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Extinction of auditory fear conditioning requires MAPK/ERK activation in the basolateral amygdala

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Abstract

Whereas the neuronal substrates underlying the acquisition of auditory fear conditioning have been widely studied, the substrates and mechanisms mediating the acquisition of fear extinction remain largely elusive. Previous reports indicate that consolidation of fear extinction depends on the mitogen-activated protein kinase/extracellular-signal regulated kinase (MAPK/ERK) signalling pathway and on protein synthesis in the medial prefrontal cortex (mPFC). Based on experiments using the fear-potentiated startle paradigm suggesting a role for neuronal plasticity in the basolateral amygdala (BLA) during fear extinction, we directly addressed whether MAPK/ERK signalling in the basolateral amygdala is necessary for the acquisition of fear extinction using conditioned freezing as a read-out. First, we investigated the regional and temporal pattern of MAPK/ERK activation in the BLA following extinction learning in C57Bl/6J mice. Our results indicate that acquisition of extinction is associated with an increase of phosphorylated MAPK/ERK in the BLA. Moreover, we found that inhibition of the MAPK/ERK signalling pathway by intrabasolateral amygdala infusion of the MEK inhibitor, U0126, completely blocks acquisition of extinction. Thus, our results indicate that the MAPK/ERK signalling pathway is required for extinction of auditory fear conditioning in the BLA, and support a role for neuronal plasticity in the BLA during the acquisition of fear extinction.

Introduction

Extinction of conditioned fear is a form of new learning in which the original memory acquired through the association of a conditioned stimulus (CS) with an unconditioned stimulus (US) is inhibited after repeated exposures to the CS alone (Rescorla, 2001; Myers & Davis, 2002; Maren & Quirk, 2004). Auditory fear conditioning and extinction of conditioned fear differ in that extinction memories are context dependent and labile giving rise to spontaneous recovery of fear memories (Bouton & King, 1983; Quirk, 2002). These dissimilarities between fear and extinction memories are likely to reflect distinct molecular or circuit mechanisms as recently suggested (Berman & Dudai, 2001; Lin *et al*., 2003a; Suzuki *et al*., 2004). Previous results indicate that consolidation of extinction is associated with a potentiation of synaptic efficacy, an increase in glucose metabolism, and in the expression of the immediate-early gene (IEG) c-fos in the medial prefrontal cortex (mPFC; Herry & Garcia, 2002; Milad & Quirk, 2002; Barrett *et al*., 2003; Herry & Mons, 2004). In addition, consolidation of extinction is disrupted by systemic NMDA receptor (NMDAR) blockade (Santini *et al*., 2001), and by inhibition of the mitogen-activated protein kinase/extracellular signal regulated kinase (MAPK/ERK) signalling pathway and inhibition of protein synthesis in the mPFC (Hugues *et al*., 2004; Santini *et al*., 2004). Together these results strongly suggest that the mPFC is critical for consolidation of extinction memory. In contrast, the neuronal mechanisms underlying acquisition of extinction are still unclear. Particular emphasis has been directed toward the basolateral amygdala (BLA) as a key structure for extinction learning, but the use of different learning paradigms has yielded conflicting results. In the fear potentiated startle paradigm, extinction is prevented by intra-BLA injection of the NMDAR antagonist, AP5 (Falls *et al*., 1992). In contrast, acquisition of extinction of auditory fear conditioning is unaffected by systemic injection of the NMDAR antagonist, CPP (Santini *et al*., 2001), but is dramatically impaired by systemic injection of L-type voltage-gated calcium channel (LVGCC) blockers (Cain *et al*., 2002). Because of methodological limitations in the fear potentiated startle paradigm, acquisition and consolidation of extinction can usually not be discriminated from each other. Therefore, it has been suggested that acquisition of extinction (or short-term extinction memory) relies on the activation of LVGCCs (Cain *et al*., 2002; Suzuki *et al*., 2004; Barad, 2005), whereas consolidation of extinction (or long-term extinction memory) depends on NMDAR activation in the mPFC and in the BLA (Santini *et al*., 2001; Cain *et al*., 2002; but see Lee & Kim, 1998). In support of this hypothesis, post-extinction activation of NMDARs using the partial agonist, D-cycloserine, facilitates consolidation of extinction memory in a MAPK/ERK- and protein synthesis-dependent manner (Ledgerwood *et al*., 2003; Yang & Lu, 2005).

Activity-dependent intracellular calcium increases mediated by NMDAR and/or VGCC activation can trigger signalling pathways, such as the MAPK/ERK pathway (Deisseroth *et al*., 1996; Dolmetsch *et al*., 2001; Hardingham *et al*., 2001; Moosmang *et al*., 2005), known to be necessary for the consolidation of fear conditioning and long-term potentiation (LTP) in the BLA (Huang *et al*., 2000; Schafe *et al*., 2000;

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Adams & Sweatt, 2002; Radwanska *et al*., 2002). Interestingly, a recent study shows that extinction of the fear potentiated startle is impaired by intra-BLA infusion of the MAPK inhibitor PD98059 (Lu *et al*., 2001). However, due to the same methodological issues described above, it is not possible to conclude whether this effect reflects an action on short- or long-term extinction memory. Also, it is unknown if extinction of auditory fear conditioning depends on the MAPK signalling pathway.

It has been recently reported that the induction of extinction rapidly stimulates calcineurin activity in the amygdala (Lin *et al*., 2003a, b). This increased activity has been proposed to weaken the original fear memory by inhibiting the conditioning-induced phosphorylation of downstream kinases including Akt and MAPK (Lin *et al*., 2003a).

As the MAPK/ERK signalling pathway has been implicated in consolidation of auditory and contextual fear conditioning (Schafe *et al*., 2000; Trifilieff *et al*., 2006), it is of particular interest to examine whether MAPK/ERK signalling in the BLA is required during extinction of conditioned fear. To investigate these issues, we first analysed by immunohistochemistry the temporal activation of MAPK/ERK in the BLA of mice following acquisition of extinction. Finally, we directly tested if inhibition of the MAPK/ERK pathway before extinction training by intra-BLA infusion of the MAPK kinase (MEK) inhibitor, U0126, prevents acquisition of extinction of auditory fear conditioning.

Materials and methods

Subjects

Adult male C57Bl/6J mice were purchased from IFFA Credo (Lyon, France). The animals were individually housed in plastic cages with *ad libitum* access to food and water with a 12-h light : 12-h dark cycle. All studies took place during the light portion of the cycle. Mice were handled gently for 2–3 min/day during 5 days, to minimize nonspecific stress. Animal care and behavioural and electrophysiological tests were conducted in accordance with the standard ethical guidelines (European Communities Guidelines on the Care and Use of Laboratory Animals: 86/609/EEC) and approved by the Department of Verterinary Services, Gironde.

Surgery

Mice were deeply anaesthetized using isoflurane (induction 5%, surgery 2.5%) in O_2. Mice were secured in a stereotaxic frame and bilaterally implanted with a 24-gauge stainless steel guide cannula (8-mm long) aimed at the BLA (according to Franklin & Paxinos (1997); 1.7 mm posterior to bregma; ±3.1 mm lateral to midline and 4–4.3 mm deep from the cortical surface). A 32-gauge dummy cannula was inserted into the guide cannula to prevent clogging. The guide cannula was fixed in place onto the skull with dental cement. Mice were then allowed to recover for one week in their home cage before the start of the experiment.

Drug infusion

The MEK inhibitor, U0126, (Cellsignal, Beverly, ME) was dissolved in 100% DMSO and stored at −20 °C. Animals were given bilateral intra-BLA injections of 0.3 μL per site 2% DMSO in artificial cerebrospinal fluid (aCSF, glucose, 5 mM; $CaCl_2$, 1 mM, NaCl, 125 mM, $MgCl_2$, 1 mM, $NaHCO_3$, 27 mM, KCl, 0.5 mM, Na_2SO_4, 0.5 mM, NaH_2PO_4, 0.5 mM, Na_2HPO_4, 1.2 mM), or 0.6 μg U0126 (2 μg/μL) in 2% DMSO in aCSF. Injections were performed in freely moving mice at a constant rate of 0.4 μL/min.

Behavioural procedures

Apparatus

Conditioning took place in a chamber (chamber A) consisting of a grey plastic cylinder (15.5 cm diameter × 14 cm high) with a shock grid floor made of stainless steel rods placed in a square transparent Plexiglas box (18 cm side × 23 cm high). The whole system was placed inside a sound-attenuating and temperature-regulated Plexiglas cubicle. A speaker was positioned on the top of the square transparent box to generate a tone (2.5 kHz, 80 dB). The shock grid was connected to a current generator and scrambler to provide a 1 s, 0.9 mA foot-shock. The conditioning box and the floor were cleaned with 70% ethanol before and after each session. During extinction training, the grey plastic cylinder was removed and a grey plastic floor replaced the grid floor. This context (chamber B) was washed with 1% acetic acid before and after each session. To maximize discrimination between the two contexts, light intensity was reduced during fear conditioning. The behaviour of each mouse was monitored and videotaped during all phases of the experiment. To score freezing behaviour we used two parallel measurements. First, we performed a classical time-sampling procedure during which an experimenter blind to the experimental conditions determined the mice to be freezing or not freezing every 2 s. Second, we compared these values to those obtained by an automatic infrared beam detection system (Immetronic, Pessac, France) placed on the bottom of the experimental chambers. The two values were 95% identical and were averaged for statistical analyses.

Fear conditioning and extinction training

On training day, mice were conditioned to acquire fear in response to a 30 s tone CS paired with a 1-s foot-shock US (five CS–US pairings; intertrial interval 20–180 s). The onset of the US coincided with the offset of the CS. For immunohistochemical experiments, fear conditioned mice (CS–US group) were divided into five groups (Fig. 1A). Mice of the Early-Ext group ($n = 8$) were submitted to the first session of extinction (Early Extinction) 5 h after conditioning, and killed for immunohistochemistry 1 h after the end of Early Extinction. The second group of mice (Late-Ext, $n = 24$) was submitted to an additional session of extinction 24 h later (Late Extinction), and killed at three different time-points (15 min, 1 and 6 h) after the end of Late Extinction. The next two conditioned groups were used as control groups and were either killed 5 h after fear conditioning just before Early Extinction (No Early-Ext, $n = 10$), or submitted to Early Extinction and then killed 24 h later just before Late Extinction (No Late-Ext, $n = 5$). To control for a possible contextual effect on MAPK/ERK phosphorylation, we used an additional conditioned group that was submitted to the extinction context alone during both extinction sessions (Ctx, $n = 4$). This group was killed 1 h following Late Extinction. To control for any effects of unconditioned fear on the level of MAPK/ERK phosphorylation, we used nonconditioned mice that were submitted to presentations of the CS alone during the conditioning and extinction session (CS group, $n = 4$), and killed 1 h after Late Extinction. Finally, mice neither submitted to fear conditioning nor extinction (Naïve, $n = 9$) were killed independently of our experimental schedule.

For pharmacological experiments, conditioned mice received bilateral intra-BLA injection of either the MEK inhibitor, U0126 (U0126 group; $n = 16$) or DMSO (DMSO group; $n = 15$) 10 min before Early or Late Extinction. Twenty four hours after Late Extinction, we evaluated the long-term effect of U0126 on fear expression by submitting injected mice to a final test consisting of four CS presentations. For all experiments, extinction sessions were conducted with 20 CS-alone presentations (intertrial interval 20–180 s).

A

Group	Conditioning	5 hr →	Early Extinction	24 hr →	Late Extinction	Perfusion delays
1/ Early-Ext	5 tone-shocks		20 tones		–	1 hr post-Early Extinction
3/ No Early-Ext	5 tone-shocks		–		–	5 hr post-Conditioning
2/ Late-Ext	5 tone-shocks		20 tones		20 tones	15 min, 1 & 6 hr post-Late Extinction
3/ No Late-Ext	5 tone-shocks		20 tones		–	24 hr post-Early Extinction
4/ Ctx	5 tone-shocks		Context alone		Context alone	1 hr post-Late Extinction
5/ Cs	5 tones		20 tones		20 tones	1 hr post-Late Extinction
6/ Naïve	–		–		–	

B

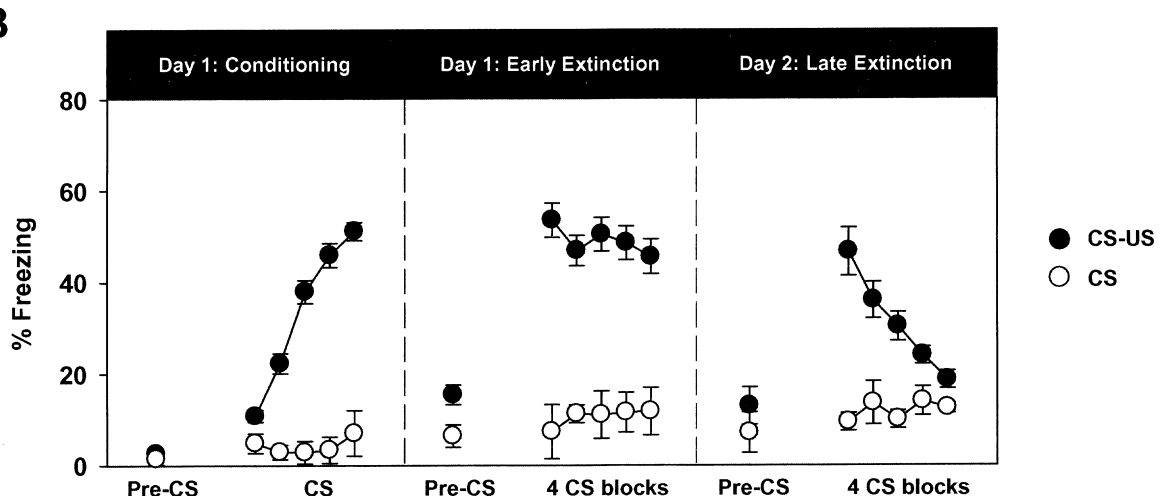

FIG. 1. Experimental protocol and behaviour of mice submitted to auditory fear conditioning and extinction. (A) During auditory fear conditioning, mice were submitted either to five tone (CS)-shock (US) presentations (Early-Ext, No Early-Ext, Late-Ext, No Late-Ext and Ctx groups) or to five tone alone presentations (Cs group) or served as naïve controls (Naïve group). Mice from the No Early-Ext group were perfused 5 h after fear conditioning whereas mice from the Early-Ext, and Ctx groups were perfused 1 h after Early Extinction. The No Late–Ext group was perfused 24 h after the Early Extinction, whereas the Late–Ext group was killed at three-time points following Late Extinction (15 min, 1 h and 6 h). The CS group was killed for immunocytochemistry 1 h following Late Extinction. The remaining Naïve group was killed independently of the experimental schedule. (B) Percentage of freezing before (Pre–CS) and during (CS and CS blocks) presentation of the CS for conditioned (CS–US) and control (CS) mice subjected to auditory fear conditioning (Day 1, Conditioning), Early- (Day 1, Early Extinction) and Late- (Day 2, Late Extinction) extinction sessions. All results are presented as means (± SEM).

Histology

On completion of the pharmacological experiment, mice were given an overdose of avertine (15 mL/kg) and transcardially perfused with 4% paraformaldehyde in 0.1 M phosphate buffer (PB). Brains were removed and postfixed overnight, sectioned (50 μm) on a vibratome (Leica), and mounted on gelatin-coated slides for thionine staining in order to verify cannulae placements.

Immunohistochemistry

At the appropriate time interval (Fig. 1A), mice submitted to immunohistochemical experiments were deeply anaesthetized with avertin (10 mL/kg, i.p.) and perfused transcardially with ice-cold 4% paraformaldehyde in 0.1 M phosphate buffer (PB). Brains were removed and postfixed overnight, sectioned (50 μm) on a vibratome (Leica) and then kept in a solution containing 30% ethylene glycol, 30% glycerol, 0.1 M PB at −20 °C until processed for immunohistochemistry. All solutions contained the phosphatase inhibitor sodium fluoride (NaF; 0.25 mM). Free-floating sections were rinsed in 0.1 M Tris buffer saline (TBS, pH 7.4), incubated with 0.5% H_2O_2 in TBS to inhibit endogenous peroxidase. After blocking in TBS containing 3% bovine serum albumin (BSA), 2% normal goat serum and 0.2% Triton -100, tissue sections were incubated at 4 °C for 48 h with primary polyclonal antibodies against the phosphorylated (anti-phospho-Thr202-Tyr204 ERK1/2, rabbit polyclonal antibody, 1 : 3000, Cell signalling, Beverly, ME) and total (nonphosphorylated and phosphorylated forms, goat polyclonal ERK1/2 antibody, 1 : 2000, Santa Cruz Biotechnology) fractions of ERK1 and ERK2. After extensive washes in TBS, sections were incubated in biotinylated goat anti-rabbit or donkey anti-goat IgG (1 : 2000; Jackson Immunoresearch) for 2 h at room temperature. Sections were again rinsed in TBS followed by

incubation in avidin-biotinylated horseradish peroxidase complex (Vectastain Elite kit, Vector Laboratories, Burlingame, CA) for 2 h at room temperature. Sections were rinsed in TBS and then in TB and the peroxidase reaction end product was visualized in 0.05 M TB (pH 7.6) containing diaminobenzidine tetrahydrochloride (DAB, 0.025%), 0.03% H_20_2, and 5% nickel ammonium sulphate for 10 min (Shu *et al.*, 1988). Finally, immunolabelled sections were washed in PB, mounted on gelatin-coated slides, dehydrated and coverslipped.

Data analysis

The quantification of phospho-MAPK/ERK immunoreactivity (pMAPK/ERK-IR) or total-MAPK/ERK immunoreactivity (tMAPK/ERK-IR) was carried out at ×20 magnification using at least three coronal serial sections (the rostro-caudal distance between consecutive sections was 0.2 mm). The number of positive cells was quantified using a computerized image analysis system (Biocom Visiolab 2000, V4.50) in the following areas of interest [according to Franklin & Paxinos (1997); – signifies posterior to Bregma]: BLA (lateral and basal nuclei of the amygdala), Bregma −1.3 to −1.9 mm. In each mouse, pMAPK/ERK-IR and tMAPK/ERK-IR was quantified within predefined boundaries delineating the BLA. Results were then expressed as the number of positive cells per mm^2. At all stages, the experimenter was blind to the experimental groups. pMAPK/ERK-IR labelling was restricted to the nucleus with no obvious cytoplasmic changes. In contrast, tMAPK/ERK-IR was located within the cytoplasmic and the somatic compartments. Statistical analysis of behavioural and immunocytochemical studies were performed by repeated measures ANOVA followed by Scheffé's *F*-tests for *posthoc* comparisons at the $P < 0.05$ level of significance. The results are presented as mean ± SEM.

Results

Extinction learning induces MAPK/ERK phosphorylation in the basolateral amygdala

CS and CS–US mice displayed low freezing levels and did not differ from each other during the pre-conditioning period ($P > 0.05$; Day 1, Pre–CS, Fig. 1B). Freezing levels remained low in the CS group during the entire session ($P > 0.05$), whereas mice in the CS–US group displayed a progressive increase of freezing behaviour as a function of CS–US presentations (Fig. 1B, Day 1 Conditioning, from 10 to 51% freezing). A two-factor repeated measures ANOVA performed on these data (group × trial) yielded a significant effect of group ($F_{1.53} = 38.751$, $P < 0.001$), trial ($F_{1.4} = 7.047$, $P < 0.001$) and interaction between group and trial ($F_{4.212} = 6.282$, $P < 0.001$). A one-factor ANOVA with repeated measures performed on the data from the CS–US group indicated a significant increase of freezing levels over fear conditioning trials ($F_{4.50} = 87.093$, $P < 0.001$). A direct comparison between CS and CS–US mice confirmed these results as both groups differs from each other from the second to the last trial of fear conditioning (all $P < 0.05$).

Five hours later, when submitted to Early Extinction mice did not display contextual fear generalization from the conditioning to the extinction context as revealed by low levels of fear during the pre-extinction period in both groups (Fig. 1B, Day 1 Early Ext, Pre–CS). In contrast, CS-alone presentations induced high level of freezing in the CS–US but not in the CS group during the entire session. A two-way ANOVA (group × blocks of extinction) performed between CS and CS–US mice revealed a significant effect of group ($F_{1.44} = 13.850$, $P < 0.001$), but not block nor interaction between group and block (all Ps > 0.05). The next day, the Late Extinction session induced a rapid decrease of the freezing response in mice of the CS–US group (Fig. 1B, Day 2 Late–Ext). A two-factor repeated measures ANOVA performed on these data (group × blocks of extinction) yielded a significant effect of group ($F_{1.26} = 11.229$, $P < 0.01$) but not block nor interaction between group and block (all Ps > 0.05). A one-factor ANOVA with repeated measures performed on these data from the CS–US group indicated a significant decrease of freezing levels over extinction trials ($F_{4.23} = 12.149$, $P < 0.001$). This was confirmed by direct comparisons showing no differences between the two groups during the last block of CS-alone presentations (Fig. 1B, Day 2 Late–Ext, Last block, CS–US vs. CS; $F_{1.26} = 1.526$, $P =$ ns). These results indicate that our protocol promotes a complete extinction of conditional fear responses at the end of the Late Extinction session.

Levels of pMAPK/ERK-IR measured following Early Extinction were not significantly different between Early-Ext and No Early-Ext control groups (Fig. 2A; $P > 0.05$). In contrast, 60 min following Late Extinction, pMAPK/ERK-IR levels in the BLA of the Late-Ext group were significantly different from those of the No Late-Ext control group (Fig. 2B, $F_{1.11} = 14.028$, $P < 0.01$). To assess the specificity of the extinction-induced pMAPK/ERK-IR in the BLA, we directly compared the values obtained in the Early Ext group to those of the Late-Ext group. Our analysis revealed a significant increase in pMAPK/ERK-IR levels for the Late-Ext group ($F_{1.14} = 9.513$, $P < 0.01$) indicating that acquisition of extinction during Late-Extinction session was associated with an increase of pMAPK/ERK-IR in the BLA. However, an unspecific tone- or context-induced activation of MAPK/ERK could also explain our results. To rule out these possibilities, we compare the levels of pMAPK/ERK-IR obtained in the Late-Ext group 60 min after Late Extinction to those of conditioned mice submitted to the context alone during extinction training (Ctx control), or unconditioned mice submitted to tone presentation (CS control), or naïve mice (Naïve; Fig. 2B). Our analysis indicated that pMAPK/ERK-IR levels for the Late–Ext group were significantly different from those of the Ctx, Cs and Naïve control groups (all Ps < 0.01). To control for the specificity of the increase in pMAPK/ERK-IR levels observed in the Late-Ext group we performed immunohistochemical analysis of the total MAPK/ERK (tMAPK/ERK, phosphorylated and nonphosphorylated forms of ERK1 and ERK2) in a subset of mice from the Late-Ext and the No Late-Ext groups (Fig. 2C). Our results indicate that tMAPK/ERK was not significantly different between No Late-Ext and Late-Ext groups. To examine further the kinetic of MAPK activation following Late-Extinction we compared pMAPK/ERK-IR levels obtained in mice killed 15, 60 and 360 min in the Late-Ext group to those of the nonextinguished control group (No Late-Ext group) (Fig. 2D). A one-way ANOVA performed on these data revealed a significant increase in pMAPK/ERK-IR within the BLA ($F_{3.20} = 5.275$, $P < 0.01$). Interestingly, direct comparison revealed a significant activation of pMAPK/ERK-IR at each time point examined after Late Extinction (all Ps < 0.05) with a maximum observed 60 min after Late Extinction. The three-fold increase in pMAPK/ERK-IR 15 min after Late-Extinction in the conditioned group suggests that MAPK/ERK activation was already initiated during Late Extinction session. Figure 2E shows representative images of pMAPK/ERK-IR within the BLA of Late- and No Late-Ext groups of mice.

Extinction learning requires MAPK/ERK activation in the BLA

Eight out of 31 animals were excluded from the statistical analysis due to misplaced injection cannulae. The placement of the cannulae for the remaining animals is shown in Fig. 3A. To address the question

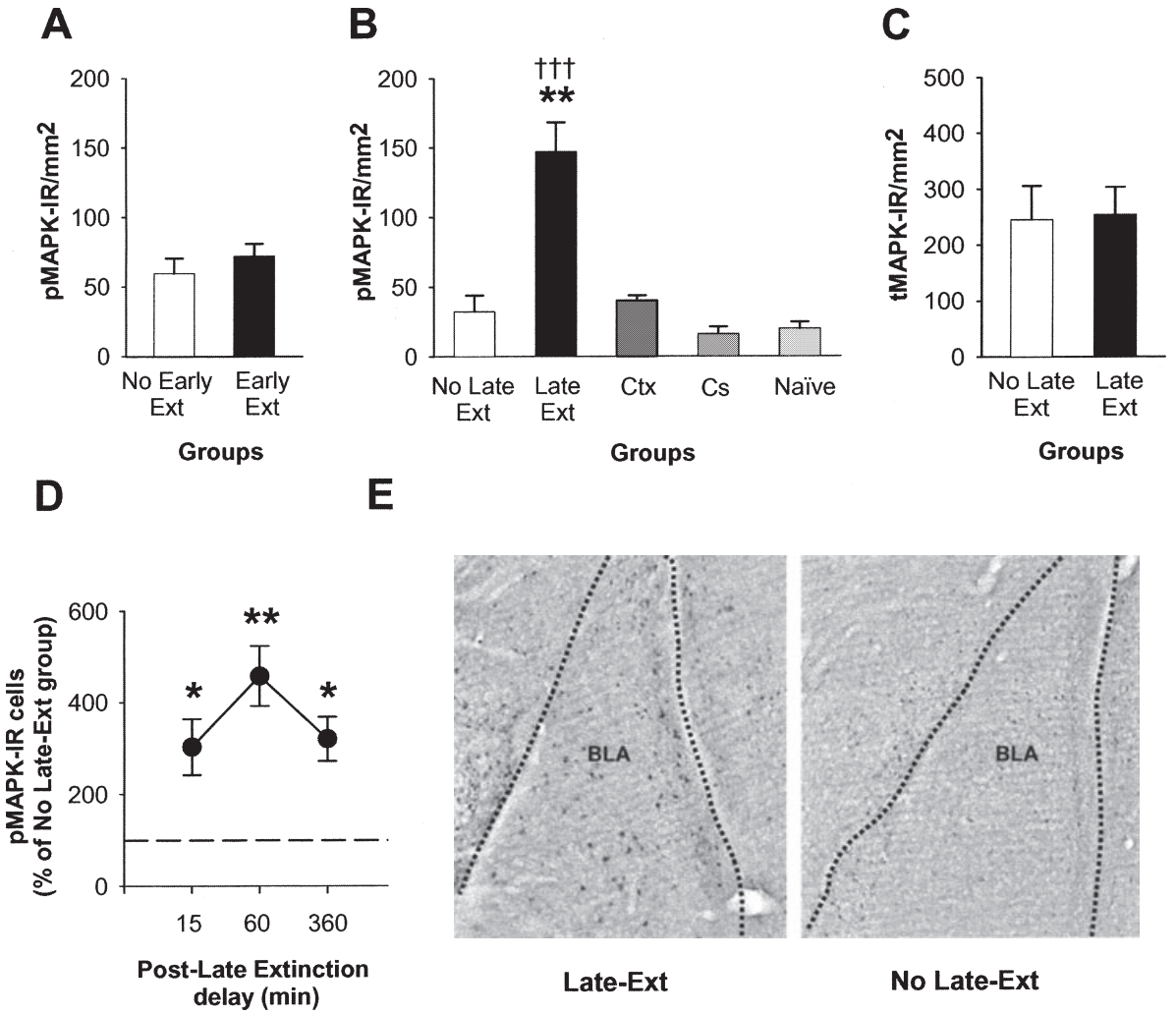

FIG. 2. Activation pattern of MAPK/ERK in the amygdala following acquisition of extinction. (A) Number of positive phosphorylated MAPK/ERK (pMAPK/ERK) cells per mm^2 in the No Early-Ext and Early-Ext groups following Early Extinction session in the BLA. (B) Number of positive pMAPK/ERK cells per mm^2 in the BLA of mice submitted to Late Extinction session [Late–Ext (60 min delay), Ctx, Cs and Naïve groups] and in non extinguished control mice (No Late-Ext). (C) Number of positive cells per mm^2 in the BLA for the total MAPK/ERK (tMAPK/ERK) in the No Late-Ext and Late-Ext groups following Late Extinction. (D) Number of positive pMAPK/ERK cells per mm^2 in the Late-Ext group expressed as a percentage of No Late-Ext control group at 15, 60 or 360 min following Late Extinction session in the BLA. (E) Representative photomicrographs showing the distribution of pMAPK/ERK positive cells in the BLA of extinguished (Late-Ext, 60-min delay) and non-extinguished (No Late-Ext) mice. Late-Ext vs. No Late-Ext, Ctx and Cs: $^{**}P < 0.01$; $^{*}P < 0.05$. Late-Ext vs. Naïve: $^{\dagger\dagger\dagger}P < 0.001$.

whether MAPK/ERK signalling is required for acquisition of extinction, the MEK inhibitor, U0126, was injected bilaterally into the BLA in one group of mice 10 min before Early (Fig. 3B, U0126 group) or Late Extinction (Fig. 3C, U0126 group). The second group was injected with DMSO serving as a control group (Fig. 3B and C, DMSO group). Mice submitted to fear conditioning and injected before Early Extinction displayed low freezing levels and did not differ from each other during the pre-conditioning period (Fig. 3B, Day 1, Pre–CS, $P > 0.05$). Following the first CS–US association, mice of both groups (U0126 and DMSO control groups) displayed a progressive increase in freezing behaviour. A two-factor repeated measures ANOVA performed on these data (group × trial) yielded a significant effect of trial (Fig. 3B, Day 1 $F_{1.4} = 26.782$, $P < 0.001$) but not group nor interaction between group and trial (all Ps > 0.05). Direct comparisons revealed no significant differences in the freezing level between U0126 and DMSO groups through the whole conditioning session (all Ps > 0.05). The injection of U0126 prior to Early Extinction did not induce notable changes in freezing level during Early, Late Extinction or during the post-extinction test in comparison to the DMSO control group (all Ps > 0.05). In addition, both groups reached the baseline freezing level at the end of Late Extinction (Pre–CS vs. last block of CS, both groups Ps > 0.05) indicating that extinction of conditioned fear was complete after Late Extinction.

Mice from the U0126 and DMSO groups submitted to fear conditioning and injected before Late Extinction did not differ from each other during the pre-conditioning period (Fig. 3C, Day 1, Pre–CS, $P > 0.05$), or during the conditioning session characterized by a progressive increase in freezing levels. A two-factor repeated measures ANOVA performed on these data (group × trial) yielded a significant effect of trial (Fig. 3C, Day 1 $F_{1.4} = 15.445$, $P < 0.001$) but not group nor interaction between group and trial (all Ps > 0.05). Direct comparisons revealed no significant differences in the freezing level between U0126 and DMSO groups through the whole conditioning session (all Ps > 0.05). Five hours later, when submitted to Early Extinction, injected mice did not display contextual fear generalization (Fig. 3C, Day 1 Early Extinction, Pre–CS). During

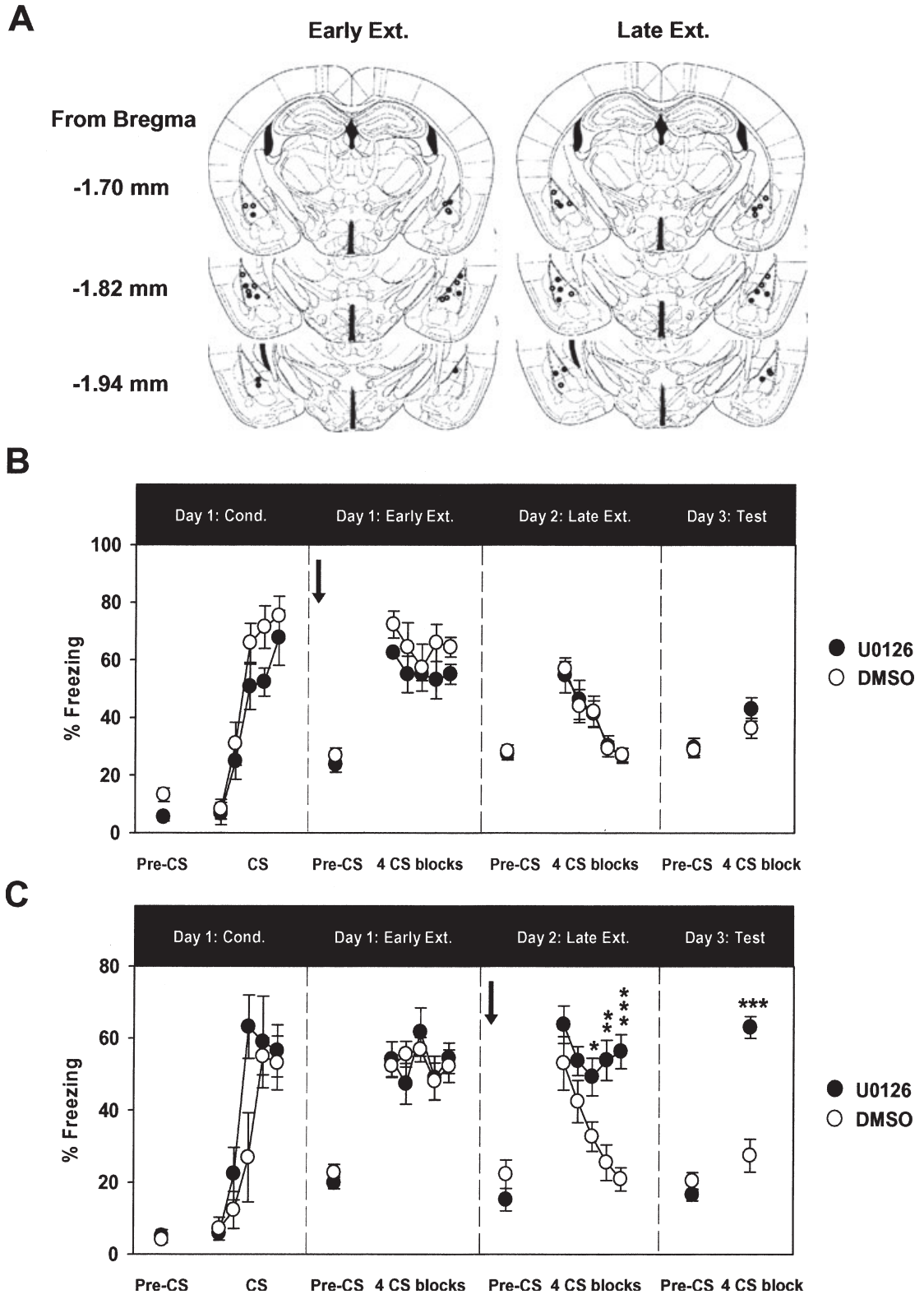

FIG. 3. Intra-BLA infusion of the MEK inhibitor, U0126, blocks acquisition of fear extinction. (A) Schematic diagram showing the location of cannulae tips in the BLA of mice injected with U0126 (filled circle) or DMSO (open circle) 10 min before Early (Early Ext.) or Late Extinction (Late Ext.) session. (B) Percentage of freezing before (Pre-CS) and during (CS and CS blocks) presentations of the CS for U0126 and DMSO groups of mice subjected to auditory fear conditioning (Day 1, Cond.), Early (Day 1, Early Ext.) and Late (Day 2, Late Ext.) Extinction, and recall test (Day 3, Test). Black arrow indicates the time-point of injection (10 min before Early Extinction). (C) Percentage of freezing before (Pre-CS) and during (CS and CS blocks) presentations of the CS for U0126 and DMSO groups of mice subjected to auditory fear conditioning (Day 1, Cond.), Early (Day 1, Early Ext.) and Late (Day 2, Late Ext.) Extinction sessions, and recall test (Day 3, Test). Black arrow indicates the time-point of injection (10 min before Late Extinction). All results are presented as means (± SEM). U0126 vs. DMSO group ***$P < 0.001$; **$P < 0.01$; *$P < 0.05$.

this session, CS-alone presentations induced high level of freezing in both groups. A two-way ANOVA (group × blocks of extinction) performed on these data revealed a significant effect of block ($F_{1.4} = 3.428$, $P < 0.01$), but not group nor interaction between groups and block (all Ps > 0.05). This later result indicates that U0126 and DMSO groups did not differ from each other in freezing level during Early Extinction. When put back in the extinction context 24 h later, mice did not display any fear generalization (Fig. 3B, Day 2 Late Extinction, Pre–CS). During the first block of CS-alone presentations, both groups of mice expressed high level of freezing responses and did not significantly differ from each other ($P > 0.05$). A two-factor repeated measures ANOVA performed on the data from Early Extinction (group × blocks of extinction) indicated a significant effect of group ($F_{1.10} = 18.827$, $P < 0.01$) and block ($F_{1.4} = 5.282$, $P < 0.01$), but not interaction between group and block ($P > 0.05$). Importantly, a one-factor ANOVA with repeated measures performed on these data indicated a significant decrease of freezing levels over extinction trials for the DMSO group only ($F_{4.20} = 8.234$, $P < 0.001$). A direct comparison between groups on each block of extinction confirmed these results as U0126 and DMSO groups differed significantly from the third to the last block of Late Extinction (all Ps < 0.05). To avoid confounding effects between acquisition and expression mechanisms, injected mice were submitted to a final test session 24 h after Late Extinction in the absence of U0126 (Fig. 3C, Day 3 Test). Whereas no significant differences were observed between the two groups during the pre-test period (Fig. 3C, Day 3 Test, Pre–CS), presentations of the CS induced a large and significantly different fear response in the U0126 group in comparison to the control DMSO group ($F_{1.10} = 34.656$, $P < 0.001$). All together these results indicate that inhibition of MAPK/ERK signalling in the BLA before Late but not Early Extinction prevents the acquisition of extinction.

Discussion

The two main findings of this study are that (i) extinction of auditory fear conditioning induces an increase in MAPK/ERK phosphorylation in the BLA, and (ii) that inhibition of the MAPK/ERK signalling pathway in the BLA prevents the acquisition of extinction memory. Thus, our results demonstrate that activation of the MAPK/ERK signalling pathway in the BLA is required for extinction of auditory fear conditioning, as previously shown for extinction of the fear potentiated startle (Lu *et al*., 2001). In addition, this study reveals that MAPK/ERK signalling in the BLA is also necessary for the acquisition of fear extinction.

Bilateral intra-BLA infusion of the MEK inhibitor, U0126, before Late but not Early Extinction prevented the acquisition of extinction. Whereas control animals exhibited a progressive decrease in freezing behaviour during repeated nonreinforced presentation of the CS, the same protocol was completely ineffective in animals injected with U0126 before Late Extinction. This effect cannot be attributed to a deficit in the expression of extinction only, because when animals were tested 24 h later in a drug free state, U0126 injected mice were unable to recall fear extinction. This indicates that inhibition of the MAPK/ERK signalling pathway is necessary for the acquisition of extinction memory. A similar deficit in the acquisition of extinction is induced by LVGCC blockers (Cain *et al*., 2002; Suzuki *et al*., 2004), but not by NMDAR antagonists (Santini *et al*., 2001; Suzuki *et al*., 2004). Indeed, recent studies indicate that LVGCC-mediated calcium entry underlies NMDAR-independent activation of MAPK and synaptic plasticity in hippocampal neurons (Thomas & Huganir, 2004; Moosmang *et al*., 2005). Thus, LVGCC-mediated activation of the MAPK/ERK signalling pathway in the BLA may underlie acquisition of fear extinction.

What might be the mechanism(s) by which MAPK/ERK activation contributes to the acquisition of fear extinction? Initially, MAPK/ERK activation might act directly on processes underlying synaptic plasticity and dendritic integration. While studies in the amygdala are largely lacking, experiments carried out in the hippocampus indicate that MAPK/ERK-dependent phosphorylation regulates synaptic AMPAR trafficking (Zhu *et al*., 2002), and activation of dendritic voltage-gated potassium channels (Yuan *et al*., 2002; Morozov *et al*., 2003). Later stages of acquisition of extinction are protein synthesis-dependent (Berman & Dudai, 2001; Lin *et al*., 2003c; Suzuki *et al*., 2004). Consistent with a role for MAPK/ERK-mediated control of local protein synthesis during the acquisition of fear extinction, recent studies implicate the MAPK/ERK pathway in controlling rapid local translation during the stabilization of newly formed hippocampal memories (Kelleher *et al*., 2004; Thomas & Huganir, 2004). Importantly, the extinction induced up-regulation of the protein phosphatase calcineurin in the BLA has been shown to depend on *de novo* protein synthesis (Lin *et al*., 2003c), and inhibition of calcineurin interferes with extinction (Lin *et al*., 2003b). Together, these observations indicate that acquisition of extinction may involve, at least in part, a MAPK/ERK-dependent control of mRNA translation in the BLA. MAPK/ERK signalling has been implicated in many forms of synaptic plasticity including long-term potentiation (LTP) and long-term depression (LTD) (for review see Sweatt, 2004; Thomas & Huganir, 2004). Accordingly, we can only speculate as to whether the present deficit in extinction learning involves an enhancement of specific synaptic circuits sustaining fear extinction, or the reversal (that is, depotentiation) of fear conditioning-induced LTP (Rosenkranz & Grace, 2002; Lin *et al*., 2003a, b; Rumpel *et al*., 2005).

There is accumulating evidence indicating that fear extinction involves activation and/or plasticity of inhibitory circuits in the amygdala (Royer & Paré, 2002; Marsicano *et al*., 2002; Rosenkranz & Grace, 2003; Berretta *et al*., 2005; Chhatwal *et al*., 2005). Given that activation of the MAPK/ERK pathway has been shown to regulate presynaptic GABA release in cortex (Matsumoto *et al*., 2006), and expression of $GABA_A$ receptor subunits in cerebellum (Xie *et al*., 2004), it will be interesting to explore how activation of MAPK/ERK signalling influences function and plasticity of inhibitory circuits in the amygdala.

During consolidation of long-term extinction memory MAPK/ERK-dependent activation of transcription may become an important factor. Indeed, we observed that MAPK/ERK phosphorylation peaked 1 h after completion of extinction training. Consistent with subsequent induction of transcriptional events, we recently reported an increase in the expression of the IEGs c-fos and zif268 in the BLA 2 h after extinction learning (Herry & Mons, 2004). Moreover, spontaneous recovery of fear one week after extinction training was associated with reduced IEG expression in the BLA. Both MAPK/ERK phosphorylation and induction of IEG expression occurred in the BLA (Herry & Mons, 2004; present study). However, the relative contribution of amygdalar subnuclei in extinction-related plasticity is still unknown. In particular, the implication of the lateral nucleus of the amygdala during extinction of conditioned fear has been difficult to assess due to the fact that lesions of this nucleus prevent acquisition of fear conditioning (Nader *et al*., 2001). Interestingly, two recent studies have investigated the effect of lesions of the basal nucleus of the amygdala (BL) on extinction of auditory fear conditioning. Pre-training lesions had no effect on extinction learning, suggesting that BL is not necessary for acquisition of extinction (Sotres-Bayon *et al*., 2004; Anglada-Figueroa & Quirk, 2005). However, in their study,

Anglada-Figueroa & Quirk (2005) also evaluated the effect of a post-training lesion of BL onto acquisition of extinction. Surprisingly, post-training BL lesions completely blocked expression of conditioned fear. Even though these results are of particular relevance for the understanding of the amygdala microcircuitry involved in fear conditioning, it prevents any firm conclusion about the implication of BL during extinction learning.

In conclusion, our results show that activation of the MAPK/ERK signalling pathway in the BLA underlies the acquisition of extinction of auditory fear conditioning. Moreover, this study unambiguously identifies a role for neuronal plasticity in the BLA during learning of extinction. Further experiments are required to fully understand the relative contributions of the different amygdala subnuclei for the acquisition and expression of fear extinction. Also, it remains to be shown, whether, and under which conditions the BLA, together with mPFC and hippocampal circuits, participates to consolidation and long-term storage of extinction memory.

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Abbreviations

BLA, basolateral amygdala; CS, conditioned stimulus; IEG, immediate-early gene; LVGCC, L-type voltage-gated calcium channel; MAPK/ERK, mitogen-activated protein kinase/extracellular-signal regulated kinase; mPFC, medial prefrontal cortex; US, unconditioned stimulus.

References

Adams, J.P. & Sweatt, J.D. (2002) Molecular psychology roles for the ERK MAP kinase in memory. *Annu. Rev. Pharmacol. Toxicol.*, **42**, 135–163.

Anglada-Figueroa, D. & Quirk, G.J. (2005) Lesions of the basal amygdala block expression of conditioned fear, but not extinction. *J. Neurosci.*, **25**, 9680–9685.

Barad, M. (2005) Fear extinction in rodents: basic insight to clinical promise. *Curr. Opin. Neurobiol.*, **15**, 1–6.

Barrett, D., Shumake, J., Jones, D. & Gonzalez-Lima, F. (2003) Metabolic mapping of mouse brain activity after extinction of a conditioned emotional response. *J. Neurosci.*, **23**, 5740–5749.

Berman, D.E. & Dudai, Y. (2001) Memory extinction, learning anew, and learning the new: dissociations in the molecular machinery of learning in cortex. *Science*, **291**, 2417–2419.

Berretta, S., Pantazopoulos, H., Caldera, M., Pantazopoulos, P. & Paré, D. (2005) Infralimbic cortex activation increases c-Fos expression in intercalated neurons of the amygdala. *Neuroscience*, **132**, 943–953.

Bouton, M.E. & King, D.A. (1983) Contextual control of the extinction of conditioned fear: Test for associative value of the context. *J. Exp. Psychol. Anim. Behav. Process*, **9**, 248–265.

Cain, C.K., Blouin, A.M. & Barad, M. (2002) L-type voltage-gated calcium channels are required for extinction, but not for acquisition or expression, of conditional fear in mice. *J. Neurosci.*, **22**, 9113–9121.

Chhatwal, J.P., Myers, K.M., Ressler, K.J. & Davis, M. (2005) Regulation of gephyrin and GABAA receptor binding within the amygdala after fear acquisition and extinction. *J. Neurosci.*, **12**, 502–506.

Deisseroth, K., Bito, H. & Tsien, R.W. (1996) Signaling from synapse to nucleus: postsynaptic CREB phosphorylation during multiple forms of hippocampal synaptic plasticity. *Neuron*, **16**, 89–101.

Dolmetsch, R.E., Pajvani, U., Fife, K., Spotts, J.M. & Greenberg, M.E. (2001) Signaling to the nucleus by an L-type calcium channel-calmodulin complex through the MAP kinase pathway. *Science*, **294**, 318–319.

Falls, W.A., Miserendino, M.J. & Davis, M. (1992) Extinction of fear-potentiated startle: blockade by infusion of an NMDA antagonist into the amygdala. *J. Neurosci.*, **12**, 854–863.

Franklin, K.J.B. & Paxinos, G. (1997) *The mouse brain: In Stereotaxic Coordinates*. Academic press, New York.

Hardingham, G.E., Arnold, F.J. & Bading, H. (2001) A calcium microdomain near NMDA receptors: on switch for ERK-dependent synapse to nucleus communication. *Nature Neurosci.*, **4**, 565–566.

Herry, C. & Garcia, R. (2002) Prefrontal cortex long-term potentiation, but not long-term depression, is associated with the maintenance of extinction of learned fear in mice. *J. Neurosci.*, **22**, 577–583.

Herry, C. & Mons, N. (2004) Resistance to extinction is associated with impaired immediate early gene induction in medial prefrontal cortex and amygdala. *Eur. J. Neurosci.*, **20**, 781–790.

Huang, Y.Y., Martin, K.C. & Kandel, E.R. (2000) Both protein kinase A and mitogen-activated protein kinase are required in the amygdala for the macromolecular synthesis-dependent late phase of long-term potentiation. *J. Neurosci.*, **20**, 6317–6325.

Hugues, S., Deschaux, O. & Garcia, R. (2004) Postextinction infusion of a mitogen-activated protein kinase inhibitor into the medial prefrontal cortex impairs memory of the extinction of conditioned fear. *Learn. Mem.*, **11**, 540–543.

Kelleher, R.J., Govindarajan, A., Jung, H.Y., Kang, H. & Tonegawa, S. (2004) Translational control by MAPK signaling in long-term synaptic plasticity and memory. *Cell*, **116**, 467–479.

Ledgerwood, L., Richardson, R. & Cranney, J. (2003) Effects of D-cycloserine on extinction of conditioned freezing. *Behav. Neurosci.*, **117**, 341–349.

Lee, H. & Kim, J.J. (1998) Amygdalar NMDA receptors are critical for new fear learning in previously fear conditioned rat. *J. Neurosci.*, **18**, 8444–8454.

Lin, C.H., Lee, C.C. & Gean, P.W. (2003a) Involvement of a calcineurin cascade in amygdala depotentiation and quenching of fear memory. *Mol. Pharmacol.*, **63**, 44–52.

Lin, C.H., Yeh, S.H., Leu, T.H., Chang, W.C., Wang, S.T. & Gean, P.W. (2003b) Identification of calcineurin as a key signal in the extinction of fear memory. *J. Neurosci.*, **23**, 1574–1579.

Lin, C.H., Yeh, S.H., Lu, H.Y. & Gean, P.W. (2003c) The similarities and diversities of signal pathways leading to consolidation of conditioning and consolidation of extinction of fear memory. *J. Neurosci.*, **23**, 8310–8317.

Lu, K.T., Walker, D.L. & Davis, M. (2001) Mitogen-activated protein kinase cascade in the basolateral nucleus of amygdala is involved in extinction of fear-potentiated startle. *J. Neurosci.*, **21**, RC162.

Maren, S. & Quirk, G.J. (2004) Neuronal signaling of fear memory. *Nature Rev. Neurosci.*, **5**, 844–852.

Marsicano, G., Wotjak, C.T., Azad, S.C., Bisogno, T., Rammes, G., Cascio, M.G., Hermann, H., Tang, J., Hofmann, C., Zieglgänsberger, W., DiMarzo, V. & Lutz, B. (2002) The endogenous cannabinoid system controls extinction of aversive memories. *Nature*, **418**, 530–534.

Matsumoto, T., Numakawa, T., Yokomaku, D., Adachi, N., Yamagishi, S., Numakawa, Y., Kunugi, H. & Taguchi, T. (2006) Brain-derived neurotrophic factor-induced potentiation of glutamate and GABA release: Different dependency on signaling pathways and neuronal activity. *Mol. Cell. Neurosci.*, **31**, 70–84.

Milad, M.R. & Quirk, G.J. (2002) Neurons in medial prefrontal cortex signal memory for fear extinction. *Nature*, **420**, 70–74.

Moosmang, S., Haider, N., Klugbauer, N., Adelsberger, H., Langwieser, N., Muller, J., Stiess, M., Marais, E., Schulla, V., Lacinova, L., Goebbels, S., Nave, K.A., Storm, D.R., Hofmann, F. & Kleppisch, T. (2005) Role of hippocampal Cav1.2 Ca^{2+} channels in NMDA receptor-independent synaptic plasticity and spatial memory. *J. Neurosci.*, **25**, 9883–9992.

Morozov, A., Muzzio, I.A., Bourtchouladze, R., Van-Strien, N., Lapidus, K., Yin, D., Winder, D.G., Adams, J.P., Sweatt, J.D. & Kandel, E.R. (2003) Rap1 couples cAMP signaling to a distinct pool of p42/44 MAPK regulating excitability, synaptic plasticity, learning, and memory. *Neuron*, **39**, 309–325.

Myers, K.M. & Davis, M. (2002) Behavioral and neural analysis of extinction. *Neuron*, **36**, 567–584.

Nader, K., Majidishad, P., Amorapanth, P. & LeDoux, J.E. (2001) Damage to the lateral and central, but not other, amygdaloid nuclei prevents the acquisition of auditory fear conditioning. *Learn. Mem.*, **8**, 156–163.

Quirk, G.J. (2002) Memory for extinction of conditioned fear is long-lasting and persists following spontaneous recovery. *Learn. Mem.*, **9**, 402–407.

Radwanska, K., Nikolaev, E., Knapska, E. & Kaczmarek, L. (2002) Differential response of two subdivisions of lateral amygdala to aversive conditioning as revealed by c-Fos and P-ERK mapping. *Neuroreport*, **13**, 2241–2246.

Rescorla, R.A. (2001) Experimental extinction. In Mower R.R. & Klein S.B. (Eds), *Handbook of Contemporary Learning Theories.* Lawrence Erlbaum Associates Publishers, London, pp. 119–154.

Rosenkranz, J.A. & Grace, A.A. (2002) Dopamine-mediated modulation of odour-evoked amygdala potentials during pavlovian conditioning. *Nature*, **417**, 282–287.
Rosenkranz, J.A. & Grace, A.A. (2003) The prefrontal cortex regulates lateral amygdala neuronal plasticity and responses to previously conditioned stimuli. *J. Neurosci.*, **23**, 11054–11064.
Royer, S. & Paré, D. (2002) Bidirectional synaptic plasticity in intercalated amygdala neurons and the extinction of conditioned fear responses. *Neuroscience*, **115**, 455–462.
Rumpel, S., LeDoux, J.E., Zador, A. & Malinow, R. (2005) Postsynaptic receptor trafficking underlying a form of associative learning. *Science*, **308**, 83–88.
Santini, E., Ge, H., Ren, K., Pena de Ortiz, S. & Quirk, G.J. (2004) Consolidation of fear extinction requires protein synthesis in the medial prefrontal cortex. *J. Neurosci.*, **24**, 5704–5710.
Santini, E., Muller, R.U. & Quirk, G.J. (2001) Consolidation of extinction learning involves transfer from NMDA-independent to NMDA-dependent memory. *J. Neurosci.*, **21**, 9009–9017.
Schafe, G.E., Atkins, C.M., Swank, M.W., Bauer, E.P., Sweatt, J.D. & LeDoux, J.E. (2000) Activation of ERK/MAP kinase in the amygdala is required for memory consolidation of pavlovian fear conditioning. *J. Neurosci.*, **20**, 8177–8178.
Shu, S.Y., Ju, G. & Fan, L.Z. (1988) The glucose oxidase-DAB-nickel method in peroxidase histochemistry of the nervous system. *Neurosci. Lett.*, **85**, 169–171.
Sotres-Bayon, F., Bush, D.E. & LeDoux, J.E. (2004) Emotional perseveration: an update on prefrontal–amygdala interactions in fear extinction. *Learn. Mem.*, **11**, 525–535.
Suzuki, A., Josselyn, S.A., Frankland, P.W., Masushige, S., Silva, A.J. & Kida, S. (2004) Memory reconsolidation and extinction have distinct temporal and biochemical signatures. *J. Neurosci.*, **24**, 4787–4795.
Sweatt, J.D. (2004) Mitogen-activated protein kinases in synaptic plasticity and memory. *Curr. Opin. Neurobiol.*, **14**, 311–317.
Thomas, G.M. & Huganir, R.L. (2004) MAPK cascade signaling and synaptic plasticity. *Nature Rev. Neurosci.*, **5**, 173–183.
Trifilieff, P., Herry, C., Vanhoutte, P., Caboche, J., Desmedt, A., Riedel, G., Mons, N. & Micheau, J. (2006) Foreground contextual memory consolidation requires two independent phases of hippocampal ERK/CREB activation. *Learn. Mem.*, **13**, 349–358.
Xie, F., Raetzman, L.T. & Siegel, R.E. (2004) Neuregulin induces GABAA receptor beta2 subunit expression in cultured rat cerebellar granule neurons by activating multiple signaling pathways. *J. Neurochem.*, **90**, 1521–1529.
Yang, Y.L. & Lu, K.T. (2005) Facilitation of conditioned fear extinction by *d*-cycloserine is mediated by mitogen-activated protein kinase and phosphatidylinositol 3-kinase cascades and requires *de novo* protein synthesis in basolateral nucleus of amygdala. *Neuroscience*, **134**, 247–260.
Yuan, L.L., Adams, J.P., Swank, M., Sweatt, J.D. & Johnston, D. (2002) Protein kinase modulation of dendritic K+ channels in hippocampus involves a mitogen activated protein kinase pathway. *J. Neurosci.*, **22**, 4860–4868.
Zhu, J.J., Oin, Y., Van Aelst, L. & Malinow, R. (2002) Ras and Rap control AMPA receptor trafficking during synaptic plasticity. *Cell*, **110**, 443–455.