Fear learning in unmedicated patients with anxiety disorders: a comparison of delay conditioning, fear reversal, and trace conditioning.

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ABSTRACT

Anxiety disorders are common and impairing, yet their underlying mechanisms remain incompletely understood. Fear learning provides a critical translational framework for investigating pathological anxiety, bridging laboratory models and clinical phenomena. Prior studies have been limited by important methodological issues, including the inclusion of non-anxiety diagnoses, high comorbidity, and medication use. Here we examined three forms of fear learning— delay conditioning, fear reversal, and trace conditioning—in unmedicated adults with minimally comorbid primary anxiety disorders and demographically matched controls. Patients showed greater psychophysiological arousal and reduced brain activation in the left dorsolateral prefrontal cortex to the learned safety cue (CS−) during the early phase of delay conditioning. Group differences were not evident for the learned threat cue (CS+) during delay conditioning or for either cue in the fear-reversal and trace-conditioning paradigms. Taken together, these observations underscore the selectivity of Pavlovian learning deficits among unmedicated individuals with anxiety disorders and highlight differences in learning or using safety-related information to adaptively regulate fear.

Keywords

Fear Conditioning; Anxiety Disorders; Trace Conditioning; Reversal Learning; Neural Response; fMRI; Psychophysiological Measures.

INTRODUCTION

Anxiety disorders, including generalized anxiety disorder (GAD), social anxiety disorder (SAD) and panic disorder (PD), affect about 12% of adults globally each year, with evidence suggesting that prevalence may be on the rise.[1,2] These disorders can severely disrupt daily functioning, including occupational and social functioning, relationships, and overall quality of life.[3,4] Given their impact, understanding the underlying mechanisms of anxiety disorders is essential for developing more effective or tolerable treatments.[2–4]

Pavlovian fear (or threat) learning paradigms have become a crucial translational tool in anxiety disorders research, bridging the gap between laboratory research and clinical practice.[5] These paradigms can be leveraged to study a variety of processes, including the acquisition (hereafter referred to as conditioning), reversal, and extinction of learned fears. In fear conditioning, a formerly neutral stimulus elicits fear (conditioned stimulus, CS+) after being associated with an innately aversive stimulus (unconditioned stimulus, US). Two key forms of Pavlovian conditioning have been distinguished. In delay conditioning, the presentation of the CS+ and US overlap in time, with onset of the US typically following that of the cue (i.e., delayed). In trace conditioning, the CS+ and US are separated by a brief interval, requiring the learner to hold a ‘trace’ of the CS in memory. In human research, responses to the CS+ are typically compared to a second cue which, because it is unpaired, is indicative of safety and remains comparatively neutral (CS-). In fear reversal, the contingencies are reversed, requiring individuals to inhibit their learned responses to previously learned threat and safety signals.[6] Across these diverse paradigms, fear responses are typically assessed using a mixture of subjective ratings; psychophysiological responses, such as the skin conductance

response (SCR); and neuroimaging measures, such as functional magnetic resonance imaging (fMRI).[78]

Both fear conditioning and fear reversal processes may play a crucial role in anxiety disorders. Increased susceptibility to conditioning (e.g., heightened fear responses to neutral stimuli) may explain persistent fear associations in patients with anxiety disorders. Conversely, impaired fear reversal may reflect difficulty adapting to changing cues, such as failing to respond to new threats or overreacting to now-safe stimuli.[9] Flexible updating of threat associations is also key to effective treatment of anxiety disorders.[10]

Studies assessing fear conditioning and fear reversal in individuals with anxiety disorders have yielded inconsistent findings. A comprehensive recent meta-analysis of delay-conditioning paradigms found no differences in threat (CS+) reactivity among individuals with mixed anxiety and trauma diagnoses, as indexed by ratings and psychophysiological responses.[11] Nevertheless, patients did show heightened responses to safety cues (CS-), suggesting aberrant safety learning rather than heightened fear conditioning.[11] While an important advance, these observations are limited by the inclusion of medicated individuals diagnosed with a wide variety of disorders (e.g., obsessive-compulsive disorder, OCD), raising the possibility that the reported safety learning deficit actually reflects a downstream consequence of treatment.[13] The meta-analysis also did not account for comorbidity, which is a critical inferential limitation given that approximately 60% of individuals with an anxiety disorder also meet criteria for a depressive or other anxiety disorder.[2]

Fear reversal in anxiety disorders remains understudied. In one of the few published studies, Savage and colleagues reported no significant differences in

ratings, psychophysiological arousal, or brain activation measures during reversal between unmedicated young patients (aged 15–25) with SAD and healthy controls.[12] In a predominantly medicated GAD sample, Roberts et al. found that those patients had a significantly higher overall SCR and a reduced differential SCR (CS+>CS-) compared to healthy controls during the early, but not the late, phase of fear reversal.[13] This study underscored the importance of temporal dynamics in human fear learning. For example, previous research suggests that learning during fear acquisition is typically stronger in early trials than in later ones.[14] In neuroimaging studies, early trials of fear acquisition are thought to more effectively capture the activation of specific brain regions.[15,16]

Trace fear conditioning has received even less empirical attention, and no prior research has specifically investigated trace conditioning in individuals with anxiety disorders. This is unfortunate because trace paradigms may better reflect real-life situations where cues and aversive outcomes are temporally separated [17] and are considered "weak" situations compared to the “strong” delay paradigms.[18,19] Weakening the situation, by reducing the certainty, proximity, or intensity of the US, may enhance sensitivity to group differences.[18]

To address these fundamental questions, the present study investigated delay conditioning, fear reversal, and trace conditioning in an unmedicated sample of 34 adults with DSM-5[20] anxiety diagnoses (primarily GAD or SAD), with minimal or no comorbidity, and 102 age and gender-matched controls. Consistent with recent recommendations,[7] we acquired a comprehensive set of fear measures, including subjective ratings, SCR, and fMRI. Based on previous research,[11,13] we anticipated that individuals with anxiety disorders would show 1) heightened responses to the

CS− during delay conditioning, indicating impaired safety learning; 2) reduced differential conditioning during fear reversal, reflecting difficulties in updating threat and safety associations; and heightened fear conditioning or deficient safety learning during delay conditioning. We generally expected group differences to be more evident during the earlier portion of each learning phase (e.g., early delay acquisition), consistent with other recent work. [7,14–16,21]

METHODS

Participants

Participants were recruited as part of a larger study focused on identifying predictors of pathological anxiety. Here we investigated potential differences in Pavlovian fear conditioning in unmedicated individuals with anxiety disorders (n=34) and gender- and age-matched healthy controls (n=102; **Table 1**). Diagnostic eligibility was determined by an experienced clinician using the MINI International Neuropsychiatric Interview. [22] For descriptive purposes, participants completed self-reported measures of anxiety, depressive symptoms, and dispositional negative affect (see "**Recruitment procedures**" and "**Self-report measures**" in Sup. Mat.). All participants provided informed written consent. The study was approved by the ethics committee at Hospital de Bellvitge in Barcelona (protocol # PR144/16).

Fear learning assessment

Participants completed two fear-learning tasks in the scanner while subjective ratings, SCR, and fMRI were assessed. The first task assessed delay fear conditioning and fear reversal, whereas the second task assessed trace fear conditioning. The order of the tasks was counterbalanced across participants. In both

tasks, the unconditioned stimulus (US) was an individually calibrated electric shock, designed to be “unpleasant but not painful”. In the delay/reversal task, the conditioned stimuli (CSs) were blue and yellow spheres presented against a black background, whereas in the trace task, the CSs were waves, dots, or triangles. Both tasks used the same procedures for subjective ratings, SCR, and fMRI data collection, and participants received identical instructions (see “**Fear learning assessment**” in the **Sup.Mat**.).

Delay fear conditioning and fear reversal task

We leveraged a previously validated delay fear acquisition/reversal task that encompassed three phases: pre-conditioning, fear conditioning, and fear reversal[23] (**Figures 1A** and **1B**). During pre-conditioning, the to-be-conditioned CS+ and CS- (2,000 ms) were each presented five times. The US (250 ms) was never presented. During conditioning, the CS+ and US co-terminated on one-third of trials, enabling us to examine skin-conductance and fMRI responses unconfounded by US presentation. The CS- was never paired with the US. During fear reversal, the CS-shock contingency was reversed (newCS+: p=33.3%; newCS-: p=0.0%). Across the conditioning and reversal phases, there were a total of 15 CS+/newCS+ trials (5 reinforced) and 10 CS-/newCS- trials (pseudorandomized). During the conditioning phase, the second CS+ trial was reinforced. During the reversal phase, the first presentation of the new CS+ was reinforced. CS stimuli were counterbalanced across participants. Across all phases, the inter-trial interval (ITI) between CS trials was 12s, during which a white fixation cross (CFix) was presented.

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Trace fear conditioning task

The trace conditioning task encompassed two phases: pre-conditioning (baseline), and trace fear conditioning (**Figure 1C and 1D**). During preconditioning, two to-be-conditioned CS+ and one CS- were each presented twice. The US (250 ms) was never presented. During conditioning, the US was presented at the end of the "trace" (blank screen), 1,600-2,000 ms following CS+ offset. One of the CS+ was reinforced on 50% of trials (CS+50) and the other was reinforced on 81% of trials (CS+81). Procedures for the CS- were similar, but it was never paired with the US. During the conditioning phase, each of the three CS was presented 16 times (order pseudorandomized; 11.6-12.0-s ITI). CS stimuli were counterbalanced across participants.

Measures of conditioned fear

Subjective ratings

Immediately after each learning phase (pre-conditioning, conditioning, and reversal for the delay/reversal task, and pre-conditioning and conditioning for the trace conditioning task), participants rated each CS on two five-point Likert scales of valence and arousal related to anxiety (Self-Assessment Manikins[24]), with higher scores indicating greater valence and increased arousal (see "**Measures of conditioned fear**" in the **Sup. Mat**).

Skin conductance responses

SCR data were acquired in the scanner during the two tasks, and the response to each CS (CS+, CS-, for the delay task; newCS+ and newCS- for the reversal task; and CS+81, CS+50, and CS-, for the trace task) calculated. The acquisition and (pre)processing of SCR data followed standard procedures.[25] (see **"Measures of conditioned fear"** in the **Sup. Mat.).**

Brain activation

Neuroimaging data were acquired using a Phillips Ingenia 3T scanner (32-channel head-coil). For details on imaging acquisition and (pre)processing, see **"Brain activation**" in the **Sup. Mat**.

The two fear learning tasks were programmed in E-Prime 2.0 and displayed on an MRI-compatible back-projection screen. Both tasks were similar in duration (~16 minutes) and separated by a 15-minute break.

First-level fMRI modeling. Each participant's preprocessed time series was entered into a first-level general linear model (GLM) analysis. The onsets of each CS event type were modeled separately for each task by convolving them with a canonical hemodynamic response function. Six motion parameters were included as nuisance covariates. For the delay task, contrast images were computed for CS+ > CS− (excluding reinforced trials to avoid contamination from the US) and CS−> CFix. Fixation-cross ITIs contributed the implicit baseline. For the reversal task, contrast images were estimated for newCS+ > newCS−, also excluding reinforced trials. For the trace conditioning task, contrasts were computed for $CS+_{50}$ > CS−, $CS+_{81}$ > CS−, and $CS+_{50}$ > $CS+_{81}$. All CS+ trials were included in these contrasts, as increasing the ITI and ISI (inter-stimuli interval) minimized the risk of US-related confounds. ISIs ranged from 5.35 to 5.75 seconds.

Statistical analyses

Two-sample Student's *t*-tests and a chi-square test were used to confirm that patients and controls were adequately matched on demographic characteristics and differed in self-report measures of anxiety. Repeated-measures tests were used to confirm the absence of significant differences between the to-be-conditioned CSs during the preconditioning phase of the delay (Student's t-tests) and trace (ANOVA) conditioning tasks (see **"Preconditioning Analyses"** in **Sup.Mat**).

Subjective ratings and SCR data were analyzed using a series of mixed-model ANOVAs with CS as a within-subject factor and group as a between-subject factor. For the acquisition phase of the delay task, there were 2 levels of CS (CS+, CS-). For the reversal phase, there were 2 levels of the CS (newCS+, newCS-). For the trace conditioning task, there were 3 levels of the CS (CS+50, CS+81, CS-). For the delay task, the 5 reinforced CS+/newCS+ trials were censored from SCR analyses to avoid US confounding. For the trace task, all trials were included. Post hoc comparisons were conducted using the Bonferroni test ($\alpha = 0.05$), and the Greenhouse-Geisser correction was applied when necessary. Effect sizes are reported using partial eta squared (η^2_p).

Neuroimaging analyses closely paralleled the approach used for SCR. Between-group differences in neuroimaging contrasts (CS+>CS- and CS->CSFix for delay conditioning; newCs+>newCS- for fear reversal; CS+81>CS-, CS+50>CS-, and CS+81>CS+50 for trace conditioning) were assessed using two-sample *t*-tests. Whole-brain statistical significance was determined using a cluster-level family-wise error (FWE) correction at $p < 0.05$, with clusters formed of contiguous voxels with $p<0.001$.

Consistent with other recent work, [7,14–16,26] we generally expected group differences to be more evident during the early portion of each learning phase. Therefore, we computed a second set of 'disaggregated' SCR and fMRI analyses that incorporated early-versus-late phase as a within-subject factor. For the delay and reversal tasks, early and late phases were defined as the first and last five unreinforced CS+/newCS+ and CS-/newCS- trials, respectively. For trace conditioning, they were defined as the first and last eight trials of CS+81, CS+50, and CS-.

RESULTS

Our patient sample (n=34) included 28 individuals with a primary diagnosis of GAD and 6 individuals with a primary diagnosis of SAD. There were no significant differences in age or biological sex distribution between patients and controls. Patients exhibited significantly higher anxiety, depressive symptoms, and dispositional negative affect. Groups did not differ in the perceived aversiveness of the shock US (**Table 1**).

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During preconditioning of the delay conditioning/reversal task, no significant differences were observed within each group in responses to the to-be CS+ and to-be CS− across any conditioned fear measures, including subjective ratings, SCR, or brain activation (see **Sup. Figure 1 and Sup. Table 2**). Similarly, during preconditioning of the trace conditioning task, no significant differences were found in arousal and valence ratings for either group or SCR for the patient group.

However, in the control group, SCR responses were greater for the to-be CS+81 compared to both to-be CS+50 and to-be CS− (see **Sup. Figure 2** and **Sup. Table 3**). Additionally, both groups exhibited increased activation in the visual cortex in the CS+81 > CS− contrast.

Delay fear conditioning

In the aggregated analyses that included all trials, both controls (**Figures 2.A, 2.B, 2.C**) and patients (**Figures 2.E, 2.F, 2.G**) showed evidence of successful delay fear conditioning in SCR and subjective ratings, with significantly larger SCR to the CS+ compared to the CS-, and significantly higher arousal and lower valence ratings for the CS+ compared to the CS- (all *ps*< 0.001). Although SCR was, on average, higher among patients than controls ($F(1,127) = 6.20$, $p < 0.05$, $\eta^2_p = 0.047$), the Group × CS type interaction was not significant for SCR, arousal, or valence (all F*s* ≤ .33, all *ps* ≥ .56; **Sup. Table 4**). Nevertheless, planned analyses showed that SCR to the CS- was greater in patients compared to controls [Patients: *M*(SD) = 0.13 (0.11); Controls: *M*(SD) = 0.07 (0.08); $t(127) = -3.39$, $p < 0.001$]. Similar effects were not evident for arousal or valence ratings.

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Incorporating phase (early and late) in SCR analyses, the ANOVA revealed a significant main effect of group ($F(1,127) = 6.20$, $p = .014$, $\eta^2p = .047$) and a three-way interaction between CS type, group, and phase ($F(1,127) = 4.18$, $p = .043$, $\eta^2p = .032$; **Sup. Table 5**). Post-hoc analyses revealed that controls exhibited successful

differential conditioning (CS vs CS-) during early conditioning (p< 0.05; **Figure 3.A**), whereas patients did not (p = 0.974; **Figure 3.B**). This reflected the fact that patients exhibited higher SCR to the CS− than controls during early conditioning [Patients:*M* (SD):0.18 (0.16); Controls: *M*(SD): 0.09 (0.10), (t(127) = −3.45, p < 0.001)] (**Figure 3.A** and **3.B**).

fMRI results including all trials provided evidence of successful conditioning in controls (**Figure 2.D**) and patients (**Figure 2.H**). Specifically, the CS+>CS− contrast revealed increased activation in regions previously associated with fear conditioning,[27] including the supramarginal gyrus, anterior insular cortices (extending into the frontal operculum), anterior and middle cingulate cortex, and thalamus (see **Sup. Tables 6 and 7**). Group differences were negligible in the aggregate analyses. In the disaggregated analyses, patients exhibited significantly reduced activation to the safety cue (CS-) in the left dorsolateral prefrontal cortex (dlPFC) during the early phase of conditioning (**Figure 3.C** and **3.D**). Groups did not differ in their reactivity to the safety cue during the late phase or the threat cue (CS+) in either phase.

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Fear reversal

Both controls (**Figures 4.A, 4.B, 4.C**) and patients (**Figures 4.E, 4.F, 4.G**) showed evidence of successful fear reversal in SCR and subjective ratings, with significantly larger SCR to the new CS+ compared to the new CS-, and significantly higher arousal and lower valence ratings scores to the new CS+ compared to the new CS- (all Fs ≥ 87.19, all *ps*< 0.001). The Group × CS type interaction was not significant

for SCR, arousal, or valence (all Fs ≤ 2.22, all *ps* ≥ .138) (**Sup. Table 8**). Similar conclusions were evident for the disaggregated SCR analyses (**Sup. Tables 9**).

fMRI results (new CS+>new CS-contrast) showed evidence of successful fear reversal in both controls (**Figure 4.D**) and patients (**Figure 4.H**), with increased brain activation across several brain regions, including the supramarginal gyrus, anterior insula (extending to the temporal pole), thalamus, and midcingulate cortex (see **Sup. Tables 10 and 11)**. Note that the regions activated during reversal are largely overlapping to those observed during conditioning. Group differences were negligible in the aggregate and disaggregated analyses.

Rather than calculating fear reversal by directly comparing CS+ and CS− responses during reversal, some recent fear reversal studies have separately assessed *threat reversal* and *safety reversal*.[23] In principle, this approach provides a more precise measure of the ability to update and inhibit conditioned fear responses as stimulus-outcome contingencies change. Nevertheless, groups did not differ in SCR, subjective ratings, or neural activation during the switch from CS− to CS+ or vice versa (SCR/Ratings: all ts ≤ |1.47|, all ps ≥ .145; fMRI: see **Sup. Mat.: Additional Analyses** and **Sup. Table 12**).

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Trace fear conditioning

There was evidence of successful trace conditioning within both groups and most measures. Both controls (**Figure 5.A, 5.B, 5.C**) and patients (**Figure 5.F, 5.G, 5.H**) exhibited significantly larger SCR, higher arousal, and lower valence to CS+81 and

CS+50 compared to CS− (all *ps* < 0.001). When comparing CS+81 to CS+50, both controls and patients showed significantly larger SCRs (**Figure 5.A, Figure 5.F**) and lower valence ratings (**Figure 5.C, Figure 5.H)** for CS+81 than CS+50 (*ps* < 0.02). In contrast, arousal ratings to CS+81 and CS+50 did not differ significantly in either the controls (**Figure 5.B**) or the patients (**Figure 5.G**) (*ps* > 0.99). The Group × CS type interaction was not significant for SCR, arousal, or valence (all Fs(2,244) < 2.26, all *ps* > 0.11) (**Sup. Table 13**). The main effect of group was also non-significant, indicating that SCR, arousal, and valence levels were generally similar across groups (all Fs(1,122) < 3.46, all *ps* > 0.07). Similar conclusions were evident for the disaggregated analyses (**Sup. Table 14**).

fMRI findings provided evidence of successful trace conditioning in both controls (**Figure 5.D, 5.E**) and patients (**Figure 5.I, 5.J**) for the contrasts CS+50 > CS− and CS+81 > CS−. Both contrasts were associated with increased brain activation across several regions, including the thalamus, supplementary motor area (SMA), supramarginal gyrus, precentral/postcentral gyri, and insula extending to the inferior frontal operculum (see **Sup. Tables 15 to 18**). In controls, the CS+81>CS+50 contrast was associated with increased activation in several regions, including the temporal/occipital middle gyri, putamen, hippocampus, thalamus, and precentral/postcentral gyri (see **Sup. Table 19** and **Sup. Figure 3**). This effect was not evident for the patients. The groups did not differ in their reactivity to any of the trace-conditioning contrasts, suggesting similar neural responses. Similar conclusions were evident for the disaggregated analyses

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Sensitivity analyses

None of our key conclusions materially changed when including gender and age as covariates (See **Sup. Tables 20 to 22**)

DISCUSSION

We compared subjective, psychophysiological, and neural measures of delay conditioning, fear reversal, and trace conditioning in unmedicated individuals with anxiety disorders and demographically matched controls. Overall, our findings suggest that individuals with anxiety disorders show impaired safety learning during the early stages of delay conditioning but do not exhibit marked alterations in either fear reversal or in trace conditioning.

Previous studies using delay fear conditioning paradigms in unmedicated individuals with anxiety disorders, as currently defined, remain limited. Pochlen et al.[28] found no significant differences in subjective (expectancy ratings) or psychophysiological (SCR, FPS, pupillometry) measures of conditioned fear (CS+ vs CS difference) when comparing patients with anxiety disorders (specific phobia, SAD, agoraphobia, and PD) to healthy controls. Similarly, several prior studies focusing on GAD, SAD and PD patients reported no differences between patients and controls in psychophysiological (FPS or SCR) or subjective (expectancy ratings) conditioned fear measures.[29–33]

In our aggregate analysis, which included all trials, we also found no group differences in cue differentiation (CS+ vs. CS-) during delay conditioning. However, patients showed heightened SCR responses to the safety cue (CS-). Although this effect was evident in our aggregate results, phase-specific analyses indicated that

this alteration largely is most pronounced during the early phase of delay fear conditioning. Mirroring this effect, fMRI results revealed decreased activation to the safety cue (CS-) in the left dlPFC) during the early phase of conditioning. The dlPFC plays a key role in emotion regulation, [34–36] and greater dlPFC activation in response to safety cues has been linked to fear inhibition. [37,38] Thus, reduced dlPFC engagement in patients may reflect difficulties in downregulating responses to the CS−, particularly during the early stages of learning Pavlovian safety associations. Methodologically, our observations underscore the importance of cue- and phase-specific analyses for understanding the alterations in fear learning that mark individuals with pathological anxiety.[39]

Contrary to our expectations, we did not find differences in fear reversal between our patient and control groups. Our results align with those of Savage et al.[12], who also found no differences in subjective ratings, SCR, or brain activation during fear reversal in unmedicated patients with SAD compared to healthy controls or patients with major depressive disorder. Roberts et al.[13] reported reduced differential SCR responses in GAD patients compared to controls during the early but not the late phase of fear reversal. This study included mostly (79%) medicated participants, and as noted in the introduction, medication may be a key confound in fear learning studies.[40] Variation in the type of CSs used could also influence these differences: unlike Savage et al. and the current study, which employed geometric figures, Roberts et al.[13] used angry faces as CS. Another notable difference is that both our study and Savage et al. assessed SCR in the scanner. The (f)MRI environment is stressful, which can alter both neural and behavioral responses.[41] This stress could lead to greater heterogeneity in the control group, potentially

444 decreasing the power to detect differences between controls and clinical
445 groups.[7,42,43]

446 We anticipated that using a trace conditioning paradigm, a "weak situation"
447 (see **Introduction**), with two CS+ stimuli featuring different pairing rates would
448 enhance the detection of fear learning differences between individuals with anxiety
449 disorders and controls. However, our findings did not support this hypothesis. To our
450 knowledge, no previous studies have directly compared trace fear conditioning
451 between patients with anxiety disorders and healthy controls. Our trace interval was
452 relatively short (1.6 to 2 seconds), and it is possible that differences may have
453 emerged with longer intervals—a possibility that warrants further exploration.

454 Overall, and in line with several previous reports,[12,28–33] our results suggest
455 that anxiety disorders, as a group, are not characterized by robust alterations in most
456 fear learning processes investigated here. However, it remains possible that
457 diagnoses not represented here (e.g., panic disorder) do show such alterations.
458 Additionally, disruptions in other fear learning mechanisms—such as fear
459 generalization [44] or fear extinction recall [26], may characterize anxiety disorders. The
460 “anxiety disorders” category has changed over the years and the current versions of
461 the most employed classification systems (DSM-5[20] and ICD-10 [45]) do not include
462 post-traumatic stress disorder (PTSD). A recent large-scale study on the neural
463 correlates of delay fear conditioning (Radua et al., submitted) found increased brain
464 activation during fear conditioning in multiple regions among patients with "anxiety-
465 related disorders" (a category including anxiety disorders, OCD, and PTSD)
466 compared to healthy controls. Using linear models and normative modeling
467 analyses,[46] the study further revealed that alterations in delay fear conditioning were
468 characteristic of PTSD and OCD but not of GAD or SAD. When considered

alongside our findings and previous research on PTSD,[47] these results suggest that fear conditioning abnormalities, at least in the context of delay fear conditioning, may be more strongly associated with PTSD than with other "anxiety-related disorders." Given that PTSD and OCD are often linked to greater severity and functional impairment compared to other anxiety disorders, [48,49] and that fear conditioning abnormalities have also been reported in a broader spectrum of mental disorders [50] (e.g., schizophrenia), we speculate that such abnormalities may serve as a transdiagnostic marker of severity rather than being specific to any single diagnosis. In this view, altered fear conditioning could reflect a general liability dimension that varies continuously with symptom burden, rather than mapping into specific psychiatric categorization. This hypothesis could be tested in future research by incorporating fear conditioning measures across individuals with various mental disorder diagnoses and assessing them using a standardized measure of severity and functional impairment.

Our study has several strengths and limitations. A key strength is our well-characterized patient sample, consisting of non-medicated adult individuals with a primary diagnosis of an anxiety disorder based on current classification systems and little-to-no comorbidity. These individuals were thoroughly phenotyped, exhibiting significantly higher anxiety symptom scores than controls across all psychometric measures. However, our sample was not entirely homogeneous, as it did not consist solely of patients with a single anxiety disorder (e.g. only GAD or SAD). Additionally, some anxiety disorders (e.g., specific phobia, PD) were not represented. However, the prevailing assumption in the field is that fear learning alterations are a common feature across *all* anxiety disorders.[11,51] Although our sample size was relatively small, the three paradigms examined—delay fear conditioning, fear reversal, and

trace fear conditioning—elicited robust fear responses at the subjective, psychophysiological, and neural levels *within each group*. This indicates sufficient assay sensitivity, except for certain measures in the CS+81 vs. CS+50 contrast in trace conditioning. Finally, for each process, we included multiple operationalizations—such as all trials, early and late phases, and an alternative approach to fear reversal. However, there are numerous other possible ways to operationalize fear learning processes.[52]

In summary, we did not find robust evidence that individuals with anxiety disorders (GAD and SAD) exhibit significant alterations in delay or trace fear conditioning or fear reversal, but they may be characterized by impaired safety learning. It is possible that other fear learning processes better characterize these disorders, or that such abnormalities are more relevant to other mental disorders. Future research should explore whether fear-learning abnormalities are more indicative of disorder severity rather than diagnostic status.

REFERENCES

1. Momen, N. C. *et al.* Mental Health Disorder Trends in Denmark According to Age, Calendar Period, and Birth Cohort. *JAMA psychiatry (Chicago, Ill.)* **82**, 161–170 (2025).
2. Craske, M. G. *et al.* Anxiety disorders. *Nat Rev Dis Primers* **3**, 17024 (2017).
3. Wu, Y. *et al.* Trends in the epidemiology of anxiety disorders from 1990 to 2021: A global, regional, and national analysis with a focus on the sociodemographic index. *J Affect Disord* **373**, 166–174 (2025).
4. Alene, K. A. *et al.* Global incidence, prevalence, years lived with disability (YLDs), disability-adjusted life-years (DALYs), and healthy life expectancy (HALE) for 371 diseases and injuries in 204 countries and territories and 811 subnational locations, 1990–2021: a systematic analysis for the Global Burden of Disease Study 2021. *The Lancet (British edition)* **403**, 2133–2161 (2024).
5. Pittig, A., Treanor, M., LeBeau, R. T. & Craske, M. G. The role of associative fear and avoidance learning in anxiety disorders: Gaps and directions for future research. *Neurosci Biobehav Rev* **88**, 117–140 (2018).
6. Schiller, D., Levy, I., Niv, Y., LeDoux, J. E. & Phelps, E. A. From fear to safety and back: Reversal of fear in the human brain. *Journal of Neuroscience* **28**, 11517–11525 (2008).
7. Lonsdorf, T. B. *et al.* Don't fear 'fear conditioning': Methodological considerations for the design and analysis of studies on human fear acquisition, extinction, and return of fear. *Neurosci Biobehav Rev* **77**, 247–285 (2017).
8. Fullana, M. A. *et al.* Neural signatures of human fear conditioning: An updated and extended meta-analysis of fMRI studies. *Mol Psychiatry* **21**, 500–508 (2016).
9. Craske, M. G. *et al.* Optimizing inhibitory learning during exposure therapy. *Behaviour Research and Therapy* **46**, 5–27 (2008).
10. Craske, M. G., Treanor, M., Conway, C. C., Zbozinek, T. & Vervliet, B. Maximizing exposure therapy: An inhibitory learning approach. *Behaviour research and therapy* **58**, 10–23 (2014).
11. Kausche, F. M., Carsten, H. P., Sobania, K. M. & Riesel, A. Fear and safety learning in anxiety- and stress-related disorders: An updated meta-analysis. *Neurosci Biobehav Rev* **169**, 105983 (2025).
12. Savage, H. S., Davey, C. G., Fullana, M. A. & Harrison, B. J. Threat and safety reversal learning in social anxiety disorder – an fMRI study. *J Anxiety Disord* **76**, 102321–102321 (2020).
13. Roberts, C. *et al.* Threat reversal learning and avoidance habits in generalised anxiety disorder. *Transl Psychiatry* **12**, 216–216 (2022).
14. Lonsdorf, T. B. & Merz, C. J. More than just noise: Inter-individual differences in fear acquisition, extinction and return of fear in humans - Biological, experiential, temperamental factors, and methodological pitfalls. *Neurosci Biobehav Rev* **80**, 703–728 (2017).
15. Cooper, S. E., Grillon, C. & Lissek, S. Impaired discriminative fear conditioning during later training trials differentiates generalized anxiety disorder, but not panic disorder, from healthy control participants. *Compr Psychiatry* **85**, 84–93 (2018).

16. Lissek, S. *et al.* Impaired discriminative fear-conditioning resulting from elevated fear responding to learned safety cues among individuals with panic disorder. *Behaviour research and therapy* **47**, 111–118 (2009).
17. Wehrli, J. M., Xia, Y., Gerster, S. & Bach, D. R. Measuring human trace fear conditioning. *Psychophysiology* **59**, e14119 (2022).
18. Lissek, S., Pine, D. S. & Grillon, C. The strong situation: A potential impediment to studying the psychobiology and pharmacology of anxiety disorders. *Biol Psychol* **72**, 265–270 (2006).
19. Allen, M. T. Weaker situations: Uncertainty reveals individual differences in learning: Implications for PTSD. *Cognitive, Affective and Behavioral Neuroscience* vol. 23 869–893 Preprint at (2023).
20. Association, A. P. *Diagnostic and Statistical Manual of Mental Disorders*. (American Psychiatric Association, 2013).
21. Skin Conductance Responses and Neural Activations During Fear Conditioning and Extinction Recall Across Anxiety Disorders. *JAMA psychiatry (Chicago, Ill.)* (2017) doi:10.1001/jamapsychiatry.2017.0329.
22. Sheehan, D. V *et al.* The Mini-International Neuropsychiatric Interview (M.I.N.I.): the development and validation of a structured diagnostic psychiatric interview for DSM-IV and ICD-10. *J Clin Psychiatry* **59 Suppl 20**, 22–33 (1998).
23. Savage, H. S., Davey, C. G., Fullana, M. A. & Harrison, B. J. Clarifying the neural substrates of threat and safety reversal learning in humans. *Neuroimage* **207**, 116427 (2020).
24. Bradley, M. M. & Lang, P. J. Measuring emotion: The self-assessment manikin and the semantic differential. *J Behav Ther Exp Psychiatry* **25**, 49–59 (1994).
25. Boucsein, W. *et al.* Publication recommendations for electrodermal measurements. *Psychophysiology* **49**, 1017–1034 (2012).
26. Marin, M.-F. *et al.* Skin Conductance Responses and Neural Activations During Fear Conditioning and Extinction Recall Across Anxiety Disorders. *JAMA Psychiatry* **74**, 622–631 (2017).
27. Fullana, M. A. *et al.* Neural signatures of human fear conditioning: an updated and extended meta-analysis of fMRI studies. *Mol Psychiatry* **21**, 500–508 (2016).
28. Pöhlchen, D. *et al.* No robust differences in fear conditioning between patients with fear-related disorders and healthy controls. *Behaviour research and therapy* **129**, 103610–10 (2020).
29. Hermann, C., Ziegler, S., Birbaumer, N. & Flor, H. Psychophysiological and subjective indicators of aversive pavlovian conditioning in generalized social phobia. *Biol Psychiatry* **52**, 328–337 (2002).
30. Michael, T., Blechert, J., Vriends, N., Margraf, J. & Wilhelm, F. H. Fear Conditioning in Panic Disorder: Enhanced Resistance to Extinction. *J Abnorm Psychol* **116**, 612–617 (2007).
31. Ahrens, L. M. *et al.* Fear conditioning and stimulus generalization in patients with social anxiety disorder. *J Anxiety Disord* **44**, 36–46 (2016).
32. Tinoco-González, D. *et al.* Conditioned Fear Acquisition and Generalization in Generalized Anxiety Disorder. *Behav Ther* **46**, 627–639 (2015).
33. Lissek, S. *et al.* Generalized Anxiety Disorder Is Associated With Overgeneralization of Classically Conditioned Fear. *Biol Psychiatry* **75**, 909–915 (2014).

34. Hermann, A. *et al.* Emotion regulation in spider phobia: role of the medial prefrontal cortex. *Soc Cogn Affect Neurosci* **4**, 257–267 (2009).
35. Brandl, F., Le Houcq Corbi, Z., Mulej Bratec, S. & Sorg, C. Cognitive reward control recruits medial and lateral frontal cortices, which are also involved in cognitive emotion regulation: A coordinate-based meta-analysis of fMRI studies. *NeuroImage (Orlando, Fla.)* **200**, 659–673 (2019).
36. Bo, K. *et al.* A systems identification approach using Bayes factors to deconstruct the brain bases of emotion regulation. *Nat Neurosci* **27**, 975–987 (2024).
37. Lau, J. Y. *et al.* Distinct neural signatures of threat learning in adolescents and adults. *Proceedings of the National Academy of Sciences - PNAS* **108**, 4500–4505 (2011).
38. Roesmann, K. *et al.* Neural correlates of fear conditioning are associated with treatment-outcomes to behavioral exposure in spider phobia - evidence from magnetoencephalography. *Neuroimage Clin* **35**, 103046–103046 (2022).
39. Morriss, J., Hoare, S. & van Reekum, C. M. It's time: A commentary on fear extinction in the human brain using fMRI. *Neurosci Biobehav Rev* **94**, 321–322 (2018).
40. Heesbeen, E. J. *et al.* The effect of SSRIs on fear learning: a systematic review and meta-analysis. *Psychopharmacology (Berl)* **240**, 2335–2359 (2023).
41. Muehlhan, M., Lueken, U., Wittchen, H.-U. & Kirschbaum, C. The scanner as a stressor: Evidence from subjective and neuroendocrine stress parameters in the time course of a functional magnetic resonance imaging session. *International journal of psychophysiology* **79**, 118–126 (2011).
42. Weldon, A. L. *et al.* Stress Response to the Functional Magnetic Resonance Imaging Environment in Healthy Adults Relates to the Degree of Limbic Reactivity during Emotion Processing. *Neuropsychobiology* **71**, 85–96 (2015).
43. Mutschler, I. *et al.* Who gets afraid in the MRI-scanner? Neurogenetics of state-anxiety changes during an fMRI experiment. *Neurosci Lett* **583**, 81–86 (2014).
44. Cooper, S. E. *et al.* A meta-analysis of conditioned fear generalization in anxiety-related disorders. *Neuropsychopharmacology (New York, N.Y.)* **47**, 1652–1661 (2022).
45. World Health Organization. *International Statistical Classification of Diseases and Related Health Problems*. (2019).
46. Marquand, A. F., Rezek, I., Buitelaar, J. & Beckmann, C. F. Understanding Heterogeneity in Clinical Cohorts Using Normative Models: Beyond Case-Control Studies. *Biol Psychiatry* **80**, 552–61 (2016).
47. Suarez-Jimenez, B. *et al.* Neural signatures of conditioning, extinction learning, and extinction recall in posttraumatic stress disorder: a meta-analysis of functional magnetic resonance imaging studies. *Psychol Med* **50**, 1442–1451 (2020).
48. Weingarden, H., Renshaw, K. D., Wilhelm, S., Tangney, J. P. & DiMauro, J. Anxiety and Shame as Risk Factors for Depression, Suicidality, and Functional Impairment in Body Dysmorphic Disorder and Obsessive Compulsive Disorder. *J Nerv Ment Dis* **204**, 832–839 (2016).
49. Rapaport, M. H., Clary, C., Fayyad, R. & Endicott, J. Quality-of-Life Impairment in Depressive and Anxiety Disorders. *Am J Psychiatry* **162**, 1171–1178 (2005).
50. Tuominen, L. *et al.* Impairment in acquisition of conditioned fear in schizophrenia. *Neuropsychopharmacology (New York, N.Y.)* **47**, 681–686 (2022).

51. Cooper, S. E. *et al.* A meta-analysis of conditioned fear generalization in anxiety-related disorders. *Neuropsychopharmacology (New York, N.Y.)* **47**, 1652–1661 (2022).
52. Lonsdorf, T. B., Gerlicher, A., Klingelhöfer-Jens, M. & Krypotos, A.-M. Multiverse analyses in fear conditioning research. *Behaviour research and therapy* **153**, 104072–104072 (2022).

687 **Table 1**. Demographic and clinical characteristics of participants.

Variable	**Healthy Controls (*n=102*) *Mean (SD)***	**Patients with Anxiety Disorders (*n=34*) *Mean (SD)***	***Significance***
Age	25.6 (4.82)	25.6 (3.8)	*n.s.*
Females (n, %)	57 (55.9%)	19 (55.9%)	*n.s.*
Self-report questionnaires			
STAI-T (0-60)	18.68 (9.66)	29.15 (11.8)	$p < 0.001$
IUS (27-135)	50.74 (15.56)	72.68 (25.76)	$p < 0.001$
LSAS (0-144)	22.23 (12.39)	32.47 (17.54)	$p < 0.05$
GAD-Screening Scale (0-12)	2.3 (2.13)	6.53 (3.14)	$p < 0.001$
PSWQ-11 (11-55)	25.72 (9.4)	36.41 (10.08)	$p < 0.001$
DASS-S (0-21)	3.41 (2.98)	7.09 (4.23)	$p < 0.001$
DASS-A (0-21)	1.38 (1.88)	3.47 (3.29)	$p < 0.05$
DASS-D (0-21)	1.77 (2.03)	4.88 (4.76)	$p < 0.001$
Shock aversiveness [a] (1-10)	9.32 (0.84)	9.35 (0.72)	*n.s.*
Diagnoses		**Number of participants (%)**	
GAD		24 (70.6)	
GAD plus another anxiety disorder		4 (11.8)[b]	
SAD		5 (14.7)	
SAD plus agoraphobia		1 (2.9)	

STAI-T: State-Trait Anxiety Inventory, trait version; IUS: Intolerance of Uncertainty Scale; LSAS: Liebowitz Social Anxiety Scale; GAD-Screening: Generalized Anxiety Disorder – Screening scale; PSWQ-11: Penn State Worry Questionnaire; DASS-21-S: Depression, Anxiety and Stress Scales – Stress subscale; DASS-21-A: Depression, Anxiety and Stress Scales – Anxiety subscale; DASS-21-D: Depression, Anxiety and Stress Scale – Depression subscale; GAD: Generalized Anxiety Disorder; SAD: Social Anxiety Disorder;; a: Average shock aversiveness for the two tasks (see Supplementary Methods); b: GAD+Panic Disorder (n=1); GAD+SAD (n=3); n.s.: non-significant.

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DECLARATION OF CONFLCITING INTERESTS

The authors declared no potential conflicts of interest with respect to the research, authorship, and/or publication of this article.

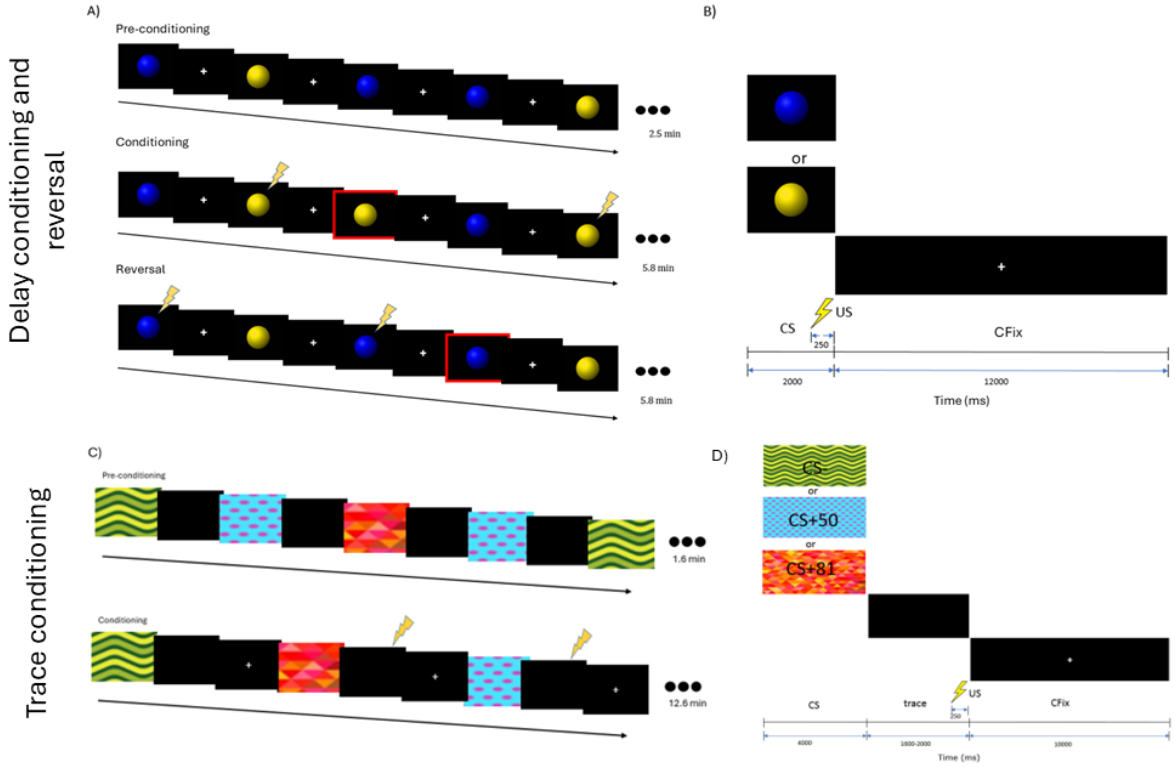


Figure 1. Fear conditioning fMRI paradigms. A) Delay fear conditioning and fear reversal task. During pre-conditioning, the US was omitted. During conditioning, the US (*lightning bolt*) was paired with one of the spheres on 33.3% trials (CS+), but not the other (CS-). During fear reversal, the CS-shock contingency was reversed to create newCS+ and newCS-. Red boxes indicate unpaired CS+ trials. B) Detailed timeline of events within delay conditioning/reversal trials. C) Trace fear conditioning task. During pre-conditioning, the US was omitted. During conditioning, the US was paired with two of the CS (CS+50: 50%; CS+81: 81%), but not the third (CS-). D) Detailed timeline of events within trace conditioning trials. Abbreviations—CFix, Cross-fixation; CS, Conditioned Stimulus; ms, milliseconds; US, Unconditioned Stimulus (US).

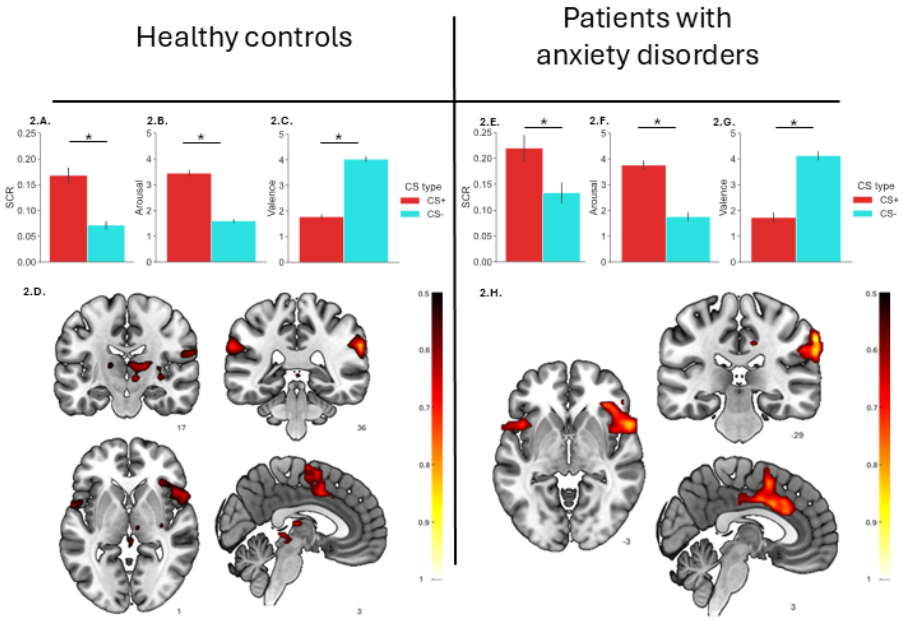


Figure 2. Delay fear conditioning in healthy controls (n=102) and patients with anxiety disorders (n=34). LEFT: Skin conductance responses (SCR) (A), subjective ratings of arousal (B) and valence (C), and functional magnetic resonance imaging (fMRI) responses (D) during delay fear conditioning in healthy controls. RIGHT: SCR (E), subjective ratings of arousal (F) and valence (G), and fMRI responses (H) during delay conditioning in patients with anxiety disorders. For subjective ratings, data refer to the responses to the CS+ or CS- at the end of the conditioning phase. For SCR, data refer to the average responses to the unreinforced CS+ trials and the CS- trials during conditioning. For fMRI, data refer to the CS+>CS- contrast, using the same trials as for the SCR. To facilitate visual comparison, the t-maps were converted to effect sizes by dividing them by the square root of the sample size. These maps were then thresholded at 0.5, representing the lower boundary of effect sizes within significant regions observed in the control group. Error bars indicate standard error of the mean (SEM). *$p<0.001$. fMRI figures display slices in the three orthogonal directions that best represent each group's results. These images are not exhaustive, and full details can be found in the referenced supplementary tables. While slice selection may vary between control and patient groups, it is aimed at highlighting the most characteristic neural activations for each group across the studied contrasts.

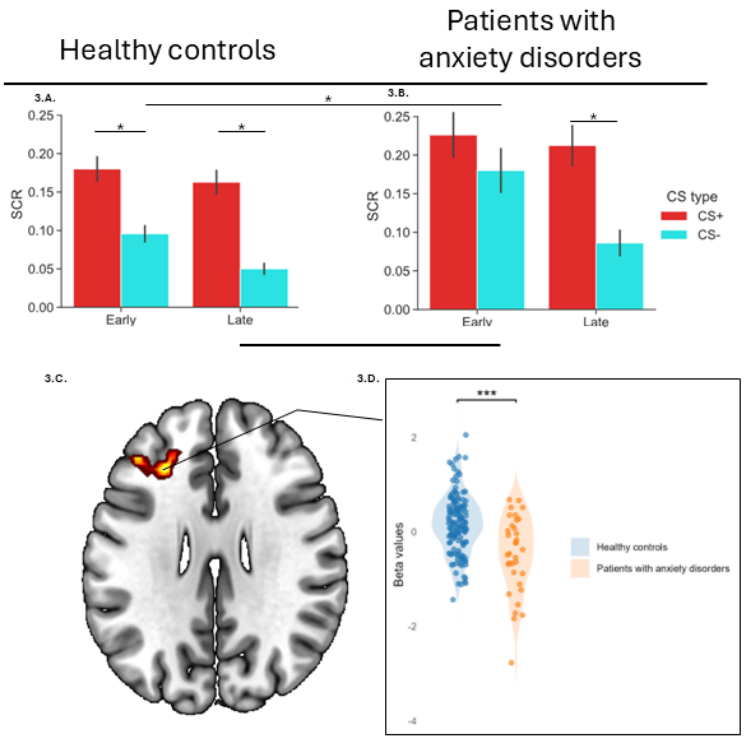


Figure 3. Delay fear conditioning responses during early and late phases in healthy controls (n = 102) and patients with anxiety disorders (n = 34). Skin conductance responses (SCR) data represent average responses to the first and last five CS+ and CS− trials (unreinforced CS+ trials only) (3.A., 3.B). fMRI results for the CS− > fixation cross contrast during the same trials show significantly less activation in patients compared to healthy controls during early conditioning within a cluster in the left dorsolateral prefrontal cortex (3.C). Violin plot depicting mean beta values within the significant cluster for each group (3.D). *p < 0.05, **p < 0.001.

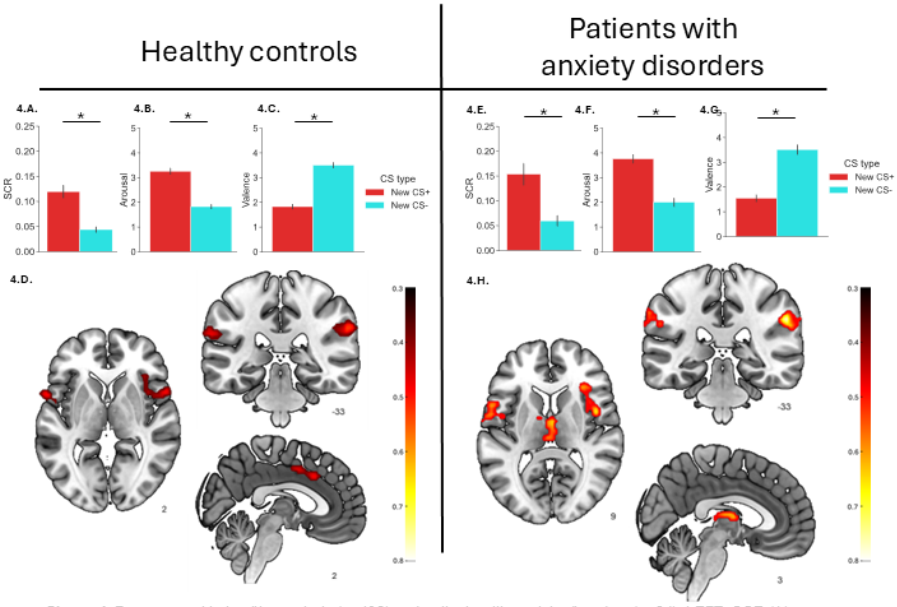


Figure 4. Fear reversal in healthy controls (n=102) and patients with anxiety disorders (n=34). LEFT: SCR (A), subjective ratings of arousal (B) and valence (C), and fMRI responses (D) during fear reversal in healthy controls. RIGHT: SCR (E) subjective ratings of arousal (F) and valence (G), and fMRI responses (H) during fear reversal in patients with anxiety disorders. For subjective ratings, data refer to the responses to the new CS+ or new CS- after fear reversal. For SCR, data refer to the average responses to the unreinforced new CS+ trials and the new CS- trials during fear reversal. For fMRI, data refer to the new CS+ > new CS- contrast using the same trials as for SCR. To facilitate visual comparison, the t-maps were converted to effect sizes by dividing them by the square root of the sample size. These maps were then thresholded at 0.5, representing the lower boundary of effect sizes within significant regions observed in the control group. Error bars indicate standard error of the mean (SEM). *$p<0.001$. fMRI figures display slices in the three orthogonal directions that best represent each group's results. These images are not exhaustive, and full details can be found in the referenced supplementary tables. While slice selection may vary between control and patient groups, it is aimed at highlighting the most characteristic neural activations for each group across the studied contrasts.

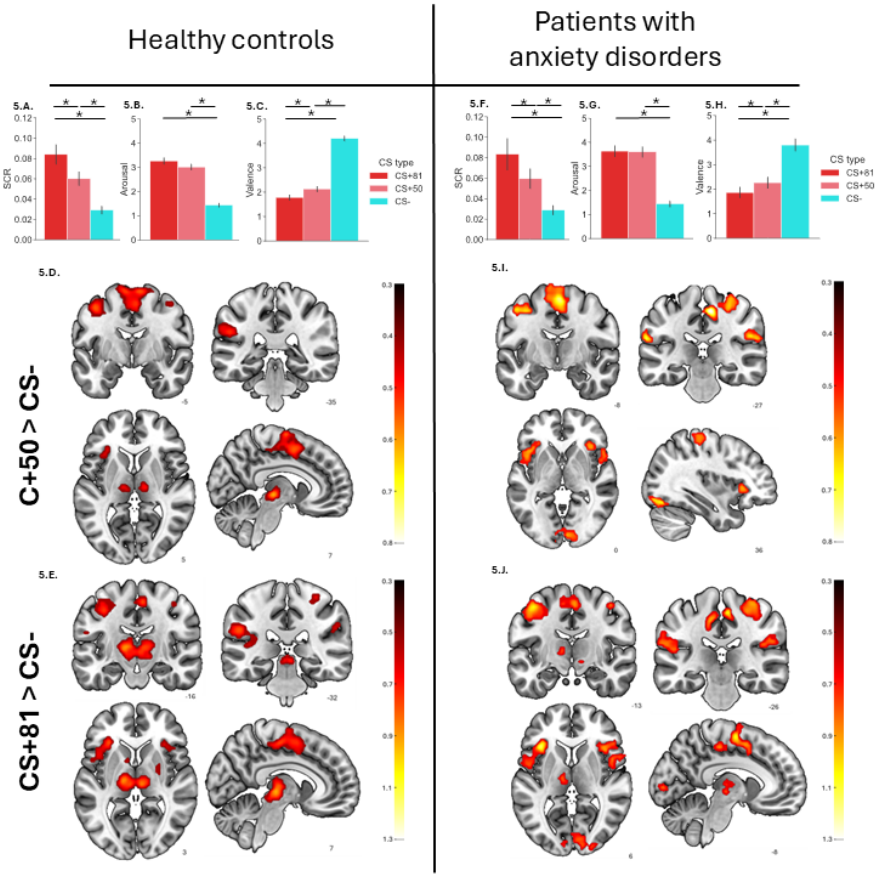


Figure 5. Trace fear conditioning in healthy controls (n=102) and patients with anxiety disorders (n=34). LEFT: SCR (A), subjective ratings of arousal (B) and valence (C), and fMRI responses for the contrasts CS+50 > CS- (D) and CS+81 > CS- (E) during trace fear conditioning in healthy controls. RIGHT: SCR (F), subjective ratings of arousal (G) and valence (H), and fMRI responses for the contrasts CS+50 > CS- (I) and CS+81 > CS- (J) during trace fear conditioning in patients with anxiety disorders. For subjective ratings, data refer to the responses to the CS+50, CS+81 or CS- at the end of the trace conditioning phase. For SCR, data refer to the average responses to the unreinforced CS+50 and CS+81 trials and the CS- trials during trace conditioning. For fMRI, data refer to the above-mentioned contrasts using the same trials as for SCR. To facilitate visual comparison, the t-maps were converted to effect sizes by dividing them by the square root of the sample size. These maps were then thresholded at 0.5, representing the lower boundary of effect sizes within significant regions observed in the control group. Error bars indicate standard error of the mean (SEM). *$p<0.001$. fMRI figures display slices in the three orthogonal directions that best represent each group's results. These images are not exhaustive, and full details can be found in the referenced supplementary tables. While slice selection may vary between control and patient groups, it is aimed at highlighting the most characteristic neural activations for each group across the studied contrasts.

Fear learning in unmedicated patients with anxiety disorders: a comparison of delay conditioning, fear reversal, and trace conditioning.

Supplementary Material

SUPPLEMENTARY METHODS

Recruitment procedures

We screened a large number (n = 840) of adult individuals (age ≥ 18) from the university community, including students and staff, using the Spanish version [1] of the State-Trait Anxiety Inventory–Trait (STAI-T) subscale[2] via a secure web system. We aimed to recruit participants with different levels of trait anxiety (including individuals with a current anxiety disorder). Therefore, STAI-T data were stratified into quartiles, and individuals who met preliminary inclusion criteria were selected from each stratum. These individuals (n = 361) were assessed during a telephone interview by an experienced clinician who administered the Spanish version [3] of the Mini International Neuropsychiatric Interview [4] and confirmed that potential participants fulfilled the inclusion/exclusion criteria. Inclusion criteria were: 1) age between 18 and 36 years, 2) owning a smartphone (because the larger study included smartphone-based assessments), and 3) being willing to participate in a neuroimaging assessment. Exclusion criteria were 1) current or previous severe medical disorder or current medication that could interfere with the study objectives (as per self-report), 2) current or past mental disorder (except current anxiety disorder, see below), or 3) current substance use (except occasional use of alcohol and other recreational drugs, or tobacco), as per the MINI, and 4) any contraindication to neuroimaging assessment. Those who met the inclusion/exclusion criteria (n = 206) gave written informed consent and participated in the laboratory session reported in this manuscript. Twenty-seven participants were excluded from MRI analysis (5 because of incidental findings, and 22 due to motion artifacts or poor image quality), leaving 179 potential participants. Thirty-four of these 179 participants were given a diagnosis of a current anxiety disorder and made up the patient group. Among the rest (n = 135), and to enhance statistical power, we selected 3 controls for each patient, ensuring that the samples had a matched gender distribution (55.9% female) and no statistically significant differences in age (mean age 25.6 years in both, $p = 1$). These participants (n = 102) made up the control group.

For the fMRI analyses of the trace conditioning task, three patients and nine controls were excluded due to poor data quality. Additionally, for the SCR analyses, one patient and four controls were excluded from the delay/reversal fear-conditioning task due to recording artifacts, and four patients and eight controls were excluded from the trace conditioning task for the same reason. The final number of participants included in each analysis/task is shown in **Sup. Table 1.**

Self-report measures

Participants completed the Spanish versions of the following measures:

-Trait subscale of the *State-Trait Anxiety Inventory* (STAI-T) a 20-item questionnaire assessing trait anxiety (dispositional negative affect) [1,2] Total scores range from 0 to 60.

-*Intolerance of Uncertainty Scale* (IUS), a 27-item questionnaire assessing the tendency to react negativelyto uncertain situations. [5,6] Total scores range from 0 to 135.

-*Liebowitz Social Anxiety Scale (LSAS),* a 24-item questionnaire assessing anxiety and avoidance of social situations.[7,8] *Total scores range from 0 to 144.*

-*Screening scale for DSM-IV Generalized Anxiety Disorder*, a 12-item questionnaire assessing Generalized Anxiety Disorder symptoms.[9,10] Total scores range from 0 to 12.

-*Penn State Worry Questionnaire-11* (PSWQ-11) a 11-item questionnaire assessing trait worry.[11,12] Total scores range from 11 to 55.

-*Depression, Anxiety and Stress Scales* (DASS-21) a 21-item scale assessing depression, anxiety, and stress symptoms.[13,14] Total scores range from 0 to 21 for each subscale.

Fear learning assessment

Instructions

Before starting the tasks (outside the scanner), participants were informed that they would, at some point during the session, view geometrical figures and experience electric shocks. They were also familiarized with the system used for recording subjective ratings.

Unconditioned stimuli

The two unconditioned stimuli (USs) were brief electric shocks, delivered as a quadratic pulse for the delay acquisition/reversal task and a sinusoidal pulse for the trace task. Shocks were administered via two MRI-compatible Ag/AgCl electrodes filled with electrolyte gel and delivered using a Biopac STMISOLA stimulator. Electrodes were placed on the left hand for the delay acquisition/reversal task and on the left forearm for the trace task. Delivering shocks to different locations helped minimize carryover or generalization effects between tasks. The intensity of both USs was individually calibrated inside the scanner using a staircase procedure to ensure the shocks were unpleasant but not painful (rated >7 on a 1–10 aversiveness scale, where 10 was maximum aversiveness). The procedure began at 30V, increasing in 10V increments until the participant indicated their maximum level of discomfort or the 100V maximum was reached.

Measures of conditioned fear

Subjective ratings

For the valence ratings, participants responded to the question "How unpleasant/pleasant did you find the [colour of the CS] sphere?". Responses ranged from 1='very unpleasant' to 5='very pleasant'. For anxious arousal ratings, participants responded to the question: "How anxious did the [colour of the CS] sphere make you feel?". Responses ranged from 1='not anxious' to 5='very anxious'. Participants' ratings were recorded using an MRI-compatible, three-button fiber-optic response box (Lumina 3G Controller, Cedrus Corporation), with which participants were familiarized prior to scanning

Skin conductance responses

Data acquisition. Skin conductance data were continuously acquired during both fear conditioning tasks using a Biopac EDA100c module, MP150 Amplifier, and AcqKnowledge 4.4.0 software (250-Hz sampling, 5 μSiemens/Volt gain, 10-Hz low-pass, DC high-pass). Skin conductance was collected from the volar surfaces of the distal phalanges of the third and fourth fingers of the left hand, using two Ag-AgCl, nonpolarizable electrodes and isotonic gel (GEL101).

Data processing and missingness. Data preprocessing was implemented using AcqKnowledge. Data were down-sampled (62.5 Hz) and smoothed to mitigate movement artifacts (63-sample median and 1-Hz low-pass filters). All SCRs were visually inspected. Trial-by-trial SCRs were quantified using custom-made MATLAB scripts as trough-to-peak responses with an onset latency of 1-6 s in the delay/reversal task and 1-5.3 s in the trace task. Trials with increases <0.02 μS or that lasted <0.5 s were scored as non-responses and set to a value of 0 μS. Trials with artifacts or excessive baseline activity were treated as missing responses. Using SCR raw data, participants showing non-valid responses in 75% of the US trials during conditioning (i.e., 7 of 10 trials for delay/reversal task and 15 of 21 trials for the trace task) were classified as physiological non-responders and all SCR trials for these participants were scored as missing values. After excluding non-responders, SCR amplitudes were normalized and range corrected separately for each task using the formula ln(1+ SCR)/ln(1 + MAX), where MAX was the individual's maximum response to the US.

Brain activation

Imaging Acquisition. T1-weighted (T1w) anatomical scans were acquired using a three-dimensional fast-spoiled gradient, inversion-recovery sequence (TR=10.43 ms; TE=4.8 ms; flip=8°; slice thickness=0.75 mm; in-plane=0.75 × 0.75 mm; matrix=320 × 320; field-of-view=240 × 240). Functional data were acquired using a single-shot gradient-echo echo-planar imaging (EPI) sequence (TR=2,000 ms; TE=25 ms; flip=90°; slice thickness=3 mm; in-plane resolution=3 × 3 mm; matrix=80 × 80). Images were collected in the AC-PC plane to minimize potential susceptibility artifacts. For the delay fear conditioning task, one run comprising 480 volumes was acquired (total acquisition time: 15m 25s). For the trace conditioning task, one run of 458 volumes was collected (total acquisition time: 14m 13s)

Anatomical data (pre)processing. T1w images were corrected for intensity non-uniformity using the ANTs (version 2.2.0,6) N4BiasFieldCorrection algorithm,[15] skull-stripped using a Nipype implementation of antsBrainExtraction.sh and the OASIS30ANTs as a target template, and segmented using the FSL (version 5.0.9 7) [16] fast algorithm. Brain surfaces were reconstructed using the FreeSurfer (version 6.0.1 8) [17] recon-all algorithm. Brain masks were refined using a variant of a previously described method for reconciling ANTS and FreeSurfer gray matter (GM) segments. [18] Volume-based spatial normalization to two standard brain-extracted templates

(MNI152NLin2009cAsym and MNI152NLin6Asym) [19] was performed using the ANTs diffeomorphic algorithm.

Functional data (pre)processing. fMRI data were preprocessed using fMRIPrep 1.4.1, [20] which is based on Nipype 1.2.0. [21] Preprocessing included skull stripping, susceptibility distortion correction using field maps, and co-registration of each BOLD reference image to the participant's T1-weighted anatomical scan using boundary-based registration (bbregister, FreeSurfer). [22] Slice timing correction was applied using AFNI's 3dTshift, [23] and motion correction was performed with FSL's mcflirt [24]. All transformations (motion correction, distortion correction, co-registration, and spatial normalization) were combined and applied in a single resampling step using ANTs with Lanczos interpolation. [25] The resulting BOLD time-series were normalized to MNI152NLin2009cAsym space and spatially smoothed with a 6 mm FWHM Gaussian kernel. For denoising, ICA-AROMA was used to identify and remove motion-related components; [26] the analyses reported here used the non-aggressively denoised time-series. Nuisance regressors included six motion parameters, their temporal derivatives and quadratic terms, as well as anatomical and temporal CompCor components. [27] These were computed after high-pass filtering (128 s cutoff), and the number of retained components was set to explain at least 50% of variance within the respective noise masks (white matter, CSF, or combined). Framewise displacement (FD) and DVARS were computed for each run. Volumes exceeding 0.5 mm FD or 1.5 standardized DVARS were flagged as motion outliers and excluded from first-level analyses. [28] Unless otherwise stated, all analyses were conducted in MNI space using the 6 mm smoothed, non-aggressively denoised BOLD time-series.

Pre-conditioning analyses

Differences between responses to each to-be CS within each group were assessed using a paired t-test for the delay/reversal task (CS+ and CS-), and one-way repeated measures ANOVAs for the trace task (CS-, CS+50, and CS+81). Specifically, we compared the last CS trial during preconditioning for SCR and brain activation, as well as arousal and valence ratings collected after the preconditioning phase.

Additional Analyses

Fear reversal – threat and safety reversal

For subjective ratings, we defined threat reversal as the difference between arousal or valence ratings for the CS+ during reversal ("new CS+") and the CS− during conditioning. Safety reversal was defined as the difference between arousal or valence ratings for the CS− during reversal ("new CS−") and the CS+ during conditioning. For SCR, threat reversal was operationalized as the sum of all SCR responses to the unreinforced new CS+ (CS+ during reversal, 10 trials) minus the sum of responses to the CS− during conditioning. Similarly, safety reversal was defined as the sum of all SCR responses to the new CS− (CS− during reversal) minus the sum of responses to unreinforced CS+

during conditioning (10 trials). For fMRI analyses, first-level contrast images were generated by comparing new CS+ vs. CS− for threat reversal and new CS− vs. CS+ for safety reversal. Both contrasts used the same trials as the SCR analysis. Threat and safety reversal for subjective ratings and SCR were analyzed separately using two sample t-tests to compare controls and patients. fMRI group differences for each contrast were assessed using two-sample t-tests.

SUPPLEMENTARY TABLES

Supplementary Table 1. Number of participants included in each analysis

	Delay /Reversal task		Trace task	
	Healthy controls	Patients	Healthy controls	Patients
fMRI analysis	102	34	93	31
SCR analysis	98	33	94	30

Supplementary Table 2. Paired t-test results for the effects of CS type on SCR, arousal, and valence during preconditioning of the delay fear-conditioning task.

Effect	t-test	*t*	*df*	*p*
Healthy Controls	SCR	0.44	95	.661
	Arousal ratings	0.58	95	.566
	Valence ratings	0.65	95	.517
Patients	SCR	-0.89	32	.377
	Arousal ratings	0.24	32	.812
	Valence ratings	-0.37	32	.712

Supplementary Table 3. ANOVAs results for the effects CS type on SCR, arousal, and valence during preconditioning of the trace fear-conditioning task.

Group	ANOVA	*F*	*df*	*p*	η^2
Healthy Controls	SCR	7.06	2, 186	.001	<.05
	Arousal ratings	0.48	2, 186	.606	<.01
	Valence ratings	1.72	2, 186	.182	<.01
Patients	SCR	0.77	2, 58	.467	<.01
	Arousal ratings	1.39	2, 58	.163	<.05
	Valence ratings	1.87	2, 58	.256	<.05

Supplementary Table 4. ANOVAs results for the effects of group and CS type on SCR, arousal, and valence during delay conditioning

ANOVA	Effect	*F*	*df*	*p*	η^2
SCR	G	6.20	1, 127	.014	.047
	CS	72.64	1, 127	<.001	.364
	CS x G	0.33	1, 127	.566	.003
Arousal ratings	G	1.91	1, 127	.169	.015
	CS	278.53	1, 127	<.001	.687
	CS x G	0.34	1, 127	.560	.003
Valence ratings	G	.004	1, 127	.837	<.001
	CS	273.56	1, 127	<.001	.683
	CS x G	0.26	1, 127	.609	.002

Note: G = Group; CS = CS type

Supplementary Table 5. ANOVA results for the effects of group, CS type, and phase (early and late) on SCR during delay fear conditioning.

Effect	*F*	*df*	*p*	η^2
G	6.20	1, 127	.014	.047
P	28.22	1, 127	<.001	.182
P x G	1.92	1, 127	.168	.015
CS	72.64	1, 127	<.001	.364
CS x G	0.33	1, 127	.566	.003
P x CS	18.18	1, 127	<.001	.125
P x CS x G	4.18	1, 127	.043	.032

Note: G = Group, P = Phase, CS = CS type

Supplementary Table 6. Brain activations during delay fear conditioning (CS+>CS-) for healthy controls.

AAL Region	Cluster *p* value - FWER	N voxels	*t*	MNI (x,y,z)	Peak *p* value uncorrected
SupraMarginal_R	<0.001	255	8.85	63, -36, 30	<0.001
			7.38	54, -30, 30	<0.001
			6.82	63, -21, 21	<0.001
SupraMarginal_L	<0.001	162	7.96	-63, -39, 30	<0.001
			6.46	-66, -24, 27	<0.001
			5.99	-60, -24, 21	<0.001
Insula_R	<0.001	189	7.55	48, 18, -3	<0.001
			7.09	57, 15, 3	<0.001
			6.5	33, 27, 0	<0.001
Thalamus_R	<0.001	163	7.38	9, -18, -3	<0.001
			7.11	18, -18, 9	<0.001
			6.68	9, -18, 9	<0.001
Temporal_Sup_R	<0.001	18	7	48, -24, -3	<0.001
Cingulum_Mid	<0.001	182	6.79	0, 6, 42	<0.001
			6.75	-6, 12, 39	<0.001
			6.5	3, 3, 60	<0.001
Rolandic_Oper_L	<0.001	62	6.77	-57, 3, 3	<0.001
			6.49	-51, 9, -3	<0.001
Insula_L			5.45	-40,19,-2	<0.001
Cerebelum_6_L	<0.001	19	6.32	-30, -63, -21	<0.001
Thalamus_L	<0.001	33	6.31	-15, -12, 9	<0.001
			5.76	-6, -9, 9	<0.001
Cerebelum_6_R	<0.001	11	6.19	36, -57, -27	<0.001
			5.61	30, -63, -24	<0.001

Note: AAL = Automated Anatomical Labeling, FWER = Family Wise Error Rate, N = Number, MNI = Montreal Neurological Institute

Supplementary Table 7. Brain activations during delay fear conditioning (CS+ vs. CS-) for patients with anxiety disorders.

AAL Region	Cluster *p* value FWER	N voxels	*t*	MNI (x,y,z)	Peak *p* value uncorrected
SupraMarginal_R	<0.001	273	6.22	66, -39, 30	<0.001
			5.47	66, -30, 39	<0.001
			5.23	66, -27, 27	<0.001
Cingulum_Ant	<0.001	466	5.26	-3, 24, 27	<0.001
Cingulum_Mid			4.65	3, 9, 33	<0.001
			4.41	6, 9, 54	<0.001
Temporal_Pole_Sup_R	<0.001	269	4.91	57, 12, -3	<0.001
			4.29	39, 30, -6	<0.001
Insula_R			4.26	45, 9, 0	<0.001
Insula_L	<0.01	171	4.35	-45, 12, -6	<0.001
			4.1	-36, 6, 6	<0.001
			3.76	-54, 9, 12	<0.001

Note: AAL = Automated Anatomical Labeling, FWER = Family Wise Error Rate, N = Number, MNI = Montreal Neurological Institute

Supplementary Table 8. ANOVAs results for the effects of group and CS type on SCR, arousal, and valence during fear reversal.

ANOVA	Effect	*F*	*df*	*p*	η2
SCR	G	1.79	1, 127	.184	.014
	CS	87.19	1, 127	<.001	.683
	CS x G	0.80	1, 127	.371	.006
Arousal ratings	G	3.10	1, 127	.081	.024
	CS	221.59	1, 127	<.001	.636
	CS x G	2.22	1, 127	.138	.017
Valence ratings	G	0.72	1, 127	.395	.006
	CS	165.94	1, 127	<.001	.566
	CS x G	1.07	1, 127	.303	.008

Note: G = Group; CS = CS type

Supplementary Table 9. ANOVA results for the effects of group, CS type, and phase on SCR during fear reversal.

Effect	*F*	*df*	*p*	η2
G	1.79	1, 127	.184	.014
P	46.64	1, 127	<.001	.269
P x G	1.43	1, 127	.233	.011
CS	87.19	1, 127	<.001	.407
CS x G	0.80	1, 127	.372	.006
P x CS	0.45	1, 127	.502	.004
P x CS x G	0.01	1, 127	.909	.000

Note*: G = Group, P = Phase, CS = CS type*

Supplementary Table 10. Brain activations during fear reversal (new CS+ vs. new CS-) for healthy controls.

AAL Region	Cluster *p* value FWER	N voxels	*t*	MNI (x,y,z)	Peak *p* value uncorrected
SupraMarginal_R	<0.001	116	7.27	60, -30, 30	<0.001
SupraMarginal_L	<0.001	60	6.33	-66, -36, 24	<0.001
			4.78	-63, -24, 24	<0.001
Temporal_Pole_Sup_L	<0.001	41	6.31	-60, 9, 0	<0.001
Temporal_Pole_Sup_R	<0.001	146	6.07	57, 12, 0	<0.001
Insula_R			6.03	36, 24, 6	<0.001
			5.91	36, 15, 6	<0.001
Cingulum_Mid	<0.001	70	6	3, 18, 36	<0.001
			5.66	0, 3, 42	<0.001

Note: AAL = Automated Anatomical Labeling, FWER = Family Wise Error Rate, N = Number, MNI = Montreal Neurological Institute

Supplementary Table 11. Brain activations during fear reversal (new CS+ vs. new CS-) for patients with anxiety disorders.

AAL Region	Cluster *p* value FWER	N voxels	*t*	MNI (x,y,z)	Peak *p* value uncorrected
SupraMarginal_R	<0.01	139	5.67	57, -33, 33	<0.001
			4.73	63, -30, 27	<0.001
			3.86	54, -21, 24	<0.001
SupraMarginal_L	<0.001	178	5.01	-57, -42, 33	<0.001
			4.2	-63, -39, 39	<0.001
			3.65	-66, -27, 18	<0.001
Insula_R	<0.001	197	4.8	45, 3, 9	<0.001
			4.69	60, 9, -3	<0.001
			4.35	36, 27, 9	<0.001
Temporal_Pole_Sup_L	<0.001	184	4.72	-60, 12, -6	<0.001
			4.25	-60, 3, 3	<0.001
			4.22	-57, 9, 9	<0.001
Thalamus	<0.05	93	4.39	3, -6, 9	<0.001
			4.22	-3, -24, 6	<0.001
			3.5	-12, -6, 12	<0.001

Note: AAL = Automated Anatomical Labeling, FWER = Family Wise Error Rate, N = Number, MNI = Montreal Neurological Institute

Supplementary Table 12. T-test results on SCR, arousal and valence for the comparison of threat reversal (new CS+ vs. CS-) and safety reversal (new CS- vs. CS+) between healthy controls and patients with anxiety disorders.

	Measure	*t*	*df*	*p*	Cohen's *d*
Threat reversal	SCR	1.429	127	.155	0.2884
	Valence	1.400	127	.164	0.2824
	Arousal	-1.466	127	.145	-0.2957
Safety reversal	SCR	1.373	127	.172	0.2770
	Valence	-0.216	127	.829	-0.0436
	Arousal	0.497	127	.620	0.1003

Supplementary Table 13. ANOVAs results for the effects of group and CS type on SCR, arousal and valence during trace conditioning

ANOVA	Effect	*F*	*df*	*p*	η2
SCR	G	<.01	1, 122	.963	<.001
	CS[a]	45.13	1.46, 178.4	<.001	.270
	CS X G[a]	<.001	1.46, 178.4	.997	<.001
Arousal ratings	G	3.47	1, 122	.065	.028
	CS[b]	127.83	1.88, 229.47	<.001	.512
	CS X G[b]	2.26	1.88, 229.47	.110	.018
Valence ratings	G	0.15	1, 122	.699	.001
	CS[c]	111.68	1.69, 205.62	<.001	.478
	CS X G[c]	1.84	1.69, 205.62	.167	.015

Note: G = Group; CS = CS type. Greenhouse-Geisser corrected values identified with lowercase letters.
[a]: Mauchly's W = .632, p < .001, Greenhouse-Geisser ε = .731
[b]: Mauchly's W = .937, p = .019, Greenhouse-Geisser ε = .940
[c]: Mauchly's W = .813, p < .001, Greenhouse-Geisser ε = .843

Supplementary Table 14. ANOVA results for the effects of group, CS type, and phase on SCR during trace fear conditioning.

Effect	*F*	*df*	*p*	η2
G	0.01	1, 122	.963	.000
P	76.83	1, 122	<.001	.386
P x G	0.39	1, 122	.533	.003
CS[a]	45.13	1.46, 178.40	<.001	.270
CS x G[a]	0.00	1.46, 178.40	.997	.000
P x CS[b]	6.87	1.83, 222.98	.002	.053
P x CS x G[b]	0.66	1.83, 222.98	.506	.005

Note: G = Group, P = Phase, CS = CS type. Greenhouse-Geisser corrected values identified with lowercase letters.

[a]: Mauchly's W = .632, p < .001, Greenhouse-Geisser ε = .731

[b]: Mauchly's W = .906, p = .003, Greenhouse-Geisser ε = .914

Supplementary Table 15. Brain activations during trace fear conditioning (CS50+ vs. CS-) for healthy controls.

AAL Region	Cluster *p* value FWER	N voxels	*t*	MNI (x,y,z)	Peak *p* value uncorrected
Thalamus	<0.001	191	9.32	6, -21, -3	<0.001
			7.09	-6, -21, -3	<0.001
			6.34	12, -18, 6	<0.001
Supp_Motor_Area	<0.001	609	7.86	-9, -9, 69	<0.001
			7.72	9, -6, 69	<0.001
			7.58	6, 3, 51	<0.001
Precentral_L	<0.001	188	7.67	-42, -6, 51	<0.001
			6.3	-36, -18, 42	<0.001
			4.8	-51, 0, 42	<0.001
SupraMarginal_L	<0.001	193	7.2	-51, -33, 27	<0.001
			6.11	-57, -39, 33	<0.001
			5.96	-60, -21, 24	<0.001
Postcentral_R	<0.001	49	6.07	30, -27, 66	<0.001
			6.02	27, -27, 57	<0.001
Insula_L	<0.001	50	5.71	-33, 27, 3	<0.001
			5.63	-33, 18, 9	<0.001
Frontal_Mid_R	<0.001	23	5.65	45, -3, 54	<0.001

Note: AAL = Automated Anatomical Labeling, FWER = Family Wise Error Rate, N = Number, MNI = Montreal Neurological Institute

Supplementary Table 16. Brain activations during trace fear conditioning (CS50+ vs. CS-) for patients with anxiety disorders.

AAL Region	Cluster *p* value FWER	N voxels	*t*	MNI (x,y,z)	Peak *p* value uncorrected
Cingulum_Mid_R	<0.001	285	7.71	9, -27, 51	<0.001
Postcentral_R			5.56	30, -30, 60	<0.001
			4.94	24, -36, 60	<0.001
Supp_Motor_Area_R	<0.001	583	6.94	3, -6, 60	<0.001
			6.06	3, 0, 72	<0.001
			5.86	9, 6, 57	<0.001
Precentral_L	<0.001	153	6.52	-36, -6, 48	<0.001
			5.68	-45, -6, 51	<0.001
			5.2	-51, 3, 42	<0.001
SupraMarginal_L	<0.001	141	6.25	-63, -27, 21	<0.001
			4.86	-60, -18, 21	<0.001
			4.5	-54, -42, 39	<0.001
Fusiform_R	<0.001	292	5.75	36, -75, -15	<0.001
			5.66	36, -84, -12	<0.001
			5.23	-6, -87, 3	<0.001
SupraMarginal_R	<0.001	109	5.67	54, -27, 24	<0.001
			4.58	48, -15, 18	<0.001
			3.67	60, -39, 27	<0.001
Frontal_Inf_Oper_L	<0.001	247	5.48	-57, 9, 15	<0.001
			5.14	-51, 0, 6	<0.001
Insula_L			5.01	-42, 18, 0	<0.001
Insula_R	<0.05	59	5.18	36, 24, 0	<0.001
Frontal_Inf_Oper_R	<0.05	46	4.73	54, 15, 0	<0.001
			4.42	51, 6, 0	<0.001
			3.99	48, 0, 6	<0.001

Note: AAL = Automated Anatomical Labeling, FWER = Family Wise Error Rate, N = Number, MNI = Montreal Neurological Institute

Supplementary Table 17. Brain activations during trace fear conditioning (CS81+ vs. CS-) for healthy controls.

AAL Region	Cluster *p* value FWER	N voxels	*t*	MNI (x,y,z)	Peak *p* value uncorrected
Thalamus	<0.001	439	10.08	6, -21, 0	<0.001
			9.35	-15, -18, 6	<0.001
			8.64	-6, -21, -3	<0.001
Precentral_L	<0.001	197	8.79	-39, -9, 48	<0.001
			5.49	-48, 3, 45	<0.001
SupraMarginal_L	<0.001	276	8.72	-51, -33, 27	<0.001
			6.58	-45, -27, 9	<0.001
			6.34	-54, -18, 24	<0.001
Insula_L	<0.001	147	8.04	-30, 27, 3	<0.001
			7.82	-33, 18, 9	<0.001
			5.45	-51, 6, 3	<0.001
Supp_Motor_Area	<0.001	403	7.99	6, -18, 54	<0.001
			7.57	6, -3, 57	<0.001
			7.06	-3, 3, 54	<0.001
Postcentral_R	<0.001	104	7.75	33, -30, 63	<0.001
			7.56	27, -27, 54	<0.001
Caudate_L	<0.001	28	7.04	-9, 6, 6	<0.001
Insula_R	<0.001	52	6.86	33, 27, 0	<0.001
			6.31	33, 24, 9	<0.001
SupraMarginal_R	<0.001	20	5.83	54, -33, 30	<0.001
Putamen_R	<0.001	20	5.65	30, 0, 0	<0.001

Note: AAL = Automated Anatomical Labeling, FWER = Family Wise Error Rate, N = Number, MNI = Montreal Neurological Institute

Supplementary Table 18. Brain activations during trace fear conditioning (CS81+ vs. CS-) for patients with anxiety disorders.

AAL Region	Cluster *p* value FWER	N voxels	*t*	MNI (x,y,z)	Peak *p* value uncorrected
Supp_Motor_Area	<0.001	460	8.94	0, -6, 60	<0.001
			7.49	6, 6, 54	<0.001
			6.67	6, -27, 54	<0.001
Insula_L	<0.001	253	7.61	-36, 24, 3	<0.001
			5.55	-57, 6, 12	<0.001
			4.67	-51, 3, 3	<0.001
Thalamus	<0.001	99	6.76	3, -21, -6	<0.001
			5.38	9, -15, -6	<0.001
			5	-9, -18, 9	<0.001
Precentral_L	<0.001	442	6.71	-36, -6, 51	<0.001
			6.68	-42, -12, 54	<0.001
			5.63	-51, -3, 42	<0.001
Parietal_Inf_L	<0.001	303	6.51	-54, -39, 39	<0.001
SupraMarginal_L			5.39	-60, -33, 27	<0.001
			5.32	-51, -27, 15	<0.001
Cingulum_Mid	<0.01	79	5.88	-12, -24, 45	<0.001
			4	-15, -39, 51	<0.001
Precentral_R	<0.001	205	5.62	33, -27, 63	<0.001
			5.25	42, -12, 57	<0.001
			4.92	33, -36, 63	<0.001
Insula_R	<0.001	183	5.58	39, 21, 6	<0.001
			5.21	48, 6, 3	<0.001
			4.93	54, 12, 9	<0.001
SupraMarginal_R	<0.01	95	5.55	51, -30, 27	<0.001
			5.03	45, -24, 21	<0.001
Calcarine	<0.001	193	5.09	-9, -90, 3	<0.001
			5.04	3, -81, 3	<0.001
			4.82	9, -87, 6	<0.001

Note: AAL = Automated Anatomical Labeling, FWER = Family Wise Error Rate, N = Number, MNI = Montreal Neurological Institute

Supplementary Table 19. Brain activations during trace fear conditioning (CS81+ vs. CS50+) for healthy controls.

AAL Region	Cluster *p* value FWER	N voxels	*t*	MNI (x,y,z)	Peak *p* value uncorrected
Temporal_Mid_R	<0.001	371	4.9	39, -51, 15	<0.001
			4.63	33, -60, 15	<0.001
			4.41	51, -57, 15	<0.001
Putamen_R	<0.01	142	4.89	30, -6, 0	<0.001
			4.74	24, -6, 9	<0.001
Hippocampus_R			4.34	30, -24, -3	<0.001
Occipital_Mid_L	<0.001	716	4.46	-39, -63, 18	<0.001
			4.37	-36, -36, 15	<0.001
Putamen_L			4.36	-21, -9, 15	<0.001
Thalamus_L			4.16	-11, -22, 6	<0.001
Precentral_R	<0.05	79	4.43	36, -18, 48	<0.001
Postcentral_R			4.3	30, -27, 54	<0.001

Note: AAL = Automated Anatomical Labeling, FWER = Family Wise Error Rate, N = Number, MNI = Montreal Neurological Institute

Additional analysis - Covariating for biological sex and age

Supplementary Table 20. ANCOVAs results for the effects of group, CS type, sex and age on SCR, arousal and valence during delay conditioning

ANCOVA	Effect	*F*	*df*	*p*	η2
SCR	G	5.86	1, 124	.017	.045
	S	4.00	1, 124	.048	.031
	A	0.29	1, 124	.594	.002
	G x S	0.45	1, 124	.505	.004
	CS	6.62	1, 124	.011	.051
	CS x G	0.60	1, 124	.439	.005
	CS x S	0.03	1, 124	.866	<.001
	CS x A	0.82	1, 124	.368	.007
	CS x G x S	3.88	1, 124	.051	.030
Arousal ratings	G	1.46	1, 124	.229	.012
	S	1.51	1, 124	.221	.012
	A	0.08	1, 124	.778	<.001
	G x S	2.92	1, 124	.090	.023
	CS	20.53	1, 124	<.001	.142
	CS x G	0.20	1, 124	.659	.002
	CS x S	3.55	1, 124	.062	.028
	CS x A	1.63	1, 124	.205	.013
	CS x G x S	3.30	1, 124	.071	.026
Valence ratings	G	0.05	1, 124	.822	<.001
	S	0.04	1, 124	.851	<.001
	A	1.11	1, 124	.293	.009
	G x S	0.09	1, 124	.767	<.001
	CS	13.68	1, 124	<.001	.099
	CS x G	0.16	1, 124	.693	<.001
	CS x S	7.39	1, 124	.007	.056
	CS x A	0.21	1, 124	.651	<.001
	CS x G x S	2.69	1, 124	.104	.021

Note: G=Group, S=Sex, A=Age, CS = CS type

Supplementary Table 21. ANCOVAs results for the effects of group, CS type, sex and age on SCR, arousal and valence during fear reversal

ANCOVA	Effect	*F*	*df*	*p*	η2
SCR	G	1.46	1, 124	.228	.012
	S	0.03	1, 124	.867	<.001
	A	0.02	1, 124	.877	<.001
	G x S	1.06	1, 124	.305	.008
	CS	6.41	1, 124	.013	.049
	CS x G	0.64	1, 124	.425	.005
	CS x S	0.31	1, 124	.579	.002
	CS x A	0.54	1, 124	.463	.004
	CS x G x S	0.95	1, 124	.331	.008
Arousal ratings	G	2.65	1, 124	.106	.021
	S	0.00	1, 124	.946	<.001
	A	2.64	1, 124	.107	.021
	G x S	1.12	1, 124	.293	.009
	CS	17.62	1, 124	<.001	.124
	CS x G	1.83	1, 124	.179	.015
	CS x S	0.16	1, 124	.687	.001
	CS x A	1.69	1, 124	.196	.013
	CS x G x S	2.22	1, 124	.139	.018
Valence ratings	G	0.47	1, 124	.492	.004
	S	1.06	1, 124	.306	.008
	A	0.71	1, 124	.401	.006
	G x S	3.13	1, 124	.079	.025
	CS	12.88	1, 124	<.001	.094
	CS x G	1.33	1, 124	.252	.011
	CS x S	0.10	1, 124	.758	<.001
	CS x A	1.19	1, 124	.277	.010
	CS x G x S	0.89	1, 124	.347	.007

Note: G=Group, S=Sex, A=Age, CS = CS type

Supplementary Table 22. ANCOVAs results for the effects of group, CS type, sex and age on SCR, arousal, and valence during trace conditioning

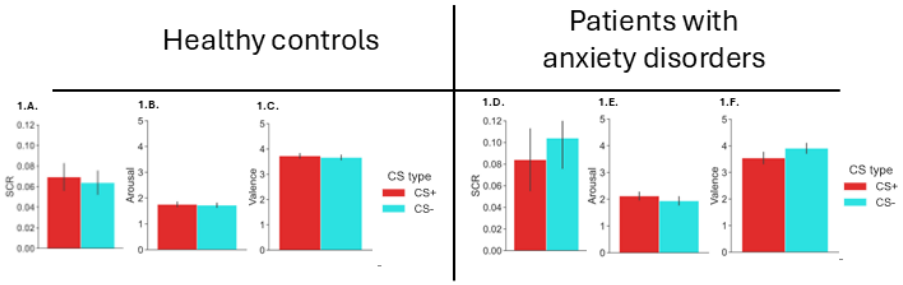
ANCOVA	Effect	*F*	*df*	*p*	η2
SCR	G	0.06	1, 119	.805	.001
	S	0.00	1, 119	.979	.000
	A	2.83	1, 119	.095	.023
	G x S	3.65	1, 119	.058	.030
	CS[a]	6.75	1.47, 175.26	.004	.054
	CS x G[a]	0.01	1.47, 175.26	.975	.000
	CS x S[a]	1.54	1.47, 175.26	.220	.013
	CS x A[a]	1.59	1.47, 175.26	.210	.013
	CS x G x S[a]	2.40	1.47, 175.26	.109	.020
Arousal Ratings	G	3.79	1, 119	.054	.031
	S	5.12	1, 119	.026	.041
	A	0.23	1, 119	.636	.002
	G x S	1.20	1, 119	.275	.010
	CS[b]	12.04	1.90, 225.95	<.001	.092
	CS x G[b]	2.19	1.90, 225.95	.117	.018
	CS x S[b]	4.72	1.90, 225.95	.011	.038
	CS x A[b]	1.55	1.90, 225.95	.215	.013
	CS x G x S[b]	2.43	1.90, 225.95	.093	.020
Valence Ratings	G	0.18	1, 119	.671	.002
	S	1.29	1, 119	.258	.011
	A	0.42	1, 119	.519	.004
	G x S	0.08	1, 119	.775	.001
	CS[c]	18.36	1.72, 204.34	<.001	.134
	CS x G[c]	1.92	1.72, 204.34	.156	.016
	CS x S[c]	1.13	1.72, 204.34	.320	.009
	CS x A[c]	4.81	1.72, 204.34	.013	.039
	CS x G x S[c]	0.12	1.72, 204.34	.857	.001

Note: G=Group, S=Sex, A=Age, CS = CS type. Greenhouse-Geisser corrected values identified with lowercase letters.
[a]: Mauchly's W = .642, p < .001, Greenhouse-Geisser ε = .736
[b]: Mauchly's W = .947, p = .039, Greenhouse-Geisser ε = .949
[c]: Mauchly's W = .835, p < .001, Greenhouse-Geisser ε = .859

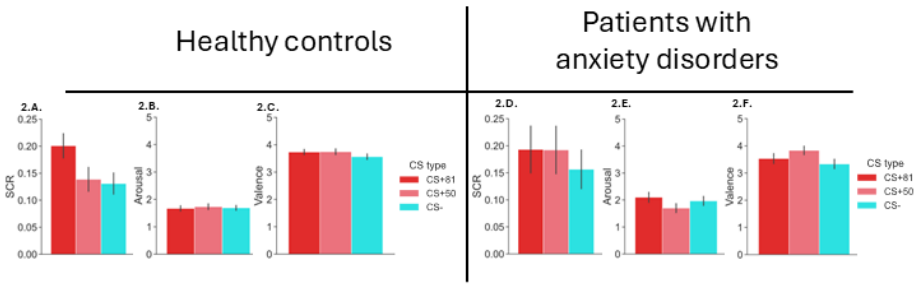
SUPPLEMENTARY REFERENCES

1. Buela-Casal, G., Guillén-Riquelme, A. & Seisdedos Cubero, N. *Cuestionario de Ansiedad Estado-Rasgo*. (TEA Ediciones, Madrid, 2016).
2. Spielberg, C. D. , Gorsuch, R. L. , & Lushene RE. *Manual for the State-Trait Inventory*. (Consulting Psychological Press, Palo Alto: CA, 1970).
3. Ferrando L *et al. MINI Entrevista Neuropsiquiátrica Internacional*. (Instituto IAP, Madrid, 1998).
4. Sheehan, D. V *et al.* The Mini-International Neuropsychiatric Interview (M.I.N.I.): the development and validation of a structured diagnostic psychiatric interview for DSM-IV and ICD-10. *J Clin Psychiatry* **59 Suppl 20**, 22–33 (1998).
5. Freeston, M. H., Rheaume, J., Letarte, H., Dugas, M. J. & Ladouceur, R. Intolerance of Uncertainty Scale. *PsycTESTS Dataset* (2012) doi:10.1037/T01110-000.
6. González Rodríguez, M., Cubas León, R., Rovella, A. T. & Darias Herrera, M. Adaptación española de la escala de intolerancia hacia la incertidumbre: procesos cognitivos, ansiedad y depresión. *Psicología y Salud* **16**, 219–233 (2006).
7. Bobes, J. , *et al.* Validation of the Spanish version of the Liebowitz social anxiety scale, social anxiety and distress scale and Sheehan disability inventory for the evaluation of social phobia. *Medicina Clínica (Barcelona)* **112**, 130–138 (1999).
8. Liebowitz, M. R. Social Phobia. *Mod Probl Pharmacopsychiatry* 141–173 (1987).
9. Bobes, J. , *et al.* Psychometric properties of the Spanish version of the screening scale for DSM-IV Generalized Anxiety Disorder of Carroll and Davidson. *Actas Esp Psiquiatr* **34**, 83–93 (2006).
10. Carroll, B. J. & Davidson, J. R. T. Screening scale for DSM-IV GAD. *Copyright* (2000).
11. Meyer, T. J. , Miller, M. L. , Metzger, R. L. & Borkovec, T. Development and validation of the Penn State Worry Questionnaire. *Behaviour research and therapy* **28**, 487–495 (1990).
12. Sandín, B., Chorot, P., Valiente, R. M. & Lostao, L. Validación española del cuestionario de preocupación PSWQ : estructura factorial y propiedades psicométricas. *Revista de Psicopatología y Psicología Clínica* **14**, 107–122 (2009).
13. Bados, A., Solanas, A. & Andrés, R. Psychometric properties of the Spanish version of Depression, Anxiety and Stress Scales (DASS). *Psicothema* **17**, 679–683 (2005).
14. Lovibond, S. H. & Lovibond, P. F. Depression Anxiety Stress Scales. *PsycTESTS Dataset* (1995) doi:10.1037/T01004-000.
15. Tustison, N. J. *et al.* N4ITK: Improved N3 Bias Correction. *IEEE Trans Med Imaging* **29**, 1310–1320 (2010).
16. Jenkinson, M., Beckmann, C. F., Behrens, T. E. J., Woolrich, M. W. & Smith, S. M. FSL. *Neuroimage* **62**, 782–790 (2012).
17. Dale, A. M., Fischl, B. & Sereno, M. I. Cortical Surface-Based Analysis: I. Segmentation and Surface Reconstruction. *NeuroImage (Orlando, Fla.)* **9**, 179–194 (1999).
18. Klein, A. *et al.* Mindboggling morphometry of human brains. *PLoS Comput Biol* **13**, e1005350–e1005350 (2017).
19. Fonov, V., Evans, A., McKinstry, R., Almli, C. & Collins, D. Unbiased nonlinear average age-appropriate brain templates from birth to adulthood. *NeuroImage (Orlando, Fla.)* **47**, S102–S102 (2009).
20. Esteban, O. *et al.* Analysis of task-based functional MRI data preprocessed with fMRIPrep. *Nat Protoc* **15**, 2186–2202 (2020).

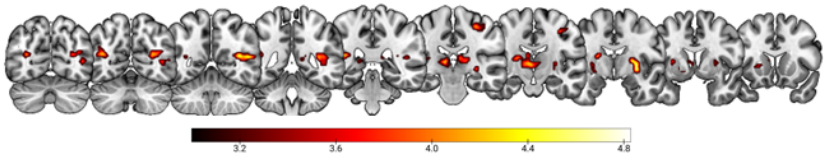
21. Gorgolewski, K. *et al.* Nipype: a flexible, lightweight and extensible neuroimaging data processing framework in python. *Front Neuroinform* **5**, 13–13 (2011).
22. Greve, D. N. & Fischl, B. Accurate and robust brain image alignment using boundary-based registration. *NeuroImage (Orlando, Fla.)* **48**, 63–72 (2009).
23. Cox, R. W. & Hyde, J. S. Software tools for analysis and visualization of fMRI data. *NMR Biomed* **10**, 171–178 (1997).
24. Jenkinson, M., Bannister, P., Brady, M. & Smith, S. Improved Optimization for the Robust and Accurate Linear Registration and Motion Correction of Brain Images. *NeuroImage (Orlando, Fla.)* **17**, 825–841 (2002).
25. Lanczos, C. Evaluation of Noisy Data. *Journal of the Society for Industrial and Applied Mathematics. Series B, Numerical analysis* **1**, 76–85 (1964).
26. Pruim, R. H. R. *et al.* ICA-AROMA: A robust ICA-based strategy for removing motion artifacts from fMRI data. *NeuroImage (Orlando, Fla.)* **112**, 267–277 (2015).
27. Behzadi, Y., Restom, K., Liau, J. & Liu, T. T. A component based noise correction method (CompCor) for BOLD and perfusion based fMRI. *NeuroImage (Orlando, Fla.)* **37**, 90–101 (2007).
28. Power, J. D. *et al.* Methods to detect, characterize, and remove motion artifact in resting state fMRI. *NeuroImage (Orlando, Fla.)* **84**, 320–341 (2014).



Supplementary Figure 1: Responses during preconditioning in the delay conditioning/reversal task in healthy controls (n=98) and patients with anxiety disorders (n=33). LEFT: SCR (last trial) (A), subjective ratings of arousal (B) and valence (C) RIGHT: SCR (last trial) (D) subjective ratings of arousal (E) and valence (F). SCR = skin conductance response. CS = conditioned stimuli. Error bars indicate standard error of the mean (SEM).



Supplementary Figure 2: Responses during preconditioning in the trace conditioning task in healthy controls (n=93) and patients with anxiety disorders (n=31). LEFT: SCR (last trial) (A), subjective ratings of arousal (B) and valence (C) RIGHT: SCR (last trial) (D) subjective ratings of arousal (E) and valence (F). SCR = skin conductance response. CS = conditioned stimuli. Error bars indicate standard error of the mean (SEM). **p<0.01.*



Supplementary Figure 3. Clusters of fMRI activations (CS81+ > CS50+) for trace conditioning in healthy controls