasticity: 10 Hz stimulation in presence of NE produced significantly less LTP in the phosphomutant mice ($129 \pm 9\%$, $n = 15$ from 5 mice) compared with their wild-type siblings ($158 \pm 13\%$, $n = 10$ from 4 mice) ($p = 0.01$) (Figures 5D and 5H). In contrast, the synaptic plasticity induced by a stronger stimulation protocol (100 Hz), and its modulation by NE, were essentially normal in the phosphomutant mice (Figure 5B). At 1 Hz stimulation, phosphomutant mice lack LTD (Figure 5E) (Lee et al., 2003). Interestingly, NE also abolishes LTD. This may be due to the fact that phosphorylation of GluR1 at Ser845 leads to synaptic reinsertion following its LTD-induced removal (Ehlers, 2000). This finding further supports phosphorylation-directed synaptic trafficking as playing a central role in the effects of NE on plasticity. Together, these results indicate that NE-induced GluR1 phosphorylation plays a critical role in NE-dependent LTP facilitation at threshold stimuli in CA1 neurons.

The Memory-Enhancing Effect of NE Is Mediated by GluR1 Phosphorylation

NE can enhance hippocampus-dependent contextual memory performance in mice as well as in human beings (Cahill et al., 1994; Frankland et al., 2004; McGaugh and Roozendaal, 2002). To test whether phosphorylation of GluR1 may mediate the memory-enhancing effect of NE, we employed a modified contextual fear-conditioning task that is known to require hippocampal function (Fanselow, 2000; Frankland et al., 2004). In this assay, mice are exposed to a cage on day 1 (pre-exposure) for 2 min, at which point they establish a contextual memory of this cage. On day 2 (conditioning), mice are placed in the same cage and immediately shocked (1 mA, 2 sec). On day 3 (test), animals are exposed to the same context again, and their activity is monitored (Figure 6A) (Frankland et al., 2004). Decreased motion on day 3 indicates the acquisition of a fear-conditioned contextual memory (Fanselow, 2000; Frankland et al., 2004; Kopec et al., 2006) (Figures 6C and 6F).

To test the facilitatory effect of NE on memory formation, we injected mice with epinephrine before placing them in the new environment on day 1, when memory of the context was formed. For wild-type mice, epinephrine injection caused a greater decrease in the mouse activity on day 3 when compared with saline-injected mice (t test, $p = 0.027$) (Figures 6D and 6G), indicating that increasing NE level enhances the formation of contextual memory. The effect of epinephrine is largely mediated by β -adrenergic receptors (Figures S1B), and injection of epinephrine itself does not have long-term effects on animals' activity (Figures S1A). In contrast to wild-type mice, GluR1 phosphomutant mice did not show enhanced memory when injected with epinephrine, as indicated by similar level of activity in epinephrine- and saline- injected groups on day 3 (t test, $p = 0.86$) (Figures 6E and 6H). Analysis of freezing response, which is another indicator of fear memory, gave similar results (data not shown).

Fear learning can be improved when exposure to the conditioning context is extended from 2 min to 8 min (Figure 6G) (Frankland et al., 2004). When such stronger learning protocol was used, epinephrine no longer enhanced the learning in both wild-type and phosphomutant animals (Figure 6H). Taken together, these experiments show that NE can improve contextual learning induced by a relatively mild learning protocol and that phosphorylation of GluR1 at Ser831 and Ser845 is required for this facilitatory effect of NE on learning.

DISCUSSION

We have investigated the role of NE in the synaptic delivery of GluR1-containing AMPA receptors, LTP, and contextual learning. We demonstrate that NE signaling and emotional stress induce phosphorylation of GluR1 at Ser845 and Ser831 sites in a transient manner. NE facilitates the induction of LTP following a similar time course of the GluR1 phosphorylation. Furthermore, the effect of NE on

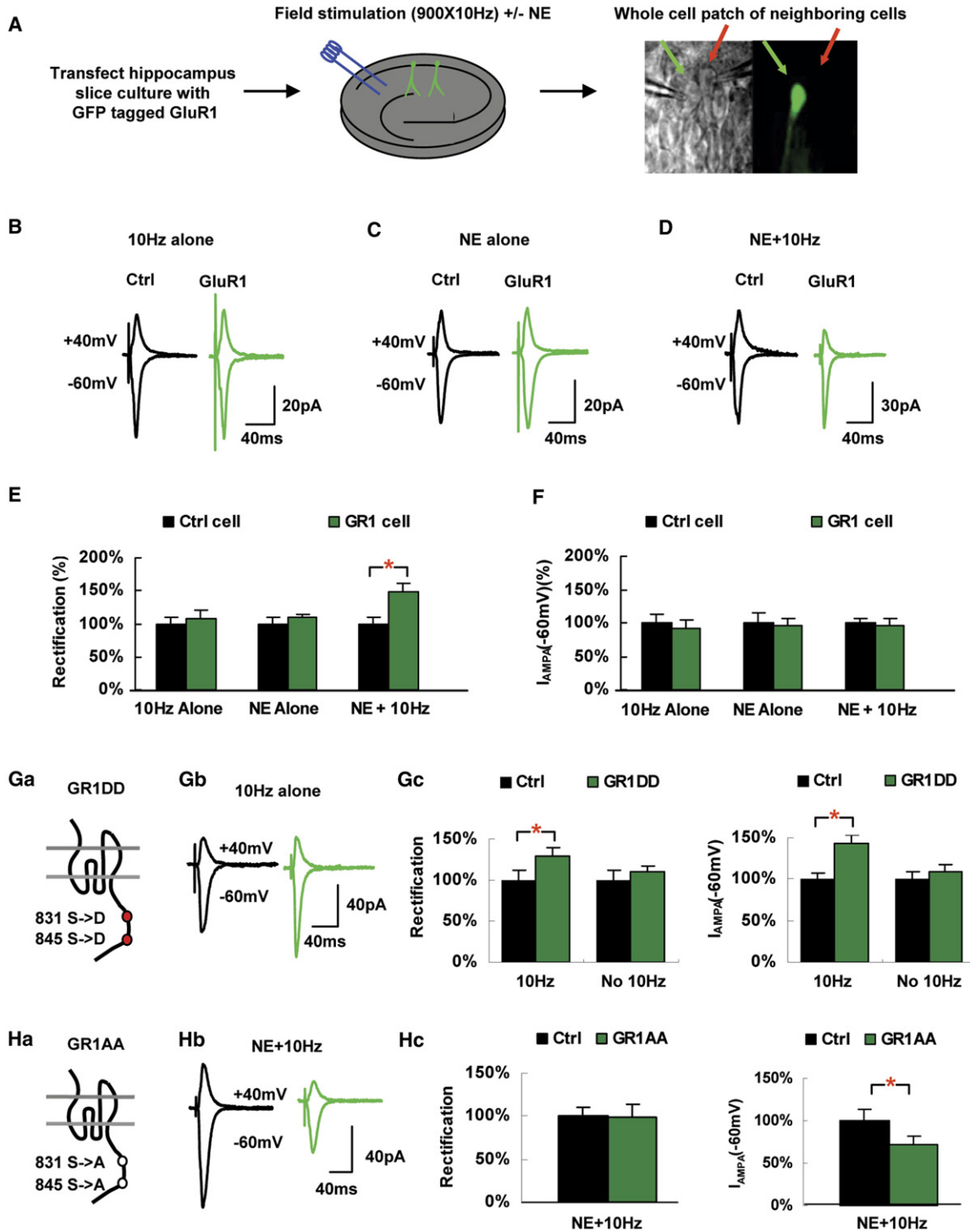


Figure 4. NE Facilitates the Synaptic Delivery of GluR1 Receptors through Phosphorylation of Ser831 and Ser845

(A) Schematic of the experimental setup and protocol.

(B–D) Ten hertz stimulation in combination with NE drives GluR1-GFP into synapses. Example of evoked AMPA receptor-mediated synaptic responses recorded at -60 mV and $+40$ mV from a neuron expressing GluR1-GFP (green) and neighboring control cell (black) not expressing the recombinant protein following indicated conditions: 10 Hz alone (10 Hz, 90 s), NE alone ($10 \mu\text{M}$, 10 min), or NE + 10 Hz (10 Hz stimulation immediately following NE washout).

(E) Average rectification values ($I_{-60 \text{ mV}}/I_{+40 \text{ mV}}$) for uninfected and infected cells. Values are normalized to uninfected cells ($p = 0.002$, t test). Error bars indicate SEM.

the synaptic delivery of GluR1 can be mimicked by a phosphomimic mutant and abolished by a phosphoblocking mutant of GluR1. Most importantly, while NE can facilitate LTP and enhance the memory performance of wild-type mice, the effects are strikingly attenuated in mice mutated in the GluR1 phosphorylation sites. The modulatory effect of NE is lost when strong, suprathreshold stimuli are delivered in wild-type or mutant animals. Our findings suggest that during emotional arousal, an elevated brain NE level leads to general phosphorylation of GluR1. This lowers the threshold for experience-driven synaptic modification, effectively facilitating the formation of memories during the period that GluR1 remains phosphorylated. Therefore, GluR1 phosphorylation induced by emotional arousal and NE may serve to tag an event as important and promote its registration into long-term memory.

In our study, NE itself is not sufficient to induce GluR1 synaptic trafficking (Figure 4), consistent with the fact that NE application alone does not induce LTP in CA1 neurons (Figure 3). The requirement of an additional mild electrical stimulation provides a plausible mechanism for the specificity of NE regulation of memory. Such a mechanism assures that not all the synaptic connections but only the synaptic pathways that receive coincident activity input within a limited time window of emotional arousal are enhanced.

How does NE-induced phosphorylation lower the threshold of GluR1 synaptic delivery? Previous work has shown that PKA activation can increase surface extrasynaptic pool of GluR1 (Oh et al., 2006). This suggested that delivery of GluR1 receptors to the extrasynaptic sites could be the intermediate step in the synaptic delivery of GluR1 receptors that is subject to NE modulation. One possible mechanism by which NE-induced phosphorylation may increase extrasynaptic GluR1 is through altering the association of GluR1 with molecules important for its delivery. e.g., either lowering the affinity of binding to an intracellular retention molecule (Esteban et al., 2003) or increasing the binding to a facilitatory molecule, such as stargazin (Chen et al., 2000; Rouach et al., 2005) or SAP97 (Leonard et al., 1998). These mechanisms could also account for increased synaptic reinsertion of GluR1 following LTD-induced removal (Ehlers, 2000). It will be interesting in the future to test whether NE may alter the interaction of GluR1 with these candidate molecules. In our initial biochemical studies, we found NE and fox urine produced a large phosphorylation effect on Ser845 (the PKA site) and a small but consistently significant effect on Ser831 (the PKC/CaMKII site). It would be interesting

to determine in the future if Ser845 is sufficient, or if Ser831 is sufficient, or if either is sufficient, or if both are necessary for the modulatory effects of NE on AMPAR trafficking and learning.

Our behavioral analysis of the GluR1 phosphomutant mice revealed an impairment of contextual learning and memory under conditions when brain NE was elevated (Figure 6). This result confirms the idea that NE-induced GluR1 phosphorylation critically contributes to the enhancement of memory formation during states with heightened NE level. In contrast, in the relatively neutral, nonstressful (saline-injected) condition, the contextual learning and memory of these phosphomutant mice was essentially normal, as indicated by a similar decrease in activity on day 3 of the GluR1 phosphomutant mice when compared with their wild-type siblings (Figure 6F). This normal basal contextual learning is in line with the largely normal spatial learning reported for the GluR1 phosphomutant mice and GluR1 knockout mice (Lee et al., 2003; Reisel et al., 2002; Zamanillo et al., 1999). One possible implication from these observations is that learning at the basal condition may not engage NE-modulated plasticity. Therefore deficits are revealed only during emotion-charged situations when NE release is above a threshold level. Another possibility is that other learning mechanisms independent of GluR1 (e.g. GluR2-long-dependent plasticity [Kolleker et al., 2003]) may be recruited and upregulated in the GluR1 phosphomutant mice. This may compensate for the malfunction of GluR1 at the basal state but may not be sufficient to mediate NE-enhanced memory.

Although our results from GluR1 phosphomutant mice established an important role of GluR1 downstream of NE in facilitating LTP and emotional learning, we do not think GluR1 accounts for all the functions of NE and emotion in these processes. Indeed the NE-facilitated LTP is not completely abolished in the CA1 neurons of the phosphomutant mice (Figure 5D), suggesting additional signaling event(s) downstream of NE have to exist in parallel to GluR1 phosphorylation. NE and β -adrenergic stimulation have been reported to decrease the activity of a calcium-activated potassium channel (Haas and Konnerth, 1983; Madison and Nicoll, 1982) and increase the activity of a voltage-dependent calcium channel (Gray and Johnston, 1987) and *N*-methyl-D-aspartate (NMDA) receptor (Huang et al., 1993; Raman et al., 1996). β -adrenergic activation can also inhibit protein phosphatase 1 and 2A (Thomas et al., 1996), which dephosphorylate GluR1 at the Ser845 site (Lee et al., 2000). Such modulatory

(F) Average EPSC current amplitude recorded at -60 mV from uninfected and infected cells. Values are normalized to uninfected cells. Error bars indicate SEM.

(G) Synaptic delivery of GluR1 with mutations that mimic phosphorylation. In (Ga) is the schematic of the GluR1DD construct, with the serine to aspartic acid mutation at residues 831 and 845. In (Gb) is an example of evoked AMPA receptor-mediated synaptic responses recorded at -60 mV and $+40$ mV from a neuron expressing GR1DD-GFP (green) and neighboring control cell (black). In (Gc), GR1DD-infected cells show increased rectification value (left, $p = 0.016$, *t* test) and EPSC amplitude at -60 mV (right, $p = 0.02$, *t* test) compared with noninfected control cells. Error bars indicate SEM.

(H) Synaptic delivery of GluR1 with mutations that block phosphorylation. (Ha)–(Hc) are similar as (Ga)–(Gc) except that the mutant construct is GluR1AA, with the serine-to-alanine mutation at residues 831 and 845 ($p = 0.04$, *t* test). Error bars indicate SEM.

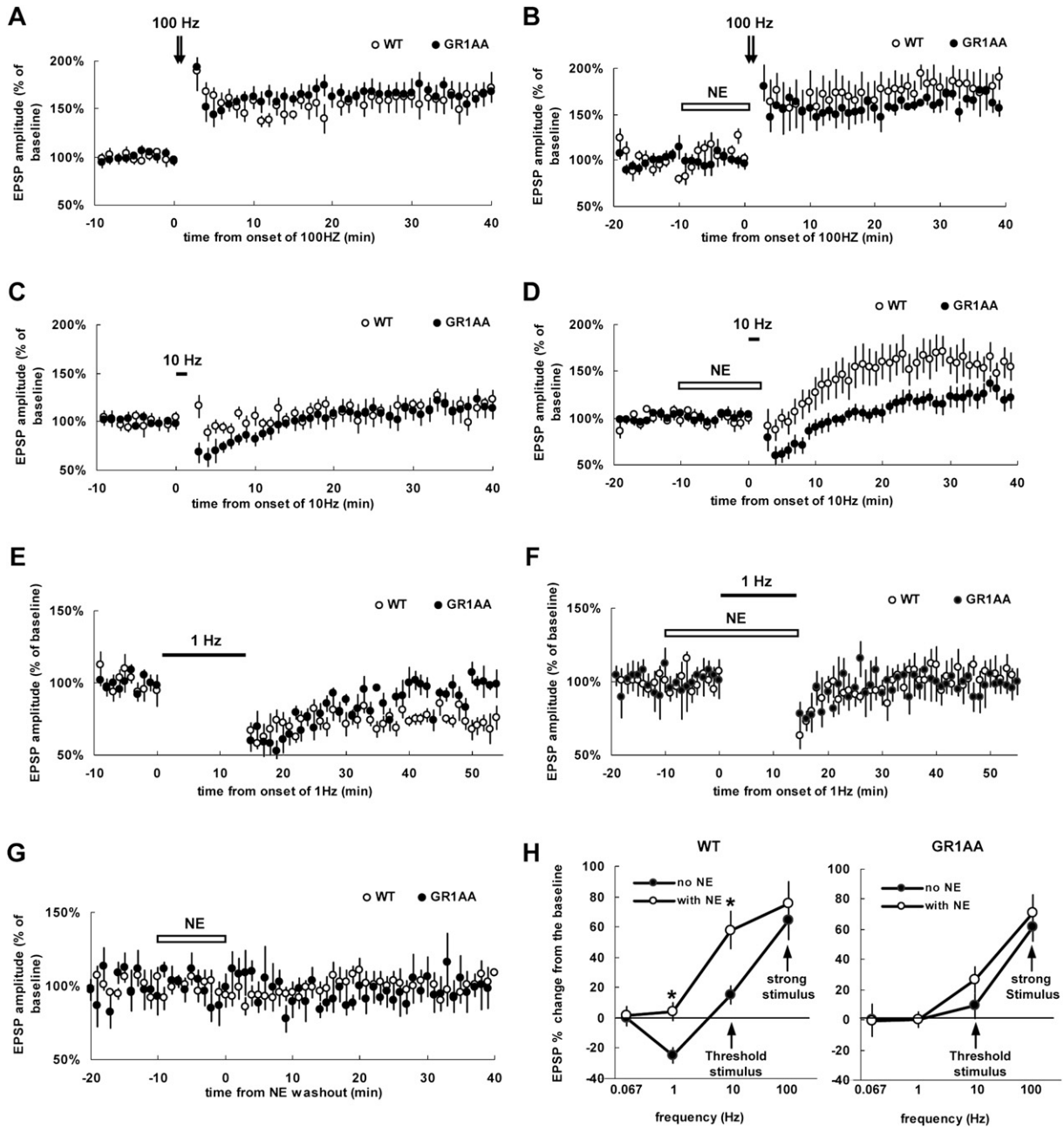


Figure 5. NE-Facilitated LTP Is Impaired in GluR1 Double-Phosphomutant Mice

(A–G) Stimuli were applied at 100 Hz (two trains of 1 s stimulation 25 s apart) (A and B), 10 Hz (900 pulses) (C and D), and 1 Hz (900 pulses) (E and F) in absence (A, C, and E) or presence (B, D, and F) of 10 μ M NE, to hippocampal slices made from GluR1 double phosphomutant mice (filled circle) or their wild-type siblings (open circle). Effect of NE alone on slices were also measured (G). In (H) are shown percent changes in normalized EPSP amplitude observed between ~36 to 40 min after 900-pulse stimulation plotted against stimulus frequency ($n = \sim 6$ –15 for each point; asterisk: $p < 0.05$ versus untreated group). Error bars indicate SEM.

processes would exert important effects on synaptic plasticity of hippocampal neurons and can presumably work in concert with the regulation of GluR1 to orchestrate the effect NE on LTP and learning.

A fundamental question in the field of LTP and memory research is how environmental factors and behavioral

states influence LTP and AMPAR trafficking and, thus, contribute to the modulation of memory (Malinow and Malenka, 2002). Our study here addresses how a particular type of behavioral state, emotional arousal, affects memory through NE regulation of GluR1 phosphorylation. It is noteworthy that phosphorylation of GluR1 can be

regulated by a number of neuromodulatory factors other than NE, including dopamine (Chao et al., 2002; Price et al., 1999; Snyder et al., 2000) and psychotropic drugs that affect neuromodulators, such as cocaine (Snyder et al., 2000), methamphetamine (Snyder et al., 2000), and fluoxetine (prozac) (Svenningsson et al., 2002). Therefore, our findings may shed light on how drugs, rewards, and other neuromodulatory factors regulate learning and memory.

EXPERIMENTAL PROCEDURES

Biochemical Analysis

For the *in vitro* experiments, hippocampal organotypic slice cultures were prepared from P6 rats, maintained in a static incubator (5% CO₂) for 5–10 days before the NE treatment. NE (10 μM) was added into slice culture medium for 10 min. When inhibitors were used, slices were preincubated with inhibitors in the following conditions: phenolamine (Sigma), 50 μM, 30 min; propranolol (Sigma), 10 μM, 30 min; KT5720 (Calbiochem), 4 μM, 2 hr; KN-93 (Calbiochem), 20 μM, 30 min.

In the *in vivo* phosphorylation experiments, mice were injected either with saline or epinephrine (0.5 mg/kg, IP). Within 15 min after drug injection, mice were anesthetized with isoflurane gas and decapitated and hippocampi were quickly isolated in cold PBS for western blot analysis. In the fox-urine exposure experiments, mice were introduced into a cage piled with tissue papers soaked with fox urine (Green Sense, TX). Mice activities were monitored and analyzed by Freezeview software (Actimetrics). Propranolol (20 mg/kg) or saline were injected (IP) ~15–30 min before mice entered the cage. After 5 min of stay in the cage, mice were sacrificed and hippocampi were isolated for western blot analysis. In all *in vivo* experiments, care was taken to minimize the handling stress of the mice.

For immunoblotting, frozen tissue samples from the *in vitro* and *in vivo* experiments were boiled for 10 min and sonicated in 1% SDS. Small aliquots of the homogenate were used to quantify the protein concentration. Equal amounts of protein were loaded onto 10% acrylamide gels, followed by transfer and blotting. Anti-phospho-GluR1 (Ser845), anti-phospho-GluR1 (Ser831) (Upstate and PhosphoSolution) and anti-GluR1 (Chemicon) antibodies were used for immunoblotting. Quantifications were done by chemiluminescence and densitometric scanning of the films under linear exposure conditions. Data on protein phosphorylation are expressed as percentage of control. Levels of phosphorylation, expressed as the ratio of phosphospecific intensity divided by total GluR intensity, were used for statistical analysis.

Acute Slice Electrophysiology

Acute hippocampal slices were prepared from ~3- to 6-week-old male rats or mice. Dissection was done using ice-cold artificial cerebrospinal fluid (ACSF, in mM: 119 NaCl, 2.5 KCl, 1 NaH₂PO₄, 26 NaHCO₃, 11 glucose, 1.3 MgCl₂, 2.5 CaCl₂) bubbled with 95%O₂/5%CO₂. Brain slices (400 μM) were cut using a manual tissue chopper, placed in a submersion-type holding chamber containing ACSF bubbled with 95%O₂/5%CO₂, recovered at 32°C for 2 hr then room temperature for ~1–4 hr. Field recordings were made in the same ACSF at 29°C ± 1.5°C. Synaptic responses were evoked using bipolar stimulating electrodes with single voltage pulse (200 μS, up to 20 V), placed in s. radiatum ~100–200 μm away from the CA1 cell bodies. Dendritic field responses were collected by a recording electrode (1–2 MΩ NaCl internal) placed in s. radiatum ~100–200 μm lateral of the stimulation electrode. Responses were evoked with a stimulation frequency of 0.067 Hz. To examine the effect of NE on LTP at different time points, slices were perfused with NE (10 μM) for 10 min (perfusion speed 1–2 ml/min). At 0, 30, and 90 min after NE washout, an electrical stimulation (10 Hz, 90 sec) was delivered to induce LTP. For LTD experiments, ~3- to 4-week-old mice were used.

Cultured Slice Electrophysiology

Organotypic hippocampal slice cultures were made from P6–7 rats and cultured for 7–10 days and infected with sindbis virus expressing GluR1-GFP, GluR1AA-GFP, or GluR1DD-GFP constructs (Shi et al., 2001). Electrophysiological recording was performed ~24–28 hr after infection. The recording chamber was first perfused with ACSF solution gassed with 5% CO₂ and 95% O₂ at 29°C ± 1.5°C. Synaptic responses were evoked using bipolar stimulating electrodes with single voltage pulse (200 μS, up to 20 V), placed over Schaffer Collateral fibers 100–150 μm away in the horizontal dimension and 200–400 μm away in the vertical dimension to the transfected CA1 cell bodies. Slices were conditioned with NE and/or 10 Hz stimulus. Field recordings were not monitored for extended periods in most experiments, since we established that these protocols provided consistent results (Figure 3), and any failure to produce plasticity would only increase the noise (SEM) in our measurements. Following conditioning, whole-cell intracellular recordings were obtained with Axopatch -1D amplifier (Axon Instruments). Rectification values were obtained between 0.5 to 1.5 hr after the induction protocol, using the same stimulating electrode used in the conditioning. Bath solution contained (in mM) 119 NaCl, 2.5 KCl, 4 CaCl₂, 4 MgCl₂, 26 NaHCO₃, 1 NaH₂PO₄, 11 glucose, 0.1 picrotoxin, 0.1 DL-APV, and 0.002 2-Chloroadenosine pH 7.4 and was gassed with 5% CO₂ and 95% O₂ at 29 ± 1.5°C. 2-Chloroadenosine was included to prevent bursting. For paired recordings, infected and neighboring uninfected cells were whole-cell accessed and synaptic responses were recorded from both cells simultaneously (Zhu et al., 2000). Patch recording pipettes (3–5 MΩ) were filled with internal solution, pH 7.25, containing (in mM) 115 CsMeSO₄, 20 CsCl, 10 HEPES, 2.5 MgCl₂, 4 Na₂ATP, 0.4 Na₂GTP, 10 Na-phosphocreatine, and 0.6 EGTA. Liquid junction potential was not corrected.

Contextual Learning Behavior Assay

Mice

GluR1 phosphomutant (Lee et al., 2003) and their wild-type siblings were ~3- to 4-month-old 129 X C57BL/6 hybrids, obtained by mating heterozygous mice. For other control experiments, mice with C57BL/6 background were used.

Setup

Preexposure, training and testing were carried out in the same chamber (Coulbourn Instruments). A video camera is positioned on top of the chamber to monitor the mice behavior. The floor of the chamber consists of 12 stainless steel rods (3 mm diameter) spaced 9 mm apart. The rods are wired to a shock generator (Coulbourn Instruments). Mice were trained or tested one at a time. Between tests, the cage floor was cleaned with a 75% ethanol solution (during test) or soap (during preexposure and training).

Procedure

The behavior protocol is a 3 day procedure, modified from Frankland et al. (2004). On day 1, mice were pre-exposed (for 2 or 8 min) to the training chamber. On day 2, mice were placed in the same chamber. Two seconds after mice entering the cage, an electrical shock (1 mA, 2 s) was delivered via the cage floor bars. Following the shock, the mice remained in the context for a further 60 s. On day 3, mice were placed back to the contextual cage and monitored over a 3 min period. Mice remain in their home cage during the rest of the time.

Measurements

For evaluation of the contextual learning, two parameters were measured. First, motion was measured by a custom-made Matlab software Mousemove (Kopec et al., 2006). Significant motion pixel (SMP) values are a linear measure of mouse motion between frames captured at 5 Hz. Second, "Freezing," defined as SMP < 10 for longer than 1 s, was quantified by Freezeview image analysis software (Actimetrics).

Drugs

Epinephrine (Sigma) was dissolved in saline solution and administered IP in a dose of (0.5 mg/kg, 10 ml/kg) ~15–30 min prior context preexposure.

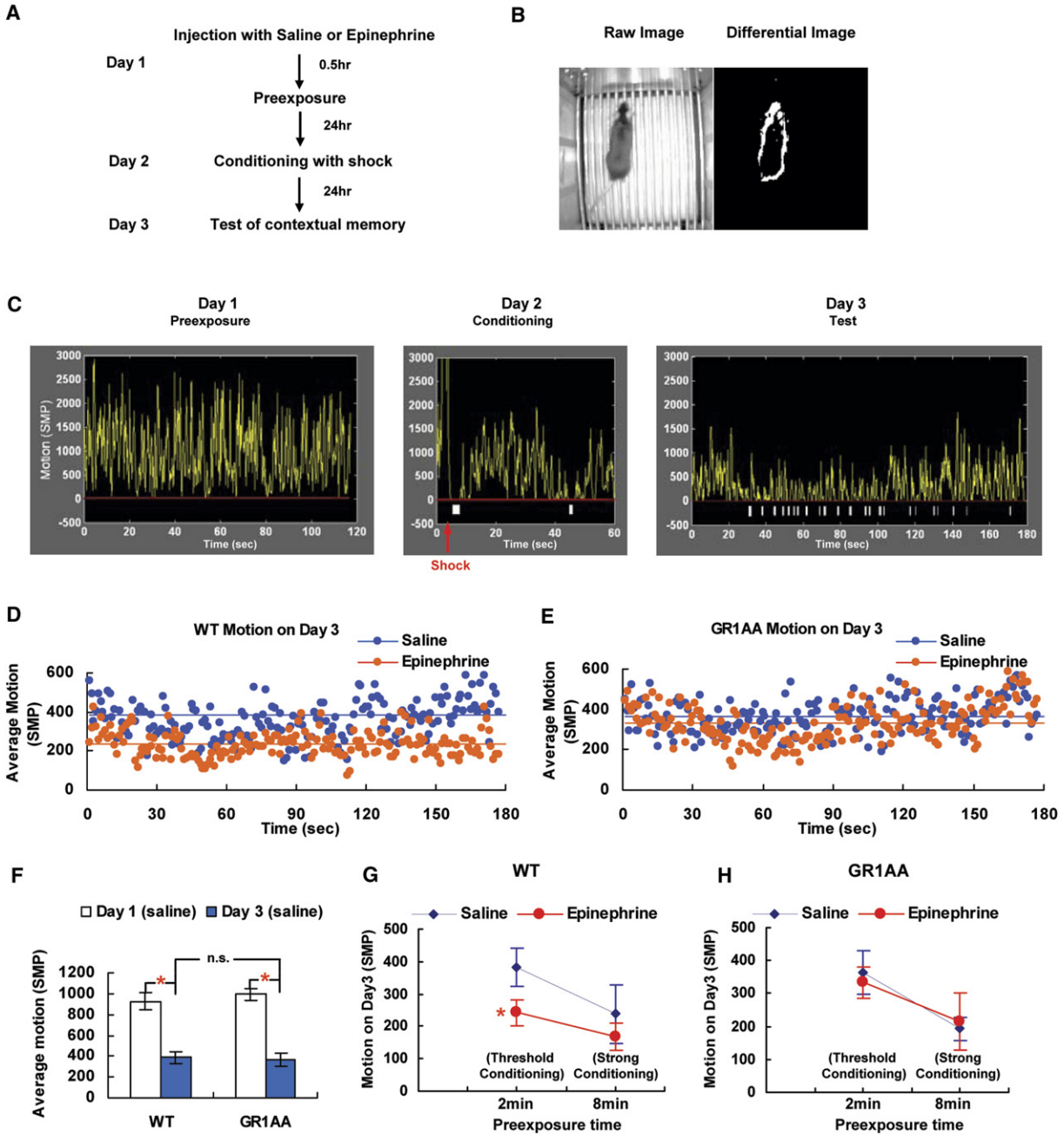


Figure 6. The Memory-Enhancing Effect of NE Is Mediated by GluR1 Phosphorylation

(A) Schematic of the 3 day behavior protocol for the preexposure-dependent contextual fear learning.

(B) Record of mouse activity. Differential images (right) derived from subtraction of two consecutive frames of the raw image (left) were used to calculate significant motion pixels (shown in white), SMP.

(C) A representative record of the activity of a mouse during the 3 day behavior protocol. Each point on graph plots SMP during 1 s epoch. Note the dramatically decreased activity (yellow lines) and increased freezing (white bars) on day 3 after conditioning learning.

(D) Epinephrine enhanced the contextual fear memory in wild-type mice, as indicated by a greater decrease of activity in epinephrine-injected group (red, $n = 13$) versus saline-injected group (green, $n = 15$) on day 3. Each point is the average of all mice in the group for a 1 s period. The lines represent the average SMP of each treatment group across the 3 min period.

(E) The epinephrine effect on contextual memory is blocked in the GluR1 double-phosphomutant mice. Note that the epinephrine- ($n = 14$) and saline-injected ($n = 14$) groups do not differ in averaged activity on day 3.

(F) Average SMP values of the GluR1 phosphomutant mice and their wild-type siblings on day 1 and day 3 ($n.s. = p = 0.81$, t test).

Supplemental Data

Supplemental Data include one figure and can be found with this article online at <http://www.cell.com/cgi/content/full/131/1/160/DC1/>.

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(G) Average SMP values of the wild-type mice on test day 3. These mice have been injected with either epinephrine or saline on day 1 before either a 2 min, or 8 min pre-exposure conditioning protocol ($p = 0.03$, t test). Error bars indicate SEM.

(H) Average SMP values of the phosphomutant mice on test day 3. Error bars indicate SEM.

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