

A Novel Retrieval-dependent Memory Process Revealed by the Arrest of ERK1/2 Activation in the Basolateral Amygdala

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Abstract (223 words)

Fully consolidated fear memories can be maintained or inhibited by retrieval-dependent mechanisms depending on the degree of re-exposure to fear cues. Short exposures promote memory maintenance through reconsolidation and long exposures promote inhibition through extinction. Little is known about the neural mechanisms by which increasing cue exposure overrides reconsolidation and instead triggers extinction. Using auditory fear conditioning in male rats, we analysed the role of a molecular mechanism common to reconsolidation and extinction of fear, ERK1/2 activation within the basolateral amygdala (BLA), after intermediate CS exposure events.

We show that an intermediate re-exposure (4 CS presentations) failed to activate ERK1/2 in the BLA, suggesting the absence of reconsolidation or extinction mechanisms. Supporting this hypothesis, pharmacologically inhibiting the BLA ERK1/2-dependent signalling pathway in conjunction with 4 CS presentations had no effect on fear expression, and the NMDA receptor partial agonist D-cycloserine, which enhanced extinction and ERK1/2 activation in partial extinction protocols (7 CSs), had no behavioural or molecular effect when given in association with 4 CS presentations.

These molecular and behavioural data reveal a novel retrieval-dependent memory phase occurring along the transition between conditioned fear maintenance and inhibition. CS-dependent molecular events in the BLA may arrest reconsolidation intracellular signalling mechanism in an extinction-independent manner. These findings are critical for understanding the molecular underpinnings of fear memory persistence after retrieval both in health and disease.

Significance statement (120 words)

Consolidated fear memories can be altered by retrieval-dependent mechanisms. Whereas a brief conditioned stimulus (CS) exposure promotes fear memory maintenance through reconsolidation, a prolonged exposure engages extinction and fear inhibition. The nature of this transition and whether an intermediate degree of CS exposure engages reconsolidation or extinction is unknown. We show that an intermediate cue exposure session (4 CSs) produces the arrest of ERK1/2 activation in the basolateral amygdala, a common mechanism for reconsolidation and extinction. Amnestic or hypermnesic treatments given in association with 4 CSs had no behavioural or molecular effects, respectively. This evidence reveals a novel retrieval-dependent memory phase. Intermediate degrees of CS exposure fail to trigger reconsolidation or extinction, leaving the original memory in an insensitive state.

Introduction

Persistent maladaptive associative memories are an essential aspect of chronic, recurrent anxiety disorders, including specific phobias and post-traumatic stress disorder (Parsons and Ressler, 2013). In human and non-human animals, retrieval of an associative memory by means of exposure to the fear conditioned stimulus (CS) can trigger either the maintenance or inhibition of stored memories, depending on the number or extent of cue presentations. A brief CS presentation, in the presence of a mismatch between what is expected and what actually occurs, leads to memory reconsolidation and the maintenance of the conditioned response (Pedreira et al., 2004; Kindt et al., 2009). By contrast, a large number of CS presentations at re-exposure triggers extinction and inhibition of the conditioned response (Pavlov and Anrep, 1927; Bouton, 2004; Hermans et al., 2006). Intriguingly, although reconsolidation and extinction have opposite behavioural effects, both can be triggered by a similar event; a CS reminder without the US. Under extreme reminder conditions (brief or prolonged) memory trace dominance is evident since either reconsolidation or extinction is exclusively engaged (Eisenberg et al., 2003; Pedreira and Maldonado, 2003). Whether an intermediate degree of CS exposure triggers reconsolidation and/or extinction in a co-existent or mutually exclusive manner is unclear.

Understanding how fear memory reconsolidation and extinction relate to each other when retrieval is triggered by intermediate amounts of CS exposure is essential fully to understand the alternative effects of retrieval on memory persistence. Moreover, since CS exposure is an essential aspect of existing treatments for anxiety disorders (Vervliet et al., 2013), with growing interest in using pharmacological agents to potentiate their effectiveness (de Kleine et al., 2013), defining the boundary conditions - the characteristics of the transition from reconsolidation to extinction and the underlying molecular mechanisms - is essential for developing more effective therapies.

Even though reconsolidation and extinction of cued fear memory are dependent upon distinct networks within the brain, the basolateral amygdala (BLA) is a locus for both processes (Nader et al., 2000; Maren, 2015), and within the BLA these memory processes rely on both exclusive and common neural mechanisms. Thus, reconsolidation specifically requires synthesis of the immediate early gene *Zif268* (Lee et al., 2005), while extinction specifically requires the synthesis of the protein phosphatase calcineurin (Merlo et al.,

2014). However, both processes rely on the antecedent activation of NMDA-type glutamate receptors (NMDAR) (Lee and Kim, 1998; Milton et al., 2013) and the extracellular signal-regulated kinase1/2 (ERK1/2) signalling pathway (Duvarci et al., 2005; Herry et al., 2006). Thus, while reconsolidation and extinction are initiated by similar extra- and intra-cellular events, the transcriptional and translational events underlying each memory process are unique (Mamiya et al., 2009; Merlo et al., 2014).

Because BLA ERK1/2 is activated by both fear memory reconsolidation and extinction, measuring CS exposure-dependent BLA pERK1/2 levels provides an ideal molecular marker to distinguish between the mutually exclusive or gradual coexistence hypotheses of these opposing processes (Perez-Cuesta and Maldonado, 2009). Thus, if the transition is gradual, with both memory processes being partially engaged, pERK1/2 should be increased by an intermediate number of CS presentations. By contrast, a failure to affect BLA pERK1/2 levels by intermediate retrieval conditions would support a mutually exclusive three-phase transition. Therefore, in the present experiments we measured BLA pERK1/2 levels and the behavioural effect of ERK1/2 signalling cascade inhibition when fear memory was retrieved by presenting an intermediate number of CSs. We hypothesised that in rats with a fully consolidated auditory fear memory an intermediate number of CSs would fail to activate ERK1/2 in the BLA, supporting the three-phase transition hypothesis.

We demonstrate here that 4 CS presentations had no effect on BLA pERK1/2 levels and left the fear memory insensitive to ERK1/2 blockade in the BLA. We thereby reveal a mutually exclusive relationship between reconsolidation and extinction and show that the transition between them as a result of increasing CS exposure is explained by a three-phase model.

Materials and Methods

Animals. Two hundred and fifty adult male Lister-Hooded rats weighing 250-300 g (Charles River, RGD_2312466) were used. All animals were kept under a 12 h light/dark cycle (lights off at 0700) and provided with food and water *ad libitum* except for during behavioural procedures. All animal procedures were conducted in accordance with the EU

legislation on the protection of animals used for scientific purposes (Directive 2010/63/EU) and the research was regulated under the Animals (Scientific Procedures) Act 1986 Amendment Regulations 2012 following ethical review by the University of Cambridge Animal Welfare and Ethical Review Body (AWERB).

Surgeries. Rats were anaesthetized with ketamine hydrochloride (100 mg/kg; Ketaset, Fort Dodge Animal Health) and xylazine (9 mg/kg; Rompun, Bayer), and implanted with 22-gauge stainless steel bilateral indwelling guide cannulae (Plastics One) aimed at the BLA. The coordinates were 2.6 mm posterior to bregma, 4.5 mm lateral to the mid line and 3.6 mm ventral to *dura mater*. Stainless-steel obturators were inserted to maintain patency during recovery and in between infusions.

Intracranial microinfusions. Infusions were carried out as before (Merlo et al., 2014), with injectors extending 4mm beyond the guide cannulae. Prior to behavioural testing, animals were habituated to the infusion procedure by the administration of 0.1 μ l of sterile saline solution per side (0.25 μ l/min). U0126 (Sigma-Aldrich) was dissolved in 5% DMSO, 6% Tween 80, in 100 mM sterile phosphate-buffered saline (PBS, pH 7.2) to a final concentration of 2 μ g/ μ l. 0.5 μ l of U0126 or vehicle (5% DMSO, 6% Tween 80 in 100 mM sterile PBS) solution per side (0.25 μ l/min) were infused 30 min before re-exposure session.

Behavioural procedures. Animals were initially individually habituated to the conditioning box (Paul Fray Ltd, UK) for 2 hours. On the training day, rats were placed in the box and after 25 minutes received an auditory conditioned stimulus (CS) presentation (60s clicker, 10Hz, 80 dB) that was coterminous with the presentation of a scrambled footshock (US, 0.5 mA, 0.5 sec) delivered through the grid floor. The training session consisted of two CS-US presentations with an inter-trial interval (ITI) of 5 minutes. Twenty four hours later the rats were returned to the box and presented with 1, 4, 7, or 10 CS presentations, ITI = 1 min. Twenty-four hours later animals were again returned to the conditioning box and presented with one CS.

Drug injection. The NMDAR partial agonist D-Cycloserine (DCS, Sigma-Aldrich) or the non-competitive receptor antagonist MK-801 were dissolved in sterile saline for intraperitoneal injection (1 ml/kg). The doses of 15 mg/kg (DCS) or 0.1 mg/kg (MK-801) were selected on the basis of their mnemonic effects on prior experiments on the

reconsolidation and extinction of fear memory (Merlo et al., 2014). Drug or saline solution injections were given 30 minutes before CS presentation sessions.

Protein extraction and western blotting. Rats were sacrificed by carbon dioxide inhalation followed by neck dislocation. The brains were rapidly removed and snap frozen on dry ice prior to storage at -80°C . Cytosolic protein preparation, quantification and separation were performed as described before (Merlo et al., 2014). Blots were probed with: mouse anti-ERK1/2 (#610124, BD Biosciences, AB_397530, 1:5000), rabbit anti-phospho-p44/42 MAPK (ERK1/2) (Thr 202/ Tyr 204) (D13.14.4E) (Cell Signaling Tech, AB_10694057, 1:500), mouse anti- β -actin [AC-15] (AbCam, AB_2223210, 1:50000), goat anti-rabbit-HRP (Sigma, 1:10000) and rabbit anti-mouse-HRP (Sigma, 1:25000) diluted in Tris-buffered saline solution containing 0.1% of Tween-20. A chemiluminescent signal was induced using an enhanced chemiluminescent reagent (Amersham) and images were captured using a cooled CCD camera (ChemiDoc-It, UVP). Signal analysis and quantification was performed using ImageJ software (v1.47a, NIH, SCR_003070). Each primary antibody working concentration was adjusted to deliver a linear relationship between the amounts of loaded protein in the blot versus signal intensity.

Experimental design and statistical analysis. All training, CS presentation and test sessions were video recorded for offline behavioural analysis. The percentage of time freezing (absence of movement except for breathing) during the 1 minute prior to and during the 1-minute CS was manually scored from the videos at 5 second intervals by an observer blind to the treatment. Statistical analyses of behavioural quantifications were performed using one-way, two-way or repeated measures ANOVAs, with Group as the between-subjects factor and CS-US or CS as the within-subjects factor. Tukey's post hoc comparisons were used for further analysis. Any deviations from sphericity were corrected using the Greenhouse-Geisser correction if $\epsilon < 0.75$, and the Huynh-Feldt correction if $\epsilon > 0.75$ (Cardinal and Aitken, 2006). For western blot data analysis, optical density (OD) values and the band areas were obtained for each microdissected basolateral amygdala cytosolic sample for both target protein (pERK1/2, ERK1/2) and the β -actin loading control. Each pERK1/2 OD value was normalized to its corresponding ERK1/2 and β -actin OD value. These normalised OD values for the experimental groups were normalised to the NR group mean OD value, and then averaged for each condition. To minimise variability across

membranes, each western blot membrane included at least three different samples of each experimental and control groups. Each western blot quantification was performed twice to ensure replicability. Molecular data were analysed using one-way ANOVA with Dunnett's test for *post hoc* comparisons (Figure 1C). For western blot quantifications presented in Figure 4, OD values for SAL/Drug treated animals were normalised to the mean OD of their respective NR-SAL or NR-Drug group. These data were analysed using Student's *t*-test. In both cases Group was the between-subject factor. Tests were carried out using SPSS (IBM, SCR_002865) and JASP (University of Amsterdam, SCR_015823).

Results

Intermediate CS exposures fail to active BLA ERK1/2

Rats were fear conditioned by two pairings of an auditory clicker (CS) and a footshock (US). Animals were divided according to the treatment received during re-exposure day into 5 experimental groups as follows: non-reactivated control group (NR), exposure to 1 (1CS), 4 (4CS), 7 (7CS) or 10 (10CS) unreinforced 1-min long CS presentations (**Figure 1A**). A mixed ANOVA indicated that all prospective groups showed similar acquisition of fear conditioning, with an effect of trial (CS-US₁ vs. CS-US₂, $F_{(1, 60)} = 468.3$, $p < 0.001$, $\eta^2 = 0.89$) but no effect of group ($F_{(4, 60)} = 0.24$, $p = 0.91$) or interaction ($F_{(4, 60)} = 0.10$, $p = 0.98$) (**Figure 1B**).

Twenty four hours later, animals were either re-exposed to the different numbers of CSs or remained in their home cages as controls (**Figure 1B**). Freezing to the first CS gives a measure of fear memory retention that can be assessed for all CS-exposed groups, to ensure that training was equivalent. All CS-exposed animals showed high levels of freezing to the first CS (preCS vs. CS₁, $F_{(1, 38)} = 378.2$, $p < 0.001$, $\eta^2 = 0.91$) with no effect of group ($F_{(3, 38)} = 0.52$, $p = 0.67$) or any interaction ($F_{(3, 60)} = 0.72$, $p = 0.55$), indicating similar levels of fear memory retention across groups. Repeated measures ANOVAs showed significant effects of trials on freezing level in 4CS ($F_{(1.52, 13.71)} = 20.18$, $p < 0.001$, $\eta^2 = 0.69$), 7CS ($F_{(1.60, 11.23)} = 5.31$, $p = 0.03$, $\eta^2 = 0.43$) and 10CS groups ($F_{(2.71, 29.86)} = 33.31$, $p < 0.001$, $\eta^2 = 0.75$).

Twenty minutes after the presentation of the first CS, or immediately following removal from the home cage (NR group), animals were sacrificed and cytosolic protein extracts from the BLA prepared. A one-way ANOVA of BLA pERK1/2 levels quantified by western blots showed that the groups differed in their pERK1/2 level ($F_{(4, 60)} = 3.55$, $p = 0.01$, $\eta^2 = 0.19$). Post hoc comparisons (Dunnett's) showed that pERK1/2 was increased after 1 or 10 CS presentations ($p = 0.01$, $p = 0.04$, respectively) but remained at basal levels after 4 or 7 CSs ($p = 0.62$, $p = 0.97$, respectively, **Figure 1C**).

We have previously shown that under these specific retrieval conditions, the presentation of one unreinforced CS leads to reconsolidation of the original memory, whereas 10 CS presentations leads to the formation of a new inhibitory extinction memory (Lee et al., 2006; Merlo et al., 2014). Thus, these results confirmed the engagement within the BLA of the ERK1/2 signalling pathway under extreme reminder conditions, with both the reconsolidation- and extinction-inducing CS protocols resulting in kinase activation. By contrast, intermediate reminders of 4 or 7 CS presentations did not result in BLA ERK1/2 activation. These observations support our main hypothesis indicating that a common synaptic plasticity mechanism engaged during either reconsolidation or extinction of fear memory is not recruited under these intermediate CS exposure conditions.

ERK1/2 signalling cascade blockade during intermediate CS exposure has no effect on memory

The lack of ERK1/2 activation after 4 or 7 CS presentations suggests that the CS exposure conditions leading to reconsolidation or extinction are separated by a degree of CS exposure during which no ERK-dependent synaptic plasticity mechanisms are engaged within the BLA. Alternatively, it is possible that during the transition period, both reconsolidation and extinction are taking place but in a gradual, or more subtle, way that is undetectable in BLA pERK1/2 levels. In order to distinguish between these two alternatives, we analysed the effect of specific inhibition of the ERK1/2 signalling pathway on fear memory after presentation of 4 or 7 CSs. In order to control for the amnesic effect of the pharmacological manipulation the experiment included groups of animals treated while undergoing either reconsolidation or extinction triggered by extreme CS exposure levels (1 or 10 CSs respectively).

Rats with bilateral cannulae targeting the BLA (**Figure 2**) were fear conditioned as before. Twenty four hours later animals received an intra BLA infusion of vehicle or the ERK1/2 inhibitor U0126 30 minutes before the presentation of 1, 4, 7 or 10 CS. One day later fear memory was assessed by the presentation of one CS (**Figure 3A**). Intra-BLA infusion of U0126 had no acute behavioural effect during CS exposure sessions, with similar freezing levels across groups for the first CS (Group: $F_{(3, 71)} = 1.55$, $p = 0.21$; Drug: $F_{(1, 71)} = 0.03$, $p = 0.87$; Group x Drug: $F_{(3, 71)} = 0.35$, $p = 0.79$), or along the session (4CS groups Drug: $F_{(1, 19)} = 0.21$, $p = 0.65$, CS x Drug: $F_{(3, 57)} = 1.13$, $p = 0.35$; 7CS groups Drug: $F_{(1, 18)} = 0.46$, $p = 0.51$, CS x Drug: $F_{(3, 28, 59.07)} = 0.94$, $p = 0.43$; 10CS Drug: $F_{(1, 15)} = 0.87$, $p = 0.37$, CS x Drug: $F_{(2.81, 42.19)} = 1.18$, $p = 0.33$; **Figure 3B**). At test, a two-way ANOVA revealed no overall effect of number of CS ($F_{(3, 71)} = 1.87$, $p = 0.14$) or of ERK1/2 inhibition ($F_{(1, 71)} = 0.03$, $p = 0.86$), but a significant CS-Drug interaction, indicating differential effects of ERK1/2 inhibition with different levels of CS presentation (CS x Drug: $F_{(3, 71)} = 4.06$, $p = 0.01$, $\eta^2 = 0.15$, **Figure 3C**). Simple main effects analysis showed an effect of drug for 1 CS ($F_{(1, 19)} = 9.93$, $p = 0.005$, $\eta^2 = 0.34$) and 10 CS conditions ($F_{(1, 17)} = 5.10$, $p = 0.04$, $\eta^2 = 0.25$), but not for 4CS ($F_{(1, 19)} = 0.004$, $p = 0.95$) or 7CS groups ($F_{(1, 18)} = 0.32$, $p = 0.58$). Given that some of our experimental conditions tested the null hypothesis (H_0), i.e. the absence of U0126 effect (4 and 7 CSs), we also analysed these data using Bayesian statistics, a method not biased against H_0 (Wagenmakers, 2007; Rouder et al., 2009). This revealed that the alternative hypothesis (H_1) was 8.37 times more likely in animals receiving 1 CS and 2.04 times more likely in animals receiving 10 CSs (1CS data: $BF_{01} = 0.12$; $BF_{10} = 8.37$. 10CS data: $BF_{01} = 0.49$; $BF_{10} = 2.04$). In contrast, in animals receiving 4 CSs H_0 was 2.55 times more likely whereas in animals receiving 7 CSs the null hypothesis was 2.25 times more likely (4CS data: $BF_{01} = 2.55$; $BF_{10} = 0.39$. 7 CS data: $BF_{01} = 2.25$; $BF_{10} = 0.45$). This analysis is consistent with the frequentist analysis presented above, further confirming the absence of an amnesic effect of U0126 when administered before 4 or 7 CS presentations.

In addition to confirming previous observations on the requirement for BLA ERK1/2 in auditory fear memory reconsolidation and extinction (Duvarci et al., 2005; Herry et al., 2006) in the 1CS and 10CS groups respectively, these data indicate that the same

intervention had no behavioural effect when animals are exposed to an intermediate number of CSs.

The molecular and behavioural data presented so far indicate that both the 4 and 7 CS exposure conditions not only fail to trigger fear memory labilisation but also are insufficient to engage extinction. In order to further test the memory process dominance or absence in these two transitional stages we conducted the next series of experiments evaluating the effect of NMDAR activity modulation on the effective or ineffective activation of pERK1/2 in the BLA.

BLA pERK1/2 enhancement by D-cycloserine (DCS) during intermediate CS presentations distinguishes between sensitive and insensitive transitional states

The experiments presented here had two objectives: 1) to study the NMDAR activity dependence of BLA ERK1/2 activation during reconsolidation or extinction of fear memory, and 2) to evaluate the effect of NMDAR activity enhancement on the lack of BLA ERK1/2 activation seen after 4 or 7 CS presentations. Systemic NMDAR activity manipulations were carried out using the NMDA receptor antagonist MK-801 or partial agonist DCS.

In order to test the requirement for NMDAR activity in reconsolidation- or extinction-induced BLA ERK1/2 activation, rats were fear conditioned as before. Twenty four hours later animals were injected systemically with saline or MK-801 30 minutes before 1 or 10 CS presentations. Twenty minutes after the first CS presentation, animals were sacrificed and their brains removed. Non-reactivated control groups (NR) received the same i.p. injections, but were returned to their home cages, and sacrificed 50 minutes later. In order to test for the effect of CS exposure on BLA pERK1/2 level depending on drug condition we performed unpaired *t*-tests on western blot data. This analysis shows that 1 CS presentation induced an increase in BLA pERK1/2 in the saline group ($t(14) = 3.08$; $p = 0.004$) whereas there was no change in the presence of MK-801 ($t(14) = 0.07$; $p = 0.47$; **Figure 4A**). Similarly, there was a pERK1/2 increase in rats injected with saline and exposed to 10CS ($t(14) = 2.83$; $p = 0.006$) but not in rats treated with MK-801 ($t(14) = 1.04$; $p = 0.16$; **Figure 4B**). These data are consistent with the disruption of reconsolidation or extinction by NMDAR blockade under extreme CS presentation conditions, and

demonstrate that NMDAR activation is required for the increase in pERK1/2 associated with both of these mnemonic processes.

In parallel we analysed the effect of NMDAR activity enhancement on the BLA ERK1/2 activation state after 4 or 7 CS presentations, hypothesising that if there is a mnemonic insensitive period engaged by these levels of CS presentation, then it should not be possible to modulate pERK1/2 level by enhancing NMDAR activity. Twenty four hours after fear conditioning, rats were injected with saline or DCS 30 minutes before 4 or 7 CS presentations. Twenty minutes after the first CS presentation, animals were sacrificed and their brains removed. Non-reactivated control groups were treated as before. Animals exposed to 4 CS presentations showed no change in BLA pERK1/2 levels in both the saline ($t(8) = 1.09$; $p = 0.16$) or DCS condition ($t(8) = 0.78$; $p = 0.23$; **Figure 4C**). By contrast, animals exposed to 7 CS presentations showed no difference in pERK1/2 levels when injected with saline ($t(8) = 0.29$; $p = 0.39$) but a significant increase in the activated kinase levels when injected with DCS ($t(8) = 2.12$; $p = 0.03$; **Figure 4D**).

These results indicate that ERK1/2 activation by reconsolidation or extinction of fear memory is dependent on NMDAR activity. Also, they show that enhancement of NMDAR activity increased pERK1/2 levels only in the 7CS group, suggesting that the two transitional states induced by 4 or 7 CSs engage qualitatively different neural mechanisms.

Discussion

We have previously proposed that the transition from reconsolidation to extinction induced by increasing CS exposure conformed to a three-phase transition model that is characterised by an intermediate insensitive memory phase (Merlo et al., 2014). Since that hypothesis was based solely on behavioural observations following systemic pharmacological manipulations, it was possible that the lack of a memory modulation effect after 4 CSs was the result of simultaneous and opposing effects on both reconsolidation and extinction mechanisms that were partially engaged in the brain. In the present study we used a complementary molecular biological and behavioural approach along with more specific intra-BLA pharmacological manipulations to investigate the existence of this novel retrieval-dependent memory process triggered by intermediate degrees of cue exposure at memory retrieval. We show that activation of the BLA ERK1/2

signalling pathway, a well-established molecular marker for reconsolidation and extinction, reveals a mutually exclusive, three-phase transition between these memory processes when non-reinforced CS exposure is increased. Whereas extreme reminder conditions that trigger either fear memory reconsolidation or extinction (1 or 10 CS) resulted in an increase in BLA pERK1/2, intermediate reminders (4 or 7 CS) failed to alter BLA pERK1/2 levels. This transition mode was corroborated by pharmacological manipulation of ERK1/2 signalling pathway or NMDA-type glutamate receptor activity. Thus, intra-BLA inhibition of the ERK1/2-dependent signalling cascade by U0126 disrupted both reconsolidation and extinction, but had no behavioural effect under intermediate CS exposure protocols. However, systemic DCS treatment revealed a distinction between 4 and 7 CS conditions, since it failed to affect BLA ERK1/2 activation at 4 CS, but increased it at 7 CS, when it is also able to enhance extinction (Merlo et al., 2014).

It is well established that the ERK1/2 signalling pathway within the BLA is an essential mechanism underlying consolidation, reconsolidation and extinction of auditory fear memory (Schafe et al., 2000; Duvarci et al., 2005; Herry et al., 2006). In particular, it has been shown that intra-BLA administration of U0126 does not permanently damage the BLA, and has an amnesic effect only if the fully consolidated fear memory is retrieved (Duvarci et al., 2005). Here we show that during an intermediate reminder this amnesic manipulation is without effect even in presence of fear memory retrieval (Fig. 3). Although retrieval is a necessary condition for ERK1/2 blockade in the BLA to have an amnesic effect, it is not sufficient. U0126 can only exert its amnesic effect when infused into the BLA when either reconsolidation or extinction has been engaged.

The effect of 4 CS presentations on the fear response towards the CS at test is not significantly different to that of the 1 CS group, but is invariably at a lower level (1CS-VEH vs. 4CS-VEH in Fig. 3C and (Merlo et al., 2014)). A similar extinction-independent decrease in a contextual fear response was observed at test after intermediate context exposure (Cassini et al., 2017). It remains to be determined how 4 CSs affect the original fear memory. They may act to reduce the stored CS-US contingency information through a mechanism independent of extinction, affecting the original memory trace in the absence of reconsolidation. Such a mechanism could produce conditioned fear reduction without reinstatement, renewal or spontaneous recovery. Interestingly, 7 CS presentations did

produce a reduction of fear at test in comparison with the 1 CS group, suggestive of the early engagement of extinction mechanisms. Even though this is an indirect observation, it supports the qualitative difference between these two intermediate reminder conditions discussed above.

As shown here, the molecular biological or behavioural analyses conducted separately do not fully reveal the complexity of memory mechanisms taking place during the transition from reconsolidation to extinction. This exemplifies the necessity of combining these approaches in order fully to understand these mechanisms. Failure to promote ERK1/2 activation in the BLA after an intermediate reminder does not provide a true biomarker of memory in limbo since this was also observed in both 4 and 7 CS conditions. The lack of a memory enhancing effect of BLA ERK1/2 positive modulation following DCS is therefore further evidence of the limbo state. These two sources of experimental evidence, while affirming the existence of the limbo state, cannot rule out the possibility of a third mnemonic process interposed between reconsolidation and extinction and this warrants further detailed experimental investigation.

Reconsolidation and extinction: mutually exclusive vs coexistence hypotheses

In an attempt to distinguish between these alternative hypotheses previous reports have investigated the pharmacological effects of amnesic treatments during intermediate CS exposure. In medaka fish, exposure to the amnesic agent 3-aminobenzoic acid ethyl ester (that can prevent both reconsolidation and extinction) during an intermediate CS exposure session had no behavioural effects (Eisenberg et al., 2003). In rats, systemic administration of an NMDAR agonist or antagonist had no effect when given in association with an intermediate fear or appetitive memory retrieval session (Flavell and Lee, 2013; Merlo et al., 2014). Finally, a period of insensitivity has been reported for a fear memory in humans after an intermediate exposure session and oral administration of the beta adrenoceptor antagonist, propranolol, which prevents reconsolidation after brief memory reactivation (Sevenster et al., 2014). Even though these are consistent observations from fish to humans, the lack of specificity of the pharmacological manipulations used in these studies makes it difficult to distinguish between the two alternative memory dominance hypotheses (Perez-Cuesta and Maldonado, 2009).

Here we present molecular and behavioural evidence indicating that reconsolidation and extinction do not co-exist and that there is a limbo memory state when BLA ERK1/2 and fear expression are immune to amnestic and hypermnestic manipulations. The combination of precise parametric control of CS exposure levels with a well characterised amnestic treatment, the ERK1/2 inhibitor U0126 (which blocks both reconsolidation and extinction under extreme CS exposure conditions) allows us to reject the co-existence hypothesis and reveal the existence of an impervious, limbo memory state.

The parametric conditions that determine the engagement of alternative retrieval-dependent memory processes also depend on memory acquisition conditions (i.e: US intensity, CS duration) or memory age. Stronger or older fear memories require an extended CS re-exposure event in order to engage memory reconsolidation when compared to a younger or weaker memory (Suzuki et al., 2004). Contextual fear memories trained with longer context exposure bouts before shock presentation showed a rightwards shift in the three-phase transition profile of reconsolidation, 'limbo' and extinction (Alfei et al., 2015). Moreover, under the experimental conditions used here, it was the intermediate number of 4 CS presentations that failed to trigger reconsolidation or extinction. It is therefore possible that the degree of CS exposure necessary for 'limbo' engagement will be sensitive to conditions such as US intensity at training, CS frequency at re-exposure or total CS exposure time.

Molecular markers of memory in limbo

Activation of the ERK1/2 signalling pathway is a conserved mechanism underlying memory consolidation, reconsolidation and extinction in key brain areas and in a variety of memory paradigms (Cestari et al., 2014). Its ubiquitous function, combined with its activation time course, suggests ERK1/2 is an ideal candidate marker to study the transition from reconsolidation to extinction by increasing the number of non-reinforced CS presentations. Our experiments confirm the requirement for BLA ERK1/2 activation in both the reconsolidation and extinction of fear memory (Duvarci et al., 2005; Herry et al., 2006) and also show for the first time that an intermediate number of CS presentations fails to activate the kinase, leaving it at the level seen in a trained, but not reminded, control

group. These data suggest that within a limited range, increasing CS exposure terminates the labilisation of the original CS-US memory, without necessarily engaging memory extinction. This intriguing finding has several theoretical implications for the neural mechanisms of memory persistence upon retrieval.

In the procedure employed here, 4 CS presentations terminated the incipient memory labilisation and restabilisation mechanisms triggered by the first CS. We speculate that one or more CS-dependent molecular events may mediate this arrest of the earliest reconsolidation intracellular signalling mechanism in an extinction-independent manner. For example, a protein-protein interaction that reduces or buffers Ca^{2+} flow through the NMDAR could be activated by 4 CSs to act as an early molecular brake that disengages an ongoing synaptic plasticity mechanisms (Cho et al., 2001). Additionally, given that memory labilisation is required for reconsolidation and depends on degradation of pre-existing post-synaptic proteins *via* activation of the ubiquitin proteasome system (Lee et al., 2008), labilisation arrest could require the activation of specific de-ubiquitinating enzymes (DUBs) within the BLA. The DUB ubiquitin carboxy-terminal hydrolase L1 is highly specific to neurons and suppresses TNF- α induced ERK1/2 activation *in vitro* (Ichikawa et al., 2010).

Animals receiving 7 CS presentations also showed lack of BLA ERK1/2 activation, but were nevertheless molecularly and behaviourally sensitive to the effect of NMDAR agonism, since DCS potentiated the emerging dominant memory process of extinction. This important difference between two intermediate states highlights both the insensitivity of the intracellular signalling cascade after 4 CS presentations and also that extinction engagement is a gradual process developing after a sufficient number of CS presentations (between 4 and 7) and requiring the concerted action of kinases and phosphatases (de la Fuente et al., 2011; Merlo et al., 2014). Systemic DCS administration increased BLA calcineurin levels after 7 CS presentations, leading to extinction enhancement, but had no effect on the phosphatase levels after 4 CSs (Merlo et al., 2014). Even though reconsolidation termination and the gradual engagement of extinction are both CS repetition-dependent events, it remains to be determined whether the molecular changes such as ERK1/2 activation occur in the same or different neuronal subpopulations within the BLA. We speculate that the CS-dependent early inactivation of ERK1/2 takes place in

the fear neuronal ensemble, which stops responding to the CS as a consequence of extinction training. As more unreinforced CSs are presented, extinction neurons may then increase their firing rate in response to the extinguished CS through an ERK1/2-dependent mechanism (Herry et al., 2008). Defining the molecular mechanisms that act to arrest reconsolidation, manifesting as ERK1/2 insensitivity to CS exposure, will be an important next step in order to test putative interactions between these opposing memory maintenance and inhibition processes.

Clinical implications of memory in limbo

A widely used and effective treatment for anxiety disorders is exposure therapy. Manipulation of memory content during exposure sessions either by enhancing memory extinction or preventing memory reconsolidation is emerging as a promising development of such treatments (Bowers and Ressler, 2015). In this context, the use of DCS during exposure sessions has shown a positive (Guastella et al., 2008; Otto et al., 2010), negative (Smits et al., 2013) or no effect in patients with anxiety disorders (de Kleine et al., 2012). Furthermore, pharmacological treatment in association with traumatic memory reactivation involving CS exposure has also shown positive (Brunet et al., 2008; Kindt and van Emmerik, 2016) or no effect (Wood et al., 2015) in PTSD patients. We suggest that this pattern of results is consistent with the three component transition between reconsolidation and extinction reported here. Depending on the strength of the maladaptive aversive memory, CS exposure protocols will affect fear memory differently, but invariably the profile will follow the same transitions occurring during the CS exposure space. A pharmacological treatment such as DCS will enhance reconsolidation or extinction when using extreme CS exposure sessions, but will have no effect when CS exposure results in a memory in limbo. Conversely, if CBT is combined with an amnestic treatment in order to disrupt the traumatic memory, unintentional limbo engagement through an extended CS exposure session will leave the target memory in an insensitive state thereby preventing clinical improvement. The degree of CS exposure resulting in limbo could vary between individuals, perhaps due to differences in learning history, but knowing of the existence of a limbo state both helps to understand contradictory findings and also promote the development of new treatments to enhance exposure therapy.

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Figure legends

Figure 1. Intermediate cue exposure fails to activate BLA ERK1/2, a molecular marker of fear memory reconsolidation and extinction. (A) Experimental design. Rats were fear conditioned with two CS-US pairings. Twenty four hours after training, animals were divided into 5 groups as follows: non-reactivated control (NR), 1, 4, 7 and 10 CS

presentations (1CS, 4CS, 7CS or 10CS, respectively). Twenty minutes after the first CS presentation, or straight from the home cage, animals were sacrificed and BLA cytosolic protein extracts prepared. (B). Mean (\pm SEM) of % time freezing during cued fear conditioning and at different number of cue exposure sessions (NR n = 23, 1CS n = 12, 4CS n = 10, 7CS n = 8, 10CS n = 12). (C) Representative western blot picture and analysis of pERK1/2 levels in the BLA for NR, 1CS, 4CS, 7CS and 10CS groups. Mean relative optical density as % of NR (\pm SEM) shows that pERK1/2 is increased after 1 or 10 CS presentations, but unchanged after 4 or 7CSs. TR: training session. *: $p < 0.05$.

Figure 2. Injector tip placements within the basolateral amygdala for each experimental condition: 1CS, 4CS, 7CS and 10CS. Vehicle: open circles; U0126: closed circles. Modified from Paxinos & Watson (Paxinos and Watson, 1998).

Figure 3. Intra-BLA administration of U0126, a specific inhibitor of ERK1/2 pathway, has no behavioural consequences during intermediate CS exposure. (A) Experimental design. Animals were trained with two CS-US pairings. Twenty four hours later they were injected with vehicle or U0126 (1 μ g per side) and within each drug condition divided into four groups (1CS, 4CS, 7CS and 10CS) depending on the number of cue presentations. Twenty four hours later all the animals were tested for fear memory with the presentation of one CS (1CS VEH and 4CS U0126 n = 11; 10CS VEH n = 7; remaining groups n = 10 per group). (B and C) Mean % of time freezing (\pm SEM) at CS exposure sessions (B) or long-term memory test (C) are shown. TS: test session. *: $p < 0.05$; **: $p < 0.01$.

Figure 4. NMDAR-dependent BLA ERK1/2 activation distinguishes between sensitive and insensitive transitional states between fear memory reconsolidation and extinction. (A) Experimental design: Twenty four hours after training animals were i.p. injected with saline or MK-801 (0.1 mg/kg) and then exposed to 1 CS (1CS groups) or returned to the home cage (NR groups). Fifty minutes after the injection the animals were sacrificed and BLA cytosolic protein extracts prepared. Representative western blot pictures. Bar graph shows the mean BLA level of pERK1/2 (\pm SEM) as a % of NR group (n = 8 per group). Open bars: saline injection; striped bars: MK-801 injection. (B) Experimental design and

bar graph: same as for A, but after i.p. injection the animals were either returned to the home cage (NR groups) or exposed to 10 CS presentations (10CS groups) (n = 8 per group). (C) Experimental design: 24h after training animals were i.p. injected with saline or DCS (15 mg/Kg) and then exposed to 4 CS (4CS groups) or returned to the home cage (NR groups). Fifty minutes after the injection the animals were sacrificed and BLA cytosolic protein extracts prepared. Representative western blot pictures. Bar graph shows the mean BLA level of pERK1/2 (\pm SEM) as a % of NR group (n = 5 per group). Open bars: saline injection; striped bars: DCS injection. (D) Experimental design and bar graph: same as for C, but after i.p. injection the animals were either returned to the home cage (NR groups) or exposed to 7 CS presentations (7CS groups) (n = 5 per group). *: $p < 0.05$ vs. NR group receiving the same i.p. injection.

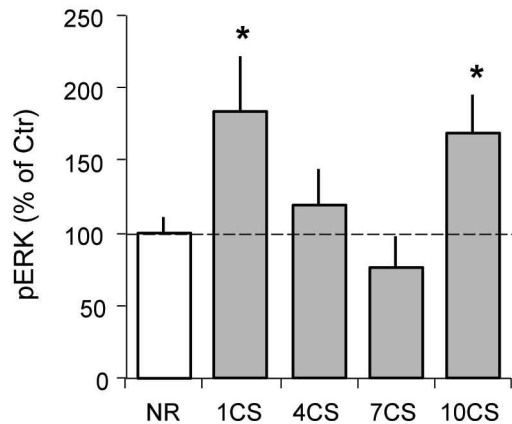
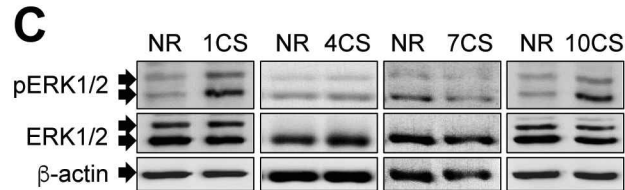
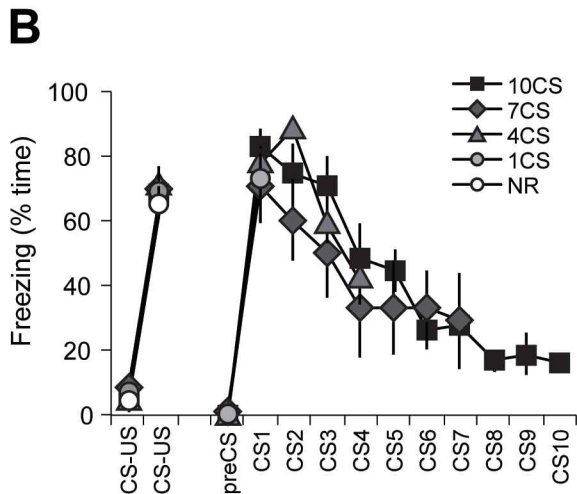
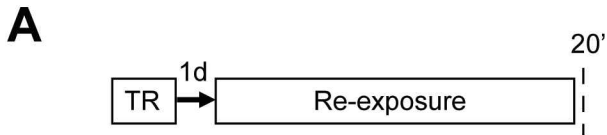


Figure 1

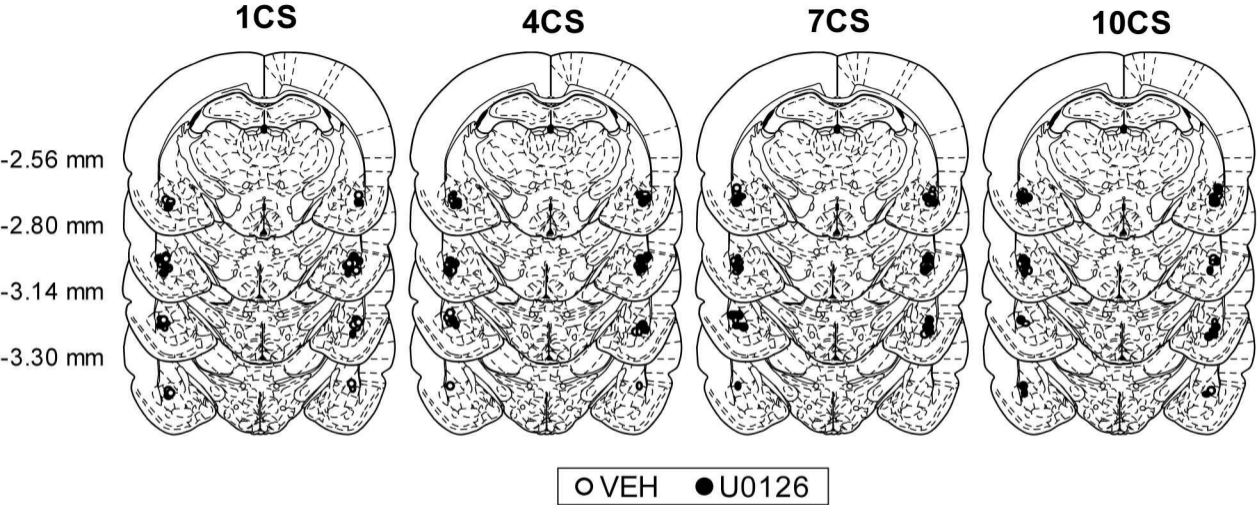


Figure 2

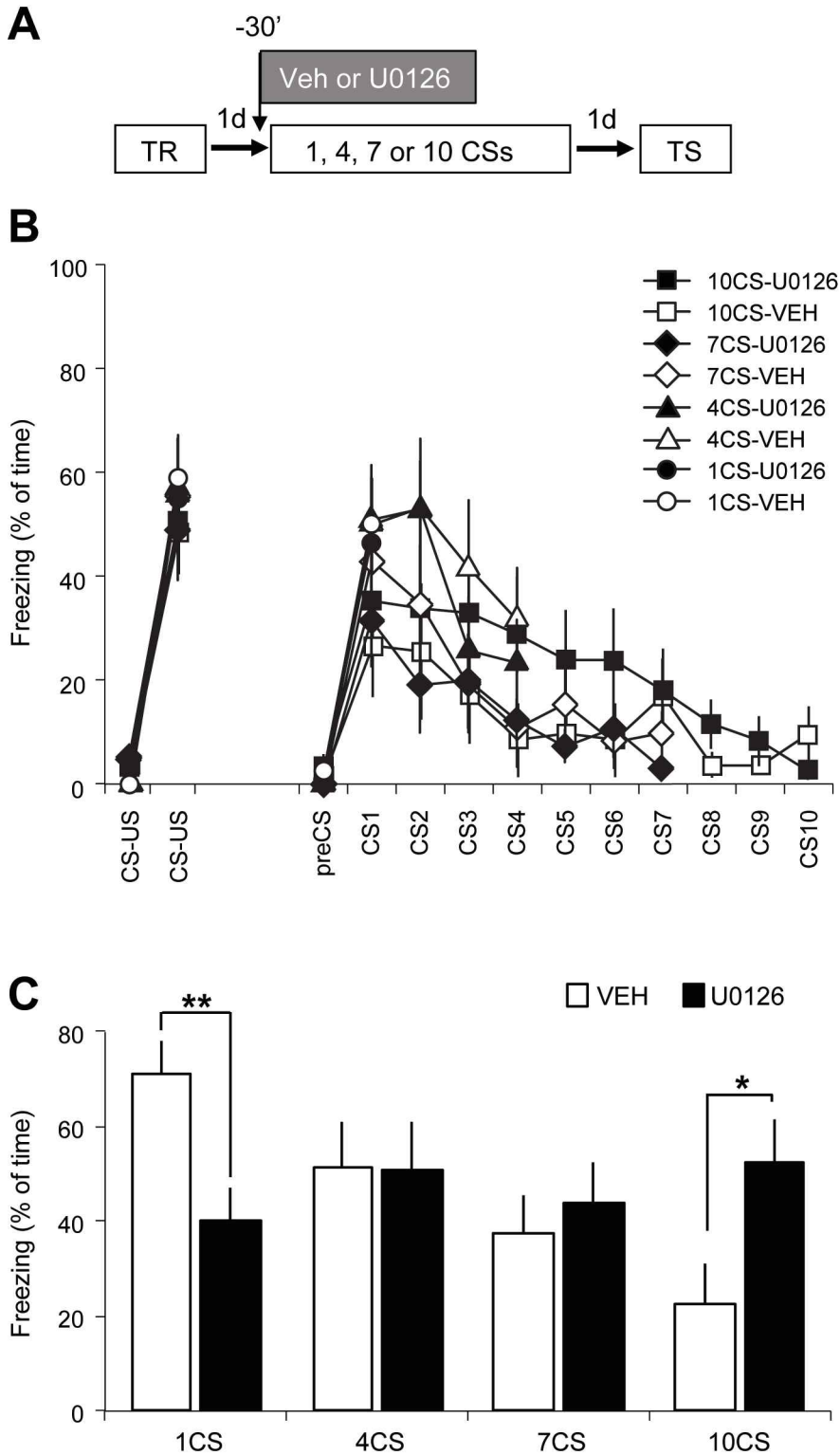


Figure 3

