How negative experiences influence the brain in pain: neuroimaging and biobehavioral insights

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Chapter 5.

An experimental investigation into the mediating role of pain-related fear in nocebo hyperalgesia.

Published as

Thomaidou MA, Veldhuijzen DS, Meulders A, Evers AWM. An experimental investigation into the mediating role of pain-related fear in boosting nocebo hyperalgesia. *PAIN 162*(1):287*, January* 2021.

Abstract

Nocebo hyperalgesia refers to increases in perceived pain that putatively result from negative expectations regarding a nocebo stimulus (e.g., an inert treatment, compared to no treatment). The precise cognitive-emotional factors contributing to the origins of nocebo effects are poorly understood. We aimed to test the effects of experimentally induced pain-related fear on the acquisition and extinction of nocebo hyperalgesia in healthy participants (N=72). Acquisition and extinction of nocebo hyperalgesia were compared between a group receiving standard nocebo conditioning (Control group) and two groups receiving distinct fear inductions: high intensity of pain stimulations (High-pain group) or a threat manipulation (High-threat group). During nocebo acquisition, the Control and High-threat groups were administered thermal-pain stimulations of moderate intensity paired with sham electrical stimulation (nocebo trials), whereas high pain intensity was administered to the High-pain group. During extinction, equivalent pain intensities were administered across all trials. Pain-related fear was measured by eyeblink startle electromyography and self-report. Nocebo hyperalgesia occurred in all groups. Nocebo effects were significantly larger in the High-pain group compared to the Control group. This effect was mediated by self-reported fear, but not by fear-potentiated startle. Groups did not differ in extinction rate. However, only the High-pain group maintained significant nocebo responses at the end of extinction. Anticipatory pain-related fear induced via a threat manipulation did not amplify nocebo hyperalgesia. These findings suggest that fear of high pain may be a key contributor to the amplification of nocebo hyperalgesia, only when high pain is experienced and not when it is merely anticipated.

Introduction

Negative expectations regarding an inert treatment stimulus have been shown to increase perceived pain intensity, as compared to perceived pain intensity in an untreated, control condition [1–5]. This phenomenon has been termed nocebo hyperalgesia [1,6]. In experimental studies negative suggestions and classical conditioning play key roles in the acquisition of nocebo hyperalgesia [7–10]. Negative suggestions regarding the effects of a (sham) treatment on pain and the pairing of this treatment with increased pain administrations can produce negative expectations about this treatment [8,11]. As a result of this learned negative expectation, an inert treatment can evoke increased pain sensitivity [9].

Expectations installed by classical conditioning and aversive (threat/fear) conditioning are closely intertwined procedurally, but nocebo research has not systematically focused on the role of fear. A focus on fear is important as cognitive-affective neural processing has been implicated in nocebo hyperalgesia [4,12–14], with numerous studies showing a specific role of the amygdala, a primary fear processing region, in nocebo, but not placebo effects [4,15,16]. Studies have used varying pain levels to induce nocebo hyperalgesia, ranging from as low as 5 to as high as 10 on 0 (no pain) to 10 (highest pain imaginable) rating scales [5,12,17–19]. These pain intensities may differentially induce fear and as such influence nocebo responses. Furthermore, the threatening nature of suggestions also varies between experimental nocebo models. For example, Geuter and Büchel [18] used the negative suggestion that a capsaicin cream would momentarily increase perceived pain, while Benedetti and colleagues [17] suggested that participants may experience severe headaches during a mountaineering trip lasting several days. Whether such differences in perceived pain intensities, threatening

suggestions, and fear-related experiences can alter induced nocebo responses remains unexplored.

Pain-related fear may arise as a result of experienced pain or from threatening information regarding upcoming pain. Fear caused directly by experiencing high pain during nocebo conditioning may augment the acquisition of negative expectations. Research indicates that stimuli paired with pain can elicit fear responses [14,20] and such pain-related fear can be acquired through associative learning [21–24]. In a more anticipatory fashion, threatening suggestions about potential pain outcomes may also induce pain-related fear which can weigh on future pain experiences and augment nocebo hyperalgesia [25]. It is therefore important to determine whether higher reported pain or threatening suggestions amplify nocebo hyperalgesia and whether pain-related fear is a mediator in this putative effect.

The study of pain-related fear in nocebo models is an important step towards a comprehensive understanding of nocebo responses. This study aimed to investigate whether high pain intensity or threatening suggestions augment the acquisition and hinder subsequent extinction of nocebo hyperalgesia. We hypothesized that, compared to lower pain, high pain would produce larger nocebo responses and that these would be more resistant to extinction. The same effects were expected for threatening verbal suggestions, compared to the absence of threatening suggestions. We further hypothesized that self-reported and psychophysiological assessments of fear would mediate these effects. Moreover, we explored whether psychological characteristics such as anxiety are related to nocebo magnitudes.

Materials and Methods

Design

This study utilized a randomized, mixed (between-within-subjects), three-group design (**Figure 1**). A randomization list was created by an independent researcher to reduce any risk of bias. All participants underwent nocebo acquisition and extinction procedures by use of classical conditioning and negative verbal suggestions. In the acquisition phase, the *Control group* and *High-threat group* were conditioned with moderate pain intensity stimuli during nocebo trials, while the *High-pain* group was conditioned with high pain intensity stimuli during nocebo trials, with the aim to additionally induce and examine increased pain-related fear in this group. The *High-threat group* received a threat manipulation, with the aim to additionally induce and examine increased pain-related fear in this group.

Participants

The required sample size for the primary analysis was calculated based on our previous nocebo study [5] comparing the magnitude of nocebo responses between three groups that received different conditioning manipulations. The analysis was conducted in G*power 3.1 [26] for a mixed model ANOVA. The effect size was f = 0.26, alpha error probability was set at $\alpha = 0.05$, and desired power was set at 0.95. The sample size indicated was 21 participants per group. Given that previous studies that included fear manipulations with similar study designs included samples of 20 to 25 participants [22] and due to the novel manipulations used in this study we aimed to include 24 participants per group. This sample size is similar to previous studies examining between-groups differences using conditioning manipulations [25,27].

Inclusion criteria were: being aged between 18 and 35 years, having a good understanding of the English language, and (corrected to) normal vision and hearing. Exclusion criteria were: pregnancy, chronic pain, serious medical or psychiatric conditions that interfere with the study of pain, painful health conditions experienced in the past 6 months, and pain or the use of analgesic medication on the day of testing. Participants would also be excluded if their pain tolerance was too high (i.e., when the thermode maximum temperature of 49.9°C was not sufficient to induce at least moderate pain). Participants were recruited via posters and the recruitment website Sona (Sona Systems, Tallinn, Estonia). Study participation involved a 1.5-hour testing session at a research laboratory of the Faculty of Social and Behavioral Sciences of Leiden University, the Netherlands. All participants provided informed consent and were reimbursed by either cash (€15) or study credits. This study was approved by the Leiden University Psychology Research Ethics Committee (CEP19-0614/347) and pre-registered on ClinicalTrials.gov.

Thermal pain stimulation

Thermal pain stimuli were delivered to participants' non-dominant volar forearm via a Thermal Sensory Analyzer with a 3×3 cm thermode probe (TSA-II; Medoc Advanced Medical Systems, Ramat Yishai, Israel). Throughout the experiment, pain intensities on the arm were rated verbally on a numeric rating scale (NRS) ranging from 0 (no pain) to 10 (worst pain imaginable on the arm). Throughout the experiment, each stimulus was initiated from a baseline of 32°C, increased to the target temperature with ramp up and return rates of 8°C per second, and presented at peak for 4 secs. The inter-stimulus interval was 10 secs.

Sensory and pain thresholds

To test warmth and pain threshold levels, heat stimuli were applied on the arm and participants were asked to indicate the first moment at which they perceived warmth and pain, respectively, from a baseline of 32°C. After a practice trial of each, the average of 3 warmth and 3 pain detection values were calculated as the threshold values for warmth and pain, respectively. This method follows published standardized and protocolled procedures [28].

Pain calibration protocol and administered stimuli

Pain calibrations and selection of pain intensities

Pain calibrations were conducted in order to select the temperatures that would be used to induce low, moderate, and high pain in the acquisition and extinction phases (similar to previous studies [5,29]). The calibrations were individually tailored, based on participants' NRS ratings of maximum 30 pain stimuli of varying intensities, ranging from 41°C to 49.9°C. Median temperatures that were rated as low, moderate, and high pain were calculated in order to select temperatures that were consistently given a certain rating. Median temperatures were selected because of the presence of outlier ratings during this early stage of participants receiving pain stimulations of varying intensities. Details of the pain calibration procedure can be found in supplementary material.

In the Control and High-threat groups, median temperatures consistently rated and experienced as NRS 1 to 3 were selected and used during control trials, while median temperatures rated as 4 to 6 were used during nocebo trials. In the High-pain group, median temperatures consistently rated as NRS 4 to 6 were used during control trials, while median temperatures rated as 7 to 9 were used during nocebo trials. Consistent with previous nocebo conditioning procedures, lower pain stimulation was administered during control trials and higher pain was

administered during nocebo trials, to condition participants to expect increased pain as a result of the inert nocebo (i.e., sham electrical stimulation).

Administered pain stimuli during nocebo acquisition and extinction

During the acquisition phase (described in detail directly below), 12 nocebo and 12 control stimuli were administered in pseudorandom order, so that no more than three trials of the same type were administered in a row. During the extinction phase (also described below), 12 nocebo and 12 control stimuli were administered in pseudorandom order. To reduce habituation or sensitization to heat-pain, the thermode was moved twice to a more proximal site on the same arm (at one third and two thirds of the paradigm).

Nocebo manipulation

A commercial Transcutaneous Electrical Nerve Stimulation (TENS) device (Beurer EM 80) was used to deliver (sham) electrical stimuli, which served as the nocebo manipulation in the nocebo acquisition and extinction procedure, as it represented an inert treatment that was not actually activated in the main experiment. A sham TENS 'treatment' was used to condition nocebo hyperalgesia that may be more ecologically valid, in that negative pain expectations are induced about the effects of a (sham) treatment stimulus. Negative verbal suggestions were used to create expectations regarding the pain-enhancing effects of administering electrical stimuli in combination with thermal pain. Two electrodes (Medi-Trace 200 EKG, 35mm) were placed in a diagonal line on the base of the thumb and the inner elbow. Prior to the start of the acquisition phase, participants underwent a short mock calibration

procedure during which they felt a light electrical pulse. This pulse was delivered in order to increase the credibility of the nocebo manipulation. The device was not actually activated during conditioning, but messages displayed on a computer screen via E-Prime 2.0 (Psychology Software Tools, Pittsburgh, PA, USA) signaled the sham activation (conditioned stimulus) of the electrical stimulation during nocebo trials. Negative suggestions indicated to all participants that when the messages "on" (in purple font; nocebo conditioned stimulus) and "off" (in yellow font; control stimulus) were displayed, their pain would be aggravated or not altered, respectively.

In the acquisition phase, the activation of sham electrical stimulation was repeatedly paired with increased pain stimulation during the 12 nocebo trials, while the 12 control trials were paired with lower pain stimulation. This is in line with previous nocebo studies [5,27] implementing classical conditioning for the experimental induction of nocebo hyperalgesia. In the extinction phase, both nocebo and control cues were paired with the same lower intensity pain stimulation. Extinction was also in line with previous studies and served to attenuate induced nocebo responses.

Fear inductions

While all groups received nocebo suggestions, the High-pain and High-threat groups were exposed to additional fear-inducing manipulations.

Pain intensity manipulation

The High-pain group received higher pain during nocebo acquisition and extinction (2-3 points higher on the NRS), which was intended to increase participants' pain-related fear, especially on nocebo trials.

Threat manipulation

The High-threat group was told that a skin sensitivity test (similar to previous studies [30], albeit not an identical threat manipulation procedure) indicated that nerves in the skin were hyper-responsive and therefore it may potentially be dangerous for them to receive the combination of heat and electrical stimuli. All groups were exposed to the skin sensitivity test, which involved attaching two electrodes to the tip of the thumb and index finger that were communicating with a monitor that displayed a scale (**Figure 2**). The mock scale was an animation that had a bar fluctuating either in the green zone, with the text "recording safe", for the Control and High-pain groups, or in the red zone, with the text "recording unsafe", for the High-threat group. The scale was visible to participants throughout the experiment.

Measures

Pain measures

Participants were provided with an 8 s window to rate their pain on the NRS, following each pain stimulation. A message, presented on the computer screen immediately after the pain stimulus returned to baseline, prompted the verbal pain rating.

Fear measures

Pain-related fear was measured via self-report and via electromyography (EMG) of startle eyeblink responses. Participants were prompted to rate their prospective fear levels of the upcoming pain stimulus in one third of acquisition and extinction trials, after visual cue presentation and

before the heat pain application. Pain-related fear was reported on a 0-10 NRS from no fear to worst fear imaginable. These measurements were similar to previous studies [30].

The startle eyeblink reflex was measured as an indicator of conditioned fear, as it is modulated by fear-evoking stimuli and by brain areas responsible for affective processing such as the amygdala and the anterior cingulate cortex [31]. Eyeblink startle response modulation was measured during the presentation of nocebo and control visual cues. Orbicularis oculi EMG activity was recorded with 3 square EL504 BIOPAC electrodes (2.5x2.5 cm diameter; BIOPAC Systems, Goleta, CA) filled with electrolyte gel. To reduce interelectrode resistance, participants' skin was scrubbed with an exfoliating gel and cleaned with an alcohol wipe. Subsequently, electrodes were placed on the right side of the face according to the specifications proposed by [32]. The raw signal was amplified by an isolated EMG100c amplifier module (BIOPAC Systems, Goleta, CA). EMG recordings were acquired through AcqKnowledge (AcqKnowledge software, Biopac Systems, Goleta, CA) at a sampling rate of 2000Hz, with a low-pass filter of 500Hz and a high-pass filter of 10Hz. The eyeblink startle response was elicited by use of a white-noise burst of 100 ms duration (i.e. startle probe), with instantaneous rise time, presented binaurally via earphones (Samsung Headset Stereo, model EHS64). The noise was calibrated at approximately 90 dBA, which is safe for hearing [32]. These auditory startle probes were delivered within a random 1 s window, 7 s after visual cue presentation and 1 s before heat pain application. The startle probes were presented in two thirds of the acquisition and extinction trials (trials during which participants were not asked to provide a fear rating), including the first and last two extinction trials, which were used to calculate the magnitude of nocebo hyperalgesia at the end of acquisition and extinction, respectively.

Manipulation check exit questions

At the end of the experiment, participants completed an exit questionnaire containing manipulation check questions, for instance regarding pain expectations, trust, and fear. The questions are described in supplementary material. All questionnaires were displayed on a computer monitor via web-based survey software (Qualtrics, Utah).

Questionnaires

A screening questionnaire containing demographic and health questions was used to screen participants for inclusion in the study. Four psychological questionnaires were administered. A short State Anxiety version of the State-Trait Anxiety Inventory, (STAI-S-s) [33,34] was administered before the start of the experiment and the State-Trait Anxiety Inventory, Trait version (STAI-T) [34] was also used. The Pain Catastrophizing Scale (PCS) [35] was used to assess catastrophizing thoughts related to pain, or pain-related worrying [36]. The Fear of Pain Questionnaire (FPQ-III) [37] was used to measure fear of minor, severe, and medical pain. Total scores were used for all questionnaires.

Experimental Procedure

On the day of the lab session (lasting approximately 90 minutes), participants received information about the experiment after which they provided written informed consent. Then, participants completed the screening for inclusion, followed by the STAI-S-s. Then, the EMG electrodes were attached [32] and the mock skin sensitivity test was performed. Warmth and pain threshold levels were then tested and individual pain stimuli were calibrated. The sham electrodes were then

attached to the hand and arm and a short mock calibration took place. Participants were asked to wear earphones and were exposed to 5 startle probes in order to achieve startle probe habituation. Then, participants underwent the nocebo acquisition and extinction procedure. After the end of the experiment participants were asked to answer the exit questions and complete the psychological questionnaires. Then, participants were debriefed and reimbursed. Reimbursement by cash or study credits was, by chance, equally distributed over groups.

Response Definition and Statistical Analyses

Behavioral data were analyzed by use of SPSS 23.0 (IBM Corp., Armonk, NY, USA). For all analyses, the threshold for significance was set at $P < 0.05$, and where multiple comparisons were performed a Bonferroni correction was used. Partial eta-squared (η_p^2) was computed as an effect size measure, with η_p^2 of 0.01 considered small, 0.06 considered medium, and 0.14 considered a large effect size [38,39].

To conduct mixed model analysis of variance (ANOVA), assumptions of normality, and homogeneity of the variances were checked. The assumption of independence was achieved by randomization of participants into groups. For mediation analyses, non-parametric and bias-corrected bootstrapping was used [40]. The independent errors assumption was checked with the Durbin-Watson statistic and multicollinearity was tested through variance inflation factor (VIF).

Pain outcome measures

Mean pain scores were calculated per trial type for each participant and nocebo magnitudes were measured within-subjects. The magnitude of

nocebo responses after acquisition (primary outcome measure) was defined as the difference between the first nocebo and the first control trial of the extinction phase. The first extinction trials were selected since the intensity of administered pain was identical in nocebo and control trials in this phase, and previous studies show the clearest effect of nocebo responses in those trials [27,41]. The magnitude of nocebo responses at the end of extinction was defined as the difference between the last nocebo and the last control trial of the extinction phase. The reduction of nocebo responses was measured as the change in magnitude of nocebo responses (nocebo minus control) between the start and the end of the extinction phase. One-way ANOVAs were used to assess mean between-groups differences in warmth and pain thresholds, temperatures used to induce pain, and NRS pain ratings during the experiment.

Fear outcome measures

The magnitude of self-reported fear levels was measured within-subjects, and was defined as the difference in fear ratings for nocebo trials compared to control trials of the acquisition or the extinction phase. Fear-potentiated eyeblink startle responses were analyzed according to typical pre-processing of EMG recordings in the PhysioData Toolbox for Matlab [42]. The EMG signal was digitized at 1000 Hz, Boxcar filtered, rectified, and each startle trial was segmented. Peak amplitudes were computed, defined as the maximum of the response curve within 21 to 300 milliseconds after startle probe onset. All startle waveforms were also manually inspected and technical abnormalities or artifacts were eliminated. Each peak amplitude was scored by subtracting it from its baseline score (averaged EMG level between 1 and 20 milliseconds after the probe onset). Finally, raw scores were transformed to T-scores, to account for inter-individual variation

in physiological reactivity. Each 4 consecutive startle probe responses of the same cue (nocebo or control) were averaged for further analyses. Trials during which baseline was higher than startle response peak (due to no eyeblink response, an occasional blink), were reject trials.

Hypothesis testing

Acquisition of nocebo hyperalgesia

First, we examined whether nocebo hyperalgesia was induced and whether it differed between the High-pain and Control groups and the High-threat and Control groups. We expected that the two fear inductions (high pain and threat manipulation) would lead to larger nocebo responses, as compared to the control group. To compare each of the fear groups to the control group, two 2x2 mixed model ANOVAs were performed, with group as the between-subjects factor and trial type as within-subjects factor (first extinction nocebo trial, first extinction control trial).

Extinction of nocebo hyperalgesia

Next, we examined whether the extinction of nocebo hyperalgesia differed significantly between the High-pain and Control groups and between the High-threat and Control groups. We expected that the two fear inductions would lead to resistance to extinction, as compared to the control group. To compare each of the fear groups to the control group, two 2x2 mixed model ANOVAs were performed with group as the between-subjects factor and time as within-subjects factor for calculated nocebo magnitudes (start of extinction, end of extinction).

In an exploratory manner, we further analyzed whether the magnitude of nocebo hyperalgesia at the end of extinction differed between groups, for High-pain vs. Control and High-threat vs. Control. To compare each of the fear groups to the control group, two 2x2 mixed model ANOVAs were conducted, with group as the between-subjects factor and trial type as within-subjects factor (last nocebo, last control extinction trials).

Mediation analyses

For the High-pain group, we expected that any effects of higher pain stimulation on the magnitude or reduction of nocebo hyperalgesia would be mediated by pain-related fear. Only when ANOVA results were significant, mediation analyses were conducted, to assess if fear mediated the relationship between pain level and the magnitude of nocebo hyperalgesia. Calculation of indirect effects and bootstrapping tests of mediation were performed, using the PROCESS macro for SPSS [43,44], with 5000 bootstrap samples. Separate mediation analyses were conducted for the self-report and startle response fear measures (mediator variables). Group (High-pain, Control) was the dichotomous predictor variable. Mediation analyses were not planned for the High-fear group, as an increase in fear is inherent to the threat manipulation.

Manipulation checks for fear levels

We examined whether increased pain levels and the threat manipulation led to higher fear levels. Mixed-model ANOVAs were performed, separately for reported fear and for startle responses, one for High-pain group vs. Control and one for High-threat group vs. Control. Group was the between-subjects factor and trial type was the within-subjects factor (nocebo, control).

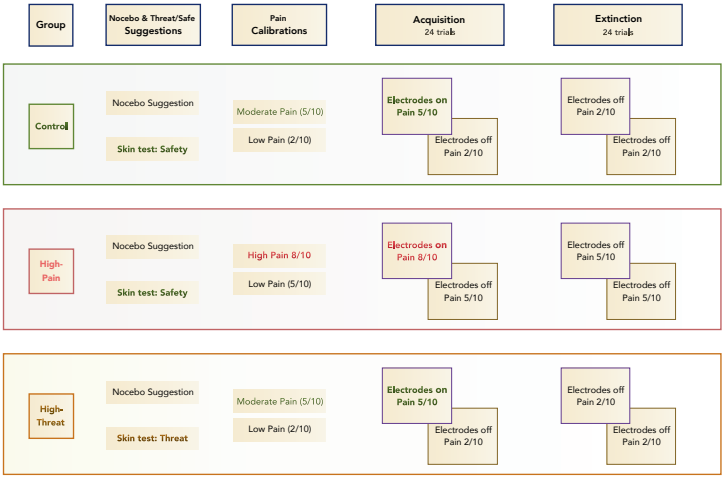

Figure 1. Illustration of the experimental design. Participants were randomly allocated to 1 of 3 groups: Control-nocebo, High-pain, High-threat. Participants in the Control group received lower pain levels during control and nocebo trials and no threat induction. Participants in the High-pain group received higher pain levels during control and nocebo trials and no threat induction. Participants in the High-threat group received lower pain levels during control and nocebo trials and a threat induction. All participants were told that (sham) electrical pulses would increase their pain sensitivity. During nocebo acquisition, higher pain stimulations were delivered during nocebo trials (electrical pulses "on") relative to control trials (electrical pulses "off"). In the extinction phase, all pain stimuli were administered at the same intensity for each participant, in order to test the acquisition and extinction of nocebo hyperalgesic responses.

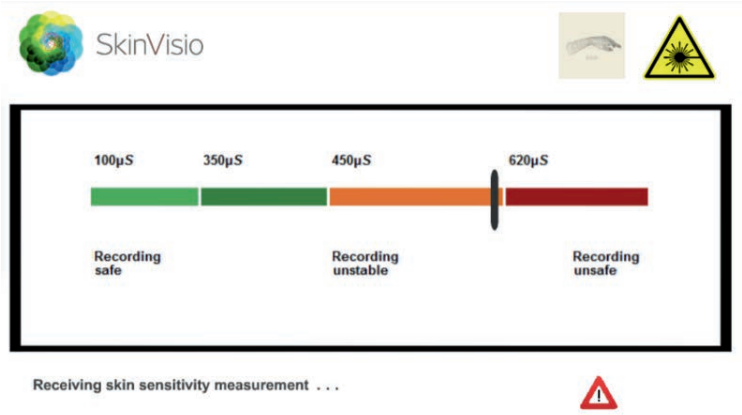

Figure 2. The mock skin sensitivity scale that participants viewed as part of the threat manipulation. The scale was displayed on a screen as an animation. For the High-threat group, the scale fluctuated within the orange and red zones. For the Control and High-pain groups, the scale fluctuated within the green zone.

Results

Participants, temperatures, pain ratings, and startle responses

A total of 75 participants were enrolled in this study. One participant was excluded for experiencing acute pain due to an injury, 1 participant was excluded due to a severe headache, and 1 participant was excluded due to a chronic pain condition (Irritable Bowel Syndrome). In total 72 participants were included in the final analyses. Exactly one-fourth of participants reported that they live as a male, stratified for (lived) gender

so that each group contained 6 male participants. Randomization resulted in a total of 24 participants in each of the three groups.

Calibrated temperature levels and pain ratings during the experiment are reported in **Table 1**. One-way ANOVAs indicated that there were no significant between-groups differences in the mean warmth and heat pain threshold levels (**Table 1**). As expected, one-way ANOVAs confirm that there were significant differences in calibrated temperatures and pain ratings during the experiment, between the High-pain group and the other two groups (**Table 1**).

The EMG[86] recordings of 6 participants were faulty (either the recording was not started due to an error or the sound probe markers were not recorded due to technical difficulties) and were excluded from the analyses. Approximately 20% of trials were marked as non-response or reject trials. While average startle responses range between 100 and 300 microvolts [45,46], in this study startle responses overall were smaller than expected across all groups and trials (**Figure 3**).

Table 1. Group means and standard deviations, as well as between-groups *P* values, for sensory thresholds, calibrated temperatures, and reported pain during the acquisition and extinction phases.

Group	Control		High-Pain		High-Fear		All groups		between-groups *P* value *
	Mean	*SD*	Mean	*SD*	Mean	*SD*	Mean	*SD*	
C° warmth threshold	33.9	0.8	33.7	0.6	33.8	0.6	33.8	0.7	0.46
C° heat pain threshold	43.8	2.4	43.3	2.7	43.3	2.2	43.5	2.4	0.65
C° moderate heat pain	45.8	1.1	47.5	0.9	45.9	1.3	46.4	1.4	< 0.001
C° high heat pain	47.7	0.7	49.1	0.7	47.7	0.9	48.1	1.0	< 0.001
NRS control trials	2.9	1.2	4.7	1.3	2.7	1.1	3.4	1.5	< 0.001
NRS nocebo trials	5.8	1.3	7.9	0.9	6.0	1.1	6.5	1.4	< 0.001

Note: Pain scores are reported on a 0-10 pain Numerical Rating Scale (NRS). Significant differences were found between the High-pain group and the other two groups ($P < 0.001$), driven by the administration of higher pain levels in this group.

Acquisition of nocebo hyperalgesia

The mean magnitudes of nocebo responses are presented in **Table 2**. **Figure 4** illustrates differences in pain ratings for the first nocebo and first control extinction trials, across all three groups.

High-pain group

Nocebo responses in the High-pain group were of almost double the magnitude compared to Control. The analysis revealed a significant interaction between group (High-pain vs. Control) and trial type (nocebo vs. control) (F (1,46) = 4.32, $P = 0.04$, $\eta_p^2 = 0.09$), indicating

significantly larger nocebo responses after higher, compared to lower pain administration (**Figure 4**).

High-threat group

The analysis showed that there was no significant interaction between group (High-threat vs. Control) and trial type (nocebo vs. control) (F (1,46) = 0.15, P = 0.69, η_p^2 = 0.003) (**Figure 4**).

Table 2. Group means and standard deviations for fear levels during acquisition and extinction, as well as magnitudes of reported nocebo hyperalgesia after acquisition and at the end of extinction.

	Group	**Control**		**High-Pain**		**High-Fear**	
		Mean	SD	Mean	SD	Mean	SD
Acquisition	Nocebo magnitude	0.9	1.4	1.8	1.4	1.1	1.1
	Fear difference (reported)	1.1	1.2	2.4	1.7	1.6	1.3
	Fear difference (EMG*)	42.6	13.4	45.9	12.3	61.6	25.6
Extinction	Nocebo magnitude	0.2	1.1	0.7	0.8	0.3	0.8
	Fear difference (reported)	0.6	0.9	1.4	1.4	0.7	1.3
	Fear difference (EMG*)	-3.10	4.8	-1.70	5.4	4.10	10.5

Note: Pain and fear scores are reported on a 0-10 pain numeric rating scale. Magnitudes of nocebo hyperalgesia are shown here as the difference between the control and the nocebo trial, at the start and at the end of extinction (i.e., after acquisition and after extinction). *SD*, Standard deviation; EMG, Electromyography.

Table 3. Correlations of nocebo magnitudes and fear magnitudes across all groups and for both the acquisition and the extinction phase.

		Nocebo magnitude					
		Control-Nocebo		High-Pain		High-Fear	
		r	*P*	*r*	*P*	*r*	*P*
Fear magnitude	Induction	0.73	<0.001	0.59	0.001	0.61	0.001
	Extinction	0.17	0.21	0.32	0.04	0.69	<0.001

Note: Pain and fear scores are reported on a 0-10 pain Numeric Rating Scale.

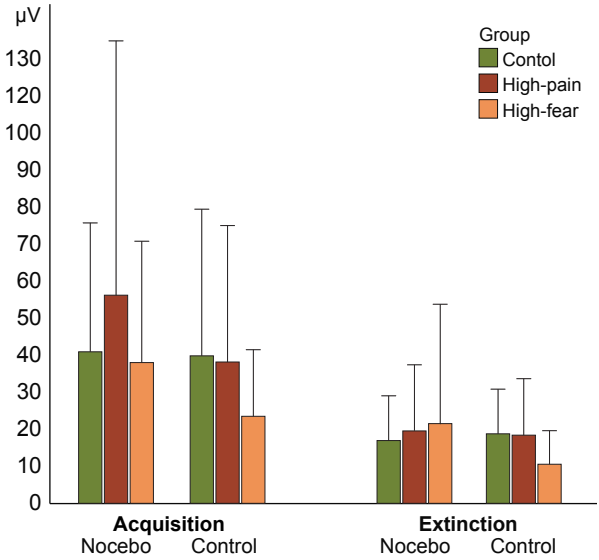

Figure 3. Means and standard deviations of startle responses as measured via electromyography. As compared to the Control group (N = 23), participants in the High-pain (N = 21) and High-threat group (N = 22) showed larger startle responses during nocebo trials as compared control trials of the acquisition phase.

Extinction of nocebo hyperalgesia

The mean magnitudes of nocebo responses at the end of extinction are presented in **Table 2**. **Figures 5a** and **5b** illustrate the reduction of nocebo hyperalgesia and the residual magnitudes of nocebo responses at the end of extinction, respectively. **Figure 6** displays the time-course of extinction for all three groups.

High-pain group

The analysis showed that there was no significant interaction between group (High-pain vs. Control) and time (nocebo magnitude at the start vs. at the end of extinction) (F (1,46) = 0.58, P = 0.45, η_p^2 = 0.01).

High-threat group

The analysis showed that there was no significant interaction between groups (High-threat vs. Control) and time (nocebo magnitude at the start vs. at the end of extinction), (F (1,46) = 0.04, P = 0.84, η_p^2 = 0.001) (**5a**).

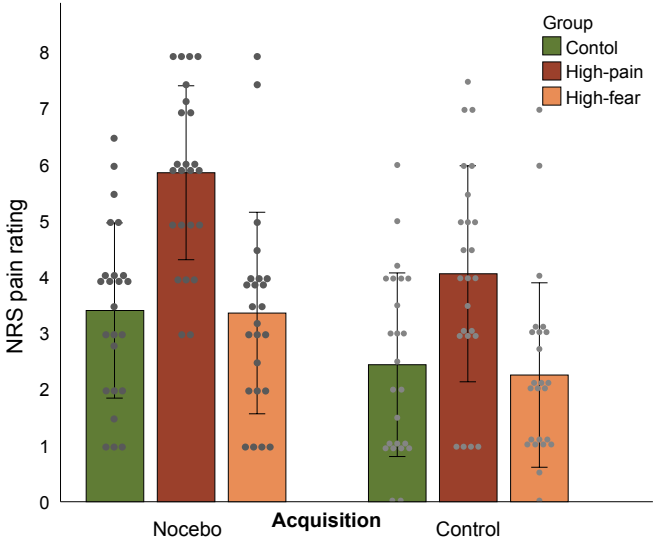

Figure 4. Acquisition of nocebo responses. Mean Numeric Rating Scale (NRS) pain ratings (sd) are depicted across all three groups (N = 72) for the first nocebo and the first control trial of extinction phase.

Residual nocebo responses

We analyzed whether the magnitude of nocebo hyperalgesia at the end of extinction differed between groups. **Figure 5b** illustrates the differences in pain ratings for the last nocebo trial and the last control trial of the extinction phase, across all groups.

High-pain group

The analysis showed a significant interaction between group and trial type, with nocebo responses at the end of extinction (nocebo vs control

trials) being significantly different between groups (High-pain vs. Control) (F (1,46) = 4.24, P = 0.04, η_p^2 = 0.09). We ran repeated-measures ANOVAs separately for the High-pain and Control groups, confirming that nocebo responses (i.e., nocebo vs. control trials) in the Control group were not significant (F (1,23) = 1.42, P = 0.25, η_p^2 = 0.08), whereas nocebo responses in the High-pain group were significant at the end of extinction (F (1,23) = 18.59, P < 0.001, η_p^2 = 0.45).

High-threat group

Nocebo responses (i.e., nocebo vs. control trials) at the end of extinction were not significantly different between the High-threat and Control groups (F (1,46) = 0.002, P = 0.98, η_p^2 < 0.001) (**Figure 5b**).

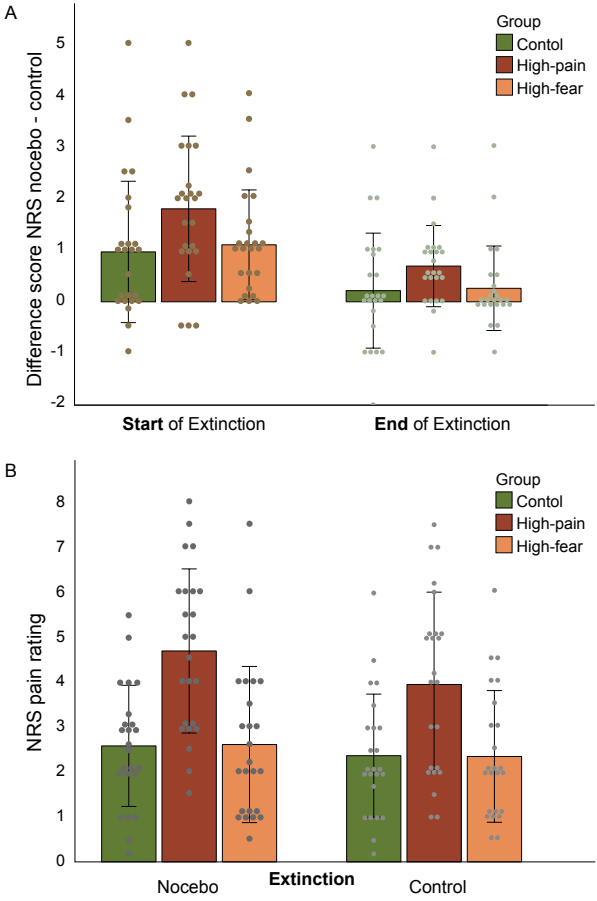

Figure 5. Extinction of nocebo hyperalgesia. A) Extinction of nocebo responses, from the start to the end of extinction. Nocebo magnitudes and standard deviations, based on Numeric Rating Scale (NRS) pain ratings, at the start and at the end extinction are depicted, between all three groups. In addition, individual scores are presented in dots. There was no significant difference in the reduction rate of nocebo magnitudes between the High-pain and Control groups, or between the High-threat and Control groups. Negative values signify an effect comparable to a placebo effect (i.e., control trials having been rated higher than the nocebo trials). B) Residual nocebo responses at the end of extinction. Mean pain ratings and standard deviations for nocebo and control trials at the end of extinction are depicted, between all three groups.

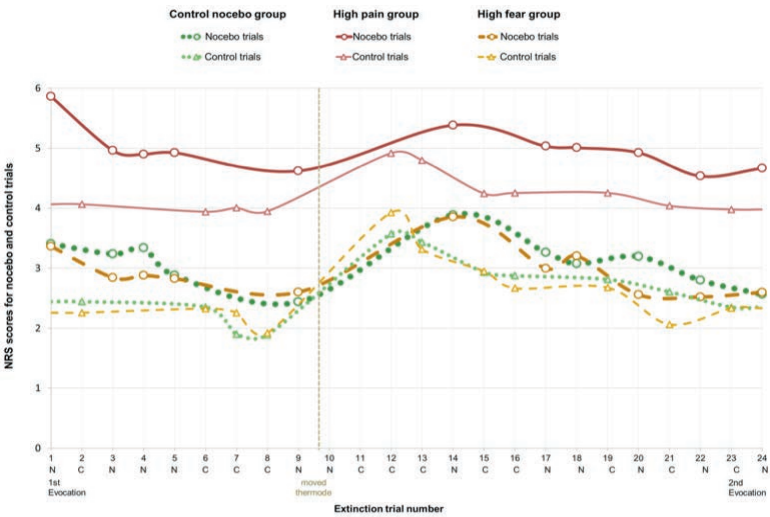

Figure 6. Pain ratings for the nocebo and control trials in the extinction phase, across all three groups. Numeric Rating Scale (NRS) pain ratings during nocebo and control trials illustrate the evocation of nocebo responses and time-course of extinction, for the Control-nocebo, High-pain, and High-threat groups. The dotted vertical line indicates the thermode moving point, after which pain ratings suddenly peak due to placing the thermode on a new location on the arm. During the entire extinction phase all pain stimuli were administered at the same intensity. It is visible that the High-pain group (red lines) consistently rated nocebo trials (thick lines) higher than control trials (thin lines), as compared to the other groups (green and orange lines).

Nocebo responses mediated by fear

To test whether the larger nocebo magnitude in the High-pain group compared to the Control group was mediated by fear, a mediation analysis was conducted using the causal steps approach suggested by Baron and Kenny [47] implemented in PROCESS [43,44]. This method uses regression analyses to determine the relationship between the predictor

variable and the outcome variable both with and without the mediator in the analysis. The regression was carried out in three steps (Figure 7). Step 1 (path c) determined that group significantly predicted nocebo magnitude (F (1,46) = 4.32, R^2 = 0.09, b = 0.83, t (46) = 2.08, P = 0.04). Step 2 (path a) determined that group significantly predicted reported fear (F (1,46) = 10.99, R^2 = 0.19, b = 1.37, t (46) = 3.32, P = 0.002). Group and reported fear together significantly predicted nocebo magnitude (F (2,45) = 19.25, $P < 0.001$, R^2 = 0.46) and step 3 (path c') determined that group did not remain a significant predictor of the nocebo magnitude after controlling for reported fear (b = -0.02, t (45) = -0.05, P = 0.96). The bootstrap analysis confirmed a significant indirect effect of group on the magnitude of nocebo responses through reported fear levels (ab = 0.85, BCa CI [0.34, 1.44]). These analyses indicate that full mediation occurred, as the relationship between the group and nocebo magnitude was no longer statistically significant when fear was entered into the model [44].

The same mediation analysis was performed with EMG fear scores as the mediator variable. EMG startle responses were not a significant mediator of relationship between the group and the nocebo magnitude, with a non-significant indirect effect of group on the magnitude of nocebo responses through EMG fear levels (ab = 0.05, BCa CI [-0.14, 0.27]).

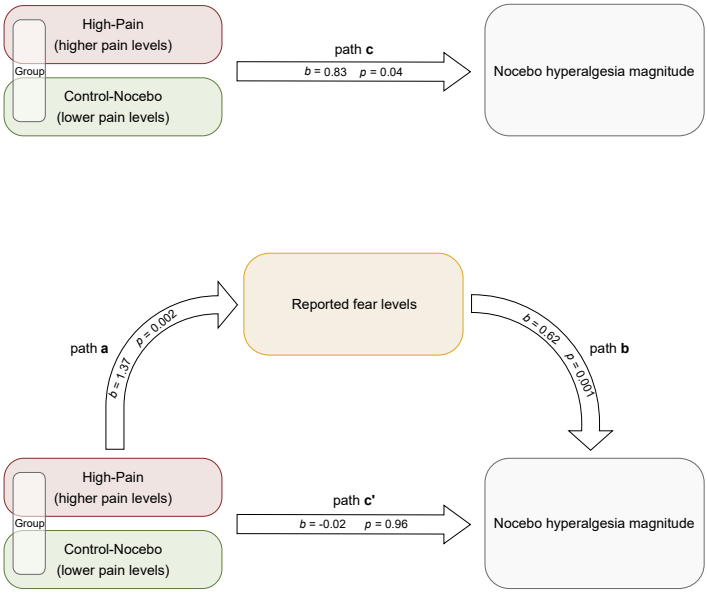

Figure 7. Diagram of the hypothesized mediation model and results. Administration of higher pain (High-pain group) compared to lower pain (Control group) significantly predicted the magnitude of nocebo responses, and this effect was mediated by reported fear levels.

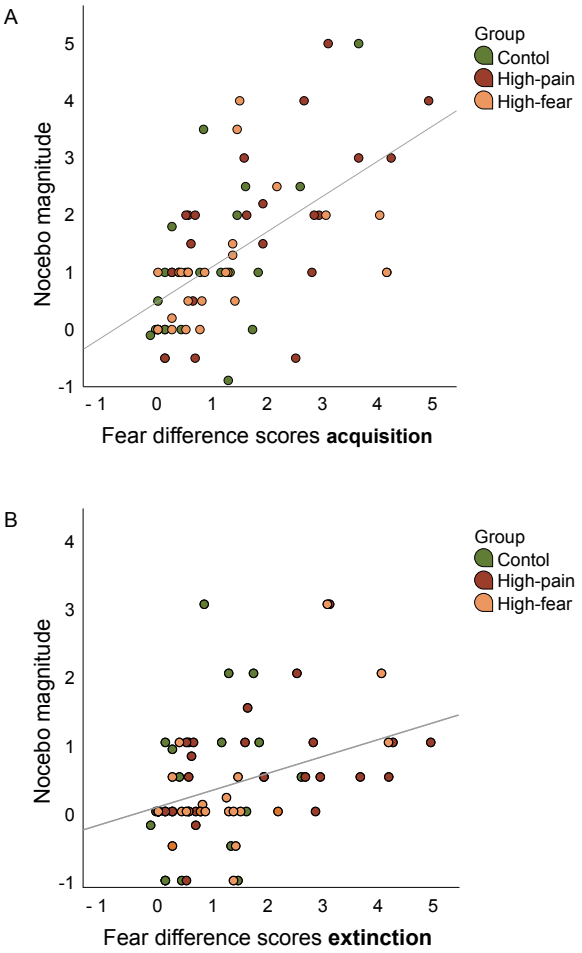

Figure 8. Correlations between the magnitude of nocebo responses and reported fear levels. A) In the acquisition phase, there was a significant high correlation across all groups between nocebo response magnitudes and reported fear levels. B) In the extinction phase, there was a significant moderate correlation across all groups between nocebo response magnitudes and reported fear. Regardless of the manipulation that participants received, pain-related fear led to larger magnitudes of nocebo hyperalgesia.

Manipulation checks for fear levels

High-pain group

Differences in reported fear in the High-pain group were more than double compared to the Control group, while startle responses were slightly higher for the High-pain compared to the Control group (Table 2). As expected, our analysis confirmed that the High-pain group reported to be more afraid than the Control group during nocebo compared to control trials (F (1,46) = 11.01, P = 0.002, η_p^2 = 0.19). No such difference occurred in eyeblink startle responses (F (1,42) = 0.75, P = 0.39, η_p^2 = 0.018).

High-threat group

Differences in reported fear in the High-threat group were more than 50% higher compared to the Control group and startle responses were higher for the High-threat group compared to the Control group (Table 2). The analysis showed that the High-threat group did not report more pain-related fear than the Control group during nocebo trials compared to control trials (F (1,46) = 3.13, P = 0.08, η_p^2 = 0.06). However, in the High-threat group startle responses were larger than in the Control group during nocebo trials compared to control trials (F (1,43) = 9.89, P = 0.003, η_p^2 = 0.19).

Furthermore, a one-way ANOVA with group (High-threat, Control) as the between-subjects group factor confirmed that the High-Threat group was significantly more frightened by the mock skin sensitivity test (based on the exit questionnaire) than the Control group, F (1,46) = 10.9, P = 0.002, η_p^2 = 0.19, suggesting that our threat manipulation worked.

Exploratory and Manipulation checks

In an exploratory manner, we examined how fear responses influenced the acquisition and extinction of nocebo hyperalgesia. Pearson's correlation analyses across all groups showed significant correlations between reported fear (difference between nocebo and control trials) and the magnitude of nocebo responses ($r = 0.59$, $P < 0.001$), as well as between reported fear and the magnitude of nocebo responses still present after extinction ($r = 0.33$, $P = 0.002$). Figure 8 illustrates the two correlations. Table 3 lists all correlations between the magnitude of reported fear and the magnitude of nocebo responses for each group and each experimental phase. Finally, we ran analyses to explore any relationships between nocebo responses, fear responses, and related psychological or cognitive factors.

Exit questions and psychological questionnaires

On average, participants believed the information they received during the study ($M = 8.6$, $SD = 1.8$), they thought the researcher was honest ($M = 8.7$, $SD = 1.5$), they were not concerned about what the researcher thought of them ($M = 3.3$, $SD = 1.7$), and they were focused on the heat tests ($M = 8.7$, $SD = 1.1$). We ran Pearson's correlations between the magnitude of nocebo hyperalgesia and manipulation check exit questions. Participants' expectations about pain during nocebo trials differed per group (Control: $M = 5.6$, $SD = 1.7$; High-pain: $M = 6.9$, $SD = 1.7$; High-threat: $M = 6.2$, $SD = 1.9$) and pain expectations across all groups were correlated to nocebo magnitudes ($r = 0.38$, $P < 0.001$). None of the other responses to exit questions where significantly correlated with the magnitude of nocebo responses (for all questions $P > 0.05$, please see supplementary material). A one-way ANOVA showed that there were no significant group differences in questionnaire scores

(for all questionnaires $P > 0.05$). Detailed questionnaire results and Cronbach's alpha scores are reported in supplementary material.

Manipulation checks for nocebo and fear responses

Pearson's correlation analyses showed significant correlations between retrospectively assessed fear of the nocebo trials (reported at the end of the experiment) and the magnitude of nocebo responses ($r = 0.25$, $P = 0.02$) as well as reported fear differences ($r = 0.63$, $P < 0.001$). There were no significant correlations between any relevant manipulation check questions or questionnaires and nocebo magnitudes or reported fear (for all questions $P > 0.05$, please see supplementary material).

Discussion

This study investigated the facilitating effects of two distinct pain-related fear manipulations on nocebo responses. We expected that higher pain levels would lead to higher pain-related fear, which would augment nocebo responses. We confirmed this by demonstrating that, compared to lower pain, conditioning with higher pain administrations produced significantly larger nocebo responses. We also showed that this effect was mediated by reported fear levels, but not by eyeblink startle responses. Contrary to our expectation, nocebo responses extinguished at a similar rate in the High-pain and Control groups. However, we found that nocebo responses at the end of extinction were significantly larger in the High-pain group. A threat manipulation did not amplify

nocebo responses. Importantly, nocebo magnitudes across all groups correlated with reported fear during conditioning. These findings bear a number of implications related to both experimental models and clinical practices.

The finding that higher pain levels produced larger nocebo responses and that this was mediated by fear may be linked to previous fear studies [20,22,23,48]. Fear is a response that can be relatively impenetrable to cognitive control [24] and can be learned via classical conditioning [49,50]. Just like nocebo conditioning models, fear-avoidance models consider pain-related fear to be a key factor in certain types of chronic pain [20]. Notably, Crombez and colleagues [48] studied a sample of chronic back pain patients and found that pain-related fear may be even more disabling than pain itself. In the current study we show that, during conditioning, fear in response to the experience of high pain may have a direct amplifying effect on the acquisition of nocebo responses. This finding may be a novel link between fear of pain and nocebo hyperalgesia, as both are postulated to play a role in pain conditions [20,51].

Studying fear in relation to the extinction of nocebo hyperalgesia may also provide insights into pain chronification. Nocebo hyperalgesia is sometimes found to be resistant to extinction [27,52,53]. In the present study, nocebo responses were extinguished in the Control group but in the High-pain group they remained statistically significant. As the extinction rate of nocebo responses was not hindered by higher pain stimulations, it is apparent that in the High-pain group the substantially larger induced effects led to residual nocebo responses. It is therefore reasonable to expect that, after a longer extinction phase, nocebo responses would eventually be extinguished even following higher pain stimulations. Nevertheless, high pain leading to residual nocebo responses bears important implications. In clinical terms this effect may indicate that, compared to lower pain, higher pain not only produces

larger nocebo responses, but these responses can also be persistently higher after an initial period of extinction.

While these findings linking higher pain levels to larger nocebo responses are in line with research into fear and pain chronification, there are some notable differences. Fear-avoidance models [22,30,54] propose that upon the experience of pain symptoms, patients with pain-related fear engage in a negative feedback loop in which fear-avoidance and reduced physical activity lead to increased disability and psychological strain [54]. In our study, participants did not engage in avoidance behaviors, yet our results support a separate pathway to pain chronification, in which fear of high pain may be conditioned in parallel with the nocebo response, thereby significantly strengthening the learning process in nocebo hyperalgesia.

In the High-threat group, only startle responses were significantly higher than in the Control group and nocebo magnitudes were not affected by the threat manipulation. Previous research also concluded that experimental threat induction is challenging [22,30]. In this study, we informed participants that they may experience sudden, intense pain due to unusual skin sensitivity. Participants were constantly exposed to a mock measurement of this skin test and were reminded to be alert to changes in their sensations. This group generally reported believing the manipulation and being significantly more frightened by it, compared to the Control group that was told that their skin was safe. This may indicate that the threat manipulation did not have a direct effect on participants' learning, not because of a lack in credibility but perhaps due to the potential negative effects being only anticipated and never actually experienced, unlike in the High-pain group. It is also possible that participants felt relatively safe and anticipated that no harm would be caused (based on their understanding of ethical standards in research). Differences in learned fear responses resulting from experienced versus anticipated threat have been highlighted in the fear literature [55] and

support the differences found in this study between the High-pain and High-threat groups.

Notably, when examining the relationship of pain-related fear with nocebo responses across all three groups, we found that fear reports almost always correlated with the magnitude of nocebo responses. This is interesting, given the substantial interindividual variation in fear of pain [56,57]. We further showed that none of the anxiety measures correlated with the magnitude of nocebo responses. This was critical in this study, as we specifically focused on the effects of fear on nocebo hyperalgesia. Fear is a response that is often difficult to disentangle from anxiety, theoretically and physiologically [58,59]. The two may produce similar responses, yet involve distinct psychobiological mechanisms, with fear involving more immediate responses to explicit danger, and anxiety presenting as a diffuse response to anticipated threat. Based on our findings, fear, as measured both during and after the experiment, produced larger nocebo responses. In contrast, anxiety, as measured after the experiment and the threat manipulation that involved anticipated threat, was not related to larger nocebo responses.

Another method for measuring fear of pain is the measurement of fear-potentiated startle responses. These responses are produced via projections from the central nucleus of the amygdala [60,61]. This role of the amygdala, as well as ample fear research, indicate that startle responses may be more specific to fear states and less to states of anxiety [62–64]. Average acoustically elicited startle responses range between 100 and 300 microvolts [45,46]. Typically, sound probes are delivered via noise-cancelling headphones, which achieve optimal auditory conditions and block sounds in the environment [65,66]. In this study, earphones were used so that participants could verbally communicate with the researcher, which was crucial in our design. Startle responses were observed; however, potentially as a result of using earphones, these were smaller than expected, on average below 100 microvolts. While trends that

followed reported fear were observed, on this smaller scale of responses most differences did not reach statistical significance. This is an apparent study limitation that should be addressed in future designs.

Another study limitation may have been the effectivity of the threat-manipulation. As mentioned earlier, participants in the High-threat group believed and were more frightened by the mock skin sensitivity test, compared to the Control group. However, this fear did not translate to increased fear during conditioning. It is possible that induced fear levels were not high or specific enough to translate into experienced fear during nocebo trials. However, it was not possible to increase threat levels without risking participants dropping out of the study or it seeming illogical for the researcher to continue the experiment. This is a common obstacle in experimental threat manipulations [22,30]. As noted, however, the threat manipulation may not have increased fear reports due to its anticipatory and obscure nature, rather than a manipulation failure, while it is also plausible that pain may have captured participants' attention and diverted it away from the potentiality of a threat.

Finally, it is important for future studies to address whether clinically relevant extinction effects are affected by fear. For instance, reinstatement of conditioned responses (after experience with unpredictable increased pain) to the conditioned stimulus has been observed in previous studies [67–70]. Reinstatement translates to clinical practice where patients may be re-exposed to exacerbated pain, even after successful treatment [70]. Similarly, patients may retrieve a previously extinguished effect, upon exposure to an aversive stimulus distinct from pain, such as fear [71]. Based on the results of this study, it is important to further examine whether high pain can also impact the return of learned effects on pain. It is worth noting that controlling for unwanted variability due to age differences in our sample, the generalizability of our findings to the general population is limited. Future studies may consider including broader age ranges.

Overall, this study implemented a novel, clinically relevant learning model that investigated the effects of fear inductions on nocebo. The findings provided evidence that experienced threat in the form of higher pain stimulations led to significantly larger nocebo hyperalgesia, compared to lower pain. Importantly, this effect was mediated by self-reported fear. The anticipation of threat, however, did not impact nocebo magnitudes. This study also indicated that higher pain stimulations induce amplified nocebo responses that persist after a period of extinction. Given the substantial impact of higher pain and pain-related fear on nocebo hyperalgesia, further assessment of these variables in relation to pain aggravation and chronification may be of value.

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