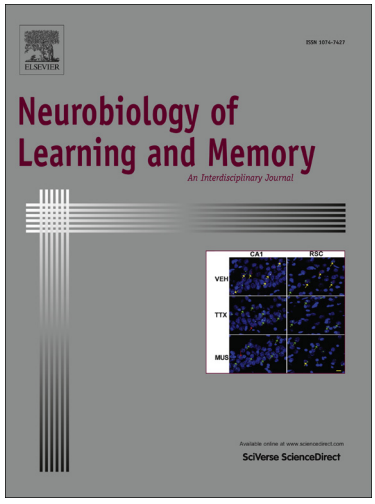

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Persistence of amygdala gamma oscillations during extinction learning predicts spontaneous fear recovery

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Abstract

Extinction of auditory fear conditioning induces a temporary inhibition of conditioned fear responses that can spontaneously reappear with the passage of time. Several lines of evidence indicate that extinction learning relies on the recruitment of specific neuronal populations within the basolateral amygdala. In contrast, post-extinction spontaneous fear recovery is thought to result from deficits in the consolidation of extinction memory within prefrontal neuronal circuits. Interestingly, recent data indicates that the strength of gamma oscillations in the basolateral amygdala during auditory fear conditioning correlates with retrieval of conditioned fear responses. In the present manuscript we evaluated the hypothesis that post-extinction spontaneous fear recovery might depend on the maintenance of gamma oscillations within the basolateral amygdala by using single unit and local field potential recordings in behaving mice. Our results indicate that gamma oscillations in the basolateral amygdala were enhanced following fear conditioning, whereas during extinction learning gamma profiles were more heterogeneous despite similar extinction learning rates. Remarkably, variations in the strength of gamma power within the basolateral amygdala between early and late stages of extinction linearly predicted the level of post-extinction spontaneous fear recovery. These data suggest that maintenance of gamma oscillations in the basolateral amygdala during extinction learning is a strong predictive factor of long term spontaneous fear recovery.

Keywords: gamma oscillations; amygdala; extinction; spontaneous recovery; fear conditioning

1. Introduction

In mammals, the mechanisms underlying emotional learning are often evaluated using auditory fear conditioning, a form of Pavlovian learning that consists of associating a conditioned stimulus (the CS, usually a tone) with a mild aversive unconditioned stimulus (the US, usually a footshock). Following fear conditioning, repeated presentation of the CS alone progressively inhibits conditioned fear responses, a phenomenon labeled fear extinction (Myers and Davis, 2007). Fear extinction is a form of new learning known to be encoded in specific neuronal networks including the basolateral amygdala (BLA) and the medial prefrontal cortex (mPFC) (Burgos-Robles, Vidal-Gonzalez, Santini, and Quirk, 2007; Courtin, Bienvenu, Einarsson, and Herry, 2013; Herry, Ferraguti, Singewald, Letzkus, Ehrlich, and Luthi, 2010; Holmes, Fitzgerald, MacPherson, DeBrouse, Colacicco, Flynn, Masneuf, Pleil, Li, Marcinkiewcz, Kash, Gunduz-Cinar, and Camp, 2012; Klavir, Genud-Gabai, and Paz, 2012; Maroun, Kavushansky, Holmes, Wellman, and Motanis, 2012; Milad and Quirk, 2002; Orsini and Maren, 2012).

Notably, several studies have consistently demonstrated that the BLA is a key structure involved in the initial stages of extinction memory formation. Several lines of evidence suggest that cellular plasticity in the BLA underlies the acquisition of extinction memory. For instance, pharmacological studies indicate that local interference with glutamatergic synaptic plasticity in the BLA, such as infusion of NMDA receptors antagonists or blockers of ERK/MAPK signaling prevents or attenuates extinction learning (Falls, Miserendino, and Davis, 1992; Herry, Trifilieff, Micheau, Luthi, and Mons, 2006; Zimmerman and Maren, 2010). Moreover, fear extinction learning is correlated with an increase in CS-evoked activity within a subpopulation of neurons in the basal nuclei of the amygdala (Herry, Ciocchi, Senn, Demmou, Muller, and Luthi, 2008). Persistence of extinction memories is usually evaluated during a post-extinction retrieval test, where the extinguished CS is presented one to several days after extinction learning. Several studies have indicated the existence of large individual differences in the response to non-reinforced CS presentations during retrieval despite similar extinction learning rates (Burgos-Robles et al., 2007; Herry and Mons, 2004; Milad and Quirk, 2002). Indeed, during post-extinction retrieval of extinction memories, some animals display spontaneous recovery of conditioned fear responses (high fear recovery), whereas others present low fear responses upon presentation of the extinguished CS (low fear recovery). The heterogeneous distribution of conditioned fear responses during post-

extinction retrieval could be mediated by specific functional changes displayed at the time of the retrieval session or acquired prior to the retrieval session.

In direct support of the first possibility, recent data indicate that enhanced correlations between BLA and mPFC theta oscillations during retrieval are correlated with the expression of previously acquired fear memories (Lesting, Narayanan, Kluge, Sangha, Seidenbecher, and Pape, 2011). In contrast, the loss of such correlations between these structures is associated with retrieval of extinction memories (Lesting et al., 2011). Moreover synaptic plasticity in the mPFC-BLA pathway has been shown to be enhanced during post-extinction fear recovery whereas synaptic plasticity in the BLA-mPFC pathway displayed the opposite pattern (Vouimba and Maroun, 2011). Only few studies have evaluated the predictive factors of long term fear recovery following extinction learning. For instance, studies have revealed that long-term retention of extinction memory was correlated with the thickness of the ventromedial prefrontal cortex in healthy humans (Milad, Quinn, Pitman, Orr, Fischl, and Rauch, 2005) and that amygdala activation during extinction in patients suffering from post-traumatic stress disorder is associated with high fear recovery (Bremner, Vermetten, Schmahl, Vaccarino, Vythilingam, Afzal, Grillon, and Charney, 2005; Milad, Pitman, Ellis, Gold, Shin, Lasko, Zeidan, Handwerger, Orr, and Rauch, 2009).

Although the neuronal mechanisms of post-extinction fear recovery are still poorly understood, recent data suggested that amygdala gamma oscillations might play a key role in this phenomenon. Indeed, several lines of evidence indicate that (i) amygdala activation during acquisition of emotional memories strongly correlates with long-term memory retrieval (Cahill, Haier, Fallon, Alkire, Tang, Keator, Wu, and McGaugh, 1996; Hamann, Ely, Grafton, and Kilts, 1999), (ii) processing of biologically significant stimuli is associated with amygdala gamma oscillations (Sato, Kochiyama, Uono, Matsuda, Usui, Inoue, and Toichi, 2011a; b; 2012) and (iii) the strength of gamma oscillations during memory acquisition predicts subsequent memory retrieval (Headley and Weinberger, 2011; Osipova, Takashima, Oostenveld, Fernandez, Maris, and Jensen, 2006; Sederberg, Kahana, Howard, Donner, and Madsen, 2003; Sederberg, Schulze-Bonhage, Madsen, Bromfield, McCarthy, Brandt, Tully, and Kahana, 2007b). Together these data suggest the possibility that the maintenance of gamma oscillations during extinction might predict spontaneous post-extinction fear recovery. The present study evaluated this hypothesis using single unit and

local field potential recordings in the BLA of mice following auditory fear conditioning and extinction learning.

2. Material and methods

2.1. Subjects

Male C57BL6/J mice (3 months old, Janvier) were individually housed for 7 days prior to all experiments, under a 12 h light/dark cycle, and provided with food and water *ad libitum*. All studies took place during the light portion of the cycle. Mice were gently handled for 2–3 min⁄day during 5 days, to minimize nonspecific stress. All animal procedures were performed in accordance with standard ethical guidelines (European Communities Directive 86/60-EEC) and were approved by the committee on Animal Health and Care of Institut National de la Santé et de la Recherche Médicale and French Ministry of Agriculture and Forestry (authorization A3312001).

2.2. Behavior

Habituation and fear conditioning took place in context A consisting of a square transparent Plexiglas box (25cm side, 80 cm high) with a shock grid floor made of stainless steel rods placed inside a sound attenuating and temperature regulated cubicle. The walls of the cubicle were made of sound attenuating black foam. Inside the cubicle the floor and walls of the Plexiglas cylinder were cleaned with a 70 % ethanol solution before and after each session. Extinction learning and post-extinction fear retrieval were performed in context B consisting of a square transparent Plexiglas cylinder (25 cm diameter, 40 cm high) with a grey plastic floor placed inside a sound attenuating and temperature regulated cubicle. The walls of the cubicle were made of sound attenuating white foam and the light intensity was reduced. The floor and walls of the Plexiglas square were cleaned with a 1 % acetic acid solution before and after each session.

To score freezing behavior an automated infrared beam detection system located on the bottom of the experimental chambers was used (Coulbourn Instruments). The animals were considered to be freezing if no movement was detected for 2 s. On day 1, 11 C57BL6/J mice were submitted to a habituation session in context A, in which they received 4 presentations of the CS^+ and of the CS^- (total CS duration: 30 s, consisting of 50 ms pips repeated at 0.9 Hz, 2 ms rise and fall, pip frequency: 7.5 kHz or white-noise, 80 dB sound pressure level, CS were counterbalanced across animals). Discriminative fear conditioning was performed on the same day by pairing the CS^+ with a US (1 s foot-shock, 0.6 mA, 5 CS^+/US pairings; inter-

trial interval: 20-180 s). The onset of the US coincided with the offset of the CS^+. The CS^- was presented after each CS^+/US association but was never reinforced (5 CS^- presentations, inter-trial interval: 20-180 s). The frequencies used for CS^+ and CS^- were counterbalanced across animals. On days 2 and 3, conditioned mice were submitted to extinction training (Early and Late Extinction sessions) in context B during which they received 4 and 12 presentations of the CS^- and CS^+, respectively. Retrieval of extinction was tested 7 days later in context B, with 4 presentations of the CS^- and the CS^+.

2.3. Surgery and recordings

Mice were anesthetized with isoflurane (induction 3%, maintenance 1.5%) in O_2. Body temperature was maintained with a temperature controller system (FHC). Mice were secured in a stereotaxic frame and unilaterally implanted in the BLA with a multi-wire electrode aimed at the following coordinates (Franklin, 1997): 1.7 mm posterior to bregma; 3.2 mm lateral to midline and 4.2 mm below the cortical surface. The electrodes consisted of 16 individually insulated nichrome wires (13 μm inner diameter, impedance 30-100 KΩ; Kanthal) contained in a 26 gauge stainless steel guide cannula. The wires were attached to an 18 pin connector (Omnetics). All implants were secured using Super-Bond cement (Sun Medical). After surgery mice were allowed 7 days to recover and habituated to handling. Analgesia was applied before, and 1 day after surgery (Metacam, Boehringer). Electrodes were connected to a headstage (Plexon) containing 16 unity-gain operational amplifiers. The headstage was connected to a 16-channel preamplifier (gain 100x, bandpass filter from 150 Hz to 9 kHz for single unit activity and from 0.7 Hz to 170 Hz for field potentials, Plexon). Spiking activity was digitized at 40 kHz and bandpass filtered from 250 Hz to 8 kHz, and isolated by time-amplitude window discrimination and template matching using a Multichannel Acquisition Processor system (Plexon).

2.4. Single unit and local field potential analysis

Single-unit spike sorting was performed using Off-Line Spike Sorter (OFSS, Plexon). Principal component scores (PC) were calculated for unsorted waveforms and plotted in a 3D PC space; clusters containing similar valid waveforms were manually defined. A group of waveforms were considered to be generated from a single neuron if the waveforms formed a discrete, isolated, cluster in the PC space and did not contain a refractory period less than 1 ms, as assessed by using auto-correlogram analyses. To avoid analysis of the same neuron recorded on different channels, we computed cross-correlation histograms. If a target neuron presented a peak of activity at a time that the reference neuron fired, only one of the two

neurons was considered for further analysis. Local field potentials (LFPs) from channels on which no obvious 50 Hz noise and movement artifacts could be detected and no units were recorded, were analyzed using NeuroExplorer software (NeuroExplorer). This prevents the possibility that spiking activity recorded simultaneously on the same channel as the LFP would lead to spurious gamma-like oscillatory activity. LFP power spectrum density was calculated using Fast Fourier Transformation (FFT) from the raw LFPs with a hanning window of 250 ms and no overlap. To calculate gamma power, we used a measure of the relative gamma power that corresponds to the ratio of power within the gamma band (30 to 80 Hz) to the total power in the power spectrum (0 to 100 Hz) of the raw LFP. This analysis corresponds to a normalization procedure that produces a measure of gamma oscillations completely independent of the large fluctuations of LFP oscillations that can occur in individual animals. For all behavioral sessions, the relative gamma power was quantified for the entire session and for each block of 4 CS^- and 4 CS^+. Relative power values were analyzed for each individual animal and then averaged for statistical analysis. To evaluate the stability of gamma power during extinction learning we defined a gamma power stability index that corresponds to the ratio of the relative gamma power recorded during Late Ext. (last block of CS^+) to the relative gamma power recorded during Early Ext. (first block of CS^+). To evaluate if gamma in the BLA was generated locally we first examined the relationship between gamma oscillations and single unit activity. We filtered the LFPs in the gamma range (30-80Hz) using a zero-phase-delay filter implemented in the NeuroExplorer software (Neuroexplorer), we then downsampled the filtered LFPs (500 Hz) and identified gamma peaks and troughs for gamma cycles with an amplitude of at least 2 standard deviations from the mean. Individual gamma phase locking was computed by calculating the instantaneous gamma phase of the filtered LFP (30-80 Hz) using the Hilbert transform. For a given neuron, each spike was assigned its corresponding LFP gamma phase value from the LFP signal recorded on the same wire. Gamma phase locking was calculated using Rayleigh's test for circular uniformity and statistical significance was assessed using Rayleigh's test *Z* value. Logarithmic transformation was applied to tighten the *Z* distribution. For significantly phased-locked neurons, the mean preferred phase was computed by using the mean circular direction. Second, to evaluate if the recorded gamma oscillations were generated in a remote structure and volume conducted to the BLA, we selected six neurons displaying at least 1000 spikes and quantified their neuronal phase locking strength to gamma oscillations recorded with electrodes located at two different depths within the BLA by using the Mean Resultant Length vector (MRL). The MRL estimates the circular concentration of

spikes. Low or high MRL values are respectively indicative of a diffuse or a concentrated circular distribution of spikes around the preferred phase. The rational behind this analysis is that the difference between the power of oscillations recorded on spatially separated electrodes will be small in the case of volume conduction, and will decrease as a function of the distance in the case of locally generated oscillations. Therefore, the absence of change in the strength of neuronal phase locking would reflect volume conduction, whereas decreased strength of neuronal phase locking would indicate locally generated oscillations.

2.5. Statistical analysis

Statistical analyses of behavioral data were performed by repeated measures ANOVA followed by Student's *t*-tests *post hoc* comparisons at the $P < 0.05$ level of significance. The results are presented as mean ± SEM. For statistical analyses using paired Student's t-tests, we systematically performed F-tests with n-1 degrees of freedom to evaluate if the variances of the two samples compared were similar. For comparisons of gamma power and MRL values, we used paired Student's *t*-tests at the $P < 0.05$ level of significance. When multiple statistical tests were performed, Bonferroni corrections were applied. To assess if the persistence and stability of BLA gamma power during extinction learning was a good predictor of spontaneous fear recovery we performed a Receiving Operator Characteristic analysis (ROC). In the context of this study, the ROC analysis was used to determine a cutoff value of gamma power that accurately predicted spontaneous recovery of conditioned fear responses. In this analysis we assumed that the animals were in a freezing or a non-freezing state during the post-extinction retrieval test. Therefore in this analysis, freezing values during CS^+ retrieval were assigned a value of 0 (for non-freezing state) or 1 (for freezing state) depending on if they were below or above 50%, respectively. Then, a measure of gamma power stability was performed during extinction for all animals and used to predict the freezing state during post-extinction retrieval. The ROC analysis calculates a cutoff value that minimizes the uncorrect predictions (i.e. the number of false positives and false negatives) while maximizing the correct predictions. Based on this value, the animals are identified as corresponding to "good predictor" or "bad predictors". We performed the ROC analysis for the gamma stability index as well as for a control and a permuted gamma stability index. The control index corresponds to the ratio of the relative gamma power recorded during Early Extinction (first block of CS^+) to the relative gamma power recorded during Habituation (CS^+). The permuted index was obtained by performing random permutations of the individual gamma stability index values binary values between animals

(50 permutations), the ROC analysis was computed for each of these permuted indexes and the obtained ROC values averaged. The area under the curve that is produced by the ROC analysis was interpreted as the probability of correct predictions, with values close to 1 indicative of a strong predictive power and values close to, or below, 0.5 indicative of low predictive power. We computed this analysis using an open source MATLAB toolbox (ROCdata function, Cardillo, 2008).

2.6. Histology

At the conclusion of the experiments, mice were given an overdose of urethane (1.4 g/kg) and recording sites were marked with electrolytic lesions before perfusion with 4% paraformaldehyde in 0.1M phosphate buffered saline. Brains were removed and post-fixed in 4% paraformaldehyde overnight, sectioned (80 µm) on a vibratome (Leica), and mounted on gelatin-coated slides for thionine staining for reconstruction of electrode locations.

3. Results

3.1. Behavioral results

During the Habituation session, tone presentations induced low freezing levels that were not significantly different between CS^- and CS^+ conditions (Fig. 1A, Day1, Habituation). Twenty-four hours following auditory fear conditioning, mice discriminated between CS^- and CS^+ as revealed by a significant increase in freezing levels evoked by CS^+ presentations, compared to CS^- presentations (Fig. 1A, Day2, Early Extinction, CS^- versus first block of CS^+: $P < 0.001$). A one-factor repeated measures ANOVA performed on CS^+ presentations during Early and Late Extinction sessions indicated a significant decrease of freezing levels over extinction trials ($F_{10,5} = 8.418$, $P < 0.001$). A direct comparison between the first and the last block of CS^+ presentations during the late extinction session confirmed these results as freezing levels were significantly lower on the last compared to the first block of CS^+ presentations ($P < 0.001$). However, a direct comparison between freezing levels evoked by CS^- and the last block of CS^+ presentations during Late Extinction session or between CS^+ presentations during habituation and the last block of CS^+ presentations during Late Extinction session revealed a significant difference, indicating that our procedure only induced a partial extinction of conditioned fear responses (all $Ps < 0.05$). Finally, when tested one week after extinction during the Retrieval session, mice displayed a significant difference in freezing levels between CS^- and CS^+ presentations ($P < 0.001$). Interestingly, despite the

fact that our behavioral procedure only induced a partial extinction, the distribution of conditioned fear responses during CS^+ presentations at Retrieval was quite heterogeneous, with mice displaying either low, moderate, or high fear responses during CS^+ presentations (Fig. 1B, C low fear: 4/11 mice < 30% freezing; moderate fear: 5/11 mice > 30% and < 60%; high fear: 2/11 mice >60 % freezing). This effect could be accounted for by variability in the effectiveness of extinction learning across animals or by the amount of freezing acquired following fear conditioning. However, correlational analyses performed between freezing levels during the first and the last blocks of 4 CS^+ presentations in the Early or Late Extinction phases and during CS^+ presentations at Retrieval failed to reveal any significant correlations (Fig 1D, E; Early Extinction versus Retrieval: $r = 0.008$, P = ns; Late Extinction versus Retrieval: $r = 0.39$, P = ns). These data clearly indicate that the heterogeneity of conditioned fear responses at Retrieval cannot be explained by a difference in fear acquisition or extinction learning across animals.

3.2. Dynamics of BLA gamma oscillations during extinction learning

Gamma oscillations (30-80 Hz) were recorded in the BLA during the behavioral procedure (Fig. 1F). We first evaluated the presence of gamma oscillations during the entire period of individual behavioral sessions using the power spectral density estimate. During the Habituation session, virtually no gamma oscillations were observed in the BLA (Fig. 2A). In striking contrast, gamma oscillations were strongly enhanced during Early and Late extinction sessions (Habituation versus Early or Late Extinction, all $Ps < 0.01$). Although there was a trend for reduced gamma oscillations during Late Extinction in comparison to Early Extinction, there were no significant differences in the strength of gamma oscillations during these phases. Power spectral analyses performed during Retrieval also revealed an increase in BLA gamma oscillations that was close to, but not significant, in comparison to Habituation (Habituation versus Retrieval, $P = 0.06$). Additionally, direct comparisons between Early and Late extinction and the Retrieval session did not reveal any significant differences (all $P > 0.05$). These results could be explained by a least two factors. First, the non-significant increase in BLA gamma power during the Retrieval session might be explained by the heterogeneous distribution of conditioned fear responses observed at Retrieval (Fig 1). Correlational analysis performed between freezing levels and gamma power during CS^+ presentations at Retrieval indeed revealed a significant positive correlation (data not shown, $r = 0.52$, $P < 0.05$). Second, the lack of significant differences between gamma oscillations during Early and Late Extinction could be related to the fact that this

analysis was performed over the entire behavioral sessions, and not specifically during CS presentation periods. To address this possibility we evaluated the dynamics of BLA gamma oscillations specifically during CS^- and CS^+ presentations during the behavioral procedure. During Habituation there were virtually no gamma oscillations during tone presentations and no significant differences in gamma oscillations power between CS^- and CS^+ presentations (Fig. 2B). In contrast, fear conditioning induced a strong increase in gamma oscillation power during Early (first block of CS^+) and Late (last block of CS^+) Extinction sessions for CS^- and CS^+ presentations (CS^-, Habituation versus Early or Late Extinction, all *Ps* < 0.05; CS^+, Habituation versus Early or Late Extinction, all *Ps* < 0.001). Although gamma oscillations were increased during CS^- presentations following fear conditioning, it was nevertheless to a lesser extent than during CS^+ presentations as revealed by significant differences between CS^- and CS^+ presentations during Early and Late Extinction (CS^- versus CS^+, Early and Late Extinction, all *Ps* < 0.05). Direct comparisons between gamma power evoked by CS^+ presentation during Early and Late Extinction session revealed a significant decrease of gamma power throughout extinction (CS+ presentations, Early versus Late Extinction: *P* <0.05). Finally, during the Retrieval session we observed a significant increase in gamma power for CS^+ presentations when compared to the habituation phase (CS^+, Habituation versus Retrieval: *P* < 0.05). The difference between CS^- and CS^+ presentations at Retrieval was also significant (Retrieval, CS^- versus CS^+: *P* <0.05). Together, these data indicate that presentations of a CS associated with footshock during fear conditioning induces gamma oscillations in the BLA and that this increase was reduced over extinction learning.

3.3. Maintenance of BLA gamma oscillations during extinction learning predicts spontaneous fear recovery

We next evaluated if the reduction of gamma oscillations induced by CS^+ presentations correlates with the inhibition of freezing behavior during extinction learning. Interestingly, there were no significant correlation between freezing levels and gamma power induced by CS^+ presentations during extinction learning (Fig 2C, *r* = 0.20 *P* = ns), although freezing and gamma power decreased significantly during extinction learning. Individual analyses of gamma power values between Early and Late Extinction revealed that the weak correlations between freezing levels and gamma power during extinction was related to a heterogeneous distribution of gamma power during Late Extinction. Indeed, we observed that some animals displayed a reduction in gamma power whereas other maintained or increased gamma power over extinction learning (Fig 2D). Because maintenance of gamma power has

been shown to correlate with long term memory (Osipova et al., 2006; Sederberg et al., 2003; Sederberg et al., 2007b) we evaluated if persistent gamma oscillations during extinction training correlated with freezing levels during post-extinction retrieval. An index of gamma power stability during extinction learning was calculated and correlated with freezing levels at Retrieval (Fig 3A, B). Strikingly, this analysis revealed a strong positive correlation between freezing levels at retrieval and gamma stability throughout extinction learning (r = 0.77, $P < 0.01$). Namely, reduced gamma power during extinction was associated with low fear recovery at Retrieval, whereas stable gamma power during extinction was associated with high fear recovery (Fig 3A, B). These results strongly suggest that the persistence and stability of BLA gamma power during extinction learning linearly predicts spontaneous fear recovery. To further evaluate this possibility, we performed a Receiving Operator Characteristic (ROC) analysis to assess the accuracy of this prediction. The ROC analysis produces a curve and the area under this curve can be interpreted as a probability of correct predictions, with values close to 1 indicative of a strong predictive power and values close to or below 0.5 indicative of low predictive power (see methods). The ROC analysis revealed a very strong predictive power of BLA gamma oscillation maintenance during extinction learning on spontaneous fear recovery at Retrieval (Figure 3C, ROC area = 0.93, $P < 0.001$). To control for this result, the same analysis was performed for randomly permuted data (the individual gamma stability index values were permuted between animals) as well as for a control gamma stability index calculated between Habituation and Early Extinction. Under these conditions the area under the ROC curve was close to 0.5 and non-significant (Figure 3C, Permuted gamma: ROC area = 0.65; control gamma index: ROC area = 0.55). Together these analyses clearly indicate that the persistence and stability of BLA gamma oscillation power during extinction learning is a predictive factor for spontaneous fear recovery at Retrieval.

3.4. Origin of BLA gamma oscillations

The gamma oscillations recorded in the BLA could be generated locally or in remote structures such as the hippocampus, and volume conducted to the BLA. To evaluate these possibilities we tested whether neurons recorded locally in the BLA during Early Extinction display phase-locked activity to BLA gamma oscillations. Phase-locking of unit spiking would indicate a local, rather than a remote, origin of gamma oscillations. We observed that the vast majority of BLA neurons were phase-locked to gamma oscillations recorded in the BLA (Fig 4A, 46/70 BLA neurons, 65.7 %) with a mean preferred phase around the

ascending phase of the gamma oscillation (Fig 4C). The same results were obtained during Late Extinction and Retrieval sessions (data not shown). Next, to further test the possibility that gamma oscillations were volume conducted from a remote structure, we quantified in a subset of animals the strength of neuronal phase-locking to gamma oscillations across electrodes that were located at different depths within the BLA. Quantification of the Mean Resultant Length Vector (MRL) a measure of spiking synchronization with ongoing oscillations, revealed that MRL values were higher when considering the electrode on which the neurons were recorded compared to a distal electrode (Fig. 4D, MRL: Same versus Distal electrode, $P < 0.001$). Together, these observations strongly suggest that the observed gamma oscillations are generated locally within the BLA.

4. Discussion

Using single unit and LFP recordings in behaving mice we found that (i) discriminative auditory fear conditioning induced an increase in the power of gamma oscillations recorded in the BLA (ii) sustained BLA gamma oscillations during extinction learning were associated with spontaneous fear recovery during post-extinction retrieval and (iii) BLA gamma oscillations are likely generated locally. Thus our results indicate that the maintenance of locally generated BLA gamma oscillations predicts long-term recovery of conditioned fear responses.

Our behavioral results indicate that despite a partial extinction of conditioned fear responses, the distribution of freezing levels one week after extinction learning was quite heterogeneous. Indeed animals displayed either low fear or high fear recovery during post extinction retrieval, an effect that cannot be accounted for by a difference in fear acquisition or extinction learning. Similar results have been previously observed in rodents using slightly different behavioral protocols (Burgos-Robles et al., 2007; Herry and Mons, 2004) and are thought to result from deficits in post-extinction consolidation (Burgos-Robles et al., 2007; Herry and Garcia, 2002; Herry, Vouimba, and Garcia, 1999), changes in mPFC to BLA or BLA to mPFC synaptic plasticity at the time of retrieval (Vouimba and Maroun, 2011), or from anatomical and functional deficits developed long before the retrieval session (Bremner et al., 2005; Milad et al., 2009; Milad et al., 2005). Our findings are in accordance with these results and further identify an interesting neuronal mechanism, namely that maintenance of gamma oscillations during extinction learning predicts spontaneous fear recovery. Gamma oscillations in the 30-80 Hz range have been observed in cortical and subcortical structures

(Buzsaki and Wang, 2012; Chrobak and Buzsaki, 1998; Collins, Pelletier, and Pare, 2001; Csicsvari, Jamieson, Wise, and Buzsaki, 2003) and have been linked to temporal encoding (Singer and Gray, 1995), sensory binding (von der Malsburg, 1995), attentional processing (Fries, Reynolds, Rorie, and Desimone, 2001), memory formation and retrieval (Montgomery and Buzsaki, 2007). In particular, the role played by gamma oscillations in memory formation and retrieval is well documented at the level of the hippocampus in rodents, humans, and non-human primates (Colgin and Moser, 2010). In particular, these studies have revealed that the development of gamma oscillations during learning strongly correlates with memory retrieval (Colgin, Denninger, Fyhn, Hafting, Bonnevie, Jensen, Moser, and Moser, 2009; Fell and Axmacher, 2011; Fell, Klaver, Lehnertz, Grunwald, Schaller, Elger, and Fernandez, 2001; Jutras, Fries, and Buffalo, 2009; Sederberg et al., 2003; Sederberg, Schulze-Bonhage, Madsen, Bromfield, Litt, Brandt, and Kahana, 2007a; Sederberg et al., 2007b). Interestingly, although BLA oscillations, particularly in the theta range, have been linked to acquisition, expression, and consolidation of fear behavior (Lesting et al., 2011; Pape and Pare, 2010; Popa, Duvarci, Popescu, Lena, and Pare, 2010; Seidenbecher, Laxmi, Stork, and Pape, 2003), very few studies have evaluated the involvement of BLA gamma oscillations in the acquisition and retrieval of fear memories. In two recent studies, Weinberger and colleagues nicely demonstrated that the strength of gamma oscillations in the auditory cortex, a neuronal structure involved in the encoding of associative fear memories (Letzkus, Wolff, Meyer, Tovote, Courtin, Herry, and Luthi, 2011; Quirk, Armony, and LeDoux, 1997; Weinberger and Diamond, 1987) predicts the acquisition of associative fear memories as measured with conditioned bradycardia (Headley and Weinberger, 2011; 2013). Our results complement these data and extend the general view of gamma oscillation involvement in aversive fear memory formation and retrieval by demonstrating that the maintenance of BLA gamma oscillations following fear conditioning is a strong predictive factor for long-term fear recovery.

From a mechanistic standpoint, gamma oscillations have been linked to $GABA_A$ receptor-mediated fast perisomatic inhibition and in particular to the involvement of fast spiking interneurons (Buzsaki and Wang, 2012; Gulyas, Szabo, Ulbert, Holderith, Monyer, Erdelyi, Szabo, Freund, and Hajos, 2010), although different mechanisms independent of fast spiking interneurons have been reported in the neocortex and the olfactory system (Kay, Beshel, Brea, Martin, Rojas-Libano, and Kopell, 2009; Rojas-Libano and Kay, 2008; Wang, 1999). Given that inhibitory post-synaptic potentials mediated by $GABA_A$ receptors have a time

constant decay ranging between 10 and 25 ms, the timing of gamma oscillations is perfectly suited to support long-term potentiation via spike timing dependent plasticity mechanisms (Campanac and Debanne, 2008; Li, Lu, Wu, Duan, and Poo, 2004). Nevertheless a strong link between fear memory encoding in the BLA, gamma oscillations, and fast spiking interneurons is still lacking and will require additional experiments. Another potential mechanism by which gamma oscillations might promote memory encoding and long-term memory is via the facilitation of synchronization between neuronal structures. For instance, gamma oscillations have been suggested to facilitate interactions between the amygdala and connected neuronal structures during appetitive conditioning (Bauer, Paz, and Pare, 2007; Popescu, Popa, and Pare, 2009). It is unclear, however, how long-range gamma synchronization of neuronal structures such as the BLA and the mPFC might contribute to the regulation of fear behavior. Finally, the identification of strong predictive factors of long-term fear recovery might have important clinical implications, particularly in the context of anxiety and post-traumatic stress disorders which are characterized by long-term recovery of traumatic memories.

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Figure legends

Figure 1 Behavioral protocol and results. A, Experimental protocol that consists of Habituation (Hab.) and Fear Conditioning sessions (FC) performed on Day 1, two Extinction sessions performed 24 and 48 hr following FC (Early and Late Ext. Day 2 and Day 3) and a Retrieval session performed on Day 10 (Ret.). During FC, the CS^+, but not the CS^- was associated with a mild foot-shock (1s, 0.6 mA). Habituation was performed in the conditioning context (▦) whereas Early/Late Extinction and Retrieval sessions were performed in the extinction context (○). **B**, Summary graph of behavioral data. During Habituation, mice (n = 11) exhibited equally low freezing levels in response to CS^- and CS^+ presentations. During Early Extinction, exposure to CS^+ (CS 1-12), but not to CS^-, evoked a significant increase in freezing behavior that was significantly reduced during Late Extinction. One week later, presentations of CS^+, but not CS^-, induced spontaneous recovery of conditioned fear responses. **C**, Distribution of conditioned fear responses during CS^+ presentations at Retrieval (n = 11). **D**, Correlation analysis performed between freezing levels recorded during the first 4 CS^+ presentations during Early Extinction and during CS^+ presentations at Retrieval. **E**, Correlation analysis performed between freezing levels recorded on the last block of 4 CS^+ presentations during Late Extinction and during CS^+ presentations at Retrieval. **F**, Locations of the recording sites in the BLA and representative

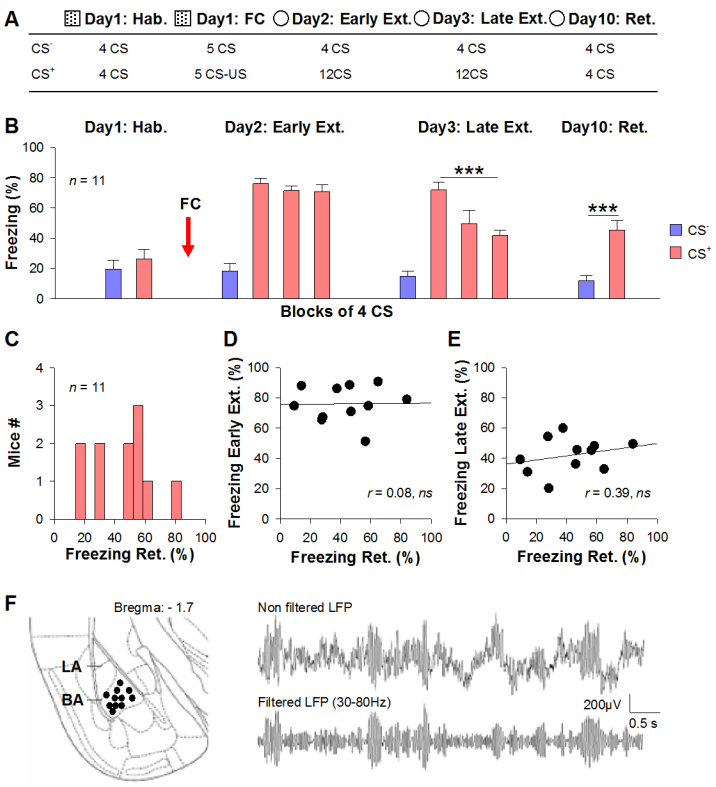
non filtered and filtered LFPs in the gamma range (30 to 80 Hz) (LA, Lateral Amygdala; BA, Basal Amygdala). Error bars indicate mean ± SEM, *** $P < 0.001$.

Figure 2 Dynamics of BLA gamma oscillations during extinction learning. A, Power spectral density of a representative LFP recorded in the BLA and quantification of the relative power of gamma oscillations for all the animals, (n = 11) during Habituation, Early and Late Extinction and Retrieval sessions. **B**, Relative power of Gamma oscillations during specific periods of CS^- (blue) and CS^+ presentations (Dark red bars during Habituation, Early Extinction and Retrieval corresponds to the first block or 4 CS^+ of the behavioral session; light red bars during Late Extinction corresponds to the last block or 4 CS^+ of the behavioral session). Fear conditioning increases gamma power during CS^+ presentations whereas extinction learning reduces it significantly. In all the behavioral sessions, CS^+ presentations evoked significantly more gamma power than CS^- presentations. During Retrieval, gamma power during CS^+ presentations was not different from the Late Extinction session. **C**, Correlation analysis performed between gamma relative power and freezing levels for the first block of 4 CS^+ during Early Extinction and the last block of 4 CS^+ during Late Extinction. **D**, Individual values of gamma power for all mice during the first block of 4 CS^+ during Early Extinction and the last block of 4 CS^+ during Late Extinction Error bars indicate mean ± SEM, * $P < 0.05$, *** $P < 0.001$.

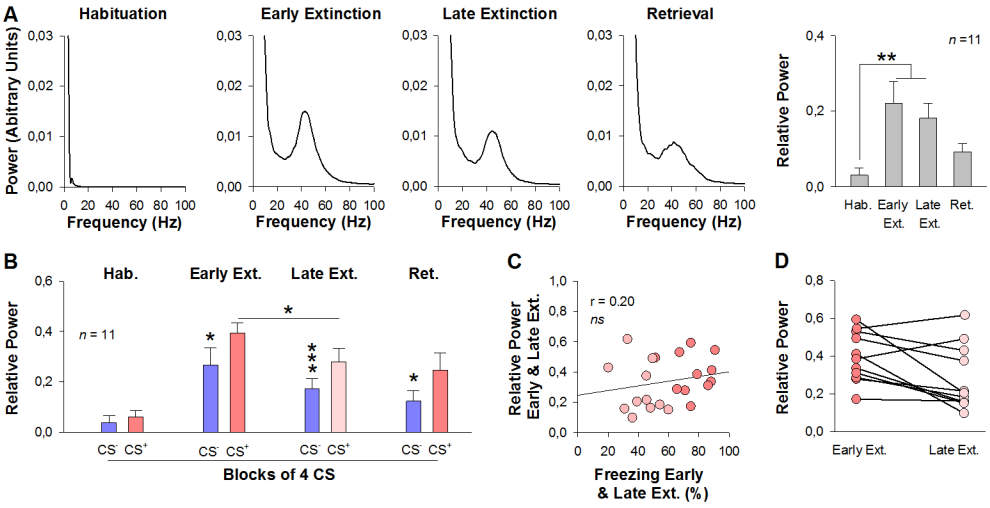
Figure 3 Maintenance of BLA gamma oscillations during extinction learning predicts spontaneous fear recovery. A, Distribution of gamma power ratio calculated between Early Extinction and Late Extinction (n = 11 mice). **B**, Correlation analysis performed between the gamma power ratio established between Late and Early Extinction sessions and freezing levels evoked CS^+ presentations during Retrieval **C**, Left: ROC curves illustrating the accuracy of gamma power stability during extinction learning to predict spontaneous fear recovery at Retrieval. The black line represents the chance level. Right: Area Under the ROC Curve (AUC) values for the different conditions. Error bars indicate mean ± SEM, *** $P < 0.001$.

Figure 4 Origin of BLA gamma oscillations. A, Raster plots and peri-stimulus time histogram of a representative BLA neuron showing a strong gamma modulation (22 min recordings, the red dashed line at 0 indicates peaks of gamma oscillations, bins of 10 ms). **B**, Firing modulation of the same BLA neuron to BLA gamma oscillations filtered in the 30-80

Hz range (0 correspond to the peak of the gamma cycle, bins of 20°). **C**, Cumulative distribution of log transformed Rayleigh's test Z of BLA neurons modulated by BLA gamma oscillations (30-80 Hz, Early Extinction, n = 70 neurons). The black dashed line indicates significant gamma phase locking threshold (Ln (Z) = 1.1, $P < 0.05$, n = 46/70 neurons). Inset: distribution of the preferred firing phases within gamma cycle for significantly gamma phase locked BLA neurons (n = 46; 0 correspond to the peak of the gamma cycle). **D**, Quantification of MRL to compare the strength of gamma phase locking for BLA neurons recorded in the same electrode as the LFP or in a distal electrode (n = 6). Error bars indicate mean ± SEM, *** $P < 0.001$.



Courtin et al., Fig. 1



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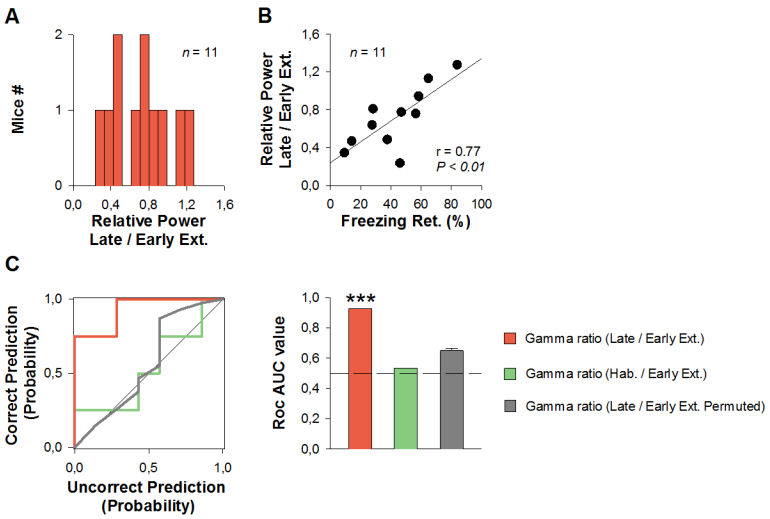

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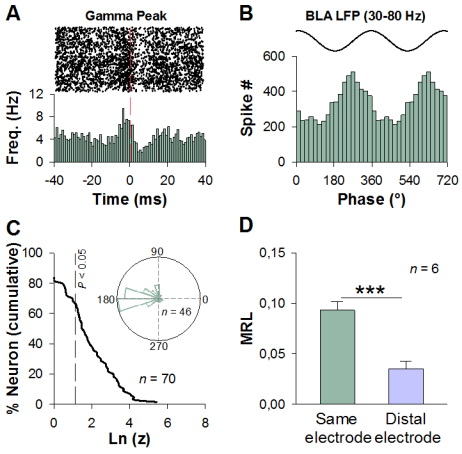

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