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Regulatory Mechanisms of Fear Extinction and Depression-Like Behavior

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Human anxiety is frequently accompanied by depression, and when they co-occur both conditions exhibit greater severity and resistance to treatment. Little is known, however, about the molecular processes linking these emotional and mood disorders. Based on previously reported phosphorylation patterns of extracellular signal-regulated kinase (ERK) in the brain, we hypothesized that ERK's upstream activators intertwine fear and mood regulation through their hippocampal actions. We tested this hypothesis by studying the upstream regulation of ERK signaling in behavioral models of fear and depression. Wild-type and ERK1-deficient mice were used to study the dorsohippocampal actions of the putative ERK activators: mitogen-activated and extracellular signal-regulated kinase (MEK), protein kinase C (PKC), and cAMP-dependent protein kinase (PKA). Mice lacking *ERK1* exhibited enhanced fear extinction and reduced depression caused by overactivation of ERK2. Both behaviors were reversed by inhibition of MEK, however the extinction phenotype depended on hippocampal, whereas the depression phenotype predominantly involved extrahippocampal MEK. Unexpectedly, inhibition of PKC accelerated extinction and decreased depression by ERK-independent mechanisms, whereas inhibition of PKA did not produce detectable molecular or behavioral effects in the employed paradigm. These results indicate that, contrary to fear conditioning but similar to mood stabilization, extinction of fear required upregulation of MEK/ERK and downregulation of ERK-independent PKC signaling. The dissociation of these pathways may thus represent a common mechanism for fear and mood regulation, and a potential therapeutic option for comorbid anxiety and depression.
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INTRODUCTION

Deregulation of acquired fear, revealed by enhanced conditioning or impaired extinction, has been regarded as a basis for the development of anxiety disorders. Human anxiety is frequently accompanied by depression, and when they co-occur, both conditions exhibit greater severity and resistance to treatment (Lydiard and Brawman-Mintzer, 1998; Dunner, 2001; Holtzheimer *et al*, 2005). Behavioral rodent models have been invaluable for the identification of neuronal genes (Lesch *et al*, 1992; Keck *et al*, 2005; Leonardo and Hen, 2006), processes (Dranovsky and Hen, 2006), and circuits (Ressler and Nemeroff, 2000), regulating trait depressive-like behavior and anxiety; however, the link between affective states and acquired fear, in particular the persistence and extinction of fear, is not well understood.

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Acquired fear responses can normally be diminished via a learned extinction process when no longer reinforced by aversive events. Impaired fear extinction has been linked to increased depression-like behaviors as recently observed in rodent models of learned helplessness (Shumake *et al*, 2005), brain-derived neurotrophic factor knockout mice (Heldt *et al*, 2007), and serotonin transporter knockout mice (Wellman *et al*, 2007). These findings suggested that depression increases susceptibility to persistent fear typical of anxiety disorders, and *vice versa*, persistent fear might contribute to depression. We hypothesized that a subset of molecular mechanisms, operating throughout the brain or its specific regions, contributes to the coregulation of mood and learned fear.

Rodent and human studies have suggested a role for the extracellular signal-regulated kinase (ERK) in depressive-like behaviors with the prefrontal cortex and hippocampus as the most likely neuroanatomical sites of ERK action (Dwivedi *et al*, 2001; Fumagalli *et al*, 2005; Qi *et al*, 2006). However, most studies performed so far have employed systemic injections of ERK inhibitors (Einat and Manji, 2006), and thus the selective contribution of ERK or its

other upstream activators in either brain region has not been elucidated.

Neuronal plasticity underlying memory formation (Atkins *et al*, 1998; Sananbenesi *et al*, 2002; Trifilieff *et al*, 2006) and stress-induced facilitation (Sananbenesi *et al*, 2003; Yang *et al*, 2004a; Revest *et al*, 2005) of contextual fear involves hippocampal ERK mechanisms regulated by several upstream kinases. ERK is also required for extinction of contextual fear (Szapiro *et al*, 2003; Fischer *et al*, 2007) and shows accelerated and more robust induction as well as increased nuclear accumulation (Chen *et al*, 2005; Fischer *et al*, 2007) when compared to fear conditioning. Thus, different upstream protein kinases likely regulate ERK activity during these learning processes.

Some of the main upstream regulators of ERK activity include the mitogen-activated and extracellular signal-regulated kinase (MEK), cAMP-dependent protein kinase (PKA), and protein kinase C (PKC) (Pouyssegur *et al*, 2002). MEK directly phosphorylates and activates ERK (Crews and Erikson, 1992), whereas PKA and PKC regulate ERK activity by indirect, MEK-dependent (Yuan *et al*, 2002) or MEK-independent mechanisms (Grammer and Blenis, 1997; Kinkl *et al*, 2001). Similarly, extensive intracellular cross-talk between hippocampal PKA, PKC, and MEK converging at ERK was shown in hippocampal slices (Roberson *et al*, 1999) and *in vivo* (Ahi *et al*, 2004). The interactions of these protein kinases in the regulation of ERK activity during fear extinction and depression-like behavior remain unexplored.

Here we examined the contribution of ERK and a subset of its upstream activators to the coregulation of mood and fear extinction. It has previously been shown that *ERK1* deletion does not affect memory acquisition, freezing behavior, or ERK2 activity after fear conditioning (Selcher *et al*, 2001). Thus, ERK1 knockout mice are uniquely suited to study ERK-dependent mechanisms specific for extinction of fear and depression-like behaviors. Given that ERK2 knockouts are not viable (Saba-El-Leil *et al*, 2003) and ERK1-deficient mice exhibit stimulus-dependent overactivation of the ERK2 isoform (Pages *et al*, 1999; Mazzucchelli *et al*, 2002), ERK1 knockouts serve as an ERK2 gain-of-function model allowing the selective role of the ERK2 isoform to be examined. Here we demonstrated that in ERK1-deficient mice, overactivation of the ERK2 isoform mediates a phenotype encompassing both enhanced fear extinction and reduced depression-like behaviors. Pharmacological experiments with inhibitors of putative upstream ERK activators revealed that downregulation of PKC and upregulation of ERK activity enhanced fear extinction and reduced depression. Whereas PKC mediated both effects via the hippocampus, the effects of ERK involved hippocampal and extrahippocampal mechanisms. On the basis of these findings, it is suggested that improved mood might lead to faster reduction of fear by accelerating the formation of extinction memories.

METHODS

Subjects

Male 9- to 11-week-old mice of the C57BL/6J (Jackson Laboratories) and C57BL/6NCrl (Charles River) strains were housed individually in Plexiglas cages. ERK1-deficient mice (Pages *et al*, 1999) were obtained from breeding couples heterozygous for the *ERK1* gene. These mice were generated in the S129/SvJ strain and backcrossed for six generations to C57BL/6J mice. Standard food pellets and water were offered *ad libitum*. Mice were habituated to the housing at least 7 days before the beginning of experiments in enclosures adjacent to the behavior testing room to minimize transportation before and after training and testing trials. Constant temperature ($22 \pm 1^{\circ}$C), humidity ($55 \pm 10\%$), 12-h light–dark cycle (0700–1900 hours), and air flow (20–40 exchanges per hour) were maintained. Genotyping was conducted as previously described (Pages *et al*, 1999). All procedures were approved by the Northwestern University Animal Care and Use Committee.

Contextual Fear Conditioning and Extinction

Contextual fear conditioning was performed as described previously (Radulovic *et al*, 1998a) by exposing the mice for 3 min to the context followed by a tone (30 s, 10 kHz, sinus 200 ms, 75 dB SPL) and electric shock (2 s, 0.7 mA, constant electric current) representing the unconditioned stimulus. This procedure resulted in a subceiling level of freezing that can be enhanced by repeated training trials (Li *et al*, 2007). Extinction began 24 h later and consisted of daily 3-min re-exposures of mice to the conditioning context in the absence of shock. Each extinction session served as the contextual memory test. This extinction procedure typically leads to reduction of fear within 4–5 days in the C57BL/6J strain (Fischer *et al*, 2004, 2007) and 8–9 days in the wild-type littermates of ERK1 knockouts. The C57BL/6NCrl mice are resistant to extinction (Stiedl *et al*, 1999; Siegmund *et al*, 2005). Freezing, characterized by lack of movement besides respiration and heart beat, associated with crouching posture (Blanchard and Blanchard, 1969), was used as an indicator of learning, and assessed by a sampling procedure every 10 s for 3 min by two trained observers. The mean number of observations indicating freezing behavior was expressed as a percentage of the total number of observations. Additionally, the activity burst to the shock was monitored by infrared beam system connected to analysis software (TSE Inc.) to ensure that all animals received the shock.

For experiments testing savings of fear extinction, mice were exposed to a reminder shock (0.7 mA, 2 s) in a novel context, serving to reinstate freezing, and then re-exposed to the context for 3 min for three consecutive days. This reminder shock is not sufficient to induce fear conditioning and specifically reinstates freezing to the conditioning but not novel contexts (Fischer *et al*, 2004). After the end of selected experiments, tone-dependent memory tests were performed by exposing the mice to the tone for 3 min in a novel context (Radulovic *et al*, 1998a). These tests were performed to exclude nonspecific effects of the manipulations on freezing to a nonextinguished conditioned stimulus (tone). Because such effects were not observed, these data are not shown.

Forced Swim Test

The forced swim test (FST) served to assess depression-like behavior (Porsolt *et al*, 1978; Petit-Demouliere *et al*, 2005).

Table 1 Drug Treatments[a] and Effects

Protein kinase	Inhibitor	Dose	Route	FST (immobility)	Fear extinction
ERK1$^{-/-}$				↓↓	↑↑
MEK	U0126	1 μg/mouse	i.h.	↓	↑
MEK	SL327	10 mg/kg	i.p.	↑	NA
Wild type					
MEK	U0126	1 μg/mouse	i.h.	↓	↓
MEK	SL327	10 mg/kg	i.p.	∅	NA
PKC	NPC15437	25 μg/mouse	i.h.	↓↓	↑↑
PKA	Rp-cAMPS	18 μg/mouse	i.h.	∅	∅

[a]The efficacy of these inhibitors *in vivo* has been documented in mouse models of conditioned fear and depression. The drugs infused i.h. were previously shown to impair fear conditioning or its stress-induced facilitation and impaired the phosphorylation of ERK along with their corresponding kinases (Atkins *et al*, 1998; Sananbenesi *et al*, 2003; Ahi *et al*, 2004) when compared to their corresponding vehicle controls. SL327 attenuated ERK-mediated depression phenotypes (Duman *et al*, 2006).

Mice were placed in glass cylinders (15 cm diameter, 30 cm height) filled with water up to 8 cm below the top. The water temperature was maintained at $20 \pm 2^{\circ}$C. After a 1-min accommodation period, the time the mice spent in an immobile posture, reflecting depression-like behavior, was recorded for 4 min and expressed as total duration of immobility (sec). We performed pilot experiments with two tests performed on consecutive days, and compared the effects of the ERK1 knockout or pharmacological treatments before the first or before the second test. Because we did not observe any differences (two-way ANOVA for factors Genotype: $F_{1,28} = 8.919$, $P < 0.01$; and Genotype × Test interaction: $F_{1,28} = 0.496$, $P = 0.491$), we only present data obtained from the first test.

Locomotor Activity

Mean activity (cm/s) was determined over a 3-min period in the same chambers used for fear conditioning, by an infrared beam system connected to the analysis software (TSE Inc.). In the extinction experiments, activity was determined concomitantly to freezing measurements. For the pharmacological studies, mice were injected with an inhibitor 15 min prior to exposure to the chamber. During these studies, shock was not delivered.

Cannulation and Drug Administration

Double-guided cannulae (C235, Plastic One, Roanake, VA) were implanted under a 1.2% avertin anesthesia (0.4 ml per mouse) as previously described (Radulovic *et al*, 1999b). The cannulae were inserted into both dorsal hippocampi of mice, anteroposterior from the bregma −1.5 mm, lateral ± 1.0 mm and 2 mm in depth (Franklin and Paxinos, 1997). The drugs, doses, and routes of administration are summarized in Table 1. The PKA inhibitor Rp-cAMPS (adenosine 3′,5′ monophosphorothiolate, RP-isomer, triethyl ammonium salt; Calbiochem, Germany) and PKC inhibitor NPC15437(*S*)-2,6-diamino-*N*-{[1′-(1″-oxotridecyl)-2′piperidinyl]methyl}hexanamide. 2Hcl (A. G. Scientific Inc., San Diego) were dissolved in artificial cerebrospinal fluid (aCSF). The aCSF contained (in mM): 130 NaCl, 3 KCl, 1.25 NaH_2PO_4, 26 $NaHCO_3$, 1 $MgCl_2$, 10 glucose, 2 $CaCl_2$. UO126 (Promega, Madison, WI), and it was dissolved 20% dimethyl sulfoxide (DMSO/aCSF). Intrahippocampal (i.h.) injections of vehicle, Rp-cAMPS (9 μg/0.25 μl/site), NPC15437 (12.5 μg/0.25 μl/site), or U0126 (0.5 μg/0.25 μl/site) were performed immediately after each nonreinforced contextual exposure. The vehicle groups compared to Rp-cAMPS and NPC15437 were injected with aCSF whereas vehicle controls for U0126 received 20% DMSO/aCSF. We have demonstrated previously that the employed doses of these inhibitors are effective *in vivo* and significantly impair the phosphorylation of their downstream targets during fear conditioning (Ahi *et al*, 2004) and its stress-induced facilitation (Sananbenesi *et al*, 2003). Cannula sites were examined for placement and site damage. Only data from mice with accurately placed cannulae (Radulovic *et al*, 1999a) were retained for analysis. The infusions did not cause damage at the cannula site, as previously shown after long-term injections of U0126 (Chen *et al*, 2005). To investigate the effects of these drugs in the FST, small i.h. injections were performed 15 min before the task. In a separate experiment, SL327, a MEK inhibitor that crosses the blood–brain barrier, was injected intraperitoneally at a dose of 10 mg/kg (10 ml/kg of 100% DMSO). In these experiments, vehicle was 100% DMSO (Atkins *et al*, 1998; Duman *et al*, 2007).

Immunohistochemistry

Mice were anesthetized with an intraperitoneal injection of 240 mg/kg of avertin, and transcardially perfused with ice-cold 4% paraformaldehyde in phosphate buffer (pH 7.4, 150 ml/mouse) as described (Stanciu *et al*, 2001; Fischer *et al*, 2007). Coronal sections (50 μm thick) were used for free-floating immunocytochemistry with primary antibodies specific for pERK-1/2 (Thr-183/Tyr-185, 1:5000, mouse monoclonal IgG_1, Sigma) or anti-phospho-cAMP response element binding protein (pCREB, Ser-133; rabbit polyclonal IgG, 1:2000, Calbiochem). Biotinylated secondary antibody and ABC complex (Vector) were used for signal amplification and DAB (Sigma) as chromogen. Quantification of somatic immunostaining was performed for the CA1

pyramidal cell layer as described (Fischer *et al*, 2007). Briefly, two CA1 areas within the dorsal hippocampus were captured and equal cutoff thresholds were applied to remove background staining. Cell somas positive for pERK-1/2 were counted within a 100 μm^2 grid in three randomly selected fields of each CA1 image using a binary mode (ImageJ). The mean number of immunopositive cells in six fields was calculated for each mouse. The same method was used to quantify pCREB-positive nuclei. Dendritic pERK levels were not quantified because they similarly increase after fear conditioning and extinction (Fischer *et al*, 2007).

Protein Extraction and Immunoblot

Individual dorsal hippocampi (the septal 2.5 mm pole) were collected from wild-type, ERK1 knockout, vehicle- or drug-injected mice 1 h after fear conditioning or extinction trials demonstrating significant group differences, as indicated. Corresponding tissue of naive mice served to prepare additional control lysates. The selected time point was earlier found to trigger maximal ERK phosphorylation in these paradigms (Sananbenesi *et al*, 2003; Fischer *et al*, 2007). Hippocampal lysates were subjected to 10% SDS-polyacrylamide gel electrophoresis, and subsequently blotted to polyvinylidene fluoride membranes (Immobilon-P, Millipore, Bedford, MA) as described previously (Fischer *et al*, 2007). Membranes saturated with I-block (Tropix) were incubated with anti-phosho-ERK (pERK, Sigma, 1 : 8000), anti-ERK (Santa Cruz, 1 : 4000), anti-MEK (Cell Signaling, MEK, 1 : 400), and anti-phospho-Elk1 (pElk1, Santa Cruz, 1 : 1000), for 1 h at room temperature. Western blots were exposed to X-ray films and developed in the range of maximal chemiluminescence emission (10 min).

Immunoprecipitation and ERK Kinase Assays

All samples for kinase assays were prepared as described above, from mice injected *in vivo* with inhibitors and vehicle or from ERK1 wild-type and knockout mice. The dorsal hippocampi used for lysate preparation were collected 1 h after the indicated extinction trials. For immunoprecipitation, 0.5 μg of total protein/lysate was incubated for 1 h at 4°C with 2 μg anti-ERK antibody followed by incubation for 30 min on ice with magnetically labeled Protein G Microbeads. Washing and elution were performed as described in the MAGmol Microbeads user's manual (Milteny Biotec).

The ERK kinase assay was performed as described previously, using Elk-1 fusion protein (Elk-1 residues 307–428 fused with glutathione *S*-transferase (GST), 41 kDa) (Cell Signaling) as the ERK substrate (Sananbenesi *et al*, 2002). Elk-1 phosphorylation was determined by immunoblot. Molecular weight and densitometric calculations were performed with the computer software ImageJ (NIH).

PKC and PKA Assays

The kinase activity of PKA and PKC were assessed in membrane fractions using PepTag Assays (Promega) based on a phosphorylation-induced fluorescence of tagged PKC- and PKA-specific peptides as described previously (Yang *et al*, 2004b). Briefly, the dorsal hippocampi of mice injected with drugs or vehicle 1 h earlier, were homogenized in ice-cold homogenization buffer (0.32 M sucrose, 10 mM HEPES, pH 7.4, 2 mM EDTA, protease inhibitors, and phosphatase inhibitors as for RIPA buffer) and centrifuged at 100 000 *g* for 30 min. The pellet was rehomogenized in homogenization buffer and sonicated, incubated with Triton X-100 (0.2%) for 30 min, and centrifuged at 100 000 *g* for 30 min. The supernatant containing membrane-bound PKC and PKA was used for the assays. An aliquot of the preparations (2 μg of total membrane protein) was incubated for 30 min at 30°C in a PepTag reaction buffer (in mM: 100 HEPES, 6.5 $CaCl_2$, 5 DTT, 50 $MgCl_2$, and 5 ATP, pH 7.4) containing 0.4 μg/μl of the PKC-specific peptide substrate PepTagC1 (P-L-S-R-T-L-S-V-A-A-K) or PKA-specific peptide L-R-R-A-S-L-G (kemptide). The reactions were stopped by heating to 95°C for 10 min. Phosphorylated and unphosphorylated PepTag peptides were electrophoretically separated using 0.8% agarose gels. Phosphorylation of the substrates, determined by fluorescence intensity, was used to measure the kinase activity. Densitometry was performed only on the part of the images where the reflection from the loading wells did not interfere with the substrate signals.

Statistical Analysis

The behavioral data were analyzed by two-way ANOVA with factors Test and Genotype or Treatment or by three-way ANOVA with factors Test, Genotype, and Treatment. The molecular studies were analyzed by one-way ANOVA with factor Genotype or Treatment or two-way ANOVA with factors Genotype and Treatment. Scheffe's test was employed for *post hoc* group comparisons. Unpaired Student's *t*-test was used for comparisons of two groups.

RESULTS

Fear Extinction and ERK2 Activity of ERK1-Deficient Mice

Mice lacking *ERK1* exhibited significantly enhanced extinction of contextual fear (Figure 1a), as revealed by an accelerated reduction of freezing behavior after nonreinforced exposures to the conditioning context. Two-factor ANOVA employing Test and Genotype as factors demonstrated a significant effect of Genotype ($F_{1,34} = 7.862$, $P < 0.01$) and a significant Genotype × Test interaction ($F_{11,374} = 3.754$, $P < 0.05$). The same mice did not show detectable alterations of fear conditioning when compared to their wild-type littermates on the training day ($t_{34} = 0.324$, $P = 0.92$). Given that the employed training protocol results in subceiling freezing (Li *et al*, 2007), the observed effects on extinction were probably not due to differences in the strength of the conditioning memory. This possibility was further excluded in the subsequent pharmacological studies that were selectively performed after extinction tests (see below).

Locomotor activity was analyzed in one of the replicates of the extinction experiments. Consistent with earlier reports (Selcher *et al*, 2001; Mazzucchelli *et al*, 2002), ERK1 knockouts exhibited increased locomotor activity over time (Genotype: $F_{7,70} = 5.396$, $P < 0.05$; Genotype ×

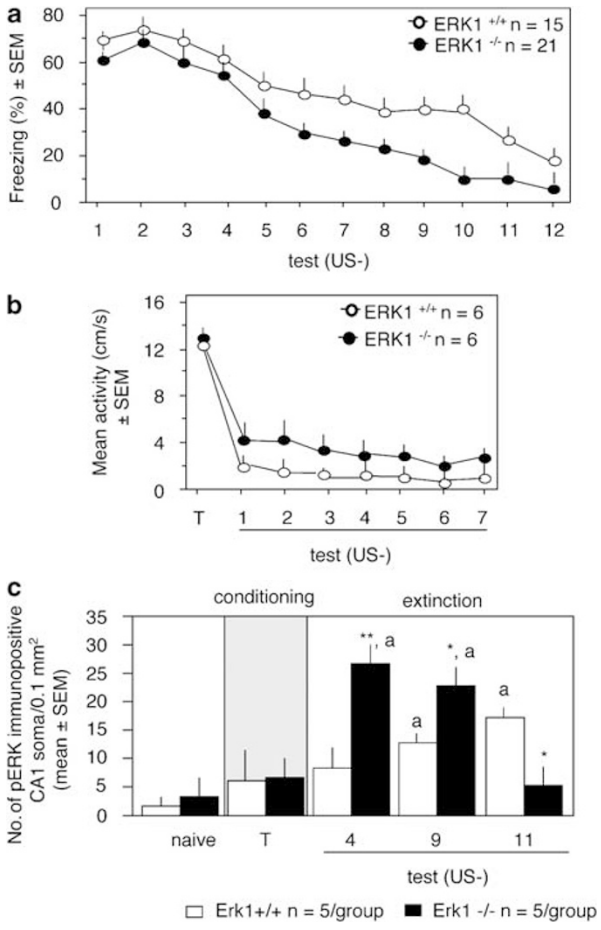

Figure 1 Contextual fear extinction and ERK2 upregulation in ERK1-deficient mice. (a) ERK1 knockouts exhibited significantly enhanced extinction ($P<0.001$) when compared to their wild-type littermates, as indicated by accelerated reduction of contextual freezing after extinction trials. (b) ERK1 knockouts show higher locomotor activity than their wild-type littermates between tests 1–7 but not during training. (c) Time course analysis of ERK phosphorylation in CA1 cells revealed an earlier upregulation of pERK2 in CA1 neurons of ERK1-deficient mice when compared to their wild-type littermates. The highest number of pERK-positive cells in ERK1 knockouts was observed between the fourth and ninth extinction test, whereas wild-type mice showed a significant increase of pERK immunostaining after the ninth and 11th test. Statistically significant differences: $^{*}P<0.01$; $^{**}P<0.001$ *vs* ERK1 $^{+/+}$, $^{a}P<0.01$ *vs* naive.

Test: $F_{7,70}=0.325$, $P=0.94$) when compared to their wild-type littermates (Figure 1b). This phenotype was not likely to contribute to their freezing behavior, as it was also suggested earlier (Selcher *et al*, 2001), given that both groups exhibited similar freezing behavior over the first three extinction tests despite these differences of activity.

Immunohistochemical analyses showed an accelerated increase of pERK in the soma and nuclei of the pyramidal CA1 layer of ERK$^{-/-}$ when compared to ERK$^{+/+}$ mice ($F_{1,40}=6.467$, $P<0.05$). The signals in ERK1-deficient mice, reflecting pERK2-positive cells, were maximal after the fourth and ninth extinction tests and subsequently declined. In the wild-type mice, pERK-1/2 levels were elevated, although to a lesser extent, after the ninth and 11th test (Figure 1c). It has previously been shown that pERK is not a consequence of FC, but is selectively induced by extinction (Fischer *et al*, 2007).

To further examine whether ERK2 is the predominantly activated isoform, we performed several analyses of dorsohippocampal lysates obtained 1 h after the ninth extinction trial. This time point was selected because after the ninth test, ERK levels increased in both genotypes but significantly more in the ERK knockout, consistent with differences in the behavioral phenotype. The level of pERK2 was elevated in the dorsal hippocampi of ERK1-deficient mice after extinction but not after fear conditioning (Figure 2a). Significant effects were observed for Genotype ($F_{1,30}=12.36$, $P<0.001$) and Group ($F_{2,30}=9.31$, $P<0.001$).

Consistent with this finding, ERK kinase activity ($t_8=13.21$, $P<0.001$, Figure 2b) and its association with MEK ($t_4=7.35$, $p<0.01$, Figure 2c) were elevated in the dorsal hippocampi of ERK1 knockouts when compared to wild-type mice. These increases were not caused by alterations of total ERK levels (Figure 2b and c), indicating enhanced activation of ERK2.

Taken together, ERK1-deficient mice exhibited enhanced fear extinction accompanied by significantly faster and stronger activation of ERK2 when compared to their wild-type littermates. As ERK2 represents the biologically more active isoform (Mazzucchelli *et al*, 2002), it appears that ERK2 did not only compensate for the lack of ERK1, but also exhibited stronger biological activity in the absence of ERK1.

MEK/ERK Signaling and Fear Extinction

In order to identify the main upstream activators of ERK during contextual fear extinction, we injected wild-type or ERK1-deficient mice with Rp-cAMPS, NPC15437, or U0126 into the dorsal hippocampus for 9 consecutive days immediately after each daily extinction trial. After the ninth test, mice were killed, their dorsal hippocampi dissected out and processed as described. The PKA inhibitor Rp-cAMPS and PKC inhibitor NPC15437 did not affect the level of pERK in the dorsohippocampal lysates of either wild-type or ERK1-deficient mice (Figure 3a and b), whereas the MEK inhibitor U0126 significantly impaired ERK phosphorylation (wild type: $F_{3,16}=8.29$, $P<0.01$; ERK1 knockout: $F_{3,16}=11.31$, $P<0.01$). There was no significant interaction for Treatment and Genotype ($F_{3,32}=0.822$, $P=0.491$). These results indicated a key role of MEK in hippocampal ERK regulation during fear extinction. Consistent with the biochemical data, intrahippocampal infusion of U0126 prevented contextual fear extinction (Figure 3c) in ERK1 knockouts and their wild-type littermates (Genotype × Treatment × Test: $F_{8,296}=9.5288$, $P<0.01$). It appears, therefore, that overactivation of the hippocampal ERK2 isoform by MEK predominantly contributed to the enhanced fear extinction phenotype of ERK1 knockouts.

MEK/ERK Signaling and Depression-Like Behavior

Given the role of MEK/ERK in fear extinction, we investigated the possible association between fear and mood regulation by interfering with this pathway. In order to examine whether the enhanced extinction phenotype of ERK1-deficient mice was associated by alterations of depression-like behavior, we tested separate groups of mice in the FST. In addition to enhanced fear extinction

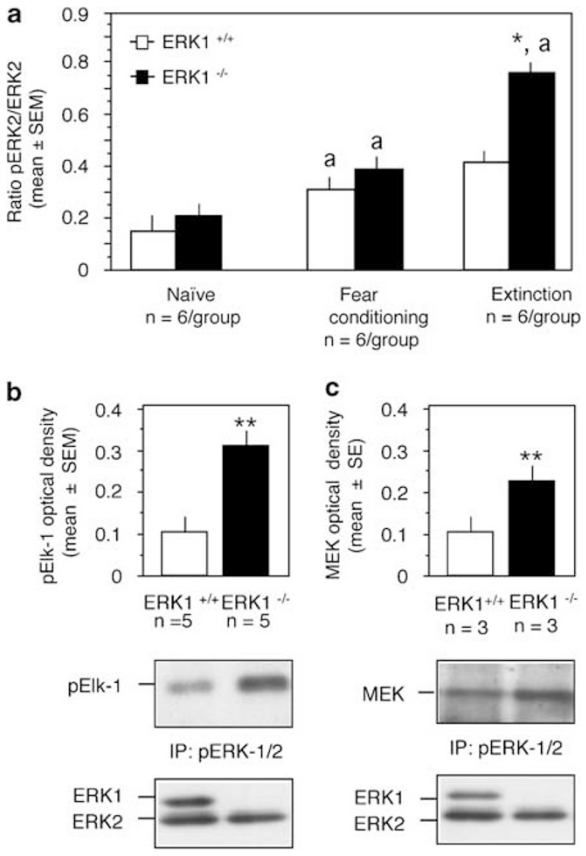

Figure 2 ERK2 activity of ERK1-deficient mice. Dorsohippocampal lysates from ERK1$^{-/-}$ and ERK1$^{+/+}$ mice were obtained from naïve mice, 1 h after fear conditioning or 1 h after the ninth extinction test. (a) The levels of pERK2 were significantly elevated in ERK1 knockouts after the ninth extinction test. Kinase assays and co-immunoprecipitation were therefore performed in dorsohippocampal lysates obtained at this time point. (b) Increased ERK2 kinase activity in ERK1$^{-/-}$ mice, as revealed by enhanced phosphorylation of the ERK downstream substrate pElk-1. (c) MEK signals in co-immunoprecipitates with pERK1/2 indicated stronger interaction between pERK2 and MEK in ERK1-deficient mice. IP: pERK1/2, immunoprecipitation with anti-pERK-1/2 antibody (mouse monoclonal). The level of total ERK in the samples was probed with a rabbit polyclonal anti ERK-1/2 antibody. Statistically significant differences: $^{*}P<0.01$; $^{**}P<0.001$ *vs* ERK1$^{+/+}$, $^{a}P<0.01$ *vs* naive.

(Figure 1a), ERK1 knockouts exhibited significant reduction of immobility in the FST ($t_{1,18}=24.996$, $P<0.01$), indicating a decrease of depression-like behavior (Figure 4a). A low dose of SL327 that does not affect locomotor activity or FST immobility in wild-type mice (Duman *et al*, 2007) was injected systemically to determine the involvement of ERK2. SL327 reversed the reduced immobility of ERK1 knockouts, but did not modify immobility in wild-type mice (Genotype × Treatment: $F_{2,35}=28.274$, $P<0.01$, Figure 4b) supporting the involvement of ERK2 in the antidepressant phenotype of ERK1$^{-/-}$ mice. The immobility time of vehicle-injected mice was similar to the measures of saline-injected mice indicating that DMSO did not exert sedative or locomotor effects. However, intrahippocampal infusions of U0126 did not reverse this antidepressant phenotype, suggesting an involvement of MEK/ERK signaling from extrahippocampal site(s) (Figure 4c). On the contrary, U0126 further decreased the immobility of both wild-type and ERK1 knockouts (Treatment: $F_{1,16}=28.579$, $P<0.01$; Figure 4c), indicating a depressant role of the hippocampal MEK/ERK pathway. The effects of U0126 were probably not due to changes of locomotion, because i.h. injections of 1 μg/mouse of this inhibitor before exposure to a novel environment did not affect mean activity (vehicle: 11.43 cm/s ± 3.2; U0126: 10.98 cm/s ± 4.3, $t_{18}=1.05$, $P=0.133$; $n=10$ mice/group). Notably, despite these regional differences, the dominant behavioral phenotype was most likely caused by extrahippocampal ERK activity counteracting the action of hippocampal ERK and resulting in a significant reduction of depression-like behavior.

PKC Signaling and Fear Extinction

Because we did not observe PKC-dependent phosphorylation of ERK either in ERK1$^{-/-}$ or ERK1$^{+/+}$ mice, we performed subsequent experiments with C57BL/6J mice to examine the role of PKC in fear extinction with larger groups of mice. The C57BL/6J strain shows faster extinction than the wild-type littermates of ERK1 knockouts originally generated on S129/SvJ background. Therefore, these studies were performed using a different time course for extinction and ERK activation as determined previously in detail (Fischer *et al*, 2007). For molecular experiments, dorsohippocampal preparations were prepared after the fourth extinction trial. At this time point, increased pERK levels accompanying extinction were observed in both C57BL/6J- and ERK1-deficient mice (Figure 1b). Dorsohippocampal injections of the PKC inhibitor NPC15437 significantly accelerated contextual fear extinction (Treatment: $F_{1,18}=4.639$, $P<0.05$, Treatment × Test: $F_{4,72}=0.744$, $P=0.565$; Figure 5a). In order to evaluate whether inhibition of PKC resulted in long-term enhancement of extinction memory, we also tested savings of extinction after reinstatement of fear under drug-free conditions. Both vehicle- and drug-injected mice showed savings of extinction, revealed by faster decline of freezing after a reminder shock ($F_{2,54}=18.136$, $P<0.01$) when compared to mice that were not exposed to extinction tests after fear conditioning (Figure 5a). However, the reduction of freezing was accelerated in the NPC15437 group, indicating enhanced extinction memory (Figure 5a). The effect of the PKC inhibitor was even stronger in the C57BL/6NCrl strain (Treatment: $F_{1,14}=88.212$, $P<0.0001$; Treatment × Test: $F_{4,56}=22.377$, $P<0.0001$) that typically exhibits persistent fear in this paradigm (Figure 5b). Kinase assays revealed that NPC15437 injected *in vivo* blocked baseline PKC activity ($t_{10}=11.23$, $P<0.001$, Figure 5c), whereas ERK activity was not affected ($t_4=0.43$, $P=0.32$, Figure 5d). These findings indicated that PKC suppresses the extinction of fear through an ERK-independent mechanism.

PKC Signaling and Depression-Like Behavior

The possibility that fear extinction and depression may share PKC-mediated mechanisms was investigated in C57BL/6J injected into the dorsal hippocampus with NPC15437. The inhibitor significantly reduced the immobility time in the FST ($t_{18}=8.32$, $P<0.01$, Figure 6b). Under similar conditions, NPC15437 did not affect loco-

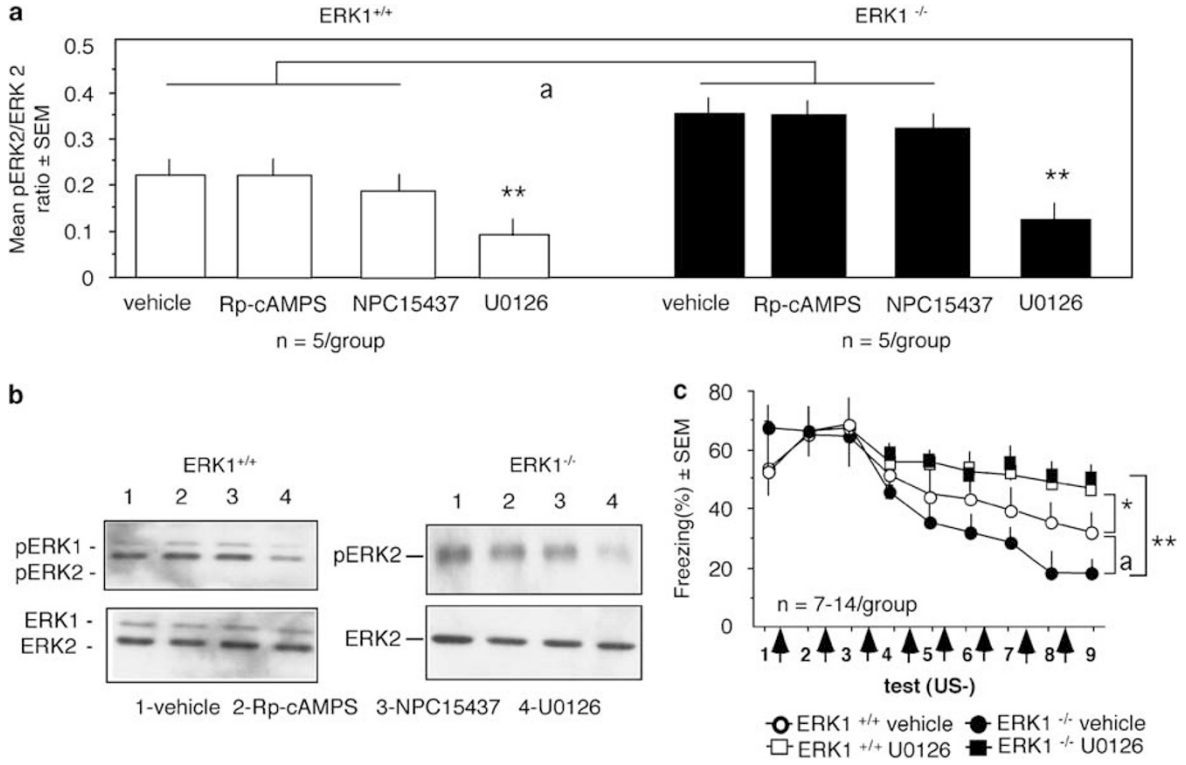

Figure 3 A selective role of the MEK inhibitor on ERK phosphorylation and fear extinction. (a) Rp-cAMPS and NPC15437 did not affect ERK phosphorylation of wild-type and ERK1 knockout mice, whereas U0126 significantly reduced pERK levels. (b) Representative immunoblots obtained from individual dorsohippocampal lysates obtained 1 h after the ninth extinction test/drug injection. (c) Intrahippocampal injection of U0126 completely reversed the extinction phenotype of ERK1 knockouts and prevented extinction of their wild-type littermates. Statistically significant differences: $^{*}P<0.01$, $^{**}P<0.001$ U0126 *vs* corresponding vehicle; $^{a}P<0.001$ *vs* ERK1$^{-/-}$ *vs* ERK1$^{+/+}$ mice. Arrows indicate drug or vehicle injections.

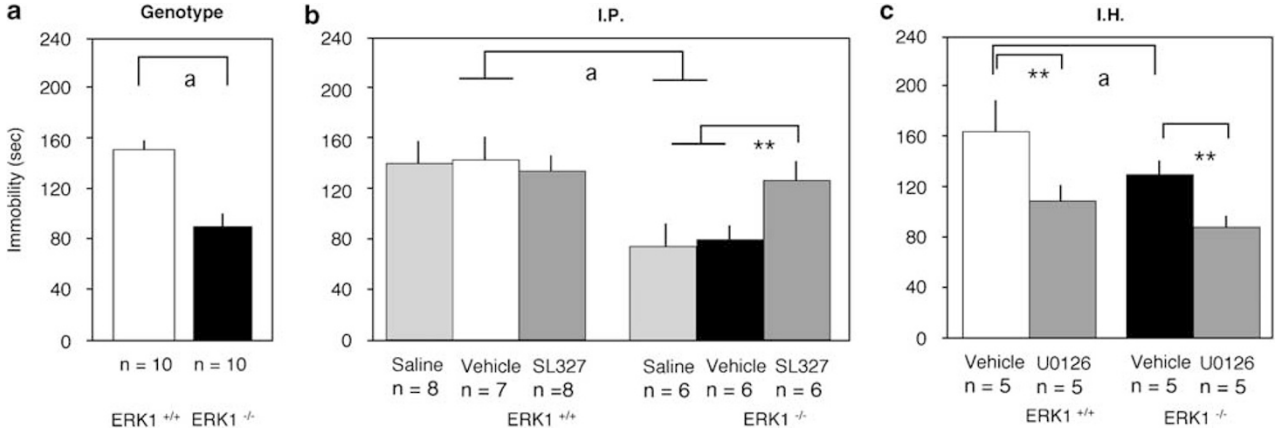

Figure 4 Effects of MEK inhibition on depression-like behavior. (a) ERK1 knockouts showed lower immobility in the FST indicating reduced depression-like behavior. (b) SL327 injected systemically reversed this behavioral phenotype. (c) U0126 injected intrahippocampally significantly reduced the immobility time in the FST in wild-type and ERK1-deficient mice. Statistically significant differences: $^{**}P<0.001$ drug *vs* vehicle; $^{a}P<0.01$ *vs* ERK1$^{+/+}$ mice.

motor activity determined 15 min post-injection (vehicle: 12.55 cm/s ± 3.8; NPC15437: 13.6 cm/s ± 3.1, $t_{16}=1.34$, $P=0.129$; $n=9$ mice/group). To test whether the antidepressant effect depended on ERK phosphorylation, separate groups of mice were injected with NPC15437 or vehicle. Hippocampi were removed either 15 min later, without any exposure to FST (before FST groups) or 20 min later after a 5-min swim session (after FST groups). The levels of pERK were significantly elevated in both vehicle- and drug-injected mice after FST when compared to the before FST group ($F_{3,13}=3.746$, $P<0.05$). NPC15437 did not affect basal or FST-induced pERK levels (Figure 6c and d), suggesting that its antidepressant actions were ERK-independent. Thus, both enhanced extinction (Figure 5a and b) and reduced depression-like behavior (Figure 6b) appeared to be mediated by inhibition of dorsohippocampal PKC via ERK-independent mechanisms.

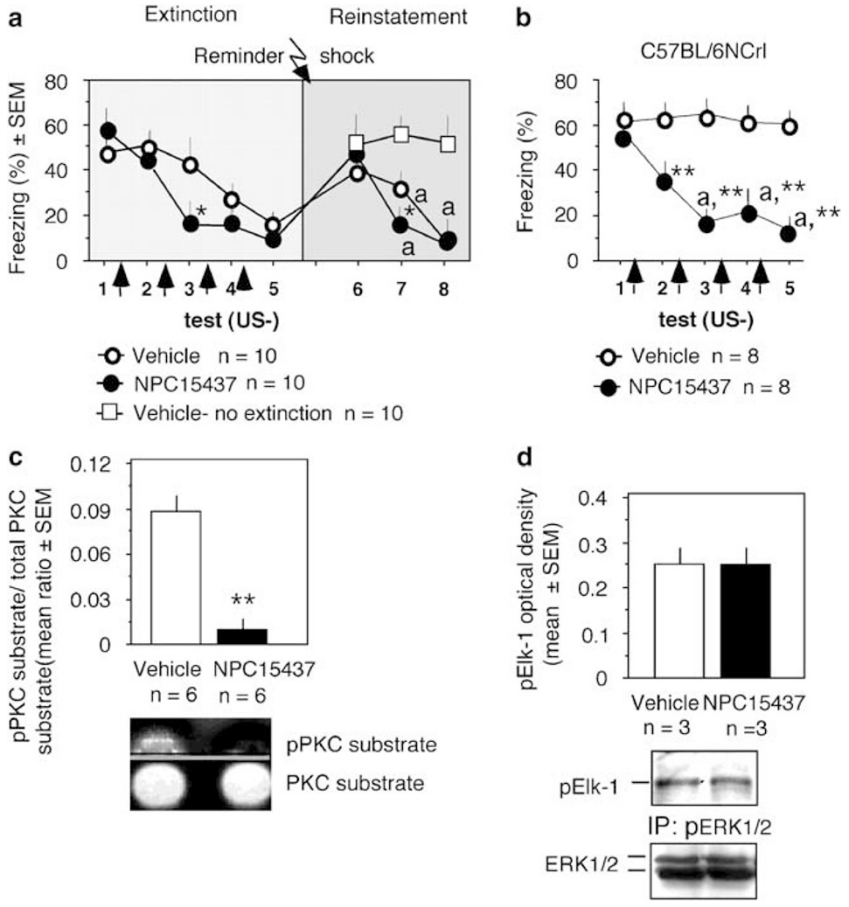

Figure 5 PKC activity and fear extinction. (a) The PKC inhibitor NPC15437 significantly facilitated fear extinction. Following a reminder shock, serving to reinstate conditioned freezing, the drug-treated mice exhibited savings of the extinction memory in the absence of further treatment. (b) NPC15437 exerted even stronger extinction enhancing effect in C57BL/6Crl mice that are resistant to contextual fear extinction. (c) In dorsohippocampal membrane preparations of mice injected with NPC15437, the inhibitor abolished membrane PKC activity. (d) ERK kinase activity in total lysates was not affected, as revealed by similar phosphorylation of pElk-1 by pERK immunoprecipitates prepared from drug- and vehicle-injected mice. Statistically significant differences: $^{*}P<0.01$; $^{**}P<0.001$ drug *vs* vehicle, $^{a}P<0.01$ drug and vehicle *vs* vehicle-no extinction group. Arrows indicate drug or vehicle injections.

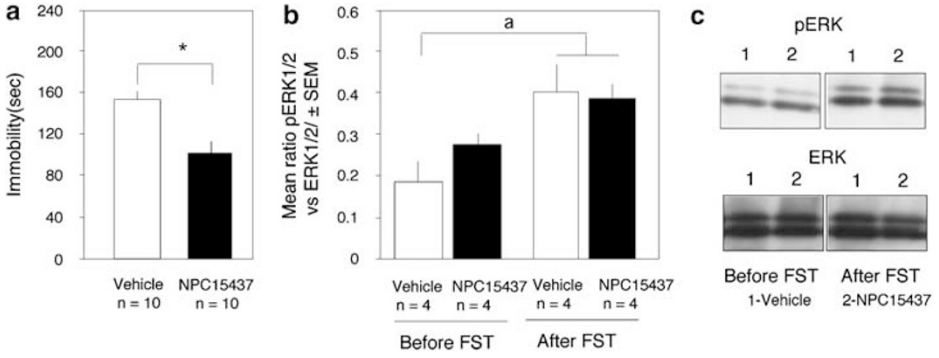

Figure 6 Effects of PKC inhibition on depression-like behavior. (a) Decreased immobility in mice injected intrahippocampally with NPC15437. (b) FST induced elevation of pERK in hippocampal lysates. NPC15437 did not affect either basal or FST-induced pERK levels. (c) Representative immunoblots. Statistically significant differences: $^{*}P<0.001$ drug *vs* vehicle; $^{a}p<0.01$ *vs* vehicle before FST.

PKA Signaling and Fear Extinction

Based on the same rationale as for the PKC studies, this set of experiments was performed with C57BL/6J mice. Acute post-extinction injections of Rp-cAMPS did not affect extinction of contextual fear (Treatment: $F_{1,18}=0.92$, $P=0.23$, Treatment × Test: $F_{4,72}=1.259$, $P=0.85$, Figure 7a) or ERK kinase activity ($t_4=0.75$, $P=0.48$, Figure 7b). To demonstrate that the inhibitor is effective in blocking PKA activity *in vivo*, we performed kinase assays with dorsohippocampal membrane preparations exhibiting readily detectable levels of baseline PKA activity. Infusions of

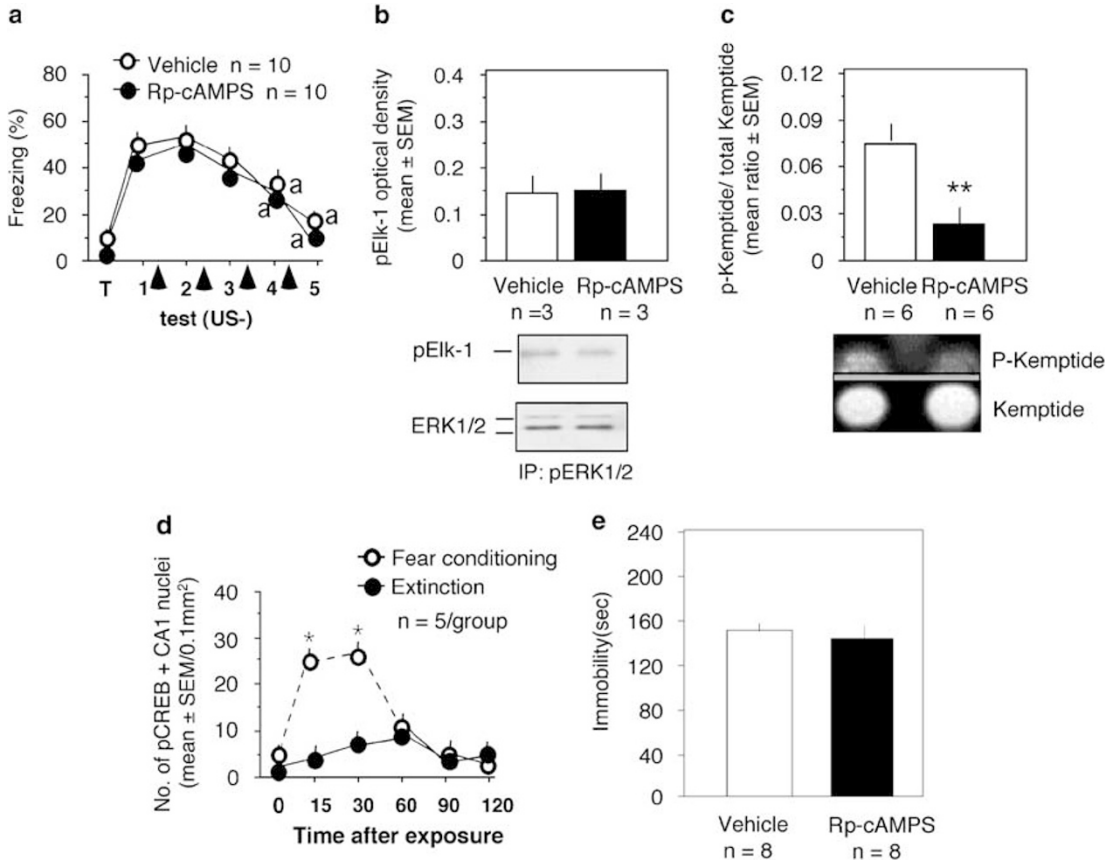

Figure 7 Effects of PKA inhibition on fear extinction and depression-like behavior. (a) The PKA inhibitor Rp-cAMPS did not significantly affect extinction as revealed by similar decrease of freezing in drug- and vehicle-injected groups. (b) In total dorsohippocampal lysates, Rp-cAMPS infused *in vivo* did not affect ERK kinase activity during extinction, as revealed by similar phosphorylation of pElk-1 by pERK immunoprecipitates prepared from drug- and vehicle-injected mice. (c) Hippocampal membrane preparations containing detectable levels of baseline PKA activity revealed reduced phosphorylation of the PKA substrate Kemptide. Thus, Rp-cAMPS was effective after dorsohippocampal injection. (d) The number of pCREB-positive nuclei, indicating inducible PKA activity, did not show a significant increase after extinction as it was observed after fear conditioning. (e) The PKA inhibitor did not affect immobility in the FST. Statistically significant differences: $^{a}P<0.01$ *vs* test 1; $^{**}P<0.001$ *vs* vehicle; $^{*}P<0.01$ fear conditioning *vs* extinction groups. Arrows indicate drug or vehicle injections.

Rp-cAMPS significantly downregulated Kemptide phosphorylation ($t_{10}=7.92$, $P<0.001$) in these preparations (Figure 7c). Thus, Rp-cAMPS blocked PKA *in vivo*. We also attempted to block inducible PKA activity by measuring the effects of Rp-cAMPS on CREB phosphorylation; however, in all samples the levels of pCREB were very low (data not shown). Therefore, we examined whether pCREB is activated at all during fear extinction. For that purpose, we compared the phosphorylation of CREB at different time points after fear conditioning or after the fourth extinction test. Two-factor ANOVA revealed significant group differences ($F_{1,48}=8.22$, $P<0.01$), reflecting an elevation of pCREB during fear conditioning but not fear extinction (Figure 7d). The inability of Rp-cAMPS to affect either ERK phosphorylation or extinction may thus be due to the lack of significant inducible activation of the PKA pathway in the employed extinction paradigm.

PKA Signaling and Depression-Like Behavior

The PKA inhibitor Rp-cAMPS injected i.h. at a dose of 18 μg/mouse did not affect depression-like behavior, as revealed by similar immobility time in the FST ($t_{14}=0.189$, $P=0.6706$; Figure 7e). Thus, although this PKA significantly impairs fear conditioning, revealed by similar pharmacological approaches (Ahi *et al*, 2004), this kinase did not seem to contribute to a significant extent to fear extinction and depression, at least under the conditions employed in this study.

DISCUSSION

In the present manuscript we provide genetic, biochemical, and neuroanatomical evidence identifying overlapping and nonoverlapping mechanisms of fear extinction and depression-like behavior. Using genetic approaches with mice lacking the ERK1 isoform, and pharmacological inhibition of the main upstream ERK activators, we demonstrated that upregulation of MEK/ERK and downregulation of PKC enhanced fear extinction and reduced depression-like behavior. The significance of the balance between MEK/ERK and PKC was demonstrated in different mouse strains and genotypes, suggesting a conserved contribution to fear and mood regulation.

The newly described phenotype of the ERK1 knockout, enhanced fear extinction and decreased immobility in the

FST, suggested that the ERK signaling pathway coregulates these behaviors. Elevated hippocampal ERK2 activity in ERK1$^{-/-}$ mice during extinction of contextual fear, as revealed by its increased phosphorylation, kinase activity, and association with MEK, suggested that ERK2 was the main mediator of the behavioral phenotype of ERK1 knockouts. Given the constitutive nature of ERK-1 deletion and significant cross-talk between signaling pathways (Pouyssegur *et al*, 2002), it cannot be excluded that additional compensatory mechanisms might also contribute to the observed behaviors. This possibility is unlikely, however, as both enhancement of fear extinction and decreased immobility were fully reversed by the specific MEK inhibitors U0126 and SL327, respectively. Notably, these specific inhibitors block only ERK-1/2 phosphorylation by MEK, without affecting p38, JNK, or other MAP kinase pathways (Favata *et al*, 1998).

The extinction phenotype of ERK1 knockouts was attenuated by intrahippocampal inhibition of MEK, supporting the view that the hippocampus may be the predominant site of ERK2 actions (Szapiro *et al*, 2003; Fischer *et al*, 2007). Consistent with earlier reports, the employed manipulations did not affect conditioning and retrieval (Selcher *et al*, 2001) or reconsolidation (Fischer *et al*, 2007; Chen *et al*, 2005; Isiegas *et al*, 2006) of the fear memory, suggesting their specific contribution to extinction learning.

The antidepressant phenotype of ERK1 knockouts was reversed by acute systemic injection of SL327 but not local injections of U0126. Rather, U0126 further decreased the immobility time both in wild-type and in knockout mice. These findings suggested that ERK mediated depressant actions (Manji and Chen, 2002; Fumagalli *et al*, 2005) via the hippocampus and antidepressant actions (Feng *et al*, 2003; Duman *et al*, 2007; Qi *et al*, 2006) via other brain sites. Using acute treatments as employed here, it was recently reported that ERK is also depressant within the amygdala (Huang and Lin, 2006). Taking into consideration that acute and subacute systemic MEK inhibition may produce opposite effects on FST behavior (Duman *et al*, 2007), it remains to be elucidated whether multiple injections or the use of different doses would consistently reveal depressant effects in the hippocampus and amygdala. The prefrontal cortex, on the other hand, is a likely site where ERK mechanisms might be antidepressant (Wellman *et al*, 2007). Independently of regional differences of ERK signaling in mood regulation, the reduced immobility of ERK1 knockouts indicated that the antidepressant actions of ERK2 were dominant for the overall behavioral phenotype.

Alterations of locomotor activity, as observed here and earlier (Selcher *et al*, 2001; Mazzucchelli *et al*, 2002) for the ERK1 knockout, is a potential confound for the evaluation of freezing behavior and immobility (Cryan *et al*, 2005). Although this possibility cannot be completely ruled out, the interference of locomotion in fear extinction is unlikely because ERK1 knockouts exhibited similar freezing behavior to their wild-type littermates over four initial extinction trials despite increased activity. Additionally, post-trial pharmacological inhibition of MEK produced converging evidence for the role of ERK in extinction without altering locomotor activity. To minimize the interference of increased locomotion in FST, and because we and others (Selcher *et al*, 2001) observed that a single, brief (minutes) exposure to a novel situation is insufficient to trigger hyperlocomotion in the ERK1 knockout, we performed the experiments during the first swimming session. Finally, locomotor effects of high doses of SL327 (Einat *et al*, 2003) were avoided by employing a low dose that did not increase locomotion (Duman *et al*, 2007), but instead selectively attenuated the antidepressant phenotype of ERK1 knockouts. Future studies using different behavioral paradigms to investigate extinction and depression-like behavior will be important to further strengthen the relationship between these processes.

Depression-like behavior caused by gene deletion of the serotonin transporter is associated with normal formation but impaired recall of fear extinction memory (Wellman *et al*, 2007). Our findings with the ERK1 knockout demonstrated that learning processes underlying fear extinction might be also coregulated with mood states at the molecular level. Thus, depression may intertwine with anxiety at different stages of cognitive processing (learning/retrieval) depending on the specific underlying molecular mechanism.

Despite their coordinated activation of ERK signaling in hippocampal slices (Roberson *et al*, 1999; Banko *et al*, 2004) and during fear conditioning (Weeber *et al*, 2000; Sananbenesi *et al*, 2003; Ahi *et al*, 2004), neither PKC nor PKA seemed to significantly contribute to ERK activation during fear extinction and FST. The mechanisms for the dissociation of PKC and PKA from the ERK pathway are not clear, but likely involve cytoskeletal rearrangement implicated in protein trafficking (Arimura and Kaibuchi, 2007) and learning processes (Fischer *et al*, 2004). We hypothesize that redistribution of these kinases, as previously observed (Olds *et al*, 1989; Van der Zee *et al*, 1995), could partly account for their lack of interaction.

PKC prevented extinction by ERK-independent mechanisms, as indicated by accelerated fear reduction upon inhibition of PKC, without associated change of ERK activity. Furthermore, intrahippocampal treatment with NPC15437 enhanced savings of extinction after reinstatement of fear, suggesting that PKC may contribute to persistent fear by directly inhibiting extinction processes. The effect of the PKC inhibitor was not restricted to one mouse strain, but was also profound in C57BL/6NCrl mice that typically acquire and generalize strong fear responses (Radulovic *et al*, 1998b) and exhibit resistance to contextual fear extinction (Stiedl *et al*, 1999; Siegmund *et al*, 2005). Together with data showing effects of long-term activation of atypical hippocampal PKC on behavioral performance (Pastalkova *et al*, 2006), our data suggest that typical PKCs might also contribute to persistent fear-motivated behavior.

In agreement with the suggested role of PKC in depression (Manji *et al*, 1993), we observed an antidepressant effect of hippocampal PKC inhibition, as revealed by decreased immobility in the FST following injection of NPC15437. These findings indicated that persistent fear and depression were linked by ERK-independent actions of PKC within the hippocampus (see Figure 8). The identification of specific PKC isoforms and their downstream targets remains an important future direction for the elucidation of PKC-mediated mechanisms. Indeed, given that PKC isoforms are specifically assigned to different effector

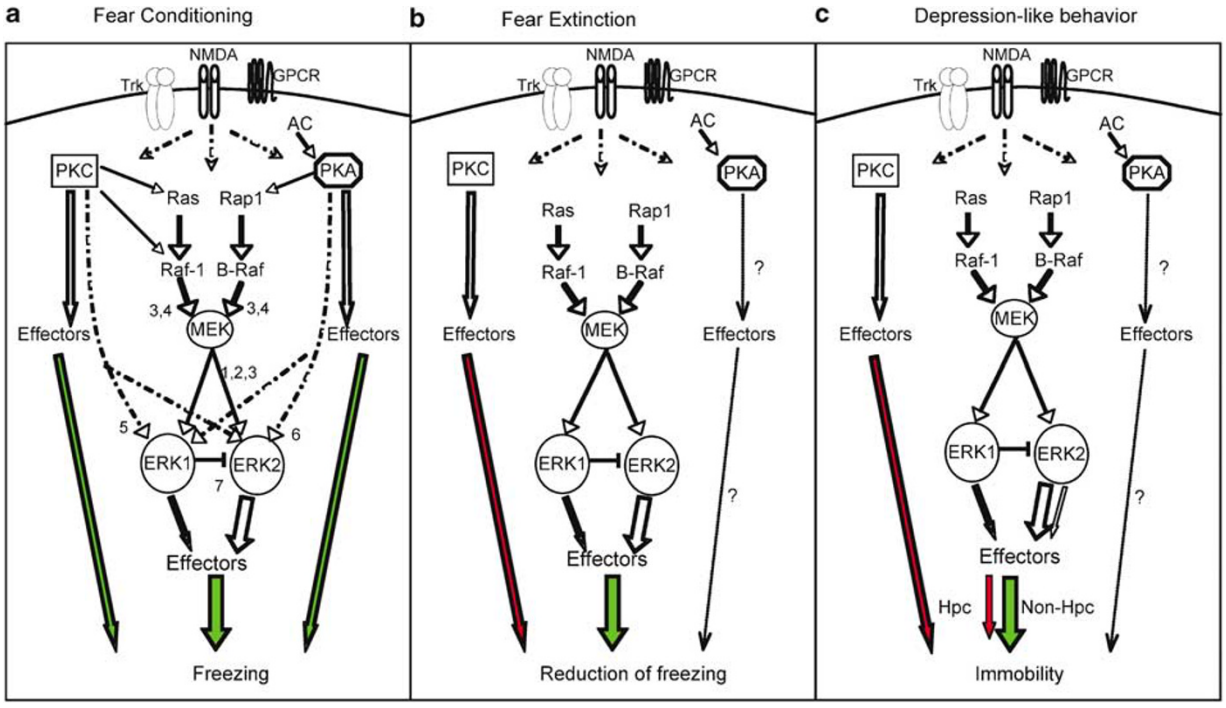

Figure 8 Schematic of ERK signaling during fear conditioning, extinction, and depression-like behaviors. (a) The upstream regulation of ERK by PKA and PKC, in fear conditioning, and (b) the dissociation of PKA and PKC from ERK in fear extinction and (c) in depression-like behaviors. Dashed lines represent MEK-independent activation of ERK. Solid lines represent MEK-dependent ERK activation. Box arrows represent activation of downstream effectors. Green arrows represent an enhancing effect and red arrows an inhibitory effect on outcome, respectively. '?' represents undetermined molecular pathways. Abbreviations: AC, adenylate cyclase; NMDA, *N*-methyl-D-aspartate receptor; Trk, tyrosine receptor kinase; GPCR, G-protein-coupled receptor; HPC, hippocampal. References: (1) Crews and Erikson (1992); (2) Ahi *et al* (2004); (3) Yuan *et al* (2002); (4) Roberson *et al* (1999); (5) Grammer and Blenis (1997); (6) Tajima *et al* (2005); (7) Mazzuchelli *et al* (2002).

mechanisms (Selcher *et al*, 2002), it is likely that diverging downstream targets of PKC and MEK contribute to the different behavioral outcomes in extinction of fear and depression-like behaviors. This dissociation of PKC from the MEK/ERK pathway could enable the formation of extinction memory (MEK/ERK) while simultaneously preserving the conditioning memory (PKC). Another interesting possibility is that these kinases also contribute to the affective load of the conditioning (fear) and extinction (fear relief) memories, thereby linking mood to learning processes.

Contrary to the requirement for multiple injections of MEK and PKC inhibitors for the disruption of fear extinction, these drugs effectively modulated depression-like behavior after a single acute administration. Such effects, also reported in earlier studies (Einat *et al*, 2003; Chen *et al*, 2005; Duman *et al*, 2007; Fischer *et al*, 2007), are probably due to the fact that although mood regulation, as tested by the FST, can be influenced by experience, it does not require prior learning (Petit-Demouliere *et al*, 2005). New learning, on the other hand, is essential for the extinction of fear (Myers and Davis, 2002), which in this paradigm requires multiple trials. Thus, the drug actions are not constrained by their effects on mood, but also by the time at which a specific signaling pathway critically contributes to the formation of an extinction memory.

In these studies, post-session inhibition of PKA did not affect fear extinction, ERK activity, or depression-like behavior. Low hippocampal levels of the PKA substrate pCREB observed after nonreinforced tests suggested that endogenous PKA was not activated to a comparable extent during extinction as during fear conditioning. Our findings are consistent with studies on extinction of tone-dependent fear demonstrating low amygdalar pCREB levels (Lin *et al*, 2003) and lack of Rp-cAMPS effects (Tronson *et al*, 2006). It should be noted, however, that unlike short-term PKA inhibition, long-term upregulation (Wang *et al*, 2004) or downregulation (Isiegas *et al*, 2006) of PKA signaling by genetic manipulations affecting both baseline and inducible PKA activity in several brain areas and over a long period of time might prevent or enhance fear extinction, respectively. Given that the effects of genetic PKA inactivation predominantly affected within session extinction (Isiegas *et al*, 2006), it is also possible that longer extinction trials are required for the induction of the PKA pathway.

It was previously suggested that lack of positive reinforcement could contribute to depression via striatal circuitry (Schulz *et al*, 2004). Our results complement those findings by showing that additional mechanisms, encompassing reduced extinction of fearful behaviors, may also contribute to depression via hippocampal mechanisms. Thus, behavioral despair, typical of increased depression-like behavior, could be associated with either enhanced extinction of positively (Nestler and Carlezon, 2006) or impaired extinction of aversively motivated behavior. Importantly, the same behavioral phenotype can be mediated by different brain circuitries and potentially distinctive molecular mechanisms. Further delineation of these

processes is of utmost importance to adequately define depression phenotypes and design correspondent treatment strategies.

In conclusion, we have shown that PKC-dependent, ERK-independent mechanisms underlying depression-like behavior are shared with fear extinction and distinct from those of fear conditioning. The observed dissociation of PKC and MEK signaling in regulation of ERK phosphorylation is highly novel considering that both kinases have been regarded as major ERK activators (Liebmann, 2001; Berkeley and Levey, 2003). So far, such effects have been only reported for mood-stabilizing agents whose mechanisms of action encompass, among others, downregulation of PKC and upregulation of ERK activity (Williams *et al*, 2002). The similar alteration of these pathways found during contextual fear extinction and depression indicated that the MEK/ERK and PKC balance is physiologically regulated, and that extinction processes may prove beneficial to alleviate depression and *vice versa*. Interference with these pathways may be of high therapeutic potential, considering that specific protein kinase inhibitors bypass the membrane receptors for neurotransmitters, act after acute administration as shown here and elsewhere (Manji and Chen, 2002; Duman *et al*, 2007) and may thus exhibit faster and biochemically more constrained actions in the brain than conventional mood-stabilizing drugs.

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DISCLOSURE/CONFLICT OF INTEREST

We declare no conflict of interest.

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