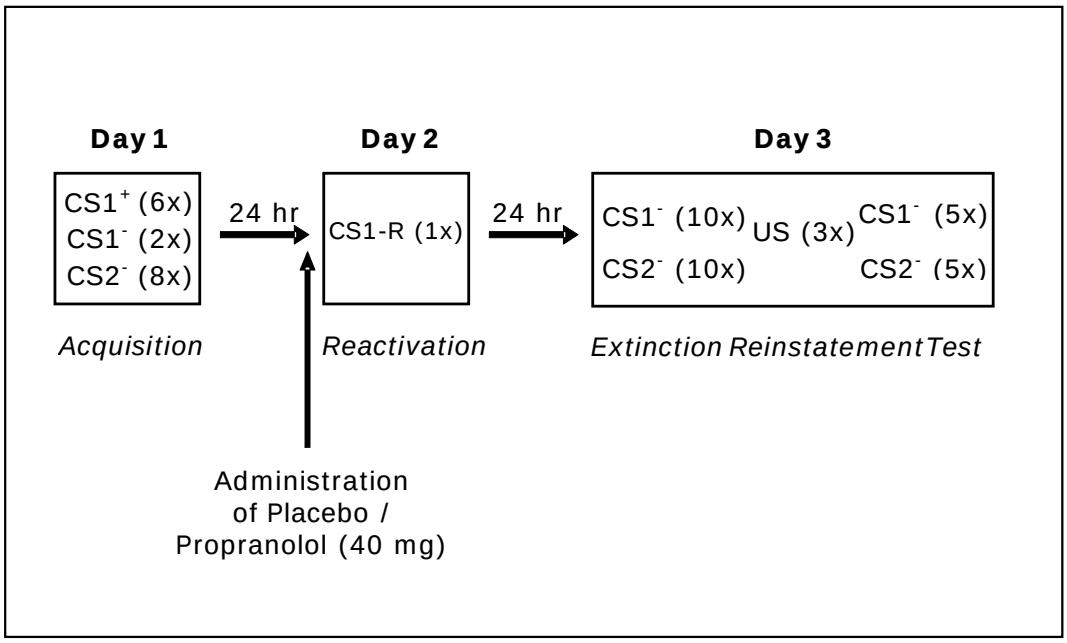
Beyond Extinction:

Erasing human fear responses and preventing the return of fear

Merel Kindt, Marieke Soeter & Bram Vervliet

University of Amsterdam

Supplementary Figures and Legends



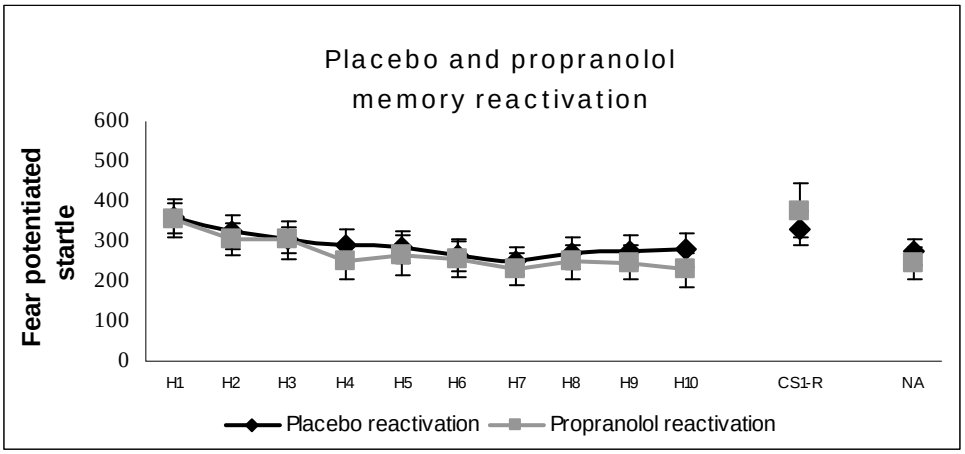
Supplementary Figure 1 Procedure for the differential fear conditioning experiment for the placebo and propranolol condition.

Nature Neuroscience: doi:10.1038/nn.2271

Day 1	***Day 2***	***Day 3***
Screening - Medical Screening - ASI - SPQ - Blood Pressure **Informed Consent** **Habituation Phase** - 10 NA Trials **Acquisition Phase** - 6 $CS1^+$ Trials - 2 $CS1^-$ Trials - 8 $CS2^-$ Trials - 10 NA Trials	**Screening / Assessments** - Blood Pressure - STAI-S **Pill Administration** - Propranolol - Placebo **1 ½ hr** ---------- ***Placebo & Propranolol*** **Habituation Phase** - 10 NA Trials **Memory Reactivation** - 1 CS1-R Trial - 1 NA Trial ---------- **Or** ---------- ***Propranolol No-Reactivation*** ---------- **Screening / Assessments** - Blood Pressure - STAI-S	**Screening** - Blood Pressure **Habituation Phase** - 10 NA Trials **Extinction Phase** - 10 $CS1^-$ Trials - 10 $CS2^-$ Trials - 10 NA Trials **Reinstatement** - 3 Unsignaled USs **Reinstatement Phase** - 5 $CS1^-$ Trials - 5 $CS2^-$ Trials - 5 NA Trials **STAI-T**

Supplementary Figure 2 Summary of assessments.

Nature Neuroscience: doi:10.1038/nn.2271



Supplementary Figure 3 Mean startle potentiation to the habituation (trial H1 through H10), CS1-R, and Noise Alone trials during memory reactivation for the Placebo Reactivation and Propranolol Reactivation group. Error bars ± s.e.m.

Nature Neuroscience: doi:10.1038/nn.2271

Supplementary Methods

Participants. Sixty undergraduate students (17 men, 43 women) from the University of Amsterdam ranging in the age of 18 to 28 (M = 20.70 ± .32 s.e.m.) participated in the study.

All participants were assessed to be free from any current or previous medical or psychiatric condition that would contraindicate taking a single 40 mg oral dose of propranolol (i.e., pregnancy, seizure disorder, respiratory disorder, cardiovascular disease, BP < 90/60, diabetes, liver-/kidney disorder, depression, and psychosis). In order to eliminate individuals who might have difficulty with any temporary symptoms induced by propranolol, an additional exclusion criterion contained a high score (i.e., index above 26) on the ASI.

Participants were randomly assigned to one of two conditions with the restriction that conditions were matched on SPQ scores as close as possible: pill placebo (n = 20; mean SPQ score 6.10 ± 1.24 s.e.m.) and propranolol (n = 20; mean SPQ score 9.10 ± 1.34 s.e.m.). For the additional control condition (propranolol without reactivation; n = 20), the SPQ score was 8.05 ± 1.54. In each propranolol condition only three participants believed that propranolol had been taken. In the placebo condition, two participants believed that they got propranolol.

Participants received either partial course credits or were paid a small amount (€ 35,–) for their participation in the experiment. The study was approved by the ethical committee of the University of Amsterdam and informed consent was obtained from all participants.

Nature Neuroscience: doi:10.1038/nn.2271

Assessments. Since propranolol may decrease blood pressure, blood pressure was taken for two reasons: *1)* as an exclusion criterion (BP < 90/60); and *2)* as a manipulation check of propranolol on day 2 (see **Supplementary Data**). State and trait anxiety were assessed with the State and Trait Anxiety Inventory (STAI-S / STAI-T)[1]. The Spider Phobia Questionnaire (SPQ)[2] was used to assess the degree of spider fear. In addition, the Anxiety Sensitivity Index (ASI)[3] was taken.

Procedure. Participants were administered a medical screening questionnaire, the ASI, and the SPQ on day 1. In addition, blood pressure was taken on day 1, day 2 (twice) and day 3.

During each session, participants sat behind a table with a computer monitor at a distance of 50 cm in a sound-attenuated room. Each phase began with a 1-min acclimation period consisting of 70 dB broadband noise, which continued throughout the session as background noise, followed by a habituation phase consisting of ten startle probes to reduce initial startle reactivity. Characteristics of the CSs, trial order, ITIs, and startle probes as well as the instructions regarding the US-expectancy measures during *memory reactivation* (day 2) and *extinction / reinstatement / testing* (day 3) were similar to *acquisition* (day 1). Assignment of the slide as $CS1^{+}$ and $CS2^{-}$ was counterbalanced across participants (**Supplementary Figs. 1** and **2**).

Day 1. After attachment of the EMG and shock electrodes, the intensity of the US was determined by gradually increasing the level of a 2-ms aversive electric stimulus delivered to the wrist of the non-preferred hand. The intensity of shock was individually set at a level defined by the participant as “uncomfortable, but not painful”. Furthermore, participants were asked to look carefully at both slides. They were told that an electric

Nature Neuroscience: doi:10.1038/nn.2271

stimulus would follow one of the slides for most of the time, while the other slide would never be followed by the US. They were also told that they should learn to predict whether an electric stimulus would occur or not on the basis of the slides. Participants were required to rate the expectancy of the electric stimulus during the presentation of each slide by shifting a cursor on a continuous 11-point rating scale and push the left mouse button.

In order to strengthen the fear association during acquisition, fear relevant stimuli served as CSs (i.e., pictures of spiders, IAPS number 1200 & 1201)[4]. In the *acquisition* phase, both the CS1 and CS2 were presented 8 times for 8 s. The startle probe was presented 7 s after CS onset and was followed by the US 500 ms later. In order to delay extinction learning, only 75 % of the presentation of the CS1[5] was reinforced. In addition, 10 startle probes were presented alone (Noise Alone; NA). Intertrial intervals (ITI) varied between 15, 20, and 25 s with a mean of 20 s. Order of trial and ITI were quasi-random, with the restriction that no more than two consecutive trials or ITIs were of the same type.

At the conclusion of the acquisition phase, participants were explicitly instructed to remember what they had learned. These instructions were included to enhance retention of the CS-US contingency on the following days[6].

Day 2. In order to substantiate consolidation of the fear memory, a break of 24 hr after acquisition was inserted before propranolol or pill placebo was administered. In view of the peak plasma levels[7], participants (double-blind) were given an oral dose of 40 mg propranolol or pill placebo 90 minutes before memory reactivation. Blood pressure and the STAI-S were taken before and after the experiment.

Nature Neuroscience: doi:10.1038/nn.2271

After electrode attachment, participants were told that the same slides of spiders would be presented. They were asked to look carefully at both slides and to remember what they had learned during acquisition. Participants were instructed as on day 1.

Reconsolidation of fear memory can be separately manipulated from extinction by a *single* as opposed to *repeated* unreinforced CS presentations[8-10]. In the *memory reactivation* phase, a single unreinforced CS1-R was presented for 8 s. In addition, 1 startle probe was presented alone.

The additional control condition only comprised assessment of blood pressure and the STAI-S before and 90 min after propranolol intake. Testing and propranolol administration took place in the same context as the other two conditions.

Day 3. In view of the elimination half-life[7] and the possible effects of propranolol on the startle response[11], extinction / reinstatement testing took place 24 hr after drug intake, allowing the drug to wash out before testing. Therefore, we could test the specific effect of propranolol on reconsolidation. Instructions regarding the CSs only revealed that the same pictures of spiders provided on day 1 would be presented.

In the *extinction* phase, the participants were exposed to both the $CS1^-$ and $CS2^-$ for 10 times without the US. Furthermore, 10 startle probes were presented alone (NA). At the conclusion of extinction, participants received three unsignaled USs. The time between the last extinction trial and the first reinstating US was 19 s. Following the unsignaled USs, participants were presented with another 5 $CS1^-$, $CS2^-$, and NA trials (*reinstatement testing*). The time between the reinstating USs and reinstatement testing was 18 s. At the end of the experiment, participants completed the STAI-T and responded to a question regarding pill intake (propranolol / placebo).

Nature Neuroscience: doi:10.1038/nn.2271

Supplementary Data

Statistical analyses

Startle responses and US-expectancies were analyzed by a mixed analysis of variance (ANOVA) with condition as a between-subject factor (Propranolol versus Placebo or Propranolol No-Reactivation versus Placebo or Propranolol versus Propranolol No-Reactivation) and stimulus (CS1 versus CS2) and trial as within-subject factors. For analysis of acquisition, we compared the first three trials (1–3) to the last 3 trials (6–8). For analysis of extinction, we compared the first three trials (1–3) to the last three trials (8–10). For analysis of the reinstatement effect we compared the last three extinction trials (8–10) to the first test trial.

Propranolol versus Placebo

The propranolol and placebo condition did not differ in terms of reported spider fear ($t_{38} < -1.7$), trait anxiety ($t_{38} < 1$) and shock intensity ($t_{38} < 1$). Consistent with other studies[12], propranolol did not affect the reported state anxiety that was assessed before and after pill intake ($F_{1,38} < 1$).

Analysis of the effect of propranolol on blood pressure revealed the expected decrease in systolic and diastolic blood pressure in comparison to placebo (two-way ANOVA's, $F_{1,38} = 6.10$, $P < 0.05$, $\eta^2 = .14$; $F_{1,38} = 5.25$, $P < 0.05$, $\eta^2 = .12$). Further analysis of blood pressure showed that, in the propranolol condition, the systolic blood pressure significantly decreased from $M = 127.95 \pm 2.31$ s.e.m. to $M = 113.50 \pm 1.76$ s.e.m. ($t_{19} = 7.50$, $P < 0.001$, two-tailed) and the diastolic blood pressure from $M = 77.15 \pm 1.58$ s.e.m.

Nature Neuroscience: doi:10.1038/nn.2271

to M = 70.35 ± 1.60 s.e.m. after pill intake (t_{19} = 4.29, P < 0.001, two-tailed). In the placebo condition, we observed no decrease of either systolic or diastolic blood pressure (t_{19} < 1.54; t_{19} < 1.15). Moreover, additional analyses of the blood pressure level on day 1, before the pill intake on day 2, and on day 3, revealed no differential effect of pill intake on the course of the systolic and diastolic blood pressure (respectively, $F_{2,37}$ < 2.13; $F_{2,37}$ < 2.43).

Analysis of the startle response to Noise Alone trials (NA) unveiled neither a significant difference between the propranolol and placebo condition in the acquisition phase (main effect of pill, $F_{1,38}$ < 1), nor to the one NA trial during memory reactivation (t_{38} <1). However, during extinction the startle response to the NA trials was slightly attenuated in the propranolol condition compared to the placebo condition though not significant (main effect of pill, $F_{1,38}$ = 3.11, P < 0.09, η^2 = .08). Moreover, the response to the first NA trial during test (after reinstatement) was reduced in the propranolol condition compared to the placebo condition (t_{36} = 2.06, P < 0.05, two-tailed), suggesting that the fear erasure effect at test (day 3) generalized to the context.

We found no effects of propranolol on the US-expectancy data (stimulus x trial x condition, $Fs_{1,38}$ < 1) (**Fig. 1b,d**). In both the propranolol and placebo condition, we observed a significant differential increase in US expectancy ($CS1^+$ versus $CS2^-$) during acquisition (stimulus x trial, $F_{1,38}$ = 190.92, P < 0.001, η^2 = .83), a significant decrease in US expectancy during extinction (stimulus x trial, $F_{1,38}$ = 111.78, P < 0.001, η^2 = .75) and a significant reinstatement effect (stimulus x trial, $F_{1,38}$ = 23.04, P < 0.001, η^2 = .38) (**Fig. 1b,d**).

Nature Neuroscience: doi:10.1038/nn.2271

Propranolol No-Reactivation versus Placebo / Propranolol

We observed no differences in terms of reported spider fear between the propranolol no-reactivation and the other two conditions ($ts_{38} < 1$). However, comparison of trait anxiety showed a marginally significant difference between the propranolol no-reactivation ($M = 37.65 \pm 2.00$) and the placebo condition ($M = 32.15 \pm 1.91$) ($t_{38} = -1.99$, $P = .055$, two-tailed). The difference between the propranolol no-reactivation and propranolol condition ($M = 32.95, \pm 1.58$) approached significance ($t_{38} = –1.85$, $P = .072$, two-tailed). Also, the intensity of shock was significantly lower in the propranolol no-reactivation ($M = 11.45 \pm .88$) as compared to the placebo condition ($M = 16.00 \pm 2.00$), ($t_{38} = 2.08$, $P < .05$, two-tailed), and compared to the propranolol condition the effect approached significance ($M = 14.10 \pm 1.25$) ($t_{38} = –1.74$, $P = .091$, two-tailed). We will discuss the differences in trait anxiety and US intensity between the conditions in the analyses of the startle response and CS-US expectancies. We observed no differences in terms of reported state anxiety before and after pill intake between the propranolol no-reactivation and the other two conditions ($Fs_{1,38} < 1.4$).

Analysis of the effect of propranolol on blood pressure in the no-reactivation condition revealed the expected decrease in systolic blood pressure in comparison to placebo (two-way ANOVA, $F_{1,38} = 9.47$, $P < 0.01$, $\eta^2 = .20$), but we observed no difference in diastolic blood pressure ($F_{1,38} < 1.5$). No differences in decrease of both systolic and diastolic blood pressure were observed between the propranolol no-reactivation and propranolol condition ($F_{1,38} < 1.7$), indicating that both propranolol conditions exerted a similar physiological effect. In the propranolol no-reactivation condition, both the systolic and diastolic blood pressure significantly decreased after pill intake from $M = 124.60 \pm 2.12$

Nature Neuroscience: doi:10.1038/nn.2271

s.e.m. to M = 107.95 ± 1.74 s.e.m (t_{19} = 8.99, P < 0.001, two-tailed) and from M = 72.55 ± 1.59 s.e.m. to M = 68.40 ± 1.19 s.e.m. (t_{19} = 3.20, P = 0.05, two-tailed), respectively. Moreover, blood pressure levels on day 1, before pill intake on day 2, and on day 3, revealed no differential effect (of pill intake) on the course of both the systolic and diastolic blood pressure between the propranolol no-reactivation and placebo condition (Fs$_{2,37}$ < 1.90) and the propranolol no-reactivation and propranolol condition (Fs$_{2,37}$ < 2.38).

Trait anxiety and shock intensity differed between the propranolol no-reactivation condition and both other conditions. In order to control for the possible effects of these variables on the startle response, we calculated Pearson correlations for both the whole sample and for the separate conditions. Only one significant correlation appeared between trait anxiety and the startle response to the control stimulus (CS2) after reinstatement in the propranolol no-reactivation condition (r = 0.49, P < 0.05, two-tailed). Therefore, only the analysis of the reinstatement effect of the differential startle response (CS1 versus CS2) included trait anxiety as a covariate. Note that the positive correlation between trait anxiety and startle response to the control stimulus (CS2) is in line with other human fear conditioning studies[13, 14].

Analysis of the differential startle response (CS1 versus CS2) on day 1 showed no difference in fear learning from trial 1–3 to trial 6–8 between the propranolol no-reactivation and placebo condition (F < 1), but a marginally significant difference was observed between the propranolol no-reactivation and propranolol condition ($F_{1,38}$ = 3.87, P = .056, η^2=.09). Further analysis showed a significant increase of the differential startle response during acquisition in both the propranolol no-reactivation ($F_{1,19}$ = 13.50, P <

Nature Neuroscience: doi:10.1038/nn.2271

0.01, η^2=.42) and the propranolol condition ($F_{1,19}$ = 28.01, P < 0.001, η^2=.60). Note that the superior acquisition observed in the propranolol condition in comparison to the propranolol no-reactivation condition (**Fig. 1c,e**) works against the hypothesis that administration of propranolol combined with active retrieval of the fear memory would reveal less fear responses at test.

Similar to the placebo condition, the differential startle response remained stable from the last acquisition trials (trial 6–8) on day 1 to the first extinction trials (trial 1–3) on day 3 ($F_{1,38}$ < 1) (**Fig. 1a,e**). Hence, we observed a normal fear response in the propranolol no-reactivation condition 48 hr after acquisition (day 3). Moreover, the reduction of the conditioned startle response in the propranolol condition 24 hr after reactivation differed significantly from the propranolol no-reactivation condition ($F_{1,38}$ = 29.02, P < .001, η^2=.43) (**Fig. 1c,e**). In contrast to the propranolol condition, the differential startle response in the propranolol-no reactivation condition even slightly increased from day 1 to day 3 ($F_{1,19}$ = 3.65, P = 0.07, η^2=.16). Thus, the decrease of the fear response in the propranolol condition is dependent on the active retrieval of the fear memory.

Analysis of extinction learning showed no difference of the startle response (CS1 versus CS2) from trial 1–3 to trial 8–10 between the propranolol no-reactivation and the placebo condition ($F_{1,38}$ < 1). In addition, the course of extinction between the propranolol no-reactivation and the propranolol condition differed significantly ($F_{1,38}$ = 13.46, P < 0.01, η^2=.26) (**Fig.1 c,e**). The extinction training significantly reduced the differential startle response in the propranolol no-reactivation condition ($F_{1,19}$ = 35.40, P < 0.001, η^2=.65), whereas we observed no differential change of the startle response in the propranolol condition ($F_{1,19}$ < 1).

Nature Neuroscience: doi:10.1038/nn.2271

Analysis of the reinstatement effect with trait anxiety as covariate showed no difference between the propranolol no-reactivation and placebo condition for the differential startle response (CS1 versus CS2) from the last extinction trials (trial 8–10) to the first reinstatement trial ($F_{1,36} < 1.2$). Hence, the fear reinstatement was not affected by the administration of propranolol without active retrieval of the fear memory. Comparison of the reinstatement effect between the propranolol no-reactivation and propranolol condition did not reveal the expected difference, ($F_{1,36} < 2.0$). However, as can be seen in **Figure 1e**, not only the startle response to the feared CS1 but also to the control CS2 increased after the US-only trials. Analysis of the reinstatement effect within the propranolol no reactivation condition, showed a significant increase of the startle response (reinstatement effect for both CS1 and CS2) from the last extinction trials (trial 8–10) to the first reinstatement trial ($F_{1,19} = 7.40$, $P < 0.05$, η^2=.28), but no increase of the differential startle response (CS1 versus CS2) ($F_{1,19} < 1.7$). The observation that the return of fear after reinstatement is not only observed for the feared stimulus (CS1), but also for the control stimulus (CS2), indicates a generalization of the previously acquired fear to the safety signal in the propranolol condition without reactivation. This generalization effect has also been observed in other studies on fear reinstatement in humans[15, 16]. Since the generalization of fear was only observed in the propranolol no-reactivation condition, further analysis of the fear reinstatement comprised the startle response to the feared CS1. Comparison of the propranolol no-reactivation and propranolol conditions revealed a significant difference of the fear reinstatement to the CS1 ($F_{1,37} = 4.46$, $P < 0.05$, η^2=.11), indicating that the absence of fear reinstatement in the propranolol condition was dependent on the active retrieval of the fear memory (**Fig.**

Nature Neuroscience: doi:10.1038/nn.2271

1c,e). Analysis of the fear reinstatement in the propranolol no-reactivation condition indeed showed a significant return of fear to the feared stimulus (CS1) ($F_{1,19}$ = 8.40, P < 0.01, η^2=.31). Interestingly, the placebo and propranolol no-reactivation condition both revealed a complete post-extinction recovery of the fear response, as is indicated by no difference in startle response to the last acquisition trial (CS1) and the first reinstatement trial (ts$_{19}$ < 1.4) (**Fig. 1a,e**). In sum, both the oral administration of propranolol and the reactivation of the fear memory seem to be necessary for the observed eradication of the fear response.

Analysis of the startle response to the Noise Alone trials (NA) unveiled no significant differences between the propranolol no-reactivation and placebo condition during acquisition and extinction (main effect of pill, Fs$_{1,38}$ < 1.5), or to the first NA trial after reinstatement (t_{36} < –1.1). Also, we observed no difference in startle response to the NA trials between the propranolol no-reactivation and propranolol condition during acquisition (main effect of pill, $F_{1,38}$ < 2.2). However, similarly to the differences between the propranolol and placebo condition, the startle response to the NA trials was lower in the propranolol condition than in the propranolol no-reactivation condition during extinction (main effect of pill, $F_{1,38}$ = 8.35, P < 0.01, η^2 = .18) and after reinstatement (t_{38} = –2.96, P < 0.01, two-tailed). Again, this suggests that the amnesic effect of propranolol not only disrupted the reconsolidation of the previously learned fear association but also its context.

Analysis of the US expectancy data revealed no differences in acquisition, extinction and fear reinstatement between the propranolol no reactivation condition and the other two conditions (three-way ANOVA's, Fs$_{1,38}$ < 2.4) (**Fig. 1b,d,f**). Separate analyses for

Nature Neuroscience: doi:10.1038/nn.2271

the propranolol no-reactivation condition, showed a significant acquisition effect ($F_{1,19}$ = 116.95, $P < 0.001$, $\eta^2 = .86$) and a significant extinction effect ($F_{1,19} = 48.61$, $P < 0.001$, $\eta^2 = .72$). In line with the startle responses, we observed a reinstatement effect for both the feared stimulus (CS1) and the control stimulus (CS2) ($F_{1,19} = 10.03$, $P < 0.01$, $\eta^2 = .35$), but no differential fear reinstatement effect ($F_{1,19} < 1$). In addition, analysis of the US expectancy to the feared CS1 stimulus alone showed also a significant reinstatement effect ($F_{1,19} = 8.73$, $P < 0.01$, $\eta^2 = .32$).

Nature Neuroscience: doi:10.1038/nn.2271

Supplementary References

1. Spielberger, C.D., Gorsuch, R.L. & Lusthene, R.E. *Manual for the State-Trait Anxiety Inventory* (Consulting Psychologists Press, Palo Alto, CA, 1970).

2. Klorman, R., Weerts, T.C., Hastings, J.E., Melamed, G.B.G. & Lang, P.J. *Behav. Ther.* **5**, 401-409 (1974).

3. Peterson, R.A. & Reiss, S. *Anxiety Sensitivity Index* (1987).

4. Lang, P.J., Bradley, M.M. & Cuthbert, B.N. International affective picture system (IAPS): Affective ratings of pictures and instruction manual. (University of Florida, Gainesville, FL, 2005).

5. LaBar, K.S., Gatenby, J.C., Gore, J.C., LeDoux, J.E. & Phelps, E.A. *Neuron* **20**, 937-945 (1998).

6. Norrholm, S.D.*, et al. Learn. Mem.* **13**, 681-685 (2006).

7. Gilman, A.G. & Goodman, L.S. *Goodman and Gilman's the pharmacological basis of therapeutics* (McGraw-Hill, New York, 1996).

8. Debiec, J. & LeDoux, J.E. *Neuroscience* **129**, 267-272 (2004).

9. Doyere, V., Debiec, J., Monfils, M.-H., Schafe, G.E. & LeDoux, J.E. *Nature Neurosci.* **10**, 414-416 (2007).

10. Duvarci, S., Mamou, C.B. & Nader, K. *Eur. J. Neurosci.* **24**, 249-260 (2006).

11. Davis, M., Falls, W.A., Campeau, S. & Kim, M. *Behav. Brain Res.* **58**, 175-198 (1993).

12. Grillon, C., Cordova, J., Morgan, C.A., Charney, D.S. & Davis, M. *Psychopharmacology* **157**, 342-352 (2004).

13. Grillon, C. *Biol. Psychiatry* **51**, 851-858 (2002).

14. Grillon, C. & Ameli, R. *Psychophysiology* **38**, 807-815 (2001).

15. Dirikx, T., Hermans, D., Vansteenwegen, D., Baeyens, F. & Eelen, P. *Learn. Mem.* **11**, 549-554 (2004).

16. Dirikx, T., Hermans, D., Vansteenwegen, D., Baeyens, F. & Eelen, P. *J. Beh. Ther. Exp. Psych.* **38**, 237-251 (2007).

Nature Neuroscience: doi:10.1038/nn.2271