Neuroscience 321 (2016) 222–235

PREVENTING LONG-LASTING FEAR RECOVERY USING BILATERAL ALTERNATING SENSORY STIMULATION: A TRANSLATIONAL STUDY

H. WURTZ, [a,b†] **M. EL-KHOURY-MALHAME,** [c†] **F. H. WILHELM,** [d] **T. MICHAEL,** [e] **E. M. BEETZ,** [f] **J. ROQUES,** [g] **E. REYNAUD,** [h] **J. COURTIN,** [a,b] **S. KHALFA** [h,i*†] **AND C. HERRY** [a,b*†]

[a] *INSERM, Neurocentre Magendie, U862, 146 Rue Léo-Saignat, 33077 Bordeaux, France*

[b] *Univ. Bordeaux, Neurocentre Magendie, U862, 146 Rue Léo-Saignat, 33077 Bordeaux, France*

[c] *Notre Dame University, Faculty of Humanities, Department of Psychology, Zouk Mikael BP 72, Lebanon*

[d] *Division of Clinical Psychology and Psychotherapy and Health Psychology, Department of Psychology, University of Salzburg, Hellbrunnerstrasse 32, A-5020 Salzburg, Austria*

[e] *Division of Clinical Psychology and Psychotherapy, Department of Psychology, Saarland University, Saarbrucken, Germany*

[f] *Department of Psychiatry and Medical Psychology, Princess Grace Hospital Center, Monaco, France*

[g] *Center for Treatment of Psychological Trauma, Montpellier, France*

[h] *Institut de Neurosciences de la Timone, UMR 7289, Marseille, France*

[i] *Aix-Marseille Université, Marseille, France*

Abstract—Posttraumatic stress disorder (PTSD) is a highly debilitating and prevalent psychological disorder. It is characterized by highly distressing intrusive trauma memories that are partly explained by fear conditioning. Despite efficient therapeutic approaches, a subset of PTSD patients displays spontaneous recurrence of traumatic memories after successful treatment. The development of animal behavioral models mimicking the individual variability in treatment outcome for PTSD patients represent therefore an important challenge as it allows for the identification of predicting factors of resilience or susceptibility to relapse. However, to date, only few animal behavioral models of long-lasting fear recovery have been developed and their predictive validity has not been tested directly. The objectives of this study were twofold. First we aimed to develop a simple animal behavioral model of long-lasting fear recovery based on auditory cued fear conditioning and extinction learning, which recapitulates the heterogeneity of fear responses observed in PTSD patients after successful treatment. Second we aimed at testing the predictive validity of our behavioral model and used to this purpose a translational approach based (i) on the demonstration of the efficiency of Eye Movement Desensitization and Reprocessing (EMDR) therapy to reduce conditioned fear responses in PTSD patients and (ii) on the implementation in our behavioral model of an electrical bilateral alternating stimulation of the eyelid which mimics the core feature of EMDR. Our data indicate that electrical bilateral alternating stimulation of the eyelid during extinction learning alleviates long-lasting fear recovery of conditioned fear responses and dramatically reduces inter-individual variability. These results demonstrate the face and predictive validity of our animal behavioral model and provide an interesting tool to understand the neurobiological underpinnings of long-lasting fear recovery.

This article is part of a Special Issue entitled: Neuropsychiatric Disease.

Key words: fear recovery, fear conditioning, PTSD, bilateral alternating stimulation, Eye Movement Desensitization and Reprocessing.

INTRODUCTION

Anxiety disorders are among the most frequent psychiatric conditions with a lifetime prevalence of around 6–8% in the population worldwide (Breslau et al., 1998; Kessler, 2000). In particular, posttraumatic stress disorder (PTSD) represents one of the most frequent anxiety disorders, which can develop following the experience of a traumatic event. PTSD patients exhibit a number of symptoms including re-experiencing of the traumatic event (flashback, nightmare), avoidance of places or objects associated with the initial trauma, fear generalization and hyperarousal (APA, 2000). Although, current therapeutic approaches for anxiety disorders are often associated with short-term improvement of these anxiety-related symptoms, a fraction of PTSD patients display long-lasting relapse of traumatic memories after successful treatment (Rachman, 1979; Foa et al., 1991; Rodriguez et al., 1999; Resick et al., 2002, 2012; Boschen et al., 2009; Vervliet et al., 2013). Thus it is of strong clinical interest to develop animal models reproducing human fear relapse to further understand and identify the underlying neurobiological mechanisms. Over the past years, several animal models of PTSD have been developed using various stressors, which

*Correspondence to: S. Khalfa, Institut de Neurosciences de la Timone, UMR 7289, Marseille, France. C. Herry, INSERM, Neurocentre Magendie, U862, 146 Rue Léo-Saignat, 33077 Bordeaux, France.

E-mail addresses: stephanie.khalfa@gmail.com (S. Khalfa), cyril.herry@inserm.fr (C. Herry).

† These authors contributed equally to this work.

Abbreviations: BAS, bilateral alternating stimulations; CS, conditioned stimulus; EMDR, Eye Movement Desensitization and Reprocessing; IL, infralimbic; ITI, intertrial interval; MPSS, Modified PTSD Symptoms Scale; PL, prelimbic; Post-FC, Post-Fear Conditioning; PTSD, posttraumatic stress disorder; SC, skin conductance; SCL, SC level; SCR, SC response; US, unconditioned stimulus.

http://dx.doi.org/10.1016/j.neuroscience.2015.06.012
0306-4522/

reproduced specific PTSD symptoms such as generalization of fear responses to non traumatic places or stimuli, resistance to extinction (an analog of exposure therapies in humans), hyperarousal, and avoidance of trauma-related stimuli (Siegmund and Wotjak, 2006; Goswami et al., 2013; Goode and Maren, 2014). To date however, only few attempts have been made to develop animal models mimicking PTSD long-lasting relapse of traumatic fear memories following successful treatment (Deschaux et al., 2011; Goode and Maren, 2014).

In the laboratory, traumatic aversive experiences are usually induced using the classical auditory fear-conditioning paradigm which consists in the repetitive association between a neutral stimulus (the conditioned stimulus (CS), usually a tone or a light) with a mild electrical footshock (the unconditioned stimulus (US)). Following conditioning, re-exposure to the CS induces a broad range of conditioned fear responses including an immobilization reaction labeled freezing, which represents a reliable behavioral measure of the learned association. Inhibition of conditioned fear behavior can be observed following repetitive exposure to the CS without the US in a context different from the original conditioning context, a process labeled fear extinction. Interestingly, following extinction learning, re-exposure to the extinction context, the original conditioning context, or unsignaled footshocks can lead to relapse of fear behavior (Myers and Davis, 2007; Herry et al., 2010; Goode and Maren, 2014), although it is not clear if this occurs in all the individuals tested. Individual variability is nowadays considered as a critical component of PTSD animal models because it allows identifying predicting factors of resilience or susceptibility to traumatization (Cohen et al., 2012; Goswami et al., 2013; Daskalakis and Yehuda, 2014). Unfortunately, most of the animal models of PTSD currently available do not evaluate individual susceptibility to relapse after successful fear extinction (but see Goswami et al., 2010).

In the present manuscript, we pursue to main objectives. First we developed a simple behavioral model of long-lasting fear recovery using auditory cued fear conditioning and extinction learning based on individual variability. Second, we validated our model using a translational approach based on (i) the identification of a valid therapeutic approach to reduce conditioned fear responses in PTSD patients and (II) on the implementation in our behavioral model of this therapeutic approach developed in humans. Our behavioral results in mice indicate that following successful extinction, mice display either maintenance of fear extinction or long-lasting fear recovery, which replicates the heterogeneity of fear responses observed in PTSD patients after successful treatment. We next tested the validity of our behavioral model and used a translational approach to this purpose. We first evaluated in PTSD patients undergoing classical fear conditioning and extinction, the efficiency of Eye Movement Desensitization and Reprocessing (EMDR) therapy to reduce PTSD symptoms and conditioned fear reactions. Among the therapeutic approaches to treat PTSD patients, EMDR is one of the most efficient and recommended therapies (Foa et al., 2009; WHO, 2013). In summary, it consists first in the assessment of cognitive, emotional and physical aspects of actual distress to traumatic scenes, and second in imaginal exposure to the traumatic event in association with bilateral alternating stimulations (BAS) (i.e. either auditory, visual, or somatosensory stimuli alternating between the two sides of the body) (Servan-Schreiber et al., 2006). The major therapeutic action of EMDR is thought to be the association of the patient traumatic memory with BAS (Shapiro, 1996; Herkt et al., 2014). In a second step, we implemented in behaving animals an electrical BAS of the eyelid applied during fear extinction, which mimics the core feature of the EMDR procedure, to evaluate if this stimulation alleviates long-lasting fear recovery, and therefore inter-individual variability to relapse, in our behavioral model.

EXPERIMENTAL PROCEDURE

Animals

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).

Surgery

Mice were anesthetized with isoflurane (induction 3%, maintenance 1.5%) in O_2. Body temperature was maintained with a temperature controller system (FHC, Bowdoin, ME, USA). Mice were secured in a stereotaxic frame and bilaterally implanted in muscle above the eyelid with stimulating electrodes. The electrodes consisted of silver wires (127-μm inner diameter, Phymep, Paris, France) and were attached to a four pins connector (Omnetics, Minneapolis, MN, USA). All implants were secured using Super-Bond cement (Sun Medical, Moriyama, Shiga, Japan). After surgery mice were allowed 7 days to recover and habituated to handling. Analgesia was applied before, and 1 day after surgery (Metacam, Boehringer, Ingelheim am Rhein, Germany).

Animal behavioral apparatus

Fear conditioning and extinction took place in two different contexts (A and B). The conditioning and extinction boxes were cleaned with 70% ethanol or 1% acetic acid before and after each session, respectively. To score freezing behavior an automated infrared beam detection system located on the bottom of the experimental chambers was used (Coulbourn Instruments, Whitehall, PA, USA). The animals were considered to be freezing if no movement was detected for 2 s.

Experiments in behaving animals

Twenty five and 41 C57BL6/J mice were used for to establish the behavioral model and validity testing, respectively. To establish the behavioral model, C57BL6/J mice were submitted on Day 1 to a discriminative fear-conditioning protocol in context A, in which they received five presentations of the CS^+ or 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). The CS^+ was paired with a US (1 s foot-shock, 0.6 mA, 5 CS^+/US pairings; inter-trial interval: 20–180 s, 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). On Days 2 and 3, conditioned mice were submitted to extinction training (Post-Fear Conditioning (Post-FC) and Extinction (Ext.) sessions) in context B during which they first received 4 CS^- presentations followed by 12 presentations of the CS^+. Retrieval of extinction was tested 7 days later in context B, with four presentations of the CS^- and the CS^+. To test the validity of our behavioral model, we used the same behavioral paradigm as above in implanted (BAS mice) and non implanted animals (Control mice) except that during extinction learning, electrical BAS of the eyelid (100 ms pulses at 100 μA delivered on each eyelid at 1 Hz, 500-ms delay between stimulation of the ipsilateral and contralateral eyelid) was applied during the last 8 CS^+ of the first extinction session (Post-FC) and during the entire last extinction session (Ext.). Electrical BAS of the eyelids mimic the somatic stimulations than can be used in EMDR therapy (Servan-Schreiber et al., 2006). We used a subthreshold 100 μA stimulation intensity which does not elicit pain reaction in mice (as assessed using vocalizations monitoring), nor noticeable changes in locomotor behavior. All mice were tested 7 days later in context B, with four presentations of the CS^- and the CS^+. A subset of BAS ($n = 9$) and control ($n = 7$) animals were also tested 57 days after the last extinction session in context B, with four presentations of the CS^- and the CS^+ to evaluate long-lasting fear recovery. To evaluate the specificity of the BAS protocol, we used an additional group of control animals ($n = 11$) that were submitted to the same protocol as BAS animals except that the stimulation was delivered unilaterally (UnS) (par100-ms pulses at 100 μA delivered unilaterally at 1 Hz).

Human subjects

A total of 23 adult outpatients (eight males and 15 females) were recruited at the medico-psychological crisis cell (CUMP) at the Psychiatry Pole of the Conception Hospital in Marseille, France. They all met the DSM-IV criteria for PTSD following a single traumatic event (12 aggressions, four road accidents, six work-related accidents, one grief) with no previous history of neurologic or psychiatric disorders. Subsequent analysis included 17 patients (seven males and 10 females, with mean age = 44 ± 15 years, mean education = 7.3 ± 2.7 years after grade 7, and mean time since trauma exposure = 18.4 months). Six patients were excluded from data analysis as two of them terminated the study prematurely, two of them had only partial symptom reduction and two of them were reluctant to undergo the electric stimulation. Five patients were on combined regimen of antidepressants and anxiolytics, two patients only took antidepressants and two only took anxiolytics. A total of 18 healthy adult controls (nine males and nine females, with mean age = 37 ± 14 years and mean education = 8.9 ± 2 years after grade 7) with no history of neurologic or psychiatric disorders, were recruited via screening lists at the clinical investigation center at the Timone Hospital (CIC-UPCET). They were matched to patients for age, sex and education.

Psychological assessment

All participants were assessed by a psychiatrist for PTSD and other mental health disorders using the structured Mini-Internal Neuropsychiatric Interview for DSM-IV. This allowed us to check for the absence of psychiatric disorders prior to the trauma in PTSD and screen for potential comorbid psychiatric disorders. Participants responded to demographic questions and completed the trauma-related scales: PTSD Check List Scale (PCL-S), and Modified PTSD Symptoms Scale (MPSS). The validated French versions were used for the two scales.

EMDR therapy

All PTSD patients underwent EMDR therapy. EMDR is based on an adaptive information processing model (Shapiro and Maxfield, 2002). The patient is asked to visualize the most salient aspect of a traumatic memory, the therapist induces bilateral stimulation (by means of ocular, sensory-motor or auditory left/right stimulation) (Shapiro, 1989). Patients were treated by one of three therapists, all trained by the French institute of EMDR. There was no fixed number of sessions. Sessions were planned every 7–15 days according to patients and therapists availabilities. The treatment was considered successful and complete when patients reported no more feelings of distress when thinking about their trauma. They were again interviewed by a psychiatrist, using the MINI. They were retested when they no longer met PTSD classification according to DSM-IV criteria. Patients required an average of 4.3 ± 1.7 treatment sessions (ranging from 1 to 7 sessions), over a period of 2.5 ± 1.3 months.

Apparatus and physiological recordings

An electrical stimulator (constant current unit, Biopac Systems, Inc., Goleta, CA, USA) was used to deliver the US through a bar electrode with concave tin-plated disks attached to participants left lower arm. This US was generated by varying the dial setting on a stimulus isolation adapter, for a current ranging from 0.1 to 5.0 mA. It was isolated from line current and used a 9-V

dry battery attached to an adjustable transformer. Stimulus delivery and physiological data acquisition were controlled by two PCs running E-Prime (Psychology Software Tools, Inc., Pittsburgh, PA, USA) and Acqknowlege software (Biopac Systems, Inc., Goleta, CA, USA) respectively. Physiological channels and rating dial information were recorded at a rate of 1000 Hz using the Biopac MP150 system. Skin conductance (SC) reflecting activation of the sympathetic nervous system was measured in microSiemens using two 5-mm inner diameter Ag/AgCl electrodes filled with isotonic paste. Electrodes were placed on the medial phalanges of the index and middle finger of the left hand. Since deep breathing and/or coughing may trigger SC artifacts, respiration pattern was recorded using a pneumographic belt with a respiration transducer at the rib cage, toward the end of the sternum.

Procedure

The investigation was carried out in accordance with the latest version of the Declaration of Helsinki. Participants provided informed consent in accordance with local ethical committee guidelines set forth by the CPP committee South Mediterranean 2. The experiment took place in a temperature-controlled, fully lit, and sound-attenuated room. Participants were comfortably seated at 60 cm viewing distance from a 17'' computer screen, with a refresh rate of 100 Hz. Electrodes were attached and the respiratory belt put in place. The conditioning task was the one developed by Blechert and colleagues (Blechert et al., 2007) and consisted of three different phases: habituation, acquisition and extinction. The US was a 500-ms electric shock previously determined by the participant to be ''highly annoying but not painful'' using the up-down staircase method. This was done by gradually incrementing shock intensity, while participants rated its averseness using a digital analog scale (from ''Not annoying'' = 0; to ''Highly annoying'' = 100). Once the shock intensity was determined, it was kept constant for the rest of the conditioning task.

The habituation phase started with written instructions telling participants that two pictures would be shown on the screen and that there would be no shock delivery. It consisted of 6 trials of each CS^+ and CS^-. CS^+ and CS^- images were obtained from the Rorschach inkblot test and were counterbalanced across participants. Images were presented for 8 s. The mean intertrial interval (ITI) was 18 s (range 16–20 s). At the *acquisition phase*, participants were instructed that two pictures will be shown on the screen and that only one would be occasionally followed by the electric shock. It consisted of 6 trials of each CS type and each CS^+ was followed by the US. No instructions were shown at the *extinction phase* that consisted of 6 CS^+ and 6 CS^-. CS valence ratings were repeatedly obtained. Six valence ratings were obtained for each CS in the middle and the end of each conditioning phase (every third CS was rated, yielding a total of 12 ratings). During these rating trials, a visual analog scale appeared on the screen, 4 s after CS offset, prompting participants to give retrospective valence ratings (''How did you find the last picture?'' ratings ranged from ''Pleasant'', 0; to ''Unpleasant'', 100). Upon completion of the rating, the ITI started. Previous research established that these ratings do not influence the psychophysiological outcome variables in a differential aversive conditioning paradigm (Blechert et al., 2008). Following extinction training, contingency awareness was assessed by a screen presenting the CS^+, the CS^- and a control stimulus and asking which of the three pictures had previously been paired with the US. This recognition measure of contingency awareness is considered more sensitive than post-experimental questionnaires, which require recall of contingency knowledge (Lovibond and Shanks, 2002). The experimental protocol was administered twice for all participants: before treatment (P_1) and immediately after symptom amelioration (P_2) for the PTSD group, and in matching time lags ($P_2 - P_1$) for the control group.

Statistical analyses

For animal data, statistical analyses of behavior was performed by a 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 or unpaired 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. To separate animals displaying low or high rebound of conditioned fear responses at the retrieval test we used an unsupervised cluster algorithm based on the Ward's method. Briefly, the Euclidian distance was calculated between all mouse pairs based on the two-dimensional space defined by each mouse rebound percentage. An iterative agglomerative procedure was then used to combine mice into groups based on the matrix of distances such that the total number of groups was reduced to give the smallest possible increase in the within-group sum of square deviation. Fear rebound values were calculated as the percentage of freezing observed during CS^+ presentations at Retrieval divided by freezing observed during the first block of CS^+ presentations (CS^+ 1–4) during the Post-FC session.

For human data, similar to Blechert et al. (2007), SC response (SCR) was quantified as a valid and sensitive indicator for the degree of arousal associated with amygdala activation (Critchley, 2002). A SCR response was calculated for each CS trial by subtracting the mean SC level (SCL) during the 2 s immediately prior to CS onset from the highest SCL recorded during the 8-s CS presentation. This method has been documented to be more adequate in a differential fear-conditioning paradigm than the alternative scoring method that consisted of measuring either the First or the Second Interval Response (Pineles et al., 2009). The present scoring method allows for the detection of the maximal increase in SCR at any point during the 8-s presentations. SCRs below 0.01 μS were scored as zero and square root transformation was applied to normalize the SC distribution. SCR to each CS-type (CS^+, CS^-) was averaged on three consecutive presentations, resulting in two blocks per conditioning

phase (e.g. first and second half of habituation). Artifact correction for SCRs consisted of a visual inspection of respiration and the manual exclusion of SCR that appeared to be influenced by coughs, sights or deep breaths (about 5% of each CS type was excluded). Two participants in each group had no electrodermal conditioning, i.e. they had no SCR greater than 0.01 for any of the CS during acquisition. Their data were removed from subsequent analyses. A two-way repeated measures ANOVA was used on SCRs and verbal assessments for each conditioning phase separately with Group (control, PTSD patients) as a between-subject factor and Session (1, 2), CS-type (CS^+, CS^-) and Time (first half, second half) as within-subject factors. Significant main effects at $P < 0.05$ were followed by post hoc *t*-tests using Bonferroni correction. All post hoc tests were tested at an alpha level $P < 0.05$.

RESULTS

Behavioral animal model of long-lasting fear recovery

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, b, Day 2, Post-FC, CS^- vs. first block of CS^+: $P < 0.001$). A one-factor repeated measures ANOVA performed on CS^+ presentations during Post-FC and Ext. sessions indicated a significant decrease of freezing levels over extinction trials ($F_{24,5} = 26.827$, $P < 0.001$). A direct comparison between the first block of CS^+ presentations during the Post-FC session and the last block of CS^+ presentations during the Ext. session confirmed these results as freezing levels were significantly lower on the last compared to the first block of CS^+ presentations ($P < 0.001$). Finally, when tested one week after extinction during the Retrieval session, mice displayed a significant difference in freezing levels between CS^- and CS^+ presentations ($P < 0.01$). This result indicates that mice display recovery of conditioned fear responses during the retrieval session. To further evaluate the distribution of conditioned fear responses among individuals during the retrieval session, we computed the rebound in conditioned fear responses by dividing freezing levels observed during retrieval (Retrieval session, CS^+ 1–4) with those calculated after conditioning (Post-FC session, CS^+ 1–4). Interestingly, during the retrieval session, mice display heterogeneous fear rebound values (Fig. 1c left, lowest value: 13.69%; highest value: 127.92%). Hierarchical cluster analyses (see method section) performed on those data revealed two main clusters of animals displaying low ($n = 12$ mice; mean value: 29.4 ± 5.3% rebound) and high ($n = 13$ mice; mean value: 91.4 ± 3.5% rebound) rebound values that were significantly different (Fig. 1c right, low vs. high rebound: $P < 0.01$). Finally we re-examined the kinetic of extinction learning for the two groups of animals displaying low or high rebound values at retrieval (Fig. 1d). Before conditioning or during CS^- presentations at the Post-Fc session, freezing levels were similar for Low- and High-fear animals. A two-factor (Group × Time) repeated measures ANOVA performed on CS^+ presentations during Post-FC and Ext. sessions indicated a significant effect of the time ($F_{23,5} = 27.282$, $P < 0.001$) but not group or interaction of time and group (all $Ps > 0.05$). Finally, when tested one week after extinction during the Retrieval session, Low-fear and High-fear mice displayed a significant difference in freezing levels for CS^+ presentations ($P < 0.001$). Although there was no significant statistical difference between Low- and High-fear animals during CS^- presentations, we noticed a tendency for lower freezing levels in Low- compared to High-fear mice. Importantly, Low- and High-fear mice exhibited similar freezing levels during baseline, CS^- or CS^+ presentations after conditioning and fear levels after extinction learning were not significantly different between low- and High-fear animals. These data indicate that the difference in fear levels observed between Low- and High-fear animals during retrieval cannot be due to basal anxiety levels, or impairment in fear conditioning and extinction learning. All together these results indicate that following successful extinction learning C57BL6/J mice displayed an heterogeneous profile in fear-conditioned responses at retrieval, with some animals displaying low-fear responses whereas others displaying high-fear responses.

Fear conditioning in PTSD and healthy controls before and after EMDR treatment

Groups did not differ in age, sex and education. Table 1 shows the psychometric measures for the two groups. Patients' scores on PTSD scales were initially higher than the cut-off for pathology and significantly dropped to normal levels after treatment termination. Patients met the criteria for the following major current comorbid diagnoses (before/after EMDR): major depression ($n = 10/4$), other anxiety disorders ($n = 15/8$) and medium-to-high suicidal risk ($n = 6/0$). We did not find any difference between the two groups for the selected intensity of stimulation, or its averseness.

During habituation, a two-factor (Group × Time) repeated measures ANOVA performed for valence scores during CS^+ presentations failed to reveal any significant differences before or after the EMDR treatment (Fig. 2a, b, all $Ps > 0.05$). During acquisition, a three-factor (Group × CS-type × Session) repeated measures ANOVA performed on CS^+ presentations revealed a significant interaction between Time, Group and Session ($F_{1,30} = 5.24$, $P < 0.05$). Post hoc analyses indicated that both groups exhibited differential ratings for CS^+ vs. CS^- during fear conditioning (C1 and C2) (all $Ps < 0.05$). Before EMDR, the PTSD group has more aversive ratings of CS^+ during fear conditioning (C1 and C2) and more pleasant ratings of CS^- during C1 than the control group (all $Ps < 0.05$), but not after treatment. During extinction, the same analysis indicated a significant Session × CS-type × Group interaction ($F_{1,28} = 4.31$, $P < 0.05$). Only the PTSD group Pre-EMDR had differential ratings of CS^+ and CS^-. Post hoc analyses showed that similarly

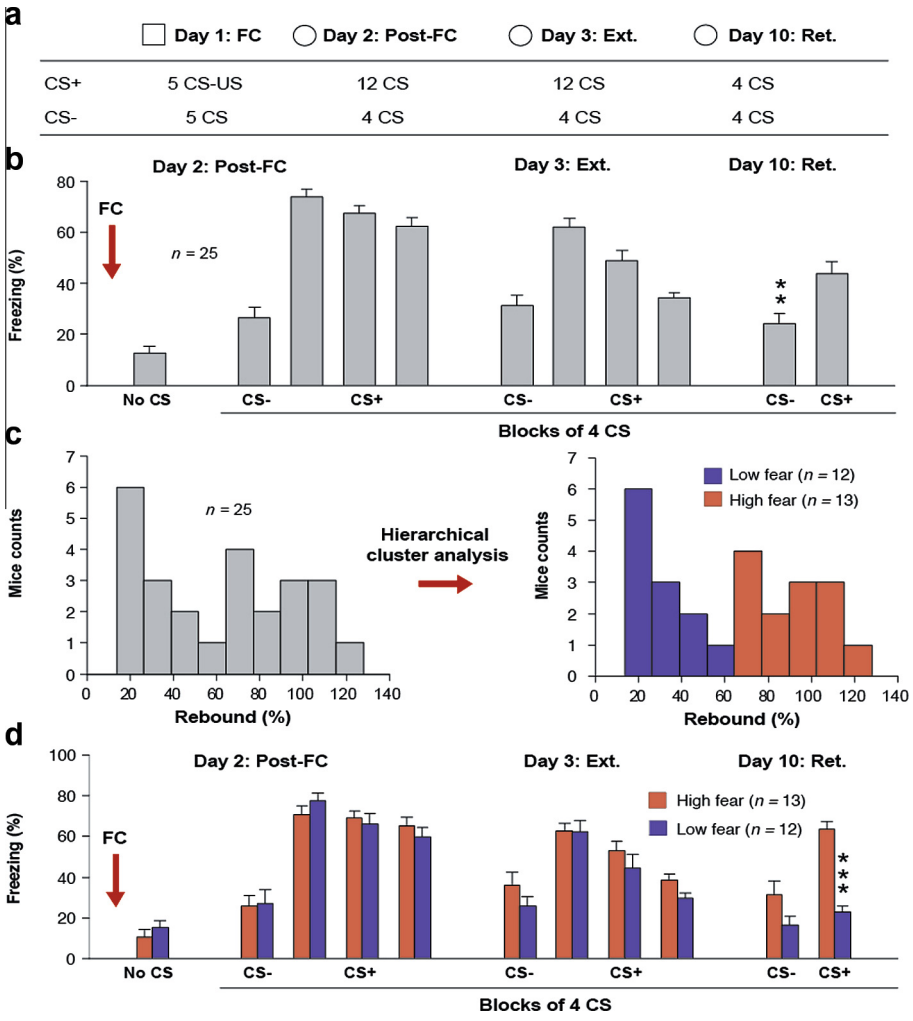

Fig. 1. Bimodal distribution of conditioned fear responses after successful extinction. (a) Behavioral protocol. (b) Average freezing behavior observed in mice (n = 25) before (no CS), and in response to CS^- and CS^+ exposure during the 2 days of extinction (Day 2: Post-FC and Day 3: Ext.) following fear conditioning (FC), and one week after extinction for the retrieval session (Day 10: Ret.). (c) Left, Distribution of freezing rebound values (calculated as freezing levels during CS^+ at retrieval divided by freezing levels during the first block of CS^+ during Post-FC) (n = 25 mice). Right, Hierarchical cluster analyses revealed two clusters of mice exhibiting high (High-fear mice, n = 13) or low (Low-fear mice, n = 12) freezing rebound values. (d) Percentage of freezing behavior observed in High-(n = 13) and Low-(n = 12) fear mice (n = 25) before (no CS), and in response to CS^- and CS^+ exposure during the 2 days of extinction (Day 2: Post-FC and Day 3: Ext.) following fear conditioning (FC), and one week after extinction for the retrieval session (Day 10: Ret.). Error bars: mean ± s.e.m. $^{**}P < 0.01$; $^{***}P < 0.001$.

Table 1. Characteristics of participants: Means (SD) for the patients PTSD Check List Scale (PCL-S), Modified PTSD Symptoms Scale (MPSS), and Impact of Event Scale (IES), stimulation level and its aversiveness. F values are the result of ANOVA for Group × Scale interaction and t values are the result of paired t-test for PTSD patients before and after EMDR. Significant p-value: $^{**}P < 0.001$ and NS: not significant

	Control 1 (n = 18)	Control 2 (n = 18)	PTSD before (n = 17)	PTSD after (n = 18)	Statistics
PCL-S	–	–	62.4 (11.7)	29.2 (6.3)	t (1,16) = 10.82**
MPSS	–	–	73.5 (23.2)	22.1 (9.5)	t (1,16) = 9.97**
Stimulation level (mA)	1.9 (1.6)	1.4 (1.4)	10.4 (19)	8.9 (15.7)	NS
US averseness rating (0–100)	5.4 (18.8)	12.16 (14.5)	10.35 (26.9)	8.9 (16.5)	NS

to acquisition, the PTSD group initially has more aversive ratings of CS^+ and more pleasant ratings of CS^- than the control group during E1 and E2 (all $Ps < 0.05$), whereas after treatment groups have comparable evaluations (Fig. 2b) (all $Ps > 0.05$).

A three-factor ANOVA of physiological responses during habituation indicated a main significant effect of Time ($F_{1,30}$ = 30.49, $P < 0.05$). Post hoc analyses revealed greater SCR for both groups at H1 compared to H2. PTSD patients had slightly larger SCR than controls during habituation (all $Ps < 0.05$) (Fig. 2c). This difference was however not significant and might be related to the generally higher levels of anxiety of PTSD patients or could reflect anticipation or attentional processes related to the novel task. During acquisition, a significant Session × CS-type × Group interaction was

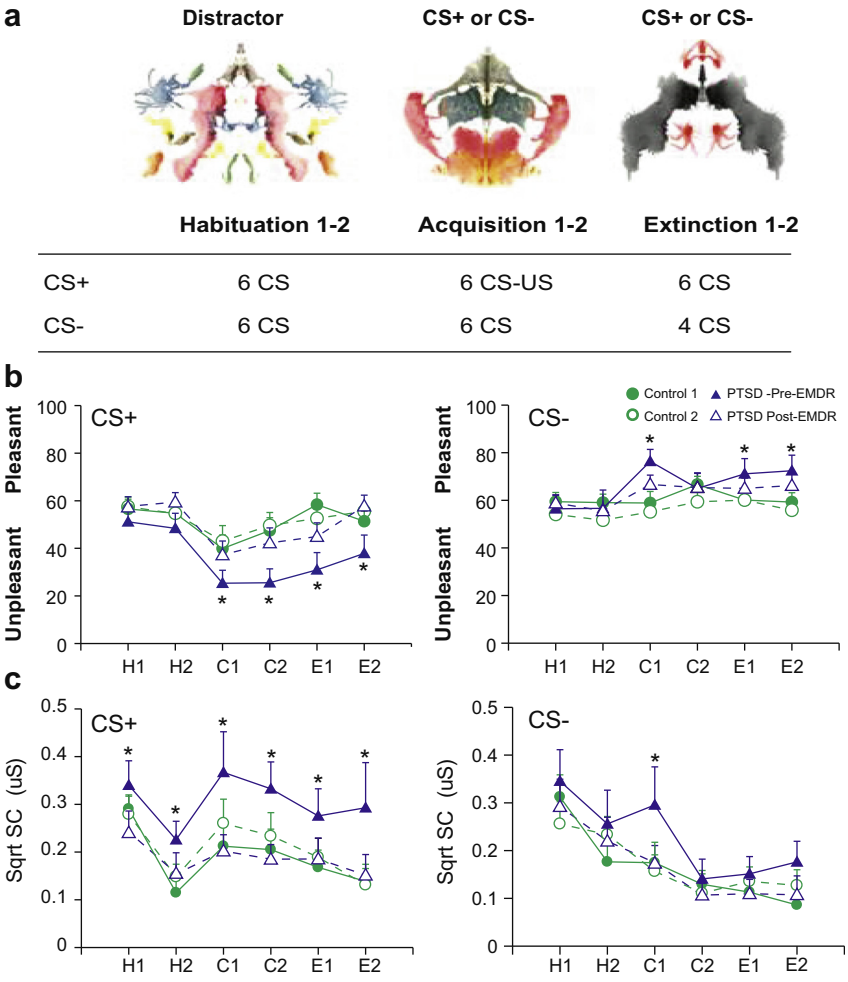

Fig. 2. Fear conditioning and extinction learning in PTSD patients before and after EMDR treatment. (a) Behavioral protocol. (b) Average verbal evaluations of CS^+ (left) and CS^- (right) valence rating in healthy controls and PTSD patients before (PTSD-Pre-EMDR (n = 18) and Control 1 (n = 17) groups) and after (PTSD-Post-EMDR (n = 18) and Control 2 (n = 17) groups) EMDR treatment during habituation (H1–H2), acquisition (C1–C2) and extinction (E1–E2) of conditioned fear responses. Ratings were done on a scale from 0 to 100. (c) Average SCR (square root transformed (Sqrt SCR) to CS^+ (left) and CS^- (right) presentations for healthy controls and PTSD patients before (PTSD-Pre-EMDR and Control 1 groups) and after (PTSD-Post-EMDR and Control 2 groups) EMDR treatment during habituation (H1–H2), acquisition (C1–C2) and extinction (E1–E2) of conditioned fear responses. Error bars: mean ± s.e.m. PTSD Pre-EMDR vs. other groups $^*P < 0.05$.

found (Fig. 2c; $F_{1,28} = 4.18$, $P < 0.05$). Both groups displayed differential SCRs and had higher responses to CS^+ compare to CS^- presentations (all $Ps < 0.05$). The PTSD group also had higher SCR to CS^+ and CS^- compared to controls, only before EMDR (all $Ps < 0.05$) but not after. During extinction, there was a significant Session × CS-type × Group interaction ($F_{1,28} = 4.07$, $P < 0.05$), which reflected higher SCR to CS^+ presentations in the PTSD Pre-EMDR group. After successful treatment, SCR was comparable in patients and controls. No significant interactions were found for CS^-. During acquisition, PTSD patients also had larger SCRs compared to controls (Fig. 2c). The SCR difference was significant, indicating that PTSD patients seemed to acquire/exhibit physiologically more intense fear responses. To ensure that subsequently higher SCR at extinction were not merely due to a generalized heightened reactivity at acquisition, we calculated the values of SCR at extinction whereby E′1 = E1-C2 and E′2 = E2-C2. A two-factor ANOVA (Session × Group) revealed a significant interaction at E′2 ($F_{1,27} = 5.24$, $P < 0.05$).

Because comorbid disorders and/or medication could possibly alter fear conditioning in PTSD, we evaluated the effect of anxiety, depression or medication on electrodermal recordings and verbal evaluations during acquisition and extinction. These factors were entered separately as covariates in the Session × CS-type × Time × Group ANOVAs. Statistical analysis revealed that all three covariates were not significantly interfering with the main interaction found for either SCR or verbal responses. Finally, statistical analysis revealed a significant correlation for SCRs and MPSS scores change from pre- to post-therapy during fear conditioning (C1) and fear extinction (E2). The difference between the MPSS scores before and after treatment, (i.e., the MPSS scores decrease), is larger when the difference in SCRs before and after treatment,

(i.e., the SCR decrease) is higher (Pearson Correlation index $r = 0.46$, $n = 15$, $P < 0.05$, $r = 0.51$, $n = 15$, $P < 0.05$). The larger the symptoms decrease the larger the SCR decrease.

Effects of eyelid bilateral alternating electrical stimulation during extinction on long-lasting fear recovery

To validate our behavioral model of long-lasting fear recovery, we implemented in behaving animals an electrical BAS of the eyelid applied during fear extinction, which mimics the core feature of the EMDR procedure. Twenty-four hours following auditory fear conditioning, BAS and control animals displayed similar levels of freezing before CS presentations and discriminated between CS^- and CS^+ as revealed by a significant increase in freezing levels evoked by CS^+ presentations, compared to CS^- presentations (Fig. 3a, b, Day 2, Post-FC, CS^- vs. first block of CS^+: all $Ps < 0.001$). BAS and control animals exhibited however significantly different freezing levels during CS^- presentations in the Post-FC session ($P < 0.05$). A two-factor (Group × Time) repeated measures ANOVA performed on CS^+ presentations during Post-FC and Ext. sessions indicated a significant effect of Group ($F_{1,39} = 8.903$, $P < 0.05$) and Time ($F_{39,5} = 70.891$, $P < 0.001$) but not of the interaction between Group and Time. A direct comparison between the first block of CS^+ presentations during the Post-FC session and the last block of CS^+ presentations during the Ext. session confirmed these results as freezing levels were significantly lower on the last compared to the first block of CS^+ presentations for each group (all $Ps < 0.001$). Moreover, the BAS group displayed reduced fear levels compared to control animals during the Post-FC session as revealed by a significant difference between the two groups on the second and third blocks of CS + presentations (all $Ps < 0.05$). Finally, when tested one week after extinction during the Retrieval session, mice from each group displayed a significant difference in freezing levels between CS^- and CS^+ presentations (all $Ps < 0.001$). Moreover, BAS animals displayed lower freezing responses to CS^- and CS^+ presentations compared to control animals (all $Ps < 0.001$). These results clearly indicate that BAS facilitated extinction learning and prevented long-lasting fear recovery during the retrieval session. To further evaluate the distribution of conditioned fear responses among individuals during the retrieval session, we computed the rebound in conditioned fear responses (see methods). Importantly, during the retrieval session, control mice displayed heterogeneous fear rebound values (Fig. 3c left, lowest value: 12.12%; highest value: 113.42%; mean: 61.17 ± 6.27%), whereas BAS animal exhibited low-fear rebound values (lowest value: 4.9%; highest value: 40.3%; mean: 22.8 ± 2.68%). A direct comparison between fear rebound values revealed a significant difference between BAS and control animals ($P < 0.001$). To make sure that the distribution of fear rebound values between BAS and control animals were not merely due to extinction learning rate or by baseline anxiety levels, we performed correlational analyses. These analyses performed between freezing levels before conditioning and during the retrieval session (CS^+ presentations), or between freezing levels at the end of extinction learning (Ext. session, last block of CS^+ presentations) and at retrieval (CS^+ presentations) failed to reveal any significant correlations (Fig. 3d, e). These data clearly indicate that low freezing values observed in BAS animals at retrieval cannot be explained by differences in anxiety or by fear extinction rate.

BAS effect is long-lasting and more efficient than unilateral eyelid electrical stimulation

To evaluate if the shift toward low-fear rebound value in BAS animals observed at Retrieval (Day 10) was persistent over time, we tested a subset of BAS animal ($n = 9$) during a second retrieval session performed 50 days later (Day 60). Although BAS animals had a tendency to display higher fear rebound values at the second retrieval session (Fig. 4a), there was however no significant differences in fear rebound values between the two sessions (Fig. 4b). Importantly, rebound values observed for BAS animals at the second retrieval session were still significantly different from values observed for control animals at Day 10 ($P < 0.05$) and Day 60 ($P < 0.01$). Finally, to control for the specificity of our electrical stimulation, we performed an additional control experiment during which the BAS was replaced by a unilateral electrical stimulation of the eyelid (UnS, $n = 11$ mice). Interestingly, whereas fear levels following conditioning (Post-FC, CS^+ 1–4) or extinction learning (Ext., CS^+ 9–12) were not significantly different between BAS and UnS animals, UnS mice displayed higher fear levels when tested during retrieval compared to BAS animals (Fig. 4c, $P < 0.05$). Consistently, analyses of the distribution of fear rebound values revealed a larger heterogeneity of fear rebound values for UnS animals compared to BAS mice (Fig. 4c right, UnS: lowest value: 9.33%; highest value: 82.07%; mean: 40.58 ± 6.17%; BAS animal lowest value: 4.9%; highest value: 40.3%; mean: 22.8 ± 2.68%; UnS vs. BAS: $P < 0.05$).

DISCUSSION

In the present study, we propose a simple behavioral model of inter-individual variability following successful extinction learning, which recapitulates the heterogeneity of fear responses in PTSD patients following successful treatment (Foa et al., 1991; Resick et al., 2002, 2012). Our data revealed that conditioned fear responses evaluated one week after extinction learning were highly heterogeneous with half of the animals displaying maintenance of fear extinction (Low-fear mice) whereas the other half exhibited recovery of conditioned fear responses (High-fear mice). This heterogeneity in conditioned fear responses at retrieval was not related to the level of conditioned fear acquired, or extinction rate, not basal anxiety levels as Low- and High-fear mice exhibited similar fear levels before fear conditioning,

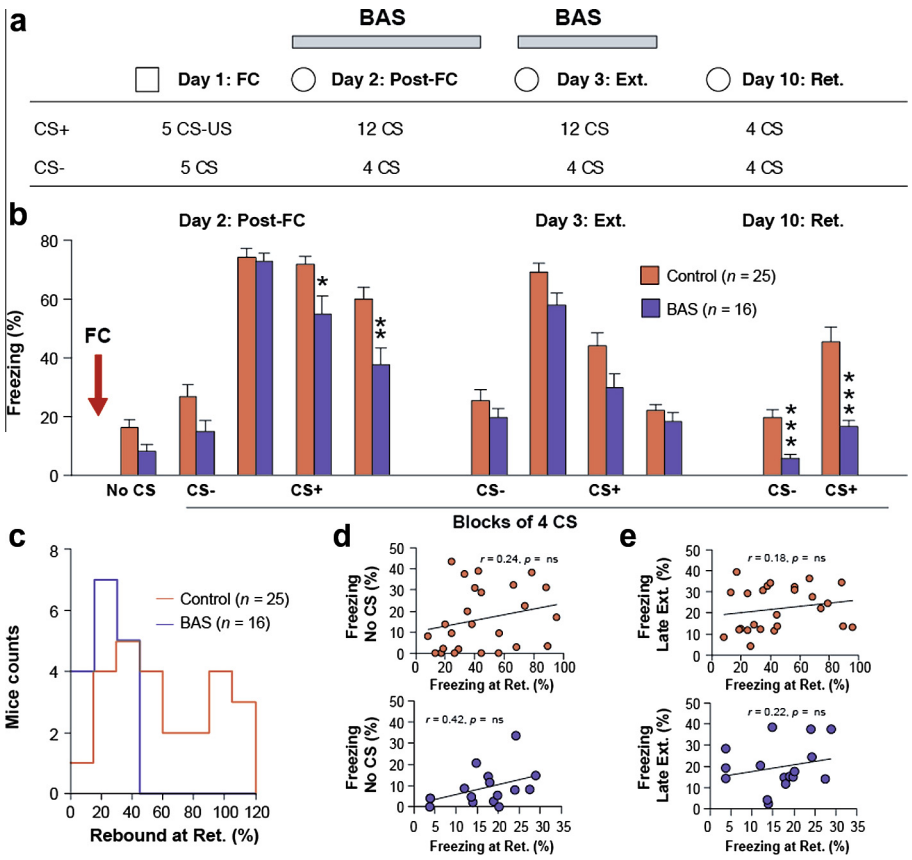

Fig. 3. Electrical bilateral alternating stimulation alleviates long-lasting fear recovery. (a) Behavioral protocol. (b) Percentage of freezing behavior observed in control mice (n = 25) and mice exposed to electrical bilateral alternating stimulation (BAS, n = 16) before (no CS), and in response to CS^- and CS^+ exposure during the 2 days of extinction (Day 2: Post-FC and Day 3: Ext.) following fear conditioning (FC), and one week after extinction for the retrieval session (Day 10: Ret.). (c) Distribution of freezing rebound values (calculated as freezing levels during CS^+ at retrieval divided by freezing levels during the first block of CS^+ during Post-FC) for control (n = 25) and BAS (n = 16) mice. (d) Correlation analyses performed between freezing values observed during CS^+ presentations at retrieval (Day 10: Ret.) and during baseline (Day 2: No CS) for control (top, n = 25) and BAS (bottom, n = 16) mice. (e) Correlation analyses performed between freezing values observed during CS^+ presentations at retrieval (Day 10: Ret.) and during the last block of CS + presentations during extinction (Day 3: Ext.) for control (top, n = 25) and BAS (bottom, n = 16) mice. Error bars: mean ± s.e.m. $^*P < 0.05$; $^{**}P < 0.01$; $^{***}P < 0.001$.

during CS^- presentations at the Post-FC session and during extinction learning.

Over the past 15 years, a number of studies have clearly identified the neuronal circuits and mechanisms leading to the maintenance of fear extinction memory or the spontaneous recovery of conditioned fear responses. For instance, it is well documented that the prelimbic (PL) and infralimbic (IL) areas of the medial prefrontal cortex (mPFC) are involved in long-lasting expression or inhibition of conditioned fear responses following extinction learning (for review see Sotres-Bayon and Quirk, 2010; Courtin et al., 2013). Lesional and inactivation of the PL were associated with a reduction in conditioned fear responses (Joel et al., 1997; Akirav et al., 2006; Blum et al., 2006; Sierra-Mercado et al., 2006; Corcoran and Quirk, 2007). In contrast, the same manipulations applied to the IL induced high-fear levels (Quirk et al., 2000; Lebron et al., 2004; Tian et al., 2011). Electrical stimulation experiments confirmed the previous results as PL and IL micro-stimulation increased and decreased conditioned fear responses, respectively (Vidal-Gonzalez et al., 2006; Burgos-Robles et al., 2009). Furthermore, extracellular recordings performed in the PL and IL identified neurons whose activity correlated with fear expression and inhibition, respectively (Herry and Garcia, 2002; Milad and Quirk, 2002; Milad et al., 2004; Vidal-Gonzalez et al., 2006; Burgos-Robles et al., 2007, 2009; Courtin et al., 2014). Finally blockade of extinction consolidation using NMDA receptor antagonists or anisomycin injections in the IL or blockage of noradrenergic and dopaminergic receptors precipitate conditioned fear recovery (Santini et al., 2004; Pfeiffer and Fendt, 2006; Burgos-Robles et al., 2007; Hikind and Maroun, 2008; Mueller et al., 2008, 2009; Sotres-Bayon et al., 2009).

Based on these available data the most parsimonious explanation of our results is that Low-fear animals consolidated fear extinction memory whereas fear recovery in High-fear animals was triggered by a lack of cellular consolidation. Importantly, although some variability in conditioned fear responses following extinction were observed in previous studies (Burgos-Robles et al., 2007; Milad et al., 2007), our study identifies two subgroups of individuals displaying opposite fear responses at retrieval. Inter-individual variability in fear responses has been previously observed in PTSD

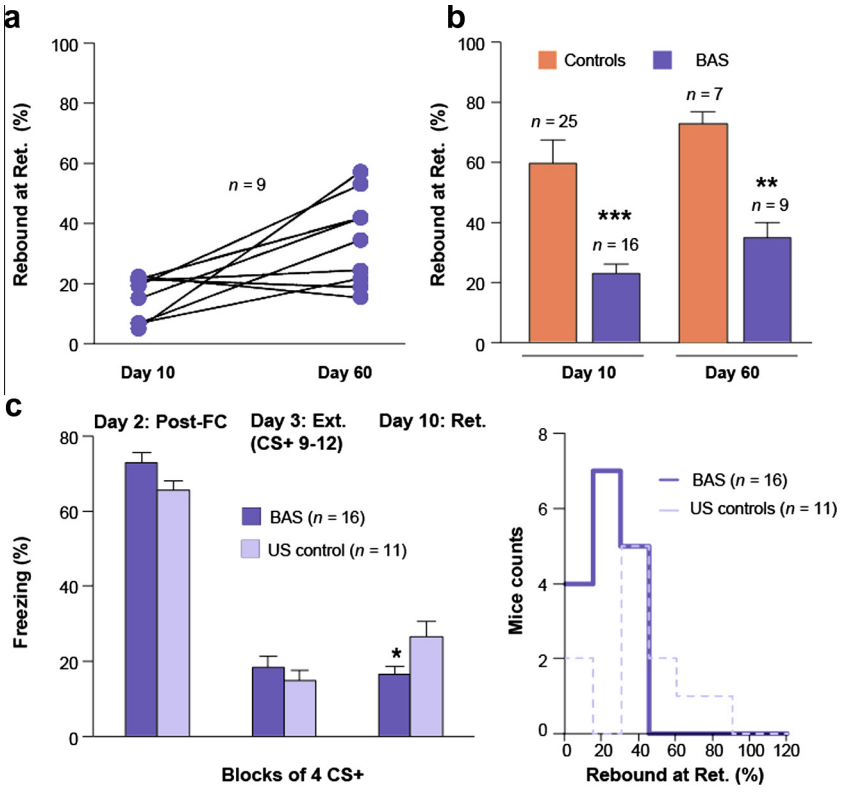

Fig. 4. BAS alleviation of fear recovery is long lasting and more efficient than unilateral electrical stimulation. (a) Distribution of freezing rebound values (calculated as freezing levels during CS^+ at retrieval divided by freezing levels during the first block of CS^+ during Post-FC) recorded at retrieval 7 (Day 10) or 57 (Day 60) days after extinction learning for BAS animals ($n = 9$). (b) Average freezing rebound values recorded at retrieval 7 (Day 10) or 57 (Day 60) days after extinction for control (Day 10: $n = 25$; Day 60: $n = 7$) and BAS (Day 10: $n = 16$; Day 60: $n = 9$). (c) Left, Average freezing behavior observed in BAS ($n = 16$) and control mice submitted to unilateral stimulation (UnS: $n = 11$) in response to CS^- and CS^+ exposure during the 2 days of extinction (Day 2: Post-FC, first block of CS^+, and Day 3: Ext., last block of CS^+) following fear conditioning, and one week after extinction for the retrieval session (Day 10: Ret.). Right, Distribution of freezing rebound values (calculated as freezing levels during CS^+ at retrieval divided by freezing levels during the first block of CS^+ during Post-FC) for BAS ($n = 16$) and UnS ($n = 11$) mice. Error bars: mean ± s.e.m. $^*P < 0.05$; $^{***}P < 0.001$.

patients following successful treatment (Rachman, 1979; Rodriguez et al., 1999; Boschen et al., 2009; Vervliet et al., 2013) or following classical fear conditioning and extinction learning (Milad et al., 2009). Individual variability is considered a critical component of several pathological conditions including addiction behavior (Piazza et al., 1989; Deroche-Gamonet et al., 2004) and PTSD (Cohen et al., 2012; Goswami et al., 2013; Daskalakis and Yehuda, 2014) because it allows identifying predicting factors of resilience or susceptibility to pathology. Thus, our behavioral model of inter-individual variability of conditioned fear responses following extinction learning captures a key feature of PTSD and thereby exhibits high face validity.

To further test if our model could predict some treatment outcome in PTSD patients (predictive validity) we first evaluated in PTSD patients submitted to classical fear conditioning and extinction, the efficiency of EMDR therapy to reduce PTSD symptoms and conditioned fear reactions. These experiments revealed major psychophysiological deficiencies in PTSD pathology in processing fear behavior using a classical fear-conditioning procedure. First and foremost we have replicated the electrodermal data and verbal assessment results presented by Blechert et al. (2007) using the same conditioning paradigm. These data confirmed previous observations of increased fear conditioning and delayed extinction in PTSD patients (Pole, 2007; Wessa and Flor, 2007; Lommen et al., 2013; Sijbrandij et al., 2013) Importantly, our results indicate that immediately after symptom amelioration by successful EMDR therapy, fear processing in PTSD patients (at acquisition and extinction) was restored to normal. More precisely, EMDR therapy decreases patients' scores on PTSD scales, from pathological to normal levels. This result is consistent with the well-established clinical and therapeutic effectiveness of EMDR (Foa et al., 2009; WHO, 2013).

Verbal ratings of CS valence gave a fair overview of the fluctuation of human perception of fear acquisition and extinction levels toward a neutral stimulus as a function of its association with an aversive shock. At habituation, PTSD patients initially had lower valence assessment likely related to their higher task-derived anxiety compared to controls. They also had higher threat expectancy and lower valence assessment at extinction, even when the CS^+ was no longer coupled to the aversive shock. PTSD patients indeed display a contingency bias in ambiguous situations (Blechert et al., 2007). This might account for instance for the tendency toward increased SCR reactivity at the second half

of extinction E2. This increased anxiety during anticipation of unpredictable stimuli is rather specific to PTSD and not to other anxiety disorders (Grillon et al., 2009), and might clinically relate to their generalized hypervigilance in the presence of aversive cues (Ehlers and Clark, 2000). In the current study, we found that unlike controls, PTSD patients also initially showed enhanced fear conditioning as revealed by verbal assessments. These results are consistent with previous findings of Orr and Roth (2000) who found that during conditioning, PTSD patients exhibited larger differential SC responses to the CS^+ vs. CS^- compared with the non-PTSD group (Orr and Roth, 2000). Results on verbal assessments reflect increased fear sensitization patterns similar to SCRs at acquisition. At extinction however, only verbal evaluations showed differential conditioning in PTSD with patients having more aversive ratings than controls. Along with verbal ratings, SCR seems to be the most sensitive maker to differential fear condition in PTSD. Similarly to Blechert et al. (2007), we found reduced extinction learning in PTSD. More particularly, PTSD patients have been shown to exhibit elevated SCR at acquisition and delayed SCR decrease at extinction (Orr and Roth, 2000; Peri et al., 2000; Blechert et al., 2007). In our study, during the first habituation session (H1) both patients and controls had higher SCR than during the second habituation session (H2), indicating that all subjects were more reactive at the beginning of the experiment; SCR being sensitive to novelty effect. Similarly to Orr and Roth (2000) and Blechert et al. (2007), we found that PTSD patients have higher SCR than controls to both CS^+ and CS^- at acquisition, and only to CS^+ at extinction (but see Grillon and Morgan, 1999). These results indicate that PTSD patient's responses to CS^-, but not CS^+ normalize by the end of the extinction session. In contrast to our data, previous studies failed to observe enhanced fear conditioning in PTSD patients (Orr and Roth, 2000; Milad et al., 2008). These discrepancies could be explained by the age of the studied population, the medications, and placebo effects. Importantly, our results indicate that EMDR therapy restored normal fear conditioning and extinction learning in PTSD patients as assessed by both implicit (physiological) and explicit (verbal) measures. These data provide preliminary evidence that fear processing alterations might be linked to PTSD symptomatology. This is further supported by significant correlations between differences in MPSS scores and differences in SCR before and after EMDR at C1 and E2. Specifically, symptom decrease strongly correlated with decreases in fear conditioning and extinction in PTSD patients. Interestingly, the elevated psychophysiological responses to fear conditioning and extinction in PTSD decreased after only an average 4.3 EMDR sessions. The biological basis of this fast process remains unknown but may rely upon structures involved both in PTSD and in fear conditioning and extinction such as the amygdala, hippocampus, and prefrontal cortex.

Although some of the PTSD patients evaluated in our study were also on medical regimen for antidepressants and/or anxiolytics (seven of 17 patients), the comorbidity profiles of patients included in this study are similar to those reported in most published studies dealing with PTSD, typically involving other anxiety and mood disorders. The group of PTSD patients studied was too small to distinguish subgroups of medicated vs. non-medicated and pure vs. heterogeneous PTSD diagnosis. Still, our results are consistent with those of non-medicated samples (Orr and Roth, 2000), and drug regimens remained stable for the study duration, and unlikely affected explicit evaluations (US expectancy, valence) of conditioning. Moreover we noted depression, generalized anxiety disorder, social anxiety and panic disorder were the most frequent comorbidities in PTSD patients. They do not seem to account for the results as we found no significant changes in the main interactions when they were entered as covariables. Nonetheless, their alterations of physiological markers cannot be totally ruled out (Lissek et al., 2005, 2008). It would be useful in future studies, to explore drug and comorbidity interaction with larger PTSD subpopulations with or without medication, and with or without comorbidities. Moreover, larger group of subjects in further experiments will also improve the strength of results. Another limitation arises by the inability to retest the drop-outs, who were mostly out of reach or refused to be retested, making it harder to assess the effect of the testing/retesting on fear conditioning and extinction in PTSD patients. On one hand, we argue against the mere effect of ''passage of time'', as patients have had PTSD symptoms for 18.4 months and showed no signs of spontaneous recovery. On the other hand, we argue against the effect of ''learning'' at the retest (i.e. by simply attending the paradigm twice). Controls do indeed show conditioned fear responses at their first and second testing as they have comparably high SCR at acquisition each time. Moreover, at both session 1 and 2, the PTSD group had similarly increased SCR at H1 and similarly elevated US aversiveness ratings indicating that repeating the paradigm did not seem to attenuate its induced anxiety. Taken together, these data indicate that EMDR treatment alleviates PTSD symptoms and normalized fear and extinction behavior in PTSD patients.

Because BAS-based EMDR treatment significantly improved fear extinction and alleviated PTSD symptoms we reasoned that applying BAS treatment during fear extinction in our animal model might impact extinction learning rate as well as long-lasting fear recovery. Importantly, in our study, we used electrical BAS of the eyelids in order to mimic the somatic stimulations than can be used in EMDR therapy (Servan-Schreiber et al., 2006) but not necessarily to promote eye movements. Interestingly, the BAS procedure applied in fear-conditioned mice facilitated extinction learning on the Post-FC session. In addition, this manipulation had a major impact on the distribution of conditioned fear responses at retrieval as revealed by low-fear responses in all animals submitted to this protocol compared to control animals. Importantly, correlational analyses revealed that these results could not be explained by higher anxiety levels or to fear levels at the end of extinction further arguing for the specificity of the BAS stimulation. However, the control group used in this study was not submitted to the

surgical procedure and this difference with BAS animals could also participate to the effect observed. Furthermore, we observed a significant effect of the BAS protocol on freezing levels to the CS- at Retrieval further suggesting that the BAS manipulation may also reduced general anxiety levels. Finally, our data revealed that this effect was lasting up to 50 days after the extinction session and that it was more pronounced using bilateral compared to unilateral eyelid stimulation.

These results demonstrate first that fear recovery following extinction learning can be significantly and efficiently reduced by application of a subthreshold BAS of the eyelid during fear extinction. Second, because BAS-based EMDR treatment is also efficient in alleviating PTSD symptoms in patients submitted to a fear conditioning and extinction protocol, this strongly suggests that our behavioral model displays high face and predictive validity. These results also open interesting questions as to what the underlying neuronal mechanisms involved in the reduction of fear recovery following BAS stimulation are. Currently, the mechanisms underlying EMDR therapy are still unknown. Even if very various hypothesis have been proposed (Bergmann, 2010; Oren and Solomon, 2012) none of them has been demonstrated. Given that not only eye movements but also auditory and somatic stimulations are effective in the treatment, the EMDR effect would not only depend upon eye movement, but may favor grounding and prevention of dissociation in patients.

Over the past years, several hypotheses have been proposed (Stickgold, 2002; Gunter and Bodner, 2009; Bergmann, 2010) although only few studies have directly explored the neuronal mechanisms associated with the EMDR treatment. From a functional perspective, EEG and fMRI studies performed during EMDR treatment indicated higher activity in prefrontal structures including the vmPFC (Richardson et al., 2009; Pagani et al., 2012), which is in line with the previously described prefrontal abnormalities in PTSD patients (Hughes and Shin, 2011). In parallel, EMDR treatment is associated with an increased activity of several structures including the amygdala and thalamus (Richardson et al., 2009), which may further suggest an important interplay between prefrontal cortex and these regions during EMDR treatment. More invasive approaches in rodents, such as extracellular single units and local field potential recordings and sophisticated genetic and molecular approaches will be required to further understand the neuronal mechanisms underlying the reduction of fear recovery following BAS stimulation. Our behavioral model in conjunction with BAS stimulation may therefore represent an interesting tool to investigate the neuronal mechanisms associated with the reduction of inter-individual variability fear responses following successful extinction learning.

Acknowledgments—We thank the members of the Khalfa and Herry laboratory for helpful discussions and comments on the manuscript. This work was supported by grants from the French National Research Agency (ANR-2010-BLAN-1442-01; ANR-10-EQPX-08 OPTOPATH; LABEX BRAIN ANR 10-LABX-43, LABEX TRAIL ANR 10-LABX-57), the European Research Council (ERC) under the European Union's Seventh Framework Program (FP7/2007-2013)/ERC grant agreement no. 281168, the Conseil Regional d'Aquitaine to C.H. and the CNRS (S.K.).

REFERENCES

Akirav I, Raizel H, Maroun M (2006) Enhancement of conditioned fear extinction by infusion of the GABA(A) agonist muscimol into the rat prefrontal cortex and amygdala. Eur J Neurosci 23:758–764.

APA (2000) Diagnostic and statistical manual of mental disorders. 4th ed, text revision. Washington, DC: American Psychiatric Association.

Bergmann U (2010) EMDR's neurobiological mechanisms of action: a survey of 20 years of searching. J EMDR Pract Res 4:22–42.

Blechert J, Michael T, Vriends N, Margraf J, Wilhelm FH (2007) Fear conditioning in posttraumatic stress disorder: evidence for delayed extinction of autonomic, experiential, and behavioural responses. Behav Res Ther 45:2019–2033.

Blechert J, Michael T, Williams LS, Purkis HM, Wilhelm FH (2008) When two paradigms meet: does evaluative learning extinguish in differential fear conditioning? Learn Motiv 39:58–70.

Blum S, Runyan JD, Dash PK (2006) Inhibition of prefrontal protein synthesis following recall does not disrupt memory for trace fear conditioning. BMC Neurosci 7:67.

Boschen MJ, Neumann DL, Waters AM (2009) Relapse of successfully treated anxiety and fear: theoretical issues and recommendations for clinical practice. Aust N Z J Psychiatry 43:89–100.

Breslau N, Kessler RC, Chilcoat HD, Schultz LR, Davis GC, Andreski P (1998) Trauma and posttraumatic stress disorder in the community: the 1996 Detroit Area Survey of Trauma. Arch Gen Psychiatry 55:626–632.

Burgos-Robles A, Vidal-Gonzalez I, Quirk GJ (2009) Sustained conditioned responses in prelimbic prefrontal neurons are correlated with fear expression and extinction failure. J Neurosci 29:8474–8482.

Burgos-Robles A, Vidal-Gonzalez I, Santini E, Quirk GJ (2007) Consolidation of fear extinction requires NMDA receptor-dependent bursting in the ventromedial prefrontal cortex. Neuron 53:871–880.

Cohen H, Kozlovsky N, Alona C, Matar MA, Joseph Z (2012) Animal model for PTSD: from clinical concept to translational research. Neuropharmacology 62:715–724.

Corcoran KA, Quirk GJ (2007) Activity in prelimbic cortex is necessary for the expression of learned, but not innate, fears. J Neurosci 27:840–844.

Courtin J, Bienvenu TC, Einarsson EO, Herry C (2013) Medial prefrontal cortex neuronal circuits in fear behavior. Neuroscience 240:219–242.

Courtin J, Chaudun F, Rozeske RR, Karalis N, Gonzalez-Campo C, Wurtz H, Abdi A, Baufreton J, Bienvenu TC, Herry C (2014) Prefrontal parvalbumin interneurons shape neuronal activity to drive fear expression. Nature 505:92–96.

Critchley HD (2002) Electrodermal responses: what happens in the brain. Neuroscientist 8:132–142.

Daskalakis NP, Yehuda R (2014) Principles for developing animal models of military PTSD. Eur J Psychotraumatol:5.

Deroche-Gamonet V, Belin D, Piazza PV (2004) Evidence for addiction-like behavior in the rat. Science 305:1014–1017.

Deschaux O, Motanis H, Spennato G, Moreau JL, Garcia R (2011) Re-emergence of extinguished auditory-cued conditioned fear following a sub-conditioning procedure: effects of hippocampal and prefrontal tetanic stimulations. Neurobiol Learn Mem 95:510–518.

Ehlers A, Clark DM (2000) A cognitive model of posttraumatic stress disorder. Behav Res Ther 38:319–345.

Foa EB, Rothbaum BO, Riggs DS, Murdock TB (1991) Treatment of posttraumatic stress disorder in rape victims: a comparison between cognitive-behavioral procedures and counseling. J Consult Clin Psychol 59:715–723.

Foa EB, Keane TM, Friedman MJ, Cohen JA (2009) Effective treatments for PTSD: practice guidelines of the international society for traumatic stress studies. New York: Guilford Press.
Goode TD, Maren S (2014) Animal models of fear relapse. ILAR J 55:246–258.
Goswami S, Rodriguez-Sierra O, Cascardi M, Pare D (2013) Animal models of post-traumatic stress disorder: face validity. Front Neurosci 7:89.
Goswami S, Cascardi M, Rodriguez-Sierra OE, Duvarci S, Pare D (2010) Impact of predatory threat on fear extinction in Lewis rats. Learn Mem 17:494–501.
Grillon C, Morgan 3rd CA (1999) Fear-potentiated startle conditioning to explicit and contextual cues in Gulf War veterans with posttraumatic stress disorder. J Abnorm Psychol 108:134–142.
Grillon C, Pine DS, Lissek S, Rabin S, Bonne O, Vythilingam M (2009) Increased anxiety during anticipation of unpredictable aversive stimuli in posttraumatic stress disorder but not in generalized anxiety disorder. Biol Psychiatry 66:47–53.
Gunter RW, Bodner GE (2009) EMDR works. But how? Recent progress in the search for treatment mechanisms. J EMDR Pract Res 3:161–168.
Herkt D, Tumani V, Gron G, Kammer T, Hofmann A, Abler B (2014) Facilitating access to emotions: neural signature of EMDR stimulation. PLoS One 9:e106350.
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, Ferraguti F, Singewald N, Letzkus JJ, Ehrlich I, Luthi A (2010) Neuronal circuits of fear extinction. Eur J Neurosci 31:599–612.
Hikind N, Maroun M (2008) Microinfusion of the D1 receptor antagonist, SCH23390 into the IL but not the BLA impairs consolidation of extinction of auditory fear conditioning. Neurobiol Learn Mem 90:217–222.
Hughes KC, Shin LM (2011) Functional neuroimaging studies of post-traumatic stress disorder. Expert Rev Neurother 11:275–285.
Joel D, Tarrasch R, Feldon J, Weiner I (1997) Effects of electrolytic lesions of the medial prefrontal cortex or its subfields on 4-arm baited, 8-arm radial maze, two-way active avoidance and conditioned fear tasks in the rat. Brain Res 765:37–50.
Kessler RC (2000) Posttraumatic stress disorder: the burden to the individual and to society. J Clin Psychiatry 61(Suppl. 5):4–12 [discussion 13–14].
Lebron K, Milad MR, Quirk GJ (2004) Delayed recall of fear extinction in rats with lesions of ventral medial prefrontal cortex. Learn Mem 11:544–548.
Lissek S, Powers AS, McClure EB, Phelps EA, Woldehawariat G, Grillon C, Pine DS (2005) Classical fear conditioning in the anxiety disorders: a meta-analysis. Behav Res Ther 43:1391–1424.
Lissek S, Levenson J, Biggs AL, Johnson LL, Ameli R, Pine DS, Grillon C (2008) Elevated fear conditioning to socially relevant unconditioned stimuli in social anxiety disorder. Am J Psychiatry 165:124–132.
Lommen MJ, Engelhard IM, Sijbrandij M, van den Hout MA, Hermans D (2013) Pre-trauma individual differences in extinction learning predict posttraumatic stress. Behav Res Ther 51:63–67.
Lovibond PF, Shanks DR (2002) The role of awareness in Pavlovian conditioning: empirical evidence and theoretical implications. J Exp Psychol Anim Behav Process 28:3–26.
Milad MR, Quirk GJ (2002) Neurons in medial prefrontal cortex signal memory for fear extinction. Nature 420:70–74.
Milad MR, Vidal-Gonzalez I, Quirk GJ (2004) Electrical stimulation of medial prefrontal cortex reduces conditioned fear in a temporally specific manner. Behav Neurosci 118:389–394.
Milad MR, Wright CI, Orr SP, Pitman RK, Quirk GJ, Rauch SL (2007) Recall of fear extinction in humans activates the ventromedial prefrontal cortex and hippocampus in concert. Biol Psychiatry 62:446–454.
Milad MR, Orr SP, Lasko NB, Chang Y, Rauch SL, Pitman RK (2008) Presence and acquired origin of reduced recall for fear extinction in PTSD: results of a twin study. J Psychiatr Res 42:515–520.
Milad MR, Pitman RK, Ellis CB, Gold AL, Shin LM, Lasko NB, Zeidan MA, Handwerger K, Orr SP, Rauch SL (2009) Neurobiological basis of failure to recall extinction memory in posttraumatic stress disorder. Biol Psychiatry 66:1075–1082.
Mueller D, Porter JT, Quirk GJ (2008) Noradrenergic signaling in infralimbic cortex increases cell excitability and strengthens memory for fear extinction. J Neurosci 28:369–375.
Mueller D, Olivera-Figueroa LA, Pine DS, Quirk GJ (2009) The effects of yohimbine and amphetamine on fear expression and extinction in rats. Psychopharmacology 204:599–606.
Myers KM, Davis M (2007) Mechanisms of fear extinction. Mol Psychiatry 12:120–150.
Oren E, Solomon R (2012) EMDR therapy: an overview of its development and mechanisms of action. Eur Rev Appl Psychol 62:197–203.
Orr SP, Roth WT (2000) Psychophysiological assessment: clinical applications for PTSD. J Affect Disord 61:225–240.
Pagani M, Di Lorenzo G, Verardo AR, Nicolais G, Monaco L, Lauretti G, Russo R, Niolu C, Ammaniti M, Fernandez I, Siracusano A (2012) Neurobiological correlates of EMDR monitoring – an EEG study. PLoS One 7:e45753.
Peri T, Ben-Shakhar G, Orr SP, Shalev AY (2000) Psychophysiologic assessment of aversive conditioning in posttraumatic stress disorder. Biol Psychiatry 47:512–519.
Pfeiffer UJ, Fendt M (2006) Prefrontal dopamine D4 receptors are involved in encoding fear extinction. NeuroReport 17:847–850.
Piazza PV, Deminiere JM, Le Moal M, Simon H (1989) Factors that predict individual vulnerability to amphetamine self-administration. Science 245:1511–1513.
Pineles SL, Orr MR, Orr SP (2009) An alternative scoring method for skin conductance responding in a differential fear conditioning paradigm with a long-duration conditioned stimulus. Psychophysiology 46:984–995.
Pole N (2007) The psychophysiology of posttraumatic stress disorder: a meta-analysis. Psychol Bull 133:725–746.
Quirk GJ, Russo GK, Barron JL, Lebron K (2000) The role of ventromedial prefrontal cortex in the recovery of extinguished fear. J Neurosci 20:6225–6231.
Rachman S (1979) The return of fear. Behav Res Ther 17:164–166.
Resick PA, Nishith P, Weaver TL, Astin MC, Feuer CA (2002) A comparison of cognitive-processing therapy with prolonged exposure and a waiting condition for the treatment of chronic posttraumatic stress disorder in female rape victims. J Consult Clin Psychol 70:867–879.
Resick PA, Williams LF, Suvak MK, Monson CM, Gradus JL (2012) Long-term outcomes of cognitive-behavioral treatments for posttraumatic stress disorder among female rape survivors. J Consult Clin Psychol 80:201–210.
Richardson P, Williams SR, Hepenstall S, Gregory L, McKie S, Corrigan F (2009) A single-case fMRI study: EMDR treatment of a patient with Posttraumatic Stress Disorder. J EMDR Pract Res 3:10–23.
Rodriguez BI, Craske MG, Mineka S, Hladek D (1999) Context-specificity of relapse: effects of therapist and environmental context on return of fear. Behav Res Ther 37:845–862.
Santini E, Ge H, Ren K, Pena de Ortiz S, Quirk GJ (2004) Consolidation of fear extinction requires protein synthesis in the medial prefrontal cortex. J Neurosci 24:5704–5710.
Servan-Schreiber D, Schooler J, Dew MA, Carter C, Bartone P (2006) Eye movement desensitization and reprocessing for posttraumatic stress disorder: a pilot blinded, randomized study of stimulation type. Psychother Psychosom 75:290–297.
Shapiro F (1989) Eye movement desensitization: a new treatment for post-traumatic stress disorder. J Behav Ther Exp Psychiatry 20:211–217.
Shapiro F (1996) Eye movement desensitization and reprocessing (EMDR): evaluation of controlled PTSD research. J Behav Ther Exp Psychiatry 27:209–218.
Shapiro F, Maxfield L (2002) Eye Movement Desensitization and Reprocessing (EMDR): information processing in the treatment of trauma. J Clin Psychol 58:933–946.

Siegmund A, Wotjak CT (2006) Toward an animal model of posttraumatic stress disorder. Ann N Y Acad Sci 1071:324–334.
Sierra-Mercado Jr D, Corcoran KA, Lebron-Milad K, Quirk GJ (2006) Inactivation of the ventromedial prefrontal cortex reduces expression of conditioned fear and impairs subsequent recall of extinction. Eur J Neurosci 24:1751–1758.
Sijbrandij M, Engelhard IM, Lommen MJ, Leer A, Baas JM (2013) Impaired fear inhibition learning predicts the persistence of symptoms of posttraumatic stress disorder (PTSD). J Psychiatr Res 47:1991–1997.
Sotres-Bayon F, Quirk GJ (2010) Prefrontal control of fear: more than just extinction. Curr Opin Neurobiol 20:231–235.
Sotres-Bayon F, Diaz-Mataix L, Bush DE, LeDoux JE (2009) Dissociable roles for the ventromedial prefrontal cortex and amygdala in fear extinction: NR2B contribution. Cereb Cortex 19:474–482.
Stickgold R (2002) EMDR: a putative neurobiological mechanism of action. J Clin Psychol 58:61–75.
Tian S, Huang F, Gao J, Li P, Ouyang X, Zhou S, Deng H, Yan Y (2011) Ventrolateral prefrontal cortex is required for fear extinction in a modified delay conditioning paradigm in rats. Neuroscience 189:258–268.
Vervliet B, Baeyens F, Van den Bergh O, Hermans D (2013) Extinction, generalization, and return of fear: a critical review of renewal research in humans. Biol Psychol 92: 51–58.
Vidal-Gonzalez I, Vidal-Gonzalez B, Rauch SL, Quirk GJ (2006) Microstimulation reveals opposing influences of prelimbic and infralimbic cortex on the expression of conditioned fear. Learn Mem 13:728–733.
Wessa M, Flor H (2007) Failure of extinction of fear responses in posttraumatic stress disorder: evidence from second-order conditioning. Am J Psychiatry 164:1684–1692.
WHO (2013) Guidelines for the management of conditions specifically related to stress. Geneva: Switzerland.

(Accepted 5 June 2015)
(Available online 16 June 2015)