

# Transcranial Ultrasonic Stimulation of the Human Amygdala during Threat Learning



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## Abstract

Transcranial Ultrasonic Stimulation (TUS) is a novel non-invasive neuromodulation technique that can achieve focal modulation of deep brain structures such as the amygdala. In this study, we combined TUS with well-validated fear conditioning procedures, exploiting the unique opportunity for a causal test of amygdala-dependent threat learning processes in healthy humans. Ten healthy participants received online bilateral amygdala-TUS during a standard fear conditioning procedure known to involve the amygdala. We stimulated at 250kHz, with a 1000Hz pulse repetition frequency and 30% duty cycle, and with an intracranial spatial-peak pulse-averaged intensity ( $I_{\text{sppa}}$ ) below 20W/cm<sup>2</sup>. TUS was delivered on a trial-by-trial basis and at the same time as presentation of the conditioned stimulus. The fear conditioning procedure was composed of an acquisition phase, followed by a distractor task, and a recall phase, which included spontaneous recall and recall following a reinstatement procedure. Neuronavigation was used throughout the entire procedure for accurate TUS targeting. We assessed the neuromodulatory effects of amygdala-TUS using a comprehensive set of physiological markers of threat learning and recall, including skin-conductance (SCR) and heart rate response (HRR). We found a significant effect of online TUS on SCR, though this did not seem to affect threat learning. No effects of TUS were found during recall or on expression to the aversive stimulus. In light of our findings, we speculate on the idea that the human amygdala might in fact not have a crucial role in threat learning, though, we strongly emphasize that more robust and consistent evidence, which the field currently lacks, is necessary.

Key words: transcranial ultrasound stimulation; TUS; threat learning; amygdala

## Introduction

The ability to recognize threat is a precondition for survival. Prompt identification of a dangerous situation makes it possible to adequately act on the threat. For example, if you were taking a nature walk and suddenly encountered a snake, identifying this as a potential threat would be necessary for responding to the situation accordingly to ensure one's own safety. However, threat can also be experienced in non-dangerous situations. In fact, an undesired expression of learned fear responses in a safe environment is what characterizes anxiety disorders (Lissek & Grillon, 2010; Shin & Liberzon, 2010). Anxiety disorders are the most prevalent class of mental disorders impacting millions of lives each year (Wittchen and Jacobi, 2005; Somers et al., 2006; Kessler et al., 2009), while a mere 40 - 60% of patients benefit from current evidence-based pharmacological and psychotherapeutic therapies, demonstrating the serious and urgent need for clinical development. To advance development of novel treatment options for anxiety disorders, we need to obtain a thorough understanding of the threat learning process: how do humans learn about threat?

The amygdala is commonly appointed as the core for processing threat. While this sub-cortical brain structure is found to be involved in emotional and learning behaviors more generally, the most consistent finding across rodent and non-human primate studies is that threat learning depends on the amygdala (Maren, 1999; Nader et al., 2001; Koo, Han & Kim, 2004; Antoniadis et al., 2007, 2009). Animal models based on these findings have contributed towards our understanding of the

underlying neural mechanisms of threat learning. Unfortunately, causal evidence in humans is very limited. Studies on patients with selective bilateral amygdala damage, also known as the extremely rare Urbach-Wiethe disease, found deficits in threat learning within this population (Bechara et al., 1995; Klumpers et al., 2015). However, amygdala damage in this population is largely heterogeneous in extent and location, which restricts generalization of these findings, in addition to these not stemming from a healthy population (Klumpers et al., 2015; Terburg et al., 2012, Terburg et al., 2018). Despite the large base of research on the role of the amygdala in threat learning, a causal role of the human amygdala in threat learning is yet to be established.

Non-invasive neuromodulation techniques can be used in humans for mapping causal brain-behavior relationships. However, because of the amygdala's location deep inside the brain, it is out of reach for most conventional non-invasive neuromodulation techniques, such as Transcranial Magnetic Stimulation (TMS) and Transcranial Electric Stimulation (TES) (Bestmann & Walsh, 2017). The novel non-invasive neuromodulation technique Transcranial Ultrasound Stimulation (TUS) can be used to effectively modulate brain activity even in deep brain structures, such as the amygdala (Folloni et al., 2019; Tufail et al., 2011; Tyler et al., 2018; Verhagen et al., 2019). In fact, a recent pilot study used amygdala-TUS on anxiety disorder patients and found a significant decrease in anxiety and depression, based on pre- and post-stimulation inventory scores (Mahdavi et al., 2021). Previously, TUS has been shown to effectively modulate amygdala activity in non-human primates (Verhagen et al., 2019). Furthermore, TUS has been

successfully employed to induce changes in cognitive processes by neuromodulation of both cortical (Reznik et al., 2020; Sanguinetti et al., 2020) and sub-cortical brain structures (Badran et al., 2020; Jeong et al., 2021).

This study intended to exploit the unique opportunity for a causal test of amygdala-dependent threat learning processes in healthy humans, using a combination of TUS and well-validated fear conditioning procedures. In the domain of threat learning, fear conditioning is the most widely studied translational model of clinical anxiety disorders (Fullana et al., 2020). With this setup, this study has been one of the first to investigate an online impact of TUS on threat learning in healthy humans.

Here, we focused on the current theory that threat learning is amygdala-dependent (see e.g. Maren, 1999; Nader et al., 2001; Koo, Han & Kim, 2004; Antoniadis et al., 2007, 2009). This would imply that disruption of amygdalar activity by TUS application should result in changes to the threat learning procedure. However, we were not able to observe these changes within the preliminary data from this study. We assessed the neuromodulatory effects on standard physiological measures of threat learning: skin-conductance response (SCR) and heart rate response (HRR) (Lonsdorf et al., 2017). Additionally, we investigated longer-term effects by including a standard fear recall phase, allowing us to assess post-TUS differences in threat-related memories and to determine whether TUS during acquisition affected subsequent recall.

## Methods

### Participants

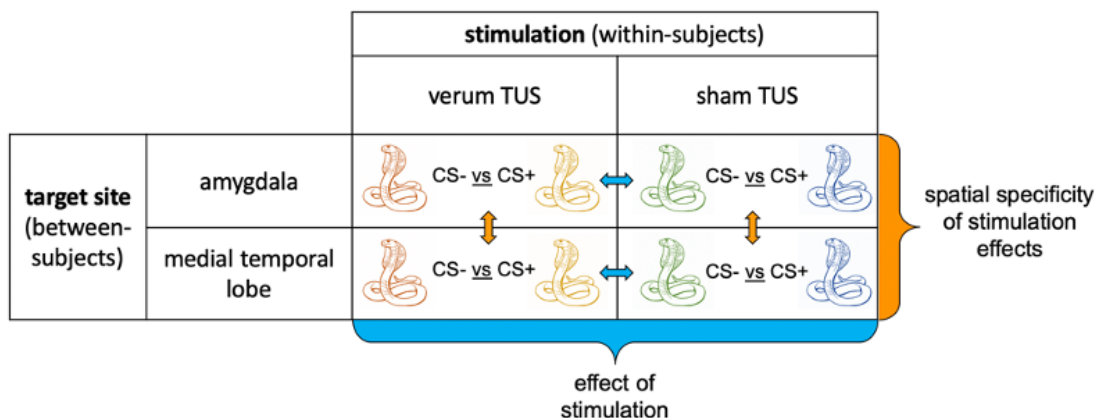
In total, 10 participants (age:  $22.3 \pm 1.4$ , males = 4, females = 6) participated in the study via the Radboud Research Participation System (SONA).

Inclusion criteria were that participants were 18 years or older, had no claustrophobia, were not pregnant, had no history of brain surgery or serious head trauma, had no history or any close relatives with epilepsy, convulsion or seizure, had no cardiac pacemaker, intra-cardiac lines or cochlear implants, had no metal in the brain, skull or elsewhere in the body, did not use psychoactive medication (excluding anti-conception), had no skin disease at the intended stimulation sites and had no ophidiophobia (fear of snakes). To verify the last requirement, participants were asked to fill out a Snake Questionnaire (Polák et al., 2016). All participants provided written informed consent prior to participation. Participants were reimbursed €40 following completion of the experiment. All procedures in this study were approved by the local ethical review board (CMO Region Arnhem-Nijmegen, The Netherlands).

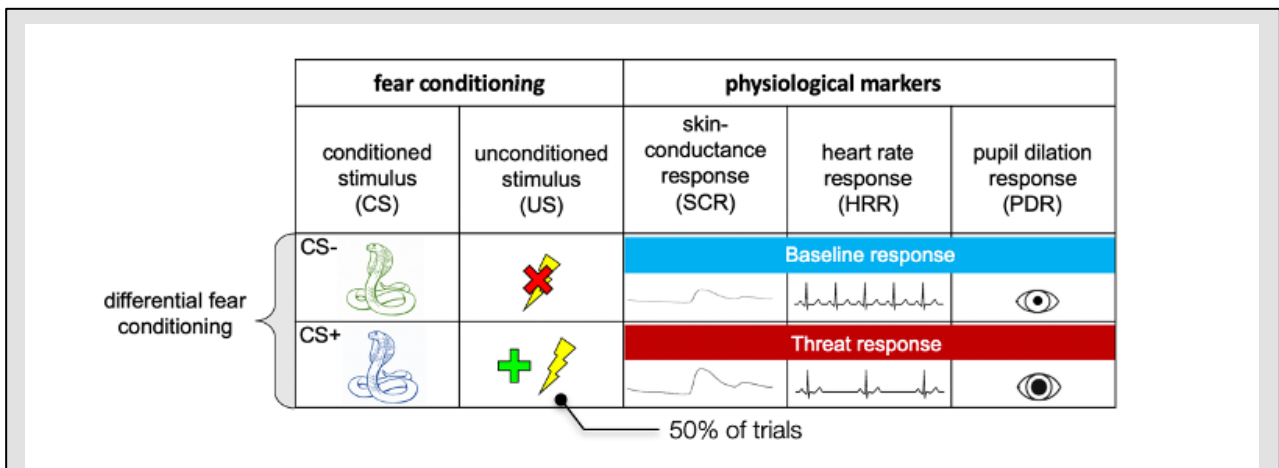
### Experimental Design and Procedure

The study employs a mixed factorial design with conditioned stimulus (CS+, CS-) and stimulation (verum TUS, sham TUS) as within-subject factors and target site (amygdala, medial temporal lobe) as between-subjects factor. The full design is shown in Fig 1. Note that the preliminary study results that we discuss here were obtained only from participants who received amygdala-TUS.

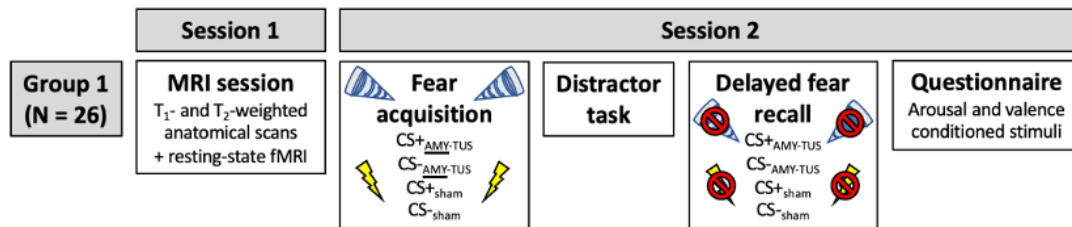
Participants were invited to complete two sessions. An overview of these sessions is provided in Fig 2. During the first session, we obtained structural and functional MRI scans from the participant. The structural scans were needed for online neuronavigation in the second session. We made use of a basolateral amygdala mask (97% threshold) from the Jülich cytoarchitectonic atlas (Eickhoff et al., 2005), which has been widely used in fMRI studies for an accurate definition of amygdala subregions (Ball et al., 2007; Roy et al., 2009; Li et



**Fig. 1. Mixed-factorial design overview showing within-subjects factors conditioned stimulus and TUS, and between-subjects factor target site.** The passive control condition, sham TUS, provides a basis for comparison and controls for potential confounds due to auditory and somatosensory sensations elicited by the application of TUS. The active control condition, medial temporal lobe verum TUS, confirms the spatial specificity of stimulation effects by delivering TUS with the same intensity to an alternate brain site within the medial temporal lobe that is not implicated in threat learning.



**The fear conditioning procedure.** A core element of our study design is the fear conditioning procedure. During fear conditioning, participants learn that a certain visual cue, such as an image of a snake, predicts the occurrence of an aversive stimulus, such as a shock. An association is formed between the conditioned stimulus (CS) and the subsequent occurrence of the unconditioned stimulus (US). Threat learning is characterized by formation and strengthening of CS-US associations. The CS- is never paired with the US, whereas the CS+ is (partially) paired with the US, in our case in 50% of the trials. Over time, the CS+ starts to elicit a physiological response in anticipation of the US. This threat-anticipatory response reflects threat learning processes and is associated with increased skin-conductance response (SCR), decelerated heart rate response (HRR), and increased pupil dilation response (PDR).



**Fig. 2. Overview of experimental procedure.** fMRI = functional magnetic resonance imaging, CS = conditioned stimulus, AMY = amygdala, TUS = transcranial ultrasound stimulation

al, 2012; Gabard-Durnam et al., 2014; Qin et al., 2014; Engman et al., 2016; Eckstein et al., 2017). This was registered from MNI space to subject-space to ensure accurate TUS targeting using neuronavigation. Additionally, resting state fMRI was acquired for future exploration of inter-individual differences in TUS intervention response based on amygdala functional connectivity. In the second session, participants underwent a fear conditioning procedure combined with TUS. This procedure consisted of an acquisition phase, a short distractor task, and a fear recall phase, which was split into spontaneous recall and recall following a reinstatement procedure. This paradigm allowed us to investigate differences in fear acquisition and potential longer-term effects during fear recall.

Throughout the session, participants were presented with four conditioned stimuli (CS), pictures of snakes. Two of these (CS+) were paired with aversive electrical stimulation (US; 50% reinforcement rate during acquisition), whereas the other two (CS-) would never be paired with aversive electrical stimulation. The 50% reinforcement rate allowed us to distinguish between responses to the

non-reinforced and the reinforced CS+, which is relevant for observation of threat learning processes that are characterized by a threat-anticipatory response to the non-reinforced CS+, but not to the CS-. The stimuli were paired (two CS+/CS- pairs) and presented in a blocked design, where a block consisted of 4 trials. One stimuli pair was always presented in verum TUS blocks and the other in sham TUS blocks. An in-house Python script was written for counterbalancing of stimuli across participants, as well as pseudo-randomization of the content and order of the blocks within and across participants. This was done with the aim to avoid snake identity effects and order effects of the stimuli. Important to note here is that counterbalanced stimulus allocation was unfortunately unsuccessful (see Discussion). In between trials and blocks a white fixation cross on a gray background was shown. These inter-trial intervals (ITI) and inter-blocks intervals (IBI) were jittered between 8-10 and 18-20 seconds respectively. The length of the ITI allows heart rate (HR) and electrodermal activity (EDA) to return to baseline between trials (Lonsdorf et al., 2017). The entire experiment was realized in Python, using the Psychopy software package for

neuroscience and experimental psychology (Peirce et al., 2019).

At the start of the experiment, participants were informed that they would be presented with four pictures of snakes, of which two would be paired with a shock. To encourage the learning process, participants were instructed to find out which snakes predicted the occurrence of a shock. The acquisition phase consisted of 120 trials, of which an equal amount of CS+ and CS- trials across 15 verum TUS and 15 sham TUS blocks. A distractor task was presented to participants between the acquisition and the recall phase. This was a 5-minute task, in which participants were shown arrows on a screen pointing either to the left (<<<<) or the right (>>>>) and asked to indicate the direction as quickly as possible by pressing a keyboard button. We included this task to avoid carry-over TUS effects into the recall phase. Finally, during the recall phase, participants were presented with the same stimuli, such that there was continuation of contextual cues from the acquisition phase, but now only receiving sham TUS and no electrical stimulation. Halfway throughout the recall phase, participants received reinstatement by electrical stimulation five times, while a different fixation point ('O') was shown on the screen, as to not associate the electrical stimulation with any of the stimuli. After this reinstatement, participants continued presentation of the stimuli with sham TUS and no electrical stimulation. The spontaneous recall phase and recall after reinstatement consisted of 40 trials each, in which all stimuli were presented equally often. Upon completion of the entire fear conditioning procedure, participants went through 200 trials. An overview of the trial level is depicted in Fig 3.

### Amygdala stimulation

Transcranial Ultrasonic Stimulation (TUS) was delivered using a NeuroFUS Pro™ system (Sonic Concepts Inc. Bothell, Washington, USA) using two piezoelectric ultrasound transducers. The transducers

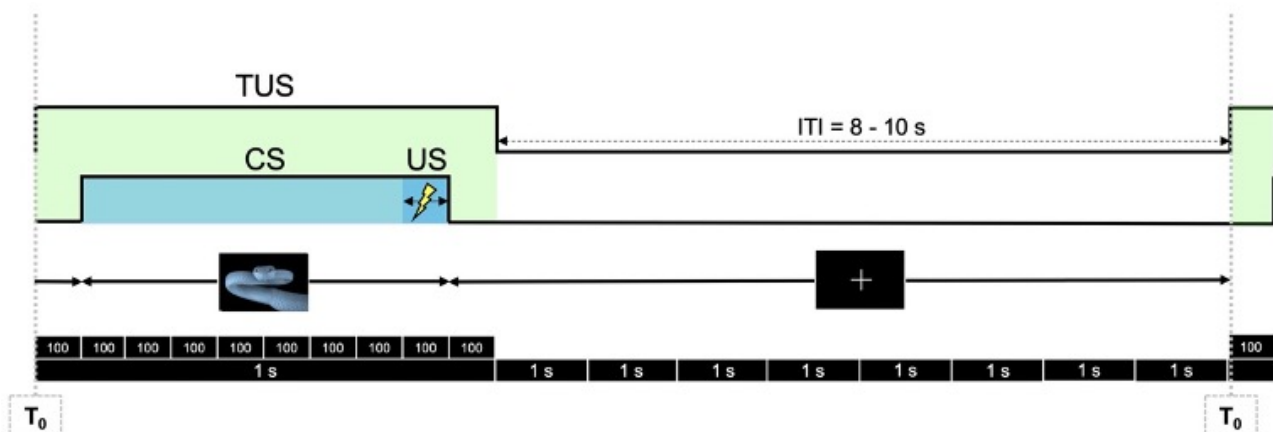
have a center frequency of 500 kHz, as commonly used for human neuromodulation (see e.g. Ai et al., 2016; Ai et al., 2018; Braun et al., 2020; Fomenko et al., 2020; Legon, Ai, et al., 2018; Legon, Bansal, et al., 2018; Legon et al., 2014; Liu et al., 2021; Mueller et al., 2014; Sanguinetti et al., 2020; Yu et al., 2020). The transducers are appropriate for neuromodulatory use in the context of our experiment (Pasquinelli et al., 2019). Two TUS transducers were bilaterally coupled to the participant's head by a trained experimenter, using a gel pad and ultrasound gel.

We replicated procedures conducted by Fomenko and colleagues' study (2020) by stimulating at 250kHz, with a 1000Hz pulse repetition frequency (PRF) at a 30% duty cycle. To avoid ultrasonic wave interference from the two stimulation sites, the TUS pulses were delivered interleaved over the left and right amygdala. The stimulation was delivered at an intracranial spatial-peak pulse-averaged intensity ( $I_{sp\text{ppa}}$ ) below 20 W/cm<sup>2</sup>. The design included a passive and active control condition (see Experimental Design and Procedure). During the passive control condition, ineffective stimulation (sham TUS) is delivered at a 1% duty cycle. This stimulation does not lead to neuromodulation but maintains vibration and humming of the device. The audible confounds of TUS were minimized using an auditory mask (see Auditory Stimulation).

### Peripheral stimulation

#### Mild electrical stimulation

Participants received electrical stimulation through the distal phalanges of the middle and ring finger of the right hand. This only happened during the acquisition phase and the reinstatement procedure. Shocks were delivered by a Transcutaneous Electrical Nerve Stimulator (TENS) unit (Prostim 2000, Bio-Protech inc., Korea) using Ag/AgCl electrodes with a shock duration of 100ms (150 Hz, pulse train of 250μs pulses). This procedure has been widely used in humans in classical aversive conditioning



**Fig. 3. Overview of a single trial.** TUS was administered for 1 second, during the entire association formation window in which the CS were shown on the screen (800 milliseconds). Electrical stimulation was administered for 100 milliseconds before the disappearance of the CS. Throughout the entire experiment, TUS is delivered for 120 seconds cumulatively, of which 60 seconds verum TUS and 60 seconds sham TUS. ITI = inter-trial interval, CS = conditioned stimulus, US = unconditioned stimulus

paradigms, in which mild electrical stimulation serves as an aversive event to be linked to a visual stimulus (Zorawski et al., 2006). Additionally, the procedure is known to reliably induce physiological responses related to threat and anxiety, such as startle reflex potentiation (Grillon et al., 1991; Hermans et al., 2006). The shock intensity level was always adjusted prior to the experiment according to a standardized staircase procedure. This allowed us to titrate the intensity to the participant's individual threshold with the aim to arrive at a level where the shocks were experienced as unpleasant, but not painful. The procedure consisted of five shock administrations, starting at 0.5 mA, whereafter the participant was asked to rate the shock on a scale from 1 (not at all painful) to 5 (very painful) and the amplitude was adjusted accordingly. The maximum number of shocks given throughout the experiment was 5 during the staircase procedure, 30 during the acquisition phase of the fear conditioning procedure (60 CS+ trials with a 50% reinforcement rate), and 5 during the reinstatement procedure.

### Visual stimulation

The fear conditioning paradigm used four images of snakes as visual stimuli, see Fig 4. The stimuli were selected on characteristics that European people have reported to be most fear eliciting (Rádlová et al., 2019). Snakes were chosen as fearful stimuli for this study, given that people naturally fear snakes (LoBue & DeLoache, 2008), which would enhance physiological threat-related responses. The images were aligned in luminance to reduce undesired pupil constriction due to the images' physical properties (Barbur, 2004).



**Fig. 4. Images of the conditioned stimuli.** These images were taken from the Geneva Affective Picture Database (GAPED) (Dan-Glauser & Scherer, 2011). From left to right, top row: Sn131, Sn123, bottom row: Sn033, Sn094.

### Auditory stimulation

TUS elicits auditory signals coming from the pulsed stimulation that are detectable in humans (Braun et al., 2020). Since we stimulate with a 1000Hz PRF and this frequency falls within the human hearing range, we needed to prevent potential confounds related to auditory and somatosensory sensations elicited by the TUS apparatus during periods of stimulation. To this end, we used a standard auditory masking procedure by delivering a masking sound at 1000Hz, which has been shown to reduce detection rates in humans (Braun et al., 2020).

### Physiological measurements

Faced with threat, the autonomous nervous system responds with increased physiological arousal (Leuchs et al., 2019). We recorded electrodermal activity (EDA) and heart rate (HR) for the offline assessment of activation of the sympathetic and parasympathetic nervous system respectively. Data was acquired using a BrainAmp EXG MR 16 channel recording system (Brain Products, Gilching, Germany). Note: pupil dilation data was also recorded during the experiment, but unfortunately, this data was not usable due to extreme noise patterns as a result of infrared light interference with the neuronavigation system (see Discussion).

### Electrodermal activity

Differential skin conductance responses (SCRs) to the conditioned stimuli (CS+, CS-) scored from electrodermal activity (EDA) is the most widely used index of human threat learning (Hamm & Weike, 2005; Lonsdorf et al., 2017, 2019; Hamm, 2020). We used this as our main study parameter. EDA was measured using Ag/AgCl electrodes placed on the palmar side of the distal phalanges of the ring and little finger of the left hand. The data was recorded with a sampling frequency of 500Hz, downsampled to 50Hz and low pass filtered (2Hz) prior to pre-processing. SCRs were pre-processed offline using the Autonomate Toolbox for Matlab (Green et al., 2014; Matlab, R2022a, The MathWorks, Inc). Event-related SCRs were calculated as the amplitude difference for trials in which the start of the increase in SCR was present between 0.5 and 8s following trial onset, with a maximum base-to-peak rise time of 6s. In case of multiple SCRs meeting these requirements, the largest response was used for analysis. This trough-to-peak scoring method is commonly applied in human fear conditioning research (Boucsein, 2012; Fowles et al., 1981; Lonsdorf et al., 2017). All event-related SCRs were baseline corrected by subtracting the average of 1s prior to stimulus onset. The distribution of SCRs is typically skewed, which can form an issue to normality assumptions of statistical tests. All amplitude difference were square root transformed to calculate normalized SCRs ( $\sqrt{\mu S}$ ) prior to

statistical analysis (Levine and Dunlap, 1982; 'Publication Recommendations for Electrodermal Measurements', 2012).

### Heart rate

Differential heart rate responses (HRRs) were used as a secondary study parameter. Electrocardiograms (ECG) were collected using three Ag/AgCl electrodes containing adhesive patches (3M Red Dot Electrode). One electrode was placed below the right clavicle, one on the left side of the chest, just below the sixth rib, and the ground electrode was attached under the left clavicle. The ECG data was recorded with a sampling frequency of 500Hz and then downsampled to 50Hz for pre-processing. The raw data was pre-processed offline using in-house software for manual artifact correction and peak detection, outputting a time-series of inter-beat intervals (IBIs) which were used to calculate the corresponding beats per minute (BPM) over time. Event-related HRRs were calculated as the net decrease in BPM, by subtracting the average BPM during 1s preceding stimulus onset from the minimum BPM during 0 – 2.5s after stimulus onset (baseline-to-trough). This window from 0 – 2.5s was chosen as this is where we typically observe threat-related bradycardia (e.g. Hamm et al., 1993; Hodes et al., 1985; Panitz et al., 2015), which is associated with activation of the parasympathetic nervous system (Klaassen et al., 2021; Lojowska et al., 2015, 2018; Matheny & Shaar 1997; Roelofs et al., 2010; Vianna & Carrive, 2005; Walker & Carrive, 2003). Event-related responses were baseline corrected by subtracting the average of 1s prior to stimulus onset.

### Statistical analyses

Statistical analyses were conducted in R (R Core Team, 2021). Directional hypotheses were tested using one-sided t-tests. Normality was assessed using the Shapiro-Wilk Normality Test. Repeated Measures Analysis of Variance (RM ANOVAs) were performed on the transformed SCR scores and HR scores, averaged across trials with CS (reinforced CS+, non-reinforced CS+, CS-), Stimulation (verum TUS, sham TUS) and phase (acquisition phase, spontaneous recall, recall after reinstatement) as within-subject factors. Alpha was set at 0.05. In the case of follow-up pair-wise comparisons, we used paired t-tests with correction for multiple comparisons (Holmes-Bonferroni). These statistical analyses are standard methods for modeling fear learning (Bach & Melinscak, 2020). Extreme outliers and data with extreme noise levels, for example due to unexpected technical issues during the experiment, was identified and excluded from analyses.

## Results

### Online TUS effect on threat learning

We investigated the online effect of TUS on differential threat learning (CS+ > CS-) during the acquisition phase. To avoid contamination of SCRs by an evoked response to the US, reinforced CS+ trials were excluded here from the used analyses.

### Skin conductance response

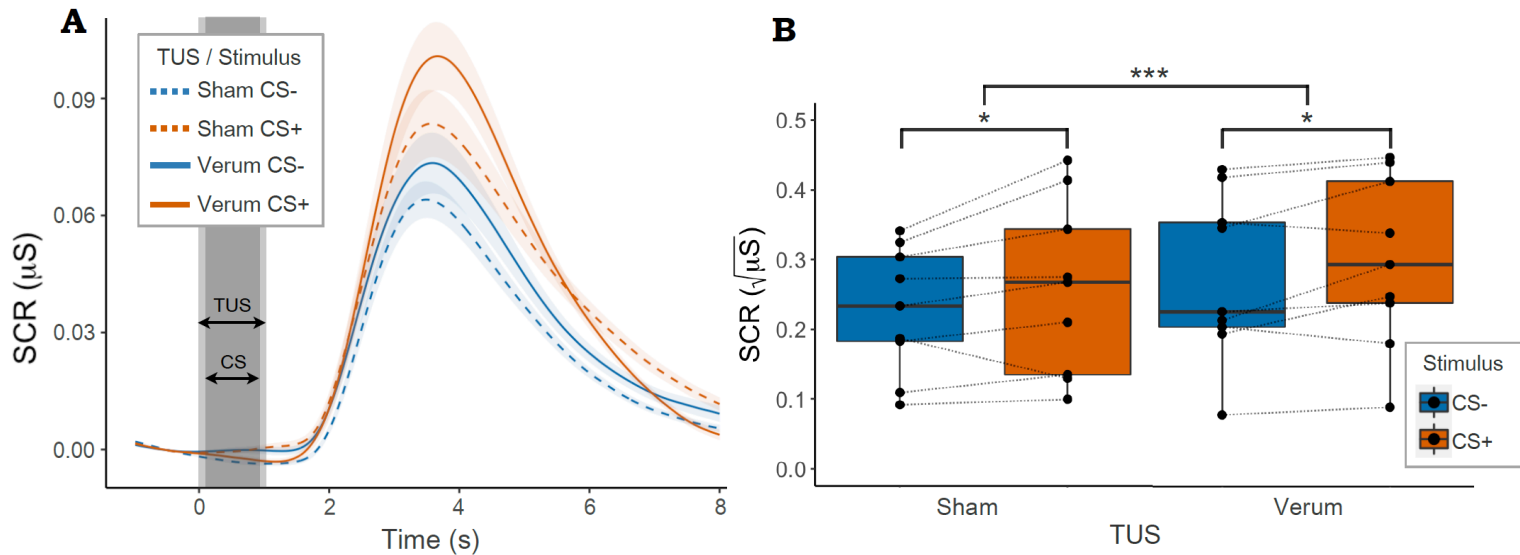
There was a significant main effect of stimulus ( $F(1,8) = 11.06, p < .01$ ) on SCR. Post-hoc analyses showed that in the sham TUS condition SCR amplitudes were significantly lower for CS- ( $M = 0.22, SD = 0.09$ ) compared to CS+ ( $M = 0.25, SD = 0.12$ ), ( $F(1,8) = 3.7, p < .05$ ). Similarly, in the verum TUS condition SCR amplitudes were significantly lower for CS- ( $M = 0.27, SD = 0.12$ ) compared to CS+ ( $M = 0.29, SD = 0.12$ ), ( $F(1,8) = 4.46, p < .05$ ) (See Fig. 5B). There was also a significant main effect of TUS ( $F(1,8) = 12.71, p < .01$ ), such that SCRs were overall significantly lower during the sham trials ( $M = 0.24, SD = 0.1$ ) compared to verum trials ( $M = 0.28, SD = 0.11$ ), ( $F(1,8) = 10.08, p < .001$ ). More specifically, SCRs to CS- were significantly smaller in the sham condition than in verum condition ( $F(1,8) = 9.1, p < .05$ ) and the same held for SCRs to CS+ ( $F(1,8) = 5.46, p < .05$ ).

### Heart rate response

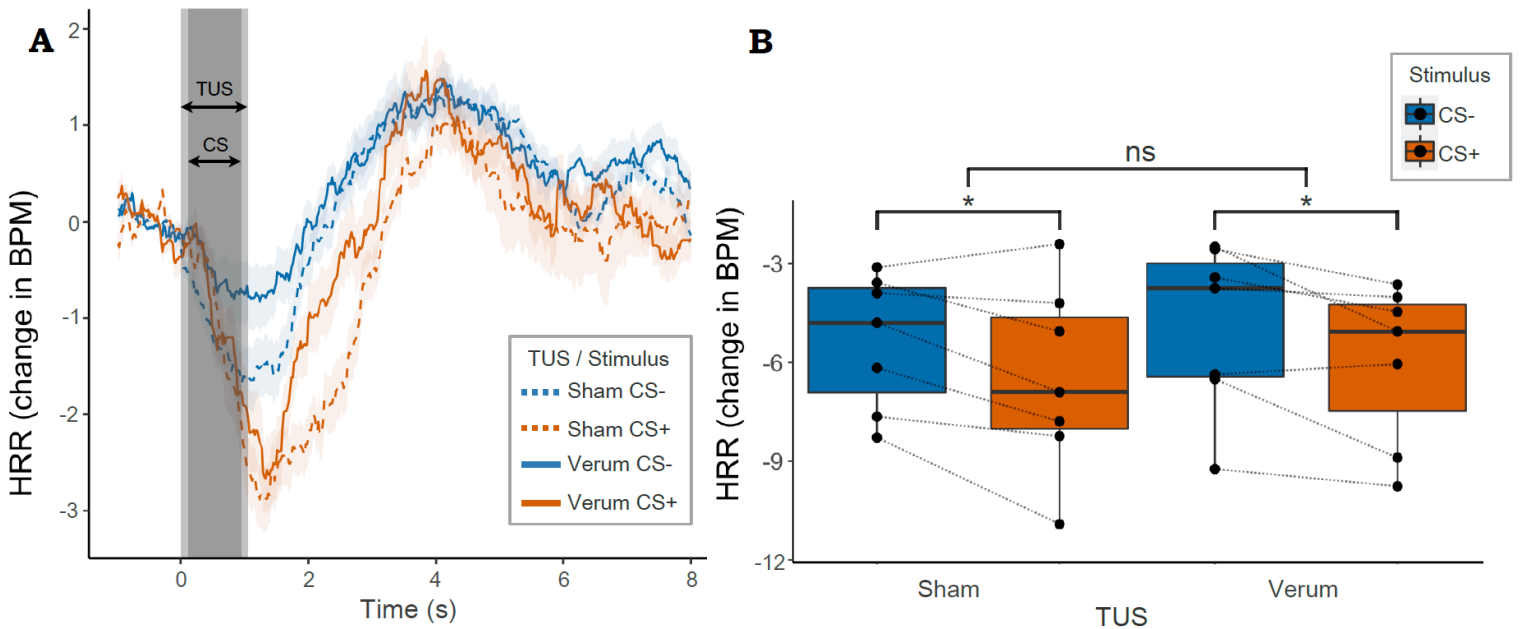
Upon closer inspection of the HRR during the acquisition phase, we found a significant main effect of stimulus ( $F(1,6) = 9.4, p < .05$ ). More specifically, results showed that in the sham condition there was a significantly stronger decrease in HR in response to the CS+ ( $M = -6.50, SD = 2.84$ ) compared to the CS- ( $M = -5.36, SD = 2.04$ ), ( $F(1,6) = 6.98, p < .05$ ), and that the same held for the CS+ ( $M = -5.98, SD = 2.42$ ) and CS- ( $M = -4.9, SD = 2.53$ ) trials during the verum condition ( $F(1,6) = 7.16, p < .05$ ) (See Fig. 6B). No significant effect of TUS could be detected, however.

### Longer-term TUS effects on threat-related memories

Moving on, we studied longer-term effects of TUS on recall of threat-related memories. Our aim here was to determine whether TUS during the acquisition phase had affected threat-related responses, measured in SCRs, during the recall phase. We could not find any significant interactions between CS, TUS, and phase. We did observe that SCRs were significantly smaller on sham CS- trials ( $M = 0.15, SD = 0.1$ ) compared to sham CS+ trials ( $M = 0.2, SD = 0.11$ ) during spontaneous recall ( $t(7) = -2.0, p < .05$ ), but not on sham trials during recall after



**Fig. 5. Skin conductance response during acquisition.** (A) Time course of SCR from 1s pre-stimulus onset to 8s after stimulus onset with confidence interval. (B) Average SCR amplitude.



**Fig. 6. Heart rate response during acquisition.** (A) Time course of HRR from 1s pre-stimulus onset to 8s after stimulus onset with confidence interval. (B) Average HR decrease measured as change in BPM.

reinstatement (CS- ( $M = 0.18$ ,  $SD = 0.13$ ) vs CS+ ( $M = 0.21$ ,  $SD = 0.13$ )), nor during verum trials in spontaneous recall (CS- ( $M = 0.15$ ,  $SD = 0.05$ ) vs CS+ ( $M = 0.19$ ,  $SD = 0.1$ )) or recall after reinstatement (CS- ( $M = 0.19$ ,  $SD = 0.15$ ) vs CS+ ( $M = 0.2$ ,  $SD = 0.13$ )). More specifically, SCRs on CS- trials in the sham condition were significantly smaller during spontaneous recall relative to recall after reinstatement ( $t(7) = -2.39$ ,  $p < .05$ ), but this was not the case for the CS+ trials ( $t(7) = -0.89$ ,  $p = 0.41$ ).

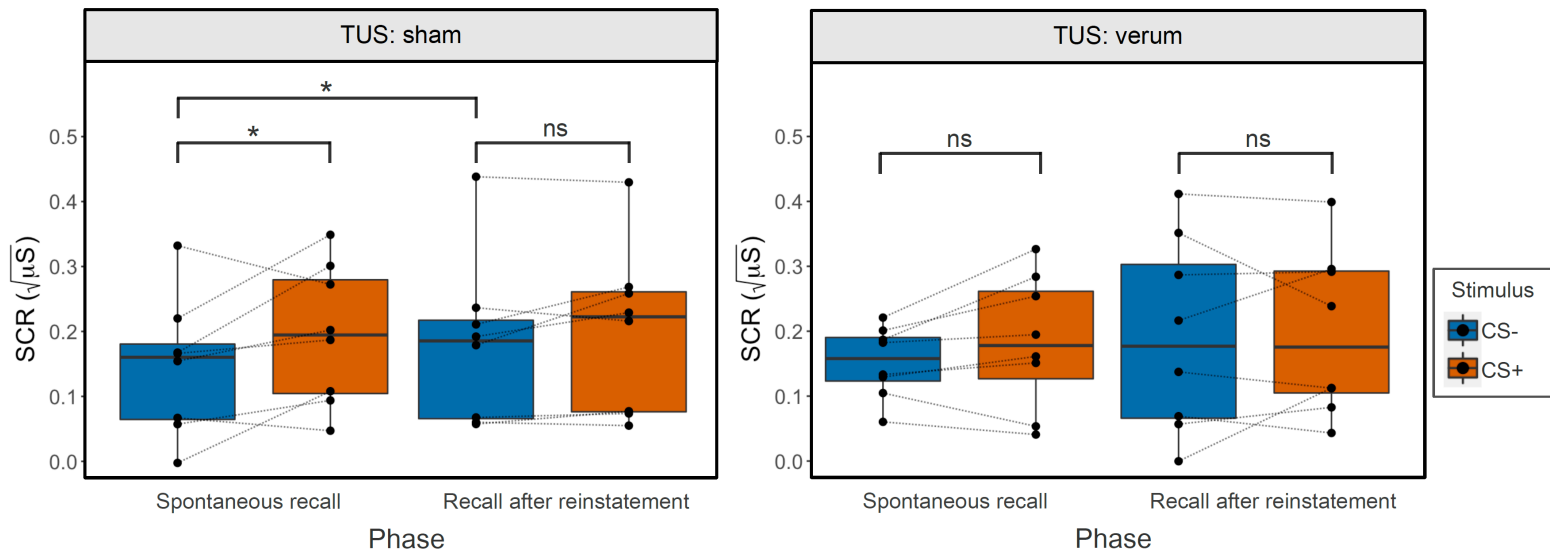
### Differential threat learning

To evaluate the success of the fear conditioning paradigm, we assessed whether differential threat

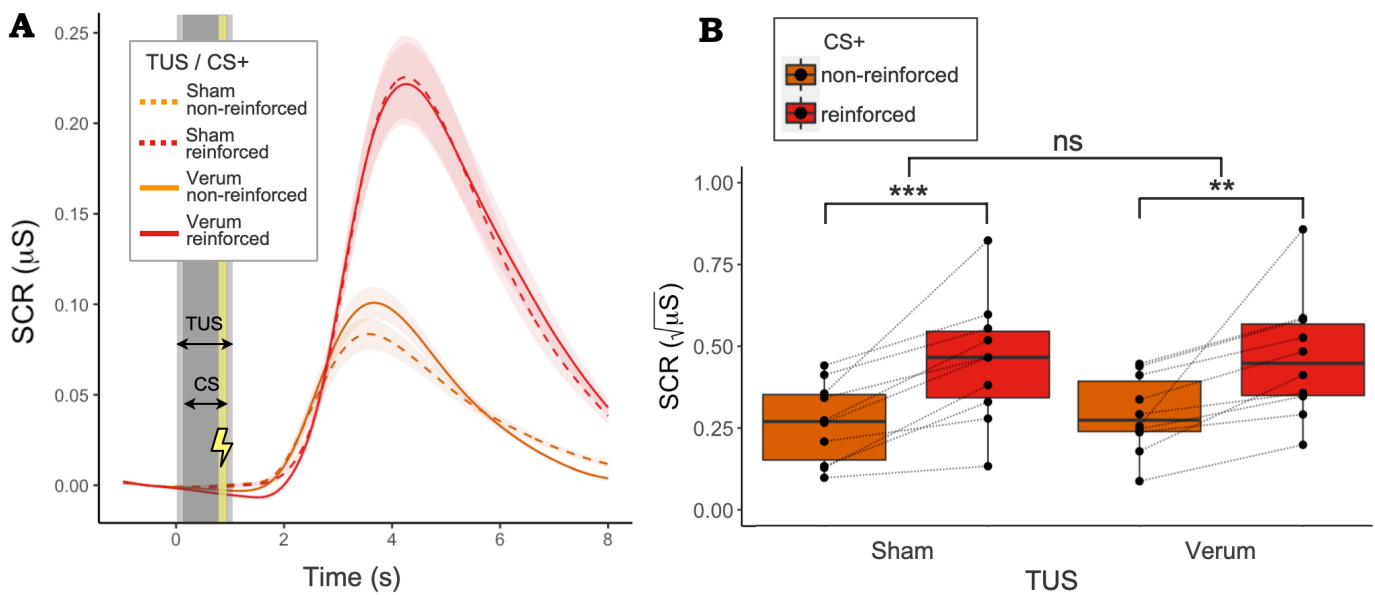
learning was present in the acquisition phase. For these analyses, we used the sham condition as a baseline to avoid potential confounds due to stimulation. To avoid contamination of SCRs by an evoked response to the US, reinforced CS+ trials were excluded from the used analyses.

### Skin conductance response

We confirmed that the SCR scores were normally distributed after square root transformation [ $W = 0.93$ ,  $p > .05$ ]. A paired t-test was conducted and showed that the SCR amplitude during CS+ trials ( $M = 0.26$ ,  $SD = 0.12$ ) was significantly higher than during CS- trials ( $M = 0.23$ ,  $SD = 0.08$ ), ( $t(9)$ ,  $p < .05$ , one-tailed). See Fig. 5B (Sham).



**Fig. 7. Skin conductance response during recall.** Average SCR amplitude during spontaneous recall and recall following reinstatement during the sham (left) and verum (right) conditions.



**Fig. 8. Skin conductance response to unconditioned stimulus.** (A) Time course of SCR from 1s pre-stimulus onset to 8s after stimulus onset with confidence interval. (B) Average SCR amplitude in response to the US (shock).

### Heart rate response

We also compared the HRR scores for CS+ trials ( $M = -6.50$ ,  $SD = 2.63$ ) and CS- trials ( $M = -6.25$ ,  $SD = 3.29$ ). A paired t-test showed that the magnitude of the heart rate deceleration was significantly higher during CS+ trials than during CS- trials, ( $t(6)$ ,  $p < .05$ , one-tailed). See Fig. 6B (Sham).

### Online TUS effect on unconditioned threat-related expression

Next, we explored whether there was any effect of TUS on expression to the US, by looking at SCR differences between reinforced and non-reinforced CS+ trials. Here, we only looked at trials from the acquisition phase, as the CS+ was never reinforced during the recall phases. Results show a significant main effect of shock ( $F(1,9) = 18.34$ ,  $p < .005$ ), but no significant main effect of TUS ( $F(1,9) = 2.30$ ,  $p = 0.16$ ). Follow-up pairwise comparisons reveal that in

the sham condition, SCRs to non-reinforced CS+ ( $M = 0.26$ ,  $SD = 0.12$ ) were significantly smaller than to reinforced CS+ ( $M = 0.45$ ,  $SD = 0.19$ ), ( $F(1,9) = 24.6$ ,  $p < .001$ ). Also in the verum condition, SCRs to non-reinforced CS+ ( $M = 0.29$ ,  $SD = 0.11$ ) were significantly smaller than to reinforced CS+ ( $M = 0.46$ ,  $SD = 0.18$ ), ( $F(1,9) = 11.5$ ,  $p < .01$ ) (See Fig. 8B).

### Discussion

In this study, we investigated the causal role of the amygdala in threat learning processes in healthy humans. To this end, ten participants received online bilateral amygdala-TUS during a standard fear conditioning procedure.

First and most importantly, we found a significant effect of online TUS on SCR, such that the average SCR is significantly smaller during sham relative to verum trials. We did not find a significant effect of online TUS on HRR, nor on differential threat

learning. Second, in assessing longer-term effects of TUS, we found SCR to be significantly smaller for sham CS- trials compared to sham CS+ trials during spontaneous recall, but not during recall after reinstatement or for the verum trials during either recall phase. Third, we found a significantly larger SCRs and stronger deceleration of HR in response to the CS+ compared to CS- during the acquisition phase, indicating that the paradigm brought about differential threat learning by inducing robust changes in participants' threat-related physiological responses. Lastly, we did not find a significant effect of TUS on threat-related expression to the aversive stimulus. Taken together, these findings suggest that in a successful threat learning paradigm, SCR was affected by TUS, indicated by differences between sham and verum conditions during acquisition, yet differential threat learning remained unaffected. However, we will say that what we see in our results is most likely to be attributed to the small sample size, in addition to the high variability in threat-related responses. Nevertheless, we would like to discuss other possible explanations for these observations.

#### **Online TUS effect on threat learning**

In line with our hypothesis, there was a significant difference between SCR in the sham and verum conditions. However, threat learning still occurred in both TUS conditions and upon closer inspection, the difference between conditions is characterized by increased SCR during the verum condition. We can therefore not conclude that TUS disrupts or alters differential threat learning during acquisition.

One possible explanation for the fact that threat learning was not affected by TUS could be that the amygdala does not play a crucial role in threat learning. In fact, recent fMRI studies have shown weak or even complete absence of amygdala activation during fear conditioning (Fullana et al., 2016; Visser et al., 2021) and extinction (Fullana et al., 2018). This is a highly debated premise, since other studies have conversely reported activation in the amygdala during threat learning (Büchel et al., 1998, LaBar et al., 1998, Reddan et al., 2018, Sjouwerman et al., 2020). Interestingly, though, a recent mega-analysis demonstrated that while there was a lack of amygdala activation relating to differential threat response, this was clearly detectable in other regions within the fear network, including the anterior insula, midcingulate cortex, and thalamus (Visser et al., 2021) Our results suggest that modulation of the amygdala does not affect the threat learning process, which is in line with this conjecture that the amygdala in fact might not be involved in threat learning.

Coming back to the difference we observed between the verum and sham conditions: there was overall

increased SCR (but not HRR) in the verum condition. How could we explain this observation? The aforementioned study by Visser et al. (2021) found that even though they saw no detectable amygdala activation in response to threat learning, large parts of the amygdala did respond more strongly to the CS- compared to the CS+, suggesting that the amygdala does play a role in processing the CS-, that is associated with the safe condition. Relating our observations to this theory, we could postulate that TUS might have disrupted the amygdala's function in fear inhibition, resulting in an increase in skin conductance.

#### **Longer-term TUS effects on threat-related memories**

Upon inspection of the recall phases, we saw differential threat learning during spontaneous recall in the sham, but not the verum condition. We could speculate on the cause of this difference. Previous studies have suggested that amygdala activation is associated with awareness of the CS-US relationship (Morris, Öhman & Dolan, 1998) and it might be possible that this awareness was more present in the sham condition than the verum condition as a long-term inhibitory effect of the stimulation. At the same time, this would contrast findings from previous studies that point at the role of the amygdala in creating novel, fear-inhibiting memory traces once presented with a change in the prior existing CS-US relationship, when the CS is no longer paired with an aversive stimulus (LeDoux, 2000; Tovote et al., 2015). Furthermore, previous studies have highlighted the importance of the amygdala in novelty detection (Wright et al., 2003; Montag-Sallaz et al., 1999) and its involvement in forming new associations following violations of current expectancies (Holland & Gallagher, 1999; Holland, Han, & Gallagher, 2000). It has also been shown that this process of forming a new association during fear extinction could be prevented by blockade of NMDA receptors within the amygdala (Baker & Azorlosa, 1996; Falls, Miserendino & Davis, 1992; Santini, Muller & Quirk, 2001). These studies all strongly suggest that the amygdala does have an important function during recall of threat-related memories, which would support alignment of the responses to CS- and CS+, as we see in the verum condition.

The most plausible explanation for not observing a TUS effect at this stage is that the adapted stimulation protocol induced no longer-term effects that would be observed during the recall phase. This is in fact in line with what we would expect, as Fomenko et al. (2020) used this protocol to suppress TMS-elicited motor cortical activity and the stimulation protocol was therefore not meant to cause longer-term effects.

What is lastly worth mentioning about the longer-term results is that we do not observe differential threat learning in recall after reinstatement in either TUS condition. This indicates that participants at this stage had successfully learned the new CS+ with no-US association, also referred to as fear extinction (Lonsdorf et al., 2017).

### **Differential threat learning**

As expected, we observe both increased SCRs and stronger HR deceleration in response to the CS+ compared to the CS- during acquisition. This confirms the success of the used fear conditioning paradigm: it was able to induce threat-related physiological changes. It is worth mentioning that these physiological measures partially reflect different processes underlying threat learning in humans. SCRs are commonly reported to reflect the activation of the sympathetic nervous system in humans (Leuchs et al., 2019). They are typically responsive to emotional arousal, such as when one is presented with salient or novel stimuli, and even more so in response to affective stimuli (Dawson et al., 2007; Wallin, 1981). Whereas for HRR, threat learning by cue conditioning is typically reflected by parasympathetically mediated bradycardia in humans (Furedy & Poulos, 1976; Headrick & Graham, 1969; Klorman & Ryan, 1980). More specifically, (negatively) arousing stimuli are known to induce bradycardia (Bradley et al., 2008; Hermans et al., 2007; Lang and Davis, 2006). Our observations are in line with previous findings from studies investigating threat learning.

### **Online TUS effect on unconditioned threat-related expression**

We saw a large significant difference between SCRs to the non-reinforced CS+ and the reinforced CS+ in both TUS conditions. This corresponds to outcomes from previous studies implementing similar fear conditioning paradigms (Dawson et al., 2007; Fullana et al., 2020). In fact, this is another indicator that our paradigm has been successful in inducing the expected robust threat-related physiological responses.

More interesting here, though, is that we did not find an effect of TUS on fear expression. This would suggest that neural activity underlying the response to the aversive stimulus was not affected by TUS. Indeed, the role of the human amygdala in fear expression has been unclear (Fullana et al., 2016; Mechias et al., 2010) and while animal studies have pointed at a well-characterized function of the amygdala in processing of noxious or painful stimuli (Neugebauer et al., 2004; Ji et al., 2010), the role of the human amygdala herein is uncertain (Wiech & Tracey, 2013). An fMRI study investigating the role of the US in threat learning found that unexpected

pain correlated with increased activity in the hippocampus, superior frontal lobe, superior parietal lobe and the cerebellum, but not the amygdala (Ploghaus et al., 2000). Our findings suggest that TUS does not affect the response to the aversive stimulus, supporting the notion that the human amygdala does not have a critical role in pain processing.

Finally, for the interpretation of our results, a number of strengths, weaknesses, and open issues related to this study are important to consider.

This study has been one of the first to investigate an online impact of TUS on threat learning in healthy humans, using a combination of TUS and well-validated fear conditioning procedures. This gave us the unique opportunity to study the causal role of the human amygdala in threat learning processes, contrasting the correlational nature of many previously carried out studies in the field. Causal evidence in humans is very limited, yet crucial for translation to novel therapies for anxiety disorders. The study further complies with recent guidelines on human fear conditioning research (Lonsdorf et al., 2019) and assesses response to threat based on a comprehensive set of physiological measures, of which SCR, the main study parameter, represents the most commonly employed outcome measure in fear conditioning research (Lonsdorf et al., 2017). All used statistical analyses are standard methods to model threat learning (Bach & Melinscak, 2020) and comply with 'Publication Recommendations for Electrodermal Measurements' (2012). A strength of our study design is that, by using a multi-factor design, the study implements both active and passive control conditions, of which the passive control controls for stimulus effects and potential confounds of stimulation effects and the active control confirms the spatial specificity of stimulation effects. The images of snakes that were used as conditioned stimuli contributed to the ecological validity of the fear conditioning procedure, as people have been shown to have a natural fear of snakes (LoBue & deLoache, 2008), as opposed to for example geometric shapes. Lastly, neuronavigation based on individual anatomical scans was used throughout the experimental session to confirm target accuracy, adding to our confidence of interpreting stimulation effects as related to amygdala-specific activity.

However, it is important to consider that the reported and analyzed study results are preliminary and account for a selection of the number of subjects to be tested. Also, identified extreme outliers have been excluded from analyses to obtain a normalized sample, even though there can be a lot of variability in individual physiological responses to the same stimuli (Hodes et al., 1985). Taken together, this means that the sample size in this study was

relatively small, which limits the statistical power of our conducted analyses. The prolongation of this study will continue to include more subjects, with up to 26 subjects receiving amygdala-TUS and another 26 receiving medial temporal lobe-TUS. Another shortcoming of this study was the absence of appropriate counterbalancing across subjects. Even though the stimuli were presented in a pseudo-random order, unfortunately the coupling of the pictures to conditioned stimuli and TUS conditions (sham, verum) was only done once for all subjects, resulting in each picture obtaining a fixed stimulus-and TUS-association across subjects. This opens up the possibility of effects based on snake identity, for example if certain snake images are inherently more salient or have a more negative/fear-related valence than others, such that they induce an increased physiological response in participants. The absence of appropriate counterbalancing prevents that such effects would be balanced out across subjects. Another misfortune is that we were not able to collect qualitative pupil dilation data. This was due to interference of infrared light from the neuronavigation apparatus that caused major disruptions in the eyetracker's signal. Pupil dilation has been shown to be sensitive to various higher-order cognitive processes, including error monitoring (Koenig et al., 2017; van den Brink et al., 2016; Preuschhoff et al., 2011), uncertainty (Lavin et al., 2014) and cognitive load (Beatty & Lucero-Wagoner, 2000; Sirois & Brisson, 2014; van der Wel & van Steenbergen, 2018). The pupil response during threat-learning, therefore, could be reflective of cognitive processes such as threat appraisal and US expectancy, besides providing another measure of arousal. And thus even though we obtained qualitative SCR and HR data, which demonstrated differential threat learning, additional assessment of pupil dilation response would have offered us complementary information on different cognitive processes involved in threat learning. Lastly, a footnote has to be made about the usage of neuronavigation during the experimental session. While this procedure aided with accurate TUS targeting, it did not actually confirm target engagement. This leaves us with some uncertainty whether the amygdala was in focus throughout the entire duration of the session and thereby, whether it was actually subject to neuromodulation. For comparison, in another study using amygdala-TUS, Folloni et al. (2019) stimulated the macaque amygdala and acquired resting state MRI from these animals, as well as from animals that received no TUS. They were able to compare the recorded activity using fMRI and observed a significant difference in the amygdala's activity coupling only for the macaques that received amygdala-TUS, that was also not apparent elsewhere in the brain and thus amygdala-specific. However, this study

employed a protocol that exerted offline TUS effects, in contrast to the protocol that we used in our study, limiting us from confirming the spatial specificity of TUS effects in a similar manner.

To conclude, our results indicated that amygdala-TUS affects threat-related SCR, but not HRR, nor threat learning. This could mean that the human amygdala does not play a crucial role in threat learning. However, this hypothesis momentarily falls short of robust evidence. Moreover, research into the amygdala's relation to threat has to-date yielded a variety of conflicting findings and it remains unclear which processes related to threat or safety drive amygdala activity. Notably, current resources of what is known about the human amygdala are predominantly of correlational nature. It will be important for future studies to accumulate clear and consistent evidence regarding the functioning of the human amygdala when the aim is to uncover the neural underpinnings of threat; a continuation of studies capitalizing on TUS, with its potential to establish a causal role of the human amygdala, could push the field into that direction.

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