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

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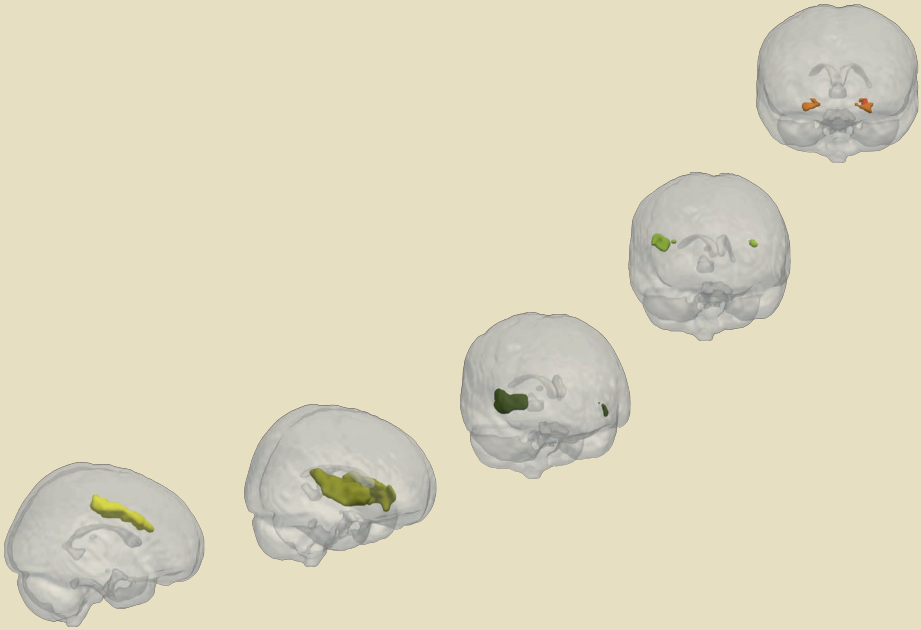

Mia A. Thomaidou

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PhD dissertation

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Dedication

For Oma Mia.

Through the darkness of the deepest ***pain***
you shine a light
so bright
for us to find the way.

Table of contents

Chapter 1.

General introduction

Learning decisively shapes the way individuals experience the world around them and how they respond to various external stimuli, including pain [1–3]. Increased pain sensitivity can result from negative experiences creating negative expectations about an environmental stimulus, such as a treatment, a phenomenon termed nocebo hyperalgesia [1–3]. Nocebo has been described as the negative counterpart to placebo. Nearly a century into the proliferation of placebo-controlled studies [4–6], research has shown that learned expectations regarding inert treatments may not only have positive placebo effects, but may also mimic negative treatment outcomes, such as medication side-effects [7–9]. Nocebo responses may thus produce deleterious effects on a variety of symptoms, as a result of learning mechanisms that are not yet fully understood. For example, it remains unclear how negative expectations on a cognitive level influence pain processing in the brain, or what the involvement of relevant emotions may be. Due to a known involvement of learning in the experience of pain [1,10–12], it is important to study and better understand the biobehavioral mechanisms that underly nocebo hyperalgesia.

In this general introduction, first, nocebo hyperalgesia will be framed as a multifaceted phenomenon that can be part of the intricate mechanisms of pain processing. Cognitive-emotional pain processing is described in the context of learned nocebo responses and as complimentary to sensory-discriminatory nociceptive processing. The state of the art in experimental nocebo research and the relevance of experimental learning mechanisms are then outlined. Experimental models that are typically implemented to investigate the cognitive and emotional aspects of nocebo hyperalgesia are described. Cognitive processes such as learning, as well as emotional processes such as fear, are then presented as major putative underlying factors in nocebo hyperalgesia, as indicated by experimental findings. Biobehavioral underpinnings of nocebo effects are then discussed in relation to current neurobiological literature; gaps in knowledge are highlighted. Finally, an outline of the current dissertation is presented.

Nocebo hyperalgesia and its involvement in pain

Nocebo hyperalgesia seems to be an intricate component of pain processing. Due to the multifaceted, subjective, and often unpredictable nature of pain, recovery from pain and chronic conditions are especially difficult to manage [13]. Pain has been described to arise in response to a nociceptive signal from the body, but this is not always a direct path. Rather, the experience of pain is heavily influenced by an array of processes within the nervous system [14]. These intricate processes do not merely rely on information regarding the nature of the nociceptive stimulus, but may include pain processing based on past experiences or cognitive-emotional factors such as fear [13]. We start by outlining the basic mechanisms of pain processing, in order to examine how these may be influenced by cognitive and emotional elements under nocebo hyperalgesic conditions.

Due to the multifactorial nature of pain, processing of nociceptive input involves a large, distributed neural network that is not yet fully understood [15]. Pain pathways have been extensively investigated via neuroimaging methods such as functional magnetic resonance imaging (fMRI) and electroencephalography (EEG) and are thought to encompass numerous brain regions and processes. One neural system that has been implicated in pain processing relays sensory-discriminatory functions that are mainly involving nociceptive stimulus information. A second, more distributed system, is thought to be involved in cognitive-evaluative processes [16,17] and these could relate to learning and behavioral underlying factors. It should be noted that these networks and systems are not clearly defined, and that the literature contains inconsistencies regarding the exact brain areas that may be included in these systems [17]. Nevertheless, sensory-discriminatory processes and cognitive-evaluative processes have been extensively researched and are often found to play important roles in pain perception [16–18] and in nocebo hyperalgesia [19–21].

Further insights come from biobehavioral studies illustrating that the experience of pain arises from a combination of bottom-up processes (for example, the type and intensity of nociceptive stimulation) [22–27] and top-down processing (for example nocebo-related processes such as learning or emotional modulation of incoming pain signals) [23,26,28–31]. As one potential product of this interplay between sensory perception, cognition, and emotion, nocebo hyperalgesia is a complex phenomenon, and sophisticated experimental methods are required to understand its influence on pain.

Experimental learning mechanisms

Research typically induces nocebo effects by use of experimental learning models. Experimental models refer to simulations of conditions or responses to treatment that resemble clinical conditions and are induced in healthy individuals, artificially, in a laboratory. In nocebo research, learning manipulations consistently induce nocebo hyperalgesic responses. Typically, conditions in which a sham treatment is associated with pain aggravation are created by use of well-established learning techniques such as classical conditioning or providing negative verbal or written information. Experimentally induced nocebo hyperalgesia in healthy subjects enables researchers to examine these effects, in order to disentangle the mechanisms by which learning can affect pain sensitivity.

In the most robust experimental models of nocebo hyperalgesia, classical conditioning forms and reinforces pain expectations through associative learning [11,32–34]. In conditioning models of nocebo hyperalgesia, an association between a high-intensity pain stimulus (unconditioned stimulus, UCS) and a nocebo (inert treatment) conditioned stimulus (CS) is formed by repeatedly pairing the two stimuli. After repeated trials, an association between the nocebo

stimulus and the worsening of pain is formed and the nocebo stimulus can evoke changes in perceived pain (conditioned response, CR), similar to the previous pain stimulus (**Figure 1**) [35,36]. The powerful associative learning mechanisms employed by classical conditioning serve to recreate learned hyperalgesic responses to a specific stimulus. Classical conditioning thus attempts to recreate a putative clinical context in which not only physical injury but also memories of previous experiences and expectations about the future could have a strong impact on pain symptoms.

Verbally delivered negative information can also alter pain expectations through instructional learning. Negative suggestions typically involve explaining the pain-enhancing effect of a (sham) treatment. Suggestions are used to induce nocebo hyperalgesia by themselves or to enhance the effectiveness of nocebo conditioning [9,37,38]. The combination of conditioning and verbal suggestions is found to create the strongest experimental model of nocebo hyperalgesia [7,39]. This points towards a complex interplay of diverse learning processes that may underlie nocebo effects.

Experimental models are thus suited for examining pain aggravation under nocebo hyperalgesic conditions, but they can also shed light on pain chronification due to nocebo. Experimental attenuation models of conditioned effects have shown that learning may be involved in the persistence of pain over time. Paradigms that employ extinction or counterconditioning methods may provide valuable insights into the factors that contribute to nocebo responses [40–43]. In a typical extinction paradigm, associations between the UCS and CS are discontinued, and learned effects would be expected to become extinct over multiple trials that are no longer negatively reinforced [7,39]. This type of behavioral paradigm represents that, even classical conditioning has been discontinued, increased pain sensitivity can persist in response to a learned nocebo stimulus, over a prolonged period of extinction [42,44]. The

clinical implications of negative pain effects that are resistant to extinction or other forms of nocebo attenuation remain unclear [1].

Cognitive-emotional factors

Experimental nocebo models that include conditioning and negative suggestions also enable the study of diverse cognitive and emotional factors that may be involved in nocebo hyperalgesia. Cognitive manipulations, such as varying the type of learning, can shed a light on the intricate processes that give rise to the experience of pain. Factors that contribute to the formation or the persistence of nocebo effects can be studied using manipulations within experimental models. For instance, studies have shown that consistent and repetitive learning methods, such as classical conditioning with continuous reinforcement of an association, induces the strongest nocebo responses [42]. However, interrupted or inconsistent learning is still able to induce nocebo hyperalgesia to some extent [42,45], which may be important from a clinical perspective, where pain and learning may be less consistent than in experimental settings. Partially reinforced learning has been shown to slow down the extinction or minimization of learned effects [45], including nocebo effects [46].

Learning as an underlying cognitive factor contributing to the formation of nocebo effects may also be investigated at a neurobiological level. Brain plasticity has long been shown to be non-unitary [47–50], with multiple mechanisms and processes being involved in different types of memorization, learning, as well as recall [48,51,52]. Given this multifaceted nature of learning, precise manipulations, targeting associative aspects of learning, hold the potential of enriching our understanding of hyperalgesic pain responses. For example, structures such as the N-Methyl-D-aspartate (NMDA) receptor and neurochemicals such as glutamate have been consistently implicated in a broad array of brain

plasticity processes, including learning by association [53–54]. Pharmacological manipulation of these receptors has been proven fruitful for enhancing learning during exposure therapy [53–55] and may also prove useful in the examination of the precise neurocognitive mechanisms, such as specific receptors and localized learning processes, that may facilitate nocebo hyperalgesia.

Emotional underlying factors, such as fear of pain symptoms, may additionally tie into the processing of pain based on prior experiences and expectations. Through the common theme of learning, nocebo hyperalgesia bares some similarities to the formation of phobias [56–58]. Nocebo and phobic responses may both be characterized by a key involvement of cognitive-affective components such as aversive learning and fear of a stimulus [12,53,59]. Indeed, neural correlates of nocebo hyperalgesia show some involvement of fear processing. This is especially evident by the consistent involvement of the amygdala in both nocebo responses [19,20,60] and fear responses [61–64]. Similarly to nocebo conditioning, pain-related fear can be acquired through associative learning [65–68]. Pain-related fear may thus be relevant to nocebo effects because it may arise in experimental models as a result of experienced pain or as a result of threatening information regarding upcoming pain. Fear experienced during nocebo conditioning may thus potentially augment the acquisition of negative expectations, but experimental studies on this are lacking so far. This makes fear an especially important factor to study in relation to nocebo hyperalgesia.

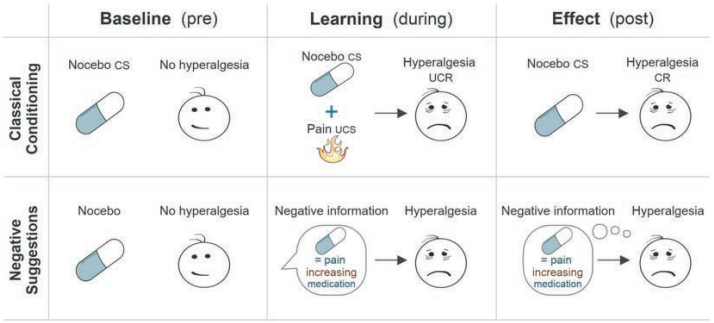

Figure 1. A representation of typical experimental procedures for delivering classical conditioning and negative suggestions. At baseline before any intervention, an inert treatment has no effect. In the case of a conditioning paradigm this becomes a conditioning stimulus, CS, but in verbal suggestion paradigms no conditioning takes place and suggestions can be delivered once, verbally or in writing. A nocebo treatment is here represented as an inert pill. During a learning paradigm, the negative association between the nocebo treatment and pain aggravation (unconditioned stimulus, UCS) is experienced by the subject (unconditioned response, UCR). If learning a negative association comes in form of a verbal suggestion, continuous learning is not required, and one suggestion can in principle suffice. Thereafter, a nocebo effect on pain (conditioned response, CR in the case of classical conditioning) is formed, as a result of learned negative expectations regarding the nocebo treatment.

Biobehavioral underpinnings

In recent years, fundamental research has focused on unravelling how nocebo responses are formed and how they may integrate in pain processing. In order to reach a better understanding of the neurocognitive components of nocebo effects, it is imperative to build on previous nocebo research, by utilizing consistent experimental models. In this way, results are comparable, and the reliability of findings can be tested over multiple studies. Theories and findings from the nocebo literature should also be connected to what is currently known about overlapping cognitive and emotional processes. Comprehensive reviews of the current state of research into the neural correlates of

nocebo hyperalgesia can thus be very valuable, next to neurobiological studies that build upon this accumulation of knowledge.

Research to date has highlighted some key areas that may be involved in nocebo hyperalgesia. Pain-specific processing has been implicated in the presentation of nocebo hyperalgesic responses. For instance, the dorsal horn of the spinal cord, the secondary somatosensory cortex, and the dorsolateral prefrontal cortex (dlPFC) all seem to be activated during nocebo responses [11,15]. This may indicate that pain reports under nocebo hyperalgesic conditions closely resemble typical pain processing. Concurrently, nocebo responses have also been shown to involve brain areas such as the anterior cingulate cortex (ACC), amygdala, and hippocampus [11,69], which supports an involvement of cognitive-emotional factors in nocebo effects, which are also involved in pain processing and integration. Nocebo hyperalgesia also seems to involve chemical systems, such as prostaglandins, cortisol, and dopamine [69,70], while electrophysiological correlates point towards an involvement of alpha and gamma brain rhythms [71–73]. It is thus evident, albeit not surprising, that nocebo hyperalgesia largely overlaps with pain processing. The involvement of a wide network of cognitive-emotional processing is also supported by neurophysiological findings. However, replicating findings from one nocebo study to the next seems challenging due to the implementation of diverse experimental models and distinct learning mechanisms between different studies. Employing diverse methods helps clarify specific aspects of nocebo hyperalgesia but also leads to inconsistencies and gaps in the literature, rendering nocebo hyperalgesia a phenomenon that is still poorly understood on a neurocognitive level.

With learning and the formation of expectations being at the heart of the most robust nocebo theories [1,11,72,74,75] it is important to observe and interpret similarities between these cognitive processes and nocebo effects. Limbic system structures, and especially the amygdala, are

known to play a crucial role in learning and memory formation [76–79]. More specifically, the amygdala and hippocampus, structures sometimes implicated in nocebo effects [20,21], play essential roles in the formation of new memories based on past experiences [80–82]. At the same time, the consistent involvement of the ACC in nocebo effects may be drawing together prior memories, expectations, and information processing. The ACC is an area that is largely interconnected to the limbic system and may play a key role in cognitive control and conflict monitoring [83,84]. These neuroscientific similarities may indicate that nocebo hyperalgesia involves a complex cognitive network that is responsible both for memory formation and for the recall and integration of learned expectations and incoming sensory information. Brain plasticity, learning, and cognitive-emotional factors, seem to form an interconnected, cortical-subcortical network that may be involved in nocebo hyperalgesia and in pain processing [21,60,62,85] (**Figure 2**).

Yet, inconsistencies and a lack of replicability between nocebo studies do not permit for firm conclusions to be drawn. Cognitive and emotional components, such as learning and fear, seem have a critical role in the formation of nocebo hyperalgesia. Nevertheless, there is uncertainty regarding the specific learning mechanisms that may be involved, how they affect brain plasticity, and how learning networks may integrate with pain processing. This is evident in the large disparity in neurophysiological findings of fMRI and EEG studies. Inconsistencies in the literature also lead to uncertainty regarding the impact of fear, as compared to related cognitive-emotional responses such as anxiety. It is important for systematic experimental research to actively manipulate states of brain plasticity, learning, memory, and other cognitive processes. While a comprehensive overview of the state-of-the-art in nocebo research is valuable, pharmacological manipulations and other methods that directly manipulate cognitive states may be central components in the strive to unravel the specific neurocognitive mechanisms of nocebo hyperalgesia.

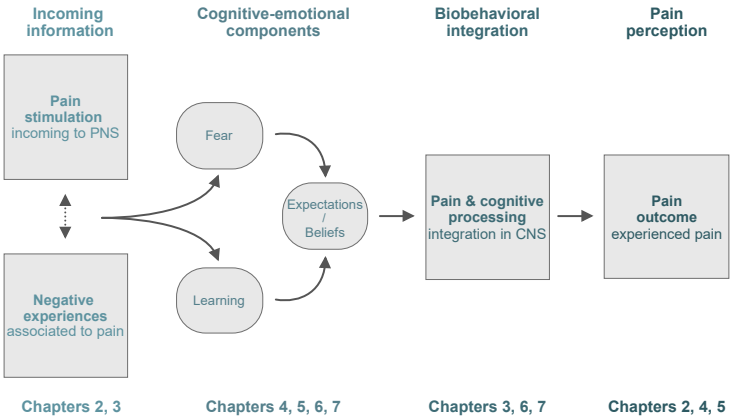

Figure 2. The working theoretical model of this dissertation. In the process of pain perception, incoming information to the Peripheral Nervous System (PNS) regarding a pain stimulus (bottom-up information), in combination with negative experiences, are influenced by nocebo-related cognitive-emotional factors (top-down information). Based on these factors of interest, integrative processing of pain takes place in the Central Nervous System (CNS), with the potential of giving rise to negative pain outcomes related to learned nocebo responses.

The current dissertation

In this dissertation, we address biobehavioral aspects of nocebo hyperalgesia, using neuroimaging as well as behavioral science methods. First, a systematic review and meta-analysis provides a novel examination of nocebo effect sizes and relevant factors in experimental studies. Thereafter, a literature review aims to comprehensively summarize what is currently known about the neurobiological correlates of nocebo hyperalgesia. Subsequently, a series of experimental studies attempt to directly manipulate and study cognitive and emotional factors involved in nocebo, by use of innovative methods. Fear and pharmacological manipulations of specific learning mechanisms are utilized for the first time in these studies, while original

electrophysiological methods reveal biomarkers of nocebo effects. The aim of this dissertation was to further the knowledge on the neurochemical, electrophysiological, and cognitive-emotional processes that underlie nocebo hyperalgesia, with a specific focus on cognition, emotion, and brain plasticity.

In **Chapter 2** we explore the state of the art in behavioral nocebo research with a systematic review and meta-analysis of nocebo literature on somatosensory sensations, including pain. We aim to address the efficacy of different experimental learning methods for the induction of nocebo effects. We systematically summarize results from dozens of studies that investigated these effects, and we discuss the implications of their findings. This meta-analysis showed that across sensations, the magnitude of nocebo responses is affected by the type of learning, with classical conditioning being more potent than verbal suggestions alone. We discuss the lack of explanatory or moderation factors found in the literature. Our analysis served to illuminate the extent to which learning processes induce nocebo effects on different sensations and what the practical and theoretical implications of a lack of moderating factors identified may be for research and clinical practice.

In **Chapter 3**, we review the neurobiological literature on nocebo hyperalgesia. This comprehensive review article summarizes neurobiological findings from studies that utilized (f)MRI, EEG, magnetoencephalography (MEG), as well as pharmacological and biochemical measures. The review provides a comprehensive overview and serves to highlight consistent neural correlates of nocebo hyperalgesia across a variety of different experimental nocebo models. In this way, this overview aims to provide an up-to-date picture of the biobehavioral correlates of nocebo effects. We outline the evidence from this field and give an overview of similarities and differences between nocebo research and learning/memory research.

Building on previous nocebo studies as described in the first chapters of this dissertation, **Chapter 4** presents a randomized controlled trial investigating distinct learning schedules. This experiment aimed to examine the role of continuous versus partial learning and the consequences of such learning schedules for the persistence of nocebo hyperalgesia. For this purpose, both the induction and the attenuation of nocebo hyperalgesia are experimentally manipulated via distinct learning methods. Healthy participants were randomized to receive conditioning on nocebo effects with continuous reinforcement, partial reinforcement, or sham conditioning. In attenuation, counterconditioning (i.e., positive conditioning of the nocebo conditioned stimulus) was compared to extinction for the attenuation of nocebo hyperalgesia. This study provided important insights into the effect that different learning schedules may have on the acquisition and attenuation of nocebo responses.

In **Chapter 5** an experimental study of cognitive-emotional processes is presented. Here, we aimed to investigate how fear can augment nocebo responses and how this may affect the persistence of these responses over time. We experimentally manipulated fear of pain during the induction of nocebo responses. We used two distinct fear inductions. One fear induction method was to manipulate pain levels with the aim of inducing fear of the high pain stimulations. The other fear induction included a threat manipulation that convinced participants that their skin is critically sensitive to pain. This study provided insights into the role of specific types of fear in the acquisition and extinction of nocebo hyperalgesia, which may be of important relevance given the relationship between fear and pain in clinical practice.

Chapter 6 presents an electrophysiological investigation into nocebo hyperalgesia. We aimed to explore alterations in EEG biomarkers during the anticipation, acquisition, and evocation of nocebo hyperalgesia. This thorough investigation served to unravel multiple electrophysiological

aspects of nocebo effects, thereby shedding light on novel aspects of brain processing under nocebo hyperalgesic conditions. We induced nocebo hyperalgesia by use of conditioning and negative suggestions and recorded EEGs before, during, and after nocebo acquisition and evocation. This EEG study enriched our understanding of the role that learning and nociceptive processing play in nocebo hyperalgesia.

In **Chapter 7** we present a pharmacological fMRI study that investigated neurochemical correlates of learning in nocebo hyperalgesia. In this randomized clinical trial, we aimed to pharmacologically manipulate NMDA receptors, known for mediating certain types of learning, such as associative learning involved in classical conditioning. fMRI methods allowed for the exploration of brain activations during nocebo acquisition and extinction. We used classical conditioning and negative suggestions to induce nocebo hyperalgesia in a group receiving a low dose of D-cycloserine (a known partial NMDA receptor agonist) and in a group receiving placebo. These manipulations and imaging methods served to explore how NMDA-dependent learning influences the formation of nocebo effects and several potentially relevant brain processes were identified.

Chapter 8 is a general discussion relating to this dissertation. In this chapter the results of the conducted studies are summarized and connected to the aims of this PhD project. We then further discuss these aims in light of theoretical and practical implications.

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Chapter 2.

Learned nocebo effects on cutaneous sensations: Meta-analysis of experimental behavioral findings.

Submitted for publication as

Thomaidou, MA, Blythe JS, Peerdeman KJ, van Laarhoven AIM, Van Schothorst MME, Veldhuijzen DS, Evers AWM. Learned nocebo effects on cutaneous sensations: A systematic review and meta-analysis of experimental behavioral findings.

Abstract

In past decades, the field of nocebo research has focused on studying how sensory perception can be shaped by learning. Behavioral conditioning processes as well as mere verbal suggestions of a negative treatment outcome may aggravate pain and itch perception. Gaining a comprehensive view of the magnitude of nocebo effects and the factors that contribute to their formation will help steer nocebo research towards fruitful directions for better understanding complex sensory phenomena. We conducted a systematic review and meta-analysis of a total of 37 distinct experimental nocebo studies on healthy participants, with four separate meta-analyses for nocebo effects on pain or itch, induced with classical conditioning and verbal suggestion, or verbal suggestion alone. We conducted subgroup analyses and meta-regression on factors such as the type and intensity of sensory stimuli, and the length of learning paradigms. This meta-analysis showed that on average, effect sizes of nocebo effects were moderate to large (Hedges *g* between 0.26-0.71 for the four primary outcomes). The combination of conditioning and verbal suggestions yielded stronger nocebo responses on pain in particular. Subgroup analyses, including factors such as the type of sensory stimulation, did not explain the moderate heterogeneity in nocebo magnitudes between different studies. Risk of bias was generally low and was not related to nocebo magnitudes either. We discuss these results in relation to the role of conditioning as well as aversive learning, and we recommend more consistency in designing and reporting nocebo experiments.

Introduction

Negative expectations regarding the effects of a treatment can result in the aggravation of cutaneous sensations such as pain and itch [1–3]. Such learned responses can be induced experimentally, allowing for the study of processes by which nocebo effects lead to symptom amplification [4–10]. In experimental studies, nocebo responses are defined as a significant increase in a sensation after a nocebo treatment, relative to no-treatment or a control treatment. Negative expectations leading to such responses are typically induced through classical conditioning, verbal suggestions, or their combination [4,5,10–13]. Classical conditioning induces nocebo effects by building implicit associations between an (inert) treatment and the worsening of sensations such as pain or itch [14–16]. Verbal suggestions explicitly provide negative information regarding the pain- or itch-increasing effects of a treatment [7]. Because nocebo studies employ diverse methods, to better understand their potential impact on nocebo outcomes these methodological features warrant a systematic investigation.

Learning has consistently been shown to underlie induced nocebo effects [5–7,9,17], and verbal suggestions seem to induce stronger nocebo responses when combined with conditioning [18]. The positive counterpart to nocebo, placebo effects, also appear to be stronger when induced through a combination of conditioning with verbal suggestions, compared to conditioning alone, both on pain [19] and itch (Bartels et al., 2014; Blythe et al., 2019). One meta-analysis included results from ten nocebo experiments published up to 2013 and reports that the overall magnitude of the nocebo effect was moderate to large and effects were generally larger when verbal suggestions were used in combination with conditioning [18]. That early meta-analysis had a limited sample of studies

available, and an up-to-date review is needed to examine how different types of learning may induce nocebo effects of different magnitudes.

At the same time, other variables, such as the type of sensation (i.e., pain or itch), stimulus modality (e.g., thermal, electrical), the intensity of pain or itch stimulations, and the length of learning in different behavioral paradigms, also require a systematic examination across studies. For example, in experimental nocebo research, some nocebo conditioning paradigms include as few as four associative learning trials (Blythe et al., 2021), while others employ much longer paradigms [6,8,21]. A diverse set of cutaneous sensory induction methods are also used, such as thermal [17], electrical [6,20], or laser pain stimulations [22]. Such methodological choices, often meant to target specific underlying processes in nocebo experiments, can potentially influence nocebo responding and thus merit further investigation.

Given the recent growth of nocebo research, we conducted a systematic review and meta-analysis of experimental nocebo studies in healthy participants to provide novel insights into distinct contributions of methodological factors in the induction of nocebo responses. We focused on cutaneous sensations, aiming to examine nocebo responses induced with comparable sensory inductions externally on the skin. First, we examined nocebo magnitudes between pain and itch and based on the learning method used. Then, we conducted subgroup analyses and meta-regression to assess how the type and intensity of stimulations, the length of learning, the timing of measurement of nocebo magnitudes, and risk of bias in studies may impact nocebo magnitudes.

Methods

Protocol and registration

The protocol for this study was pre-registered on ClinicalTrials.gov (ID: NCT04387851) and conducted based on the PRISMA statement (**Appendix A**) and Cochrane recommendations (2020). The protocol was registered based on a single search strategy for both nocebo and placebo studies, which, due to the volume of the studies returned, is now divided in two separate papers. Here, we report only the nocebo (arms of) experimental studies.

Databases and selection criteria

PubMed, PsycINFO, EMBASE, and the Cochrane CENTRAL Methodology Library were searched to identify studies. Languages were a-priori restricted to English, Dutch, and German and the publication period was not restricted. Searches were initially conducted on March 18th, 2019. Repeated searches for studies published after this time were conducted in June 2020 and July 2021. The detailed key-word strategy for each database will be made available online upon publication (**Appendix B**).

We searched for original, peer-reviewed, controlled experimental studies (or study arms) on healthy human participants that aimed to experimentally induce placebo and/or nocebo effects. Patient samples were not included, to improve the homogeneity of the results, and for the same reason we focused on cutaneous sensations (i.e., pain and/or itch stimulations that were administered on the skin), excluding for

example visceral pain studies. For the purposes of in- and exclusion, studies were considered to have induced a placebo or nocebo effect if a learning paradigm was used to induce positive or negative outcome expectations about an inert treatment. We considered as *nocebo* learning paradigms only those that aimed to induce negative expectations regarding an intervention, such as sham electrical stimulation or an inert cream. This meant that most conditioning without verbal suggestion studies were excluded from this review, as they did not include treatment associations, and were considered to be pain-conditioning, not nocebo-conditioning studies (albeit explicit mention of the terms *nocebo* and *placebo* was not a specific inclusion criterion). Additionally, we only included studies that had a control group or a control condition within-subjects, so that nocebo effects could be calculated as the difference between nocebo and control/no treatment on self-reported scores. We excluded studies that excluded or did not report data from nocebo non-responders. Post-hoc, we excluded observational learning studies as they were too few for a meaningful analysis. Studies that did not fulfill one or more of the criteria mentioned above were excluded from the meta-analysis (see **Figure 1** for a flow diagram).

Study selection

Eligibility assessment for the inclusion of studies was performed independently by two authors in each of the following steps. Titles and abstracts of articles retrieved using the search strategy were screened by two authors independently (M.M.E.v.S. and J.S.B.). The full text of articles to be included and articles about which doubts existed were then retrieved and assessed for eligibility by two authors independently (M.A.T. and J.S.B.). The reference lists of all included articles were also screened for study inclusion by two authors (M.A.T. and J.S.B.) and included articles were also entered in Web of Science to identify articles

that have cited them and should potentially be included in the meta-analysis. When necessary, authors of studies were contacted in order to provide full-text articles that were not accessible online. Any disagreements regarding study inclusion were resolved by consultation with a third author (K.J.P.).

Data extraction

One author (J.S.B.) used a standardized form to independently extract data from the included studies to derive data for analyses. Another author (M.A.T.) checked 25% of extracted values for accuracy. Extracted information included details of the intervention such as the learning method used, the control condition, study population, sensation type, pain/itch rating data, type of cutaneous stimulation (e.g., heat pain, pressure pain), type of outcome expected (i.e., placebo or nocebo), information for quality assessment, and outcome data for meta-analysis (e.g., sample size, pain/itch rating means and standard deviations). Doubts regarding data-extraction were resolved through discussion with a third review author (K.J.P.). Missing data were requested directly from the study authors. When there was no response from authors, but data could be extracted from published figures, this was done using WebPlotDigitizer version 4.4 (Rohatgi, 2020).

Risk of bias

Risk of bias (RoB) was assessed and checked by two authors (M.A.T. and J.S.B.) using the method developed by Marcuzzi and colleagues specifically for quantitative sensory testing studies [23]. This method assesses whether the sample was clearly described and was representative of the population, whether the somatosensory assessment

methods are standardized, validated, and well described, if potential confounders were considered, and adequate blinding. Each category was scored as being satisfied (0 points), not satisfied (2 points), partially satisfied (unclear; 1 point), or not applicable. Scores were selected based on criteria described in Marcuzzi and colleagues [23]. We additionally concocted numerical scores (0-34) for each study, by summing each item score, with higher scores indicating higher risk of bias (please see **Appendix C** for an example of the RoB scoring.

Statistical analyses and results synthesis

All analyses were conducted and checked by two reviewers (J.S.B. and M.A.T), using the Comprehensive Meta-Analysis software (version 3.3.070; Biostat, Englewood, USA) and R programming software for visualizations [24]. Funnel plots were inspected for outliers (i.e., studies falling outside the funnel of expected results), and to assess publication bias across studies we checked for number of imputed missing studies with Duval and Tweedie's trim and fill method [25]. Heterogeneity between studies was assessed with the I^2 statistic and visual inspection of the forest plot. I^2 is a measure of the proportion of observed variance reflecting real differences in effect sizes [26] with values of 25%, 50%, and 75% considered as low, moderate, and high degrees of heterogeneity, respectively [27]. For forests plots, we calculated study weights in *R*, by inversing the variance of each effect size.

Given the heterogeneity of study designs, random effects models were used for all meta-analyses. Effect sizes were calculated using means and standard deviations for each group (between subjects) or trial type (within subjects). [26]. We selected nocebo and control conditions based on what was reported in studies: some reported nocebo magnitudes between groups, other within groups in the first pair of evocation trials,

and others reported nocebo magnitudes as the mean difference of all control and evocation trials. When only nocebo/control difference scores were reported, these were used instead. When only standard errors were reported, they were converted to standard deviations by multiplying the standard error by the square root of the group size (n). For each study, an effect size Hedges's *g*, weighted to the sample size (N), was computed as the mean response in the nocebo condition minus the mean response for control in the evocation phase of experiments. Positive *g* values indicate a nocebo response, with values around .2 considered small, .5 medium, and .8 large.

For studies that used within-subjects comparisons, the nocebo-control condition correlation coefficient could not be derived, therefore an average r of .5 was imputed [28]. Meta-analysis was only conducted when the data of at least 4 studies were available in total. In subset analyses, the effect sizes were compared descriptively rather than with statistical tests when 2 or less studies were available per group. Studies with multiple eligible conditions were treated as separate subgroups and averaged across in CMA (e.g., when cheap vs. expensive inert treatments were used as nocebo, we averaged the results and treated this as one group (see **Table 1** for results synthesis per study).

Primary outcome measures and subgroup analyses

Our primary outcomes were the overall magnitude of nocebo responses (i.e., the difference in self-reported pain/itch between a nocebo and a control trial in the evocation phase) separately for pain and itch studies employing verbal suggestions with or without classical conditioning. We thus computed 4 pooled effect sizes: verbal suggestions in pain, conditioning with verbal suggestions in pain, verbal suggestions in itch, conditioning with verbal suggestions in itch. Whenever possible, the

mean of pain or itch ratings across the entire evocation phase was used. If only values from the first trial(s) were reported, these were used instead, and sensitivity analyses tested for differences in magnitudes between studies reporting the mean versus the first trials.

We also did subgroup analyses to compare Hedge's *g* between nocebo responses based on the type of learning (verbal suggestion or combination with conditioning) and type of sensory stimulation (e.g., thermal, electric) and the timing of nocebo measurement (as the mean of evocation or only the first evocation trials, by trial type). Meta-regression assessed the impact of the length of learning, (quantified as the number of learning trials during induction, while we also separately examined number of trials evocation), the timing of the measurement of nocebo hyperalgesia in the evocation phase (first trials versus mean of evocation trials), the stimulus intensity (calculated as the calibrated difference in pain intensity for control vs. nocebo trials) and the Risk of Bias score on nocebo magnitudes for the included studies.

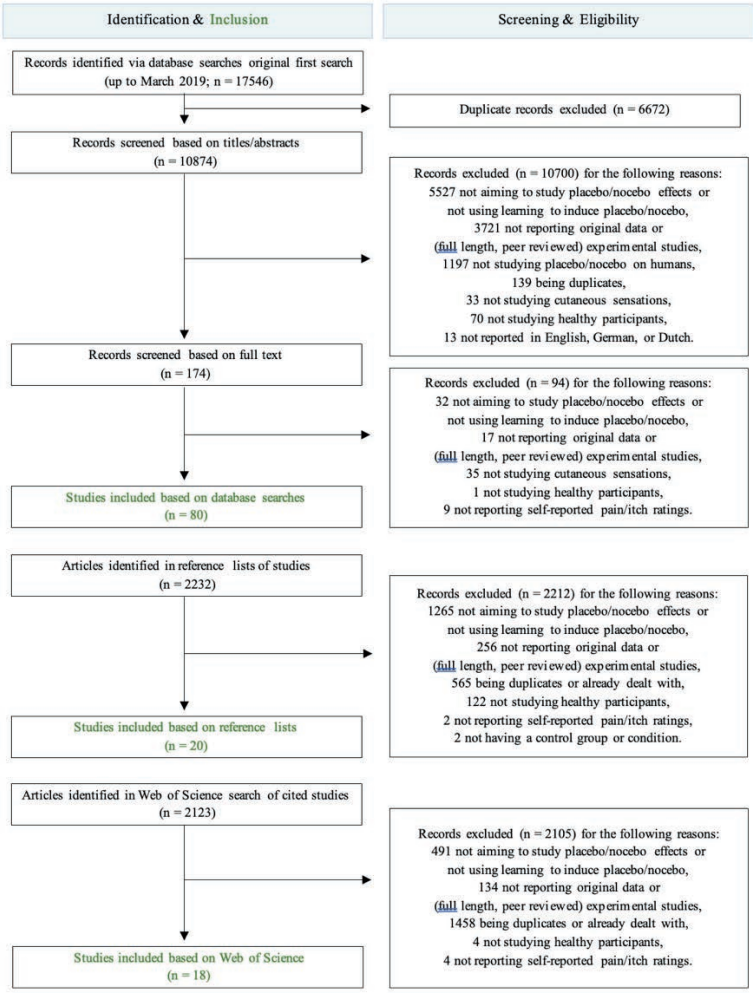
Results

Study selection

Figure 1 shows the flow of the study selection process including the reasons for exclusion at each stage. A total of 17546 nocebo and placebo papers were initially identified through the database searches. We searched for more eligible studies through reviewing the reference lists as well as web of science for each included study, as well as conducting repeat database searches in June 2020 and July 2021. At each stage of

study inclusion, duplicates were removed, and remaining articles were considered based on title and abstract, or full text. In total, we identified 24814 articles through our searches, of which 24687 were excluded.

We did not follow a strict hierarchical approach in marking exclusion criteria, but selected criteria based on what was deemed to be the major exclusion reason, for example when screening abstracts where limited information is available, therefore the following exclusion numbers provide less than precise estimates of exclusion reasons. We excluded articles for the following reasons: 8302 articles for not aiming to study nocebo or placebo effects or not using a learning paradigm to induce placebo or nocebo effects (explicit use of the terms *nocebo* or *placebo* was not an inclusion criterion), 4328 for not reporting original data or (full length, peer reviewed) experimental studies, 1229 studies for not being conducted in humans, 10440 because they were duplicates or already screened during a previous round, 101 articles for not studying (placebo/nocebo on) cutaneous sensations, 242 articles for not studying (placebo/nocebo in) healthy human participants, 20 articles because they did not report self-reported pain/itch intensity ratings, 13 for not being in English, Dutch, or German, 2 studies for not using a within- or between-subjects controlled design, 5 studies for not responding to requests for data, and 5 for excluding data from participants that were considered placebo/nocebo non-responders. A total of 127 articles were selected of which 108 included placebo conditions and 39 nocebo conditions. Of these articles, we excluded 2 observational learning studies as they were too few for a meaningful analysis. Thus, in total, **37** studies were included in this meta-analysis on nocebo effects.


Identification & Inclusion
Screening & Eligibility
Records identified via database searches original first search (up to March 2019; n = 17546)
Duplicate records excluded (n = 6672)
Records screened based on titles/abstracts (n = 10874)
Records excluded (n = 10700) for the following reasons:
5527 not aiming to study placebo/nocebo effects or not using learning to induce placebo/nocebo,
3721 not reporting original data or (full length, peer reviewed) experimental studies,
1197 not studying placebo/nocebo on humans,
139 being duplicates,
33 not studying cutaneous sensations,
70 not studying healthy participants,
13 not reported in English, German, or Dutch.
Records screened based on full text (n = 174)
Records excluded (n = 94) for the following reasons:
32 not aiming to study placebo/nocebo effects or not using learning to induce placebo/nocebo,
17 not reporting original data or (full length, peer reviewed) experimental studies,
35 not studying cutaneous sensations,
1 not studying healthy participants,
9 not reporting self-reported pain/itch ratings.
Studies included based on database searches (n = 80)
Articles identified in reference lists of studies (n = 2232)
Records excluded (n = 2212) for the following reasons:
1265 not aiming to study placebo/nocebo effects or not using learning to induce placebo/nocebo,
256 not reporting original data or (full length, peer reviewed) experimental studies,
565 being duplicates or already dealt with,
122 not studying healthy participants,
2 not reporting self-reported pain/itch ratings,
2 not having a control group or condition.
Studies included based on reference lists (n = 20)
Articles identified in Web of Science search of cited studies (n = 2123)
Records excluded (n = 2105) for the following reasons:
491 not aiming to study placebo/nocebo effects or not using learning to induce placebo/nocebo,
134 not reporting original data or (full length, peer reviewed) experimental studies,
1458 being duplicates or already dealt with,
4 not studying healthy participants,
4 not reporting self-reported pain/itch ratings.
Studies included based on Web of Science (n = 18)

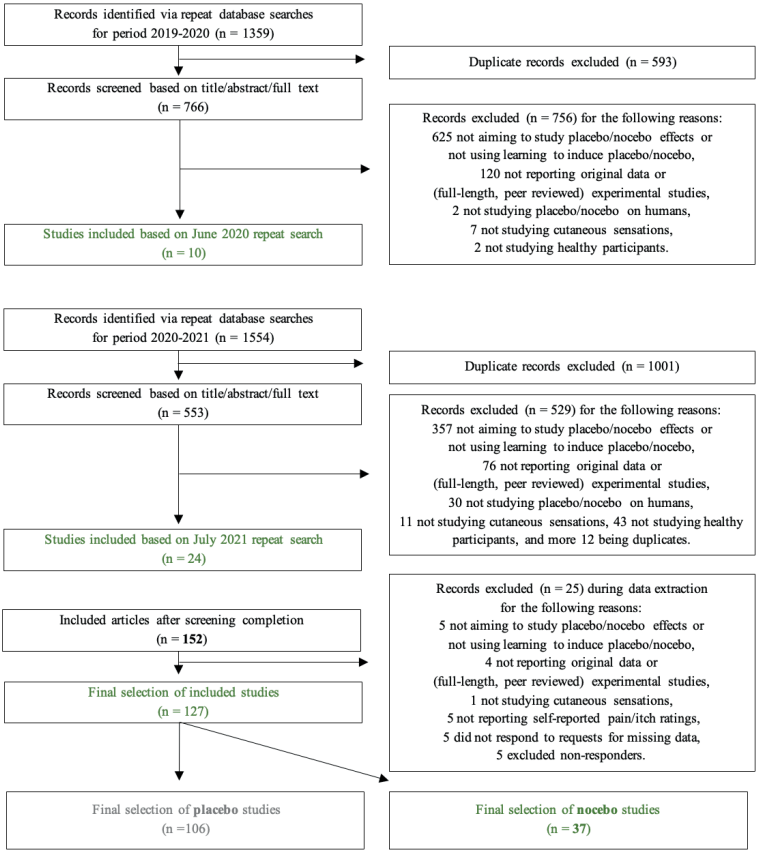


Figure 1. Flow diagram detailing the inclusion and exclusion of studies. The final sample included 127 articles, of which 106 investigated placebo effects, and 37 investigated nocebo effects (i.e., 16 studies overlapped as they investigated both placebo and nocebo).

Study characteristics

Table 1 displays study characteristics. We included 37 distinct nocebo studies, published between 2008 and 2021. Including additional

experimental conditions in a number of studies (see **Table 1**) in total we analyzed 40 study arms (30 pain and 10 itch). Thermal pain inductions were used in 19 arms, electrical pain was used in 6, pressure pain was used in 1, and mechanical, cold pressor, hot water bath, and histamine methods were each used in 1 study arm. Only 7 studies (10 arms) induced nocebo effects on itch, one of which also included pain (this study, van Laarhoven et al., 2011, is listed under *Pain* in **Table 1**). Electrical itch was used in 3 studies, one of which (van Laarhoven et al., 2011) used additional mechanical and histamine inductions in both the pain and itch groups (see **Table 1**). Histamine was used in 3 more itch studies and cowhage was used in 1 study.

For nocebo induction, most studies (18 pain and 4 itch studies) used a combination of classical conditioning and negative verbal suggestions, and for 3 we included additional study arms that employed verbal suggestions alone (**Table 1**). Verbal suggestions alone were used as the main manipulation in 10 pain studies (in total 12 arms) and 3 itch studies (in total 6 arms). Risk of bias was low within all studies, with most studies showing low risk of bias (max. 5/34) and only one study scoring in the low-moderate range with a score of 6/34 (**Table 1**). The funnel plots as well as a trim and fill method that suggested a small number of imputed studies (**Figure 3**) indicated that overall, there was a low degree of potential publication bias across all studies, with a total estimated 7 studies missing.

Magnitude of nocebo responses

See **Figures 2** and **3** for forest and funnel plots, respectively, that display effect sizes per study and pooled effects. For pain (**Figures 2A** and **2B**), the magnitude of nocebo responses on a standardized scale of 0-10 (with higher scores indicating larger nocebo magnitudes) across studies using classical conditioning with verbal suggestions ranged from 0.28 to 1.42, with the mean standardized response being M = **0.79** (SE = 0.24). Verbal suggestions alone induced effects on pain ranging from 0.00 to 1.27 (M = **0.70**, SE = 0.30). For itch, the magnitude of nocebo responses in studies that used conditioning with verbal suggestions ranged from 0.21 to 0.47 (M = **0.35**, SE = 0.24). Verbal suggestions alone induced effects on itch ranging from 0.41 to 0.75 (M = **0.58**, SE = 0.26). Based on these results, on average our meta-analysis indicated medium effects of the nocebo manipulations (Hedges g between 0.26-0.71 for each of the four pooled effects), a moderate degree of heterogeneity (I^2 average 41% across the four pooled effects), with the study effect sizes ranging between g = 0.00 and g = 1.34.

Table 1. Study characteristics for all included articles.

	Authors	Year	Sample size nocebo group	Sample size control group	Mean age (SD)	Stimulat. type	Learn. method	Results synthesis where applicable	Number of conditioning trials (N/C)	Risk of Bias score (0-34)
PAIN										
1	Colagiuri, Quinn, et al	**2015**	37	42	20.3 (4.0)	Electrical	CC+VS		32 (16/16)	3
2	Colagiuri & Quinn	**2018**	20	20	20.2 (4.0)	Electrical	CC+VS		32 (16/16)	5
3	Colagiuri, Park, et al.	**2021**	20 + 20	21 + 20	20.7 (3.6)	Electrical	CC+VS	Lengthier learning condition treated and analyzed as a separate study arm	32 (16/16)	3
4	Colloca, Petrovic, et al.	**2010**	23 + 23	n/a	22.8 (3.4)	Electrical	CC+VS	Four vs. of one learning sessions averaged together	20 (10/10) or 80 (40/40)	3
5	Colloca, Sigaudo, et al	**2008**	42 VS & 45 CC+VS	n/a	22.3 (2.4)	Electrical	CC+VS & VS	Three pain intensities averaged across VS and CC+VS conditions and analyzed as two separate study arms	24 (12/12)	3
	Corsi & Colloca	**2017**	46	n/a	27.4 (1.1)	Thermal	CC+VS		12 (6/6)	3
7	Egorova, Benedetti, et al	**2020**	24	n/a	n/a	Thermal	CC+VS		48 (24/24)	5
8	Feldhaus, Horing, et al.	**2021**	624	n/a	24.6 (3.6)	Thermal	CC+VS		16 (8/8)	3
9	Freeman, Yu, et al.	**2015**	24	n/a	21 to 49	Thermal	CC+VS		18 (9/9)	5
10	Geuter & Büchel	**2013**	20	n/a	26.4	Thermal	CC+VS		24 (12/12)	3
11	Kong, Gollub, et al.	**2008**	13	n/a	26.3 (3.6)	Thermal	CC+VS		48 (24/24)	5
12	Pazzaglia, Testani, et al.	**2016**	9 + 9	n/a	29 (5.0)	Laser	CC+VS &VS	VS condition treated and analyzed as a separate study arm	60 (30/30)	5

13	Skvortsova, Veldhuijzen, et al.	**2019**	37	n/a	23.1 (2.9)	Thermal	CC+VS		24 (12/12)	0
14	Thomaidou, Blythe, et al.	**2021b**	36	n/a	22.9 (2.2)	Thermal	CC+VS		32 (16/16)	5
15	Thomaidou, Veldhuijzen, et al.	**2020**	48	25	21.8 (2.1)	Thermal	CC+VS		30 (15/15)	5
16	Thomaidou, Veldhuijzen, et al.	**2021a**	24	n/a	22.2 (1.9)	Thermal	CC+VS		24 (12/12)	5
17	Tinnermann, Geuter, et al.	**2017**	25 + 24	n/a	25.4 (3.8)	Thermal	CC+VS	Cheap vs. expensive conditions were averaged together	16 (8/8)	6
18	Tu, Wilson, et al.	**2021**	27	n/a	27.4 (6.4)	Thermal	CC+VS		48 (24/24)	3
19	Wei, Zhou, et al.	**2018**	18	n/a	20.9 (1.4)	Electrical	CC+VS		40 (20/20)	3
20	Weng, Peerdeman, et al.	**2021**	33	n/a	21.6 (3.0)	Thermal	CC+VS		30 (15/15)	1
21	Albu & Meagher	**2016**	15	15	19.1 (1.2)	Thermal	VS		n/a	3
22	Aslaksen & Lyby	**2015**	57	54	22.2 (3.1)	Thermal	VS		n/a	3
23	Aslaksen, Åsli, et al.	**2016**	15	16	21.6 (3.3)	Thermal	VS		n/a	0
24	Aslaksen, Zwarg, et al.	**2015**	25	25	23.4 (4.1)	Thermal	VS		n/a	0
25	Camerone, Piedimonte, et al.	**2021**	19	21	23.1 (2.1)	Electrical	VS	We analyzed the 5-min condition	n/a	1
26	Geers, Close, et al.	**2019**	36	36	19.7 (3.2)	Cold pressor	VS		n/a	3
27	Nir, Yarnitsky, et al.	**2012**	12	12	25.8 (3.2)	Hot water bath	VS		n/a	3
28	van den Broeke, Geene, et al.	**2014**	15	15	23.5 (2.2)	Mechanical stimulation	VS		n/a	4
29	Vögtle, Barke, et al	**2013**	26	26	22.5 (4.4)	Pressure	VS		n/a	2
30-31	van Laarhoven, Vogelaar, et al.	**2011**	33pain & 36itch	16pain & 20itch	21.8 (2.2)	Electrical, Mechanical, Histamine	VS	Three types of stimulations averaged together across pain and across itch	n/a	1

ITCH										
32	Bartels, van Laarhoven, et al.	**2014**	23 + 23	25	22.7 (3.2)	Electrical	CC+VS & VS	VS condition treated and analyzed as a separate arm	12 (6/6)	4
33	Bartels, van Laarhoven, et al.	**2017**	99	n/a	20.3 (2.5)	Electrical	CC+VS		16 (10/6)	4
34	Blythe, Peerdeman, et al.	**2021**	19	19	21.9 (2.4)	Cowhage	CC+VS		4 (2/2)	2
35	van de Sand, Menz, et al.	**2018**	30	30	25.5	Histamine skin scrub	CC+VS		40 (20/20)	5
36	Meeuwis, van Middendorp, et al.	**2019**	24	n/a	21.8 (2.7)	Histamine iontophor.	VS		n/a	4
37	Meeuwis, van Middendorp, et al.	**2021**	28	n/a	21.9 (2.8)	Histamine iontophor.	VS		n/a	4

Note: the study by van Laarhoven et al., 2011, included both itch and pain manipulations and is listed under pain. When the sample size of a control group is listed as n/a, this suggests that the study used a within-subjects controlled design. Studies are listed separately for pain and itch and first based on the learning manipulation (VS, verbal suggestions, or CC+VS, combination of classical conditioning and verbal suggestions) and then alphabetically. N, Nocebo; C, Control; M, Male; F, Female.

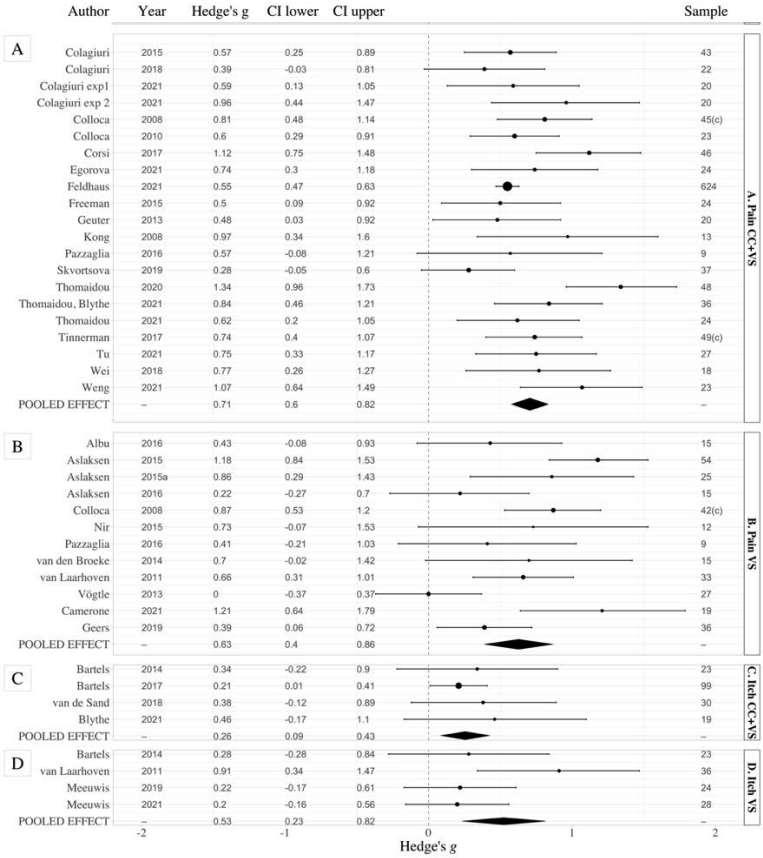


Figure 2. Forest plot of the meta-analysis indicating the magnitudes of nocebo responses following a combination of classical conditioning and verbal suggestions (CC+VS) or verbal suggestions alone (VS) on pain (**A, B**) and itch (**C, D**). Sample sizes marked with (c) indicate the combined sample from different study arms.

Classical conditioning and verbal suggestions in pain and itch

A range of different verbal suggestions were used to induce nocebo responses on pain and itch. Most studies used either an inert cream or inactive electrodes as the nocebo stimulus that would supposedly increase pain/itch sensitivity. For example, studies suggested to participants that their pain will be increased upon the activation of electrodes on their skin because these electrodes "enhance the conductivity of the pain signal being sent to the brain" [29] or "the cream that will be applied to your arm increases the effect of the heat pain and you will feel more pain after the application." [17]. Most such suggestions were delivered orally by a researcher, with few studies providing such information in writing [9,21,29–31].

For **pain**, a somewhat larger pooled nocebo effect of the combination of **conditioning with verbal suggestions** (k = 21, g = **0.71**, 95% CI 0.60 – 0.82, I^2 = 50.71%; **Figure 2A**) was observed than of **verbal suggestions** alone (k = 12, g = **0.63**, 95% CI 0.40 – 0.86, I^2 = 55.59%; **Figure 2B**). In **itch**, however, **conditioning with verbal suggestions** yielded a smaller pooled effect on the magnitude of nocebo responses (k = 4, g = **0.26**, 95% CI 0.09 – 0.43, I^2 = 0%; **Figure 2C**) compared to a medium pooled effect of **verbal suggestions** alone (k = 4, g = **0.53**, 95% CI 0.23 – 0.82, I^2 = 53.81%; **Figure 2D**) on nocebo responses. Overall, nocebo responses (see Table 1 for the relevant studies) were thus associated with medium pooled effects in pain, while in itch they were associated with slightly smaller pooled effects overall.

Magnitude of nocebo responses based on the type of stimulation

For **pain** studies that used **conditioning with verbal suggestions**, we compared effects of different pain administration methods (k = 13 thermal, k = 7 electrical) excluding the single study using laser. Thermal

pain yielded a somewhat larger pooled effect on the magnitude of nocebo responses (k = 13, g = 0.75, 95% CI 0.59 – 0.91) compared to medium pooled effects of electrical pain (k = 7, g = 0.65, 95% CI 0.51 – 0.79) on nocebo responses. For **pain** studies that used only **verbal suggestions**, we examined effects of different pain administration methods (k = 4 thermal, k = 5 electrical, k = 2 mechanical) excluding the single studies using laser, cold pressor, hot water bath, pressure, and histamine. Electrical pain yielded slightly larger pooled effect on the magnitude of nocebo responses (k = 5, g = 0.91, 95% CI 0.65 – 1.17) compared to medium effects of thermal (k = 4, g = 0.69, 95% CI 0.21 – 1.16) and mechanical (k = 2, g = 0.60, 95% CI 0.14 – 1.06).

For **itch** studies that used **conditioning with verbal suggestions**, there were too few studies to analyze (cowhage k = 1, electrical itch k = 2, and histamine k = 1). For **itch** studies that used only **verbal suggestions**, there were again too few studies (k = 2 electrical, k = 3 histamine, k = 1 mechanical).

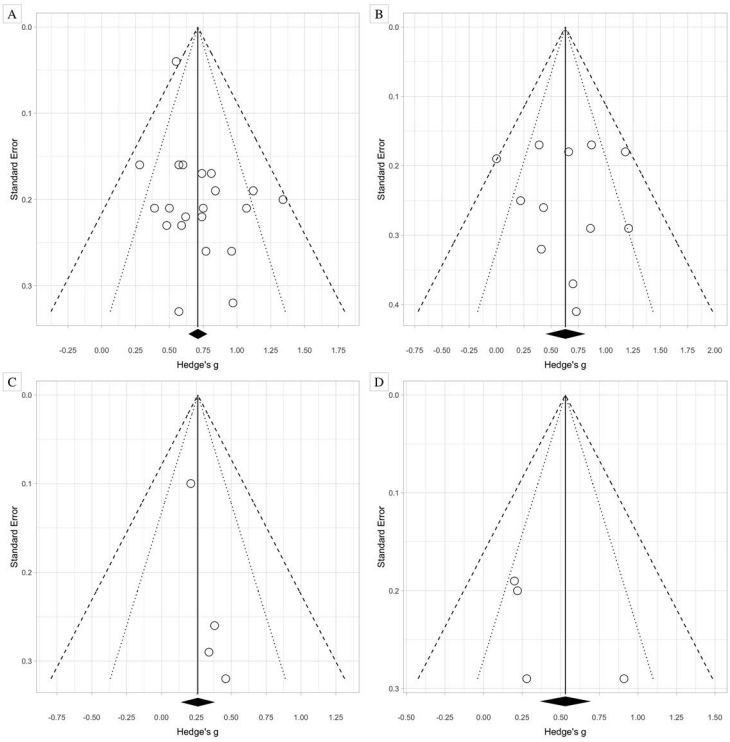


Figure 3. Funnel plots displaying studies within and outside of 95% (dotted line) and 99% (dashed line) CI, for pain verbal suggestions with (A) and without (B) conditioning, and for itch verbal suggestions with (A) and without (B) conditioning.

Magnitude of nocebo hyperalgesia based the timing of measurement

All itch conditioning studies measured the nocebo effect as the mean of all evocation trials. Among **pain** studies that employed a combination of **conditioning with verbal suggestion**, however, 13 paradigms

measured nocebo responses as the mean of all evocation (testing) trials, 6 measured the magnitude of responses in the first pair of evocation trials, and 2 studies specified different timing such as pre-post measures. Studies in which first evocation trials were used yielded a large pooled effect on the magnitude of nocebo responses (k = 6, g = 0.82, 95% CI 0.57 – 1.07) compared to medium pooled effects of measuring the effect as the mean of all evocation trials (k = 13, g = 0.66, 95% CI 0.54 – 0.79) and non-specified (k = 2, g = 0.67, 95% CI 0.23 – 1.11).

Magnitude of nocebo hyperalgesia based on the number of learning trials

Studies that employed **classical conditioning** used varying numbers of learning and evocation trials. For **pain only**, there were sufficient studies to examine the effects of different lengths of conditioning and different lengths of evocation (i.e., the length of extinction) on nocebo magnitudes. The shortest pain learning paradigm used 6 nocebo and 6 control trials, while the longest paradigms used up to 30 nocebo and 30 control trials. Evocation phases ranged from 3 nocebo and 3 control trials to 30 nocebo and 30 control trials. A meta-regression of different lengths of conditioning showed no association with the magnitude of nocebo responses (Q = 0.81, p = 0.37). Similarly, there was no association between the length of evocation and nocebo magnitudes (Q = 0.19, p = 0.67).

Magnitude of nocebo hyperalgesia based on the pain stimulus intensity

For **pain** studies that employed **classical conditioning with verbal suggestions** we had a sufficient sample to examine any relationship between differences in intensity of pain stimulations in the learning

phase and the magnitude of nocebo responses, but a meta-regression found no significant association ($Q = 0.89$, $p = 0.35$).

Magnitude of nocebo responses based on the Risk of Bias score

Lastly, we examined how RoB scores may be related to nocebo magnitudes. A meta-regression showed no significant relationship between RoB scores and the magnitude of nocebo responses for **pain** studies that used **conditioning and verbal suggestions** ($Q = 0.75$, $p = 0.39$), for **pain** studies that used only **verbal suggestions** ($Q = 0.00$, $p = 0.95$), for **itch** studies that used **conditioning and verbal suggestions** ($Q = 0.08$, $p = 0.77$), or for **itch** studies that used **verbal suggestions** alone ($Q = 1.9$, $p = 0.05$).

Discussion

We conducted a systematic review and meta-analysis of a total of 37 distinct nocebo studies on healthy participants. This meta-analysis showed that on average, nocebo effects were moderate to large in magnitude. The combination of verbal suggestions with classical conditioning yielded stronger nocebo responses on pain, but this may not necessarily be the case in the small number of itch studies. Measures of the type or intensity of pain or itch, and length of learning, did not explain the moderate heterogeneity in nocebo magnitudes between different studies. Timing of nocebo measurement in the first evocation trials yielded slightly larger nocebo magnitudes. Risk of bias was

generally low and was not related to nocebo magnitudes either. We discuss these results in relation to the role of conditioning as well as aversive learning, and we speculate of the reasons why none of the factors collected in the nocebo literature appear to consistently explain variations in the magnitudes of learned nocebo effects on pain and itch.

In experimental inductions of nocebo effects on pain, we found the magnitudes of responses across studies to be moderate to large, with a moderate heterogeneity. Often conceptualized as the counterpart of nocebo responses, placebo effects appear to be comparable in magnitude to the overall nocebo magnitude found in the current meta-analysis, but heterogeneity in placebo responses may be higher [19]. In a more recent meta-analysis, placebo responses were found to yield small to moderate effects, with moderate to large heterogeneity in results [32]. We speculate that this may indicate that the negativity of suggestions and experiences in nocebo paradigms may result in stronger learned effects, as compared to the positive expectations induced in placebo excrements. Indeed, aversive learning has consistently be shown to be prioritized over the learning of neutral or positive information in the brain [33–36], something that is thought to have an evolutionary basis [37].

Magnitudes of nocebo responses were found to be moderate to large in pain studies when looking at both verbal suggestions and combination with conditioning. As expected, in pain experiments the addition of classical conditioning yielded somewhat larger nocebo responses, suggesting that learning by experience during behavioral conditioning may be more potent than mere negative suggestions regarding pain outcomes. For itch, however, verbal suggestions alone yielded moderate effects whereas combination with conditioning resulted in small effects across studies. The number of studies included in each of the two itch conditions (k = 4 in each) may be insufficient to allow for further conclusions to be drawn regarding this apparent distinction between learned pain and itch effects.

While the number of itch studies included in this meta-analysis was small (8) compared to pain (30), overall effects on pain appear to be larger than those on itch across both learning methods, based on the present findings. Itch has been shown to be prone to suggestions and can be influenced by expectations [4] with one study that compared placebo effects induced with verbal suggestions for either pain or itch indicating that itch might be more prone to suggestions [38]. Our finding that pain resulted in larger nocebo magnitudes across the studies included here, could suggest that compared to itch, the learning of pain associations may be facilitated to a larger degree. In other words, we speculate that, as pain is perhaps more threatening and aversive than itch, it may signal a more vital threat to the person and thereby, from an evolutionary perspective [37], result in stronger learning. Further research into nocebo effects is needed, however, to reach a sufficient sample size for reliable comparison results between pain and itch.

The variability found in nocebo response magnitudes was not explained by differences between the type or intensity of pain or itch stimulation, or the length of learning. A moderate dispersion of effect sizes across the studies analyzed is important to note, especially when the measures that are systematically reported in studies, such as the duration of learning or the intensity of pain, are unable to explain such variability in nocebo response magnitudes. The large differences in applied experimental models of nocebo effects (e.g., different types of verbal suggestions, whether the experiment was conducted in a hospital or university setting, or types of nocebo and conditioned stimuli presented) may explain some of this variability in results [39]. Similarly to the efforts for aligning experimental paradigms in animal models of disease [40,41], it is essential for the field of nocebo to focus on replicating experimental paradigms and aligning paradigms according to ecologically valid models that yield comparable results across studies.

One of the most consistent differences between experimental nocebo studies seems to be the type of verbal suggestion delivered to participants. No two studies administered the same verbal suggestion. Different verbal suggestions could contain distinct emotional loads and be perceived as more or less threatening, which may in turn influence nocebo responses [13,17]. While beyond the scope and reach of the current meta-analysis, a future systematic review of distinct verbal suggestions, for example using content analysis approaches borrowed from linguistics [42,43], could shed a light on how different verbal suggestions could impact nocebo responses.

There are other variables that could explain variability of induced nocebo responses, such as sampling, demographics, and the inclusion criteria for participation, but a limitation is that these factors are not consistently reported in papers and could not be investigated in the current meta-analysis. Additionally, studies do not systematically measure fear, which is shown repeatedly to be involved in nocebo responses [13,17,44–46]. Other variables relevant to the emotional context of studies, such as the demeanor of the experimenter [47] or whether the experiment is set in an academic building or hospital, are also often not clearly documented, and could not be analyzed here. Finally, risk of bias was low across all studies and showed no relationship to nocebo magnitudes. However, the assessment tool used for this meta-analysis is designed for quantitative sensory testing studies [23] but could have missed bias aspects, such as potential publication bias for significant results, which meta-analyses studies should consider addressing.

This systematic review and meta-analysis quantified magnitudes of nocebo responses on cutaneous sensations (pain and itch) for distinct learning paradigms in experimental studies (classical conditioning with verbal suggestion, or verbal suggestion alone). We replicated previous findings that classical conditioning combined with negative verbal suggestions was strongest for inducing nocebo responses on pain.

Subgroup analyses indicated that factors related to the length of learning paradigms or intensity and type of sensory stimuli did not explain the moderate heterogeneity in nocebo effect sizes. This review provides a comprehensive summary of current findings in the field of nocebo research. We have ruled out some factors that were consistently reported in papers and could not explain the variability in results across studies, and we recommended some important directions for the field, such as increased consistency between study designs for inducing nocebo effects, as well as a systematic examination of the effects of different verbal suggestions on magnitudes of learned nocebo effects.

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Chapter 3.

How negative experience influences the brain: A comprehensive neurobiology review.

Published as

Thomaidou MA, Peerdeman KJ, Koppeschaar MI, Evers AWM, Veldhuijzen DS. How negative experience influences the brain: A comprehensive review of the neurobiological underpinnings of nocebo hyperalgesia**.** *Frontiers in Neuroscience 15*(1):2552, March 2021.

Abstract

This comprehensive review summarizes and interprets the neurobiological correlates of nocebo hyperalgesia in healthy humans. Nocebo hyperalgesia refers to increased pain sensitivity resulting from negative experiences and is thought to be an important variable influencing the experience of pain in healthy and patient populations. To comprehend and utilize current knowledge, an up-to-date, complete review of this literature is necessary. PubMed and PsychInfo databases were searched to identify studies examining nocebo hyperalgesia while utilizing neurobiological measures. The final selection included 22 articles. Electrophysiological findings pointed towards the involvement of cognitive-affective processes, e.g., modulation of alpha and gamma oscillatory activity and P2 component. Findings were not consistent on whether anxiety-related biochemicals such as cortisol plays a role in nocebo hyperalgesia but showed an involvement of the cyclooxygenase-prostaglandin pathway, endogenous opioids, and dopamine. Structural and functional neuroimaging findings demonstrated that nocebo hyperalgesia amplified pain signals in the spinal cord and brain regions involved in sensory and cognitive-affective processing including the prefrontal cortex, insula, amygdala, and hippocampus. These findings are an important step towards identifying the neurobiological mechanisms through which nocebo effects may exacerbate pain. Results from the studies reviewed are discussed in relation to cognitive-affective and physiological processes involved in nocebo and pain. By summarizing and interpreting the challenging and complex neurobiological nocebo studies this review contributes, not only to our understanding of the mechanisms through which nocebo effects exacerbate pain, but also to our understanding of current shortcomings in this field of neurobiological research.

Introduction

Negative thinking, such as having negative outcome expectations, can blunt the effect of active treatments, enhance the experience of aversive side-effects, and even produce deleterious effects in relation to recovery from symptoms such as pain, all leading to a phenomenon known as nocebo hyperalgesia [1–3]. Nocebo hyperalgesia refers to increased pain sensitivity and increased pain reports that mainly result from negative outcome expectations. Nocebo effects have been shown to be most relevant for alterations in pain tolerance or intensity and lead to higher pain reports when compared to baseline or control stimulations [4–6]. The neurobiological correlates of nocebo hyperalgesia are gaining research attention, but in lack of a comprehensive summary of findings, the neurobiology of these effects remains poorly understood. Gaining a better understanding of nocebo hyperalgesia on pain and its neural signature is an important step in the detection and prevention of these effects in patients, as well as the development of methods that may potentially counteract nocebo hyperalgesia.

Studies examining the neural correlates of nocebo hyperalgesia utilize (combinations of) different learning processes to induce nocebo hyperalgesia experimentally in order to explore the various mechanisms by which pain circuitry and experienced pain can be modulated (Figure 1) [7–9]: classical conditioning, instructional learning (i.e. through verbal suggestions), and social observational learning [10,11]. Classical conditioning forms and reinforces expectations through associative learning [12]. In conditioning models of nocebo hyperalgesia, an association is formed by repeated pairing between a high pain stimulus and an initially neutral stimulus (e.g., an inert treatment), that later becomes the nocebo stimulus. After repeated trials, an association is formed between the nocebo stimulus and the worsening of pain. Due to

this negative expectation, the nocebo stimulus evokes increases in perceived pain, similar to the high pain intensity previously paired to the nocebo, even in the absence of high pain applications. Negative verbal suggestions can also alter pain expectations, through more explicit, instructional learning. Suggestions may induce negative expectations explicitly (i.e. explaining the potential negative effects of a treatment) and can also induce nocebo hyperalgesia for example by enhancing conditioning effects [13,14]. Social observational learning can moreover induce nocebo hyperalgesia, for example when one sees someone else experiencing increased pain after a treatment [15,16]. These learning processes may result in nocebo responses that may play a detrimental role in shaping pain responses following a given event, stimulus, or treatment [3,17].

Over the past two decades, neuroimaging and pharmacological studies have begun to address the neurobiological underpinnings of nocebo experiences. Electrophysiological and neuroimaging methods such as electroencephalography (EEG) and magnetic resonance imaging (MRI) have provided valuable insights into the specific functional brain processes and underlying brain structures that are involved in nocebo hyperalgesia [5,18–21]. Moreover, the neurochemical systems underlying nocebo hyperalgesia have been explored via pharmacological administrations, blood or salivary measurements, or via imaging techniques such as Positron Emission Tomography (PET) [18,22,23]. Inconsistencies and gaps in the literature, however, render nocebo hyperalgesia a phenomenon that is still poorly understood. The nocebo literature is characterized by very diverse methods. For this reason, a comprehensive and detailed account of studies that examined neurobiological correlates of nocebo hyperalgesia may significantly aid in a better understanding of this phenomenon and can provide suggestions for improvements in the consistency of selected methodologies and reporting of results.

Through this review, we intend to provide a detailed overview of current neurobiological nocebo studies on pain and their findings. While a systematic review on this topic was not preferable due to the scarcity and diversity of neurobiological nocebo studies, a comprehensive and detailed account of these studies could be very valuable. First, in Table 1, we briefly list the different experimental models used to induce nocebo hyperalgesia across the included studies. We then provide a comprehensive overview of electrophysiological, neurochemical, and structural and functional correlates of nocebo hyperalgesia in healthy humans. These findings are discussed in relation to the sensory and cognitive processes involved, thereby providing a clear and comprehensive overview of the multitude of brain correlates involved in nocebo hyperalgesia. Finally, recommendations are provided to use more consistent methodologies and reporting of results and for replication studies.

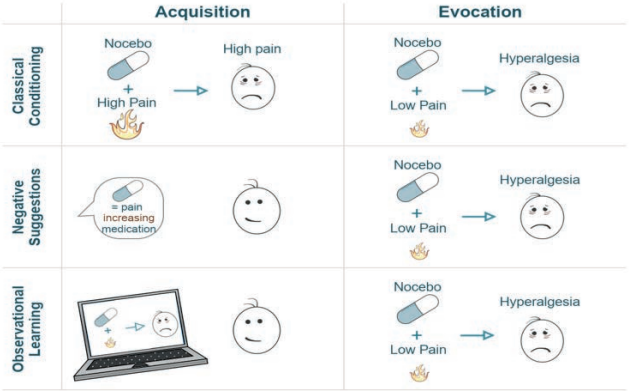

Figure 1. Illustration of three typical experimental paradigms for the induction of nocebo hyperalgesia. The acquisition column refers to the learning phase, whether conditioning-mediated, verbal, or observational learning. The evocation column refers to the evocation of the learned effect. Typically, an acquisition phase serves to induce negative expectations via conditioning, negative suggestions, observational learning, or any combination of these methods. Negative expectations are induced by combining an inert treatment with a surreptitious increase in pain stimulation (conditioning), by being told that a treatment will lead to increased pain sensitivity (negative suggestion), and/or by observing this negative treatment effect on someone else (observational learning).

Selection of studies

A search strategy was used to identify studies on nocebo hyperalgesia on PubMed and PsychInfo, published up to July 2020, using detailed key terms related to nocebo hyperalgesia, nocebo conditioning, and neurobiological methods (Appendix A). The abstracts of 1761 articles were screened for inclusion by the first author. When there was doubt about inclusion of a study, a decision was made in consultation with the last author. This review focuses only on nocebo hyperalgesia induced in healthy humans, in order to summarize and compare findings that are not influenced by underlying pain or psychological conditions and as such most clearly present the underlying mechanisms of nocebo hyperalgesic effects. Exclusion criteria were: 1) not using an experimental learning paradigm for the induction of nocebo hyperalgesia, 2) not utilizing a healthy human sample 3) not utilizing at least one neurobiological measure, such as brain imaging or a pharmacological manipulation, 4) not inducing significant nocebo responses (as neurobiological responses in relation to nocebo hyperalgesia can only be studied following a successful nocebo manipulation). The articles that fit the inclusion criteria were considered relevant for understanding the neurobiological underpinnings of nocebo hyperalgesia and are described in detail in this narrative review. The final selection included 22 articles (Table 1), of which 6 articles reported electrophysiological measures, 3 focused on chemical correlates, 1 reported structural brain correlates, and 12 reported functional brain correlates using fMRI results. Because we selectively focused on the neurobiology of nocebo hyperalgesia, we report only the nocebo arms of studies (for example, we do not report placebo manipulations). Figure 2 provides an illustration of all major findings of the studies reviewed here.

Table 1. Overview of study characteristics included in the review and key findings.

First author	Year	Sample size per nocebo group (n)	Experimental paradigm per nocebo group	Pain administration method	Nocebo (high pain) acquisition trials*	Neuro-physiological measure	Key neurobiological outcome measures	Key findings
Albu	2016	15	NVS	Thermal	n/a	EEG	Resting-state EEG with classical frequency band analyses	Enhanced low alpha (8–10 Hz) power
Thomaidou, Blythe, Houtman	2021	36	Conditioning with NVS	Thermal	16 trials (10-second plateau)	EEG	Continuous and resting-state EEG with classical frequency band and biomarker analyses	Increases in complexity of oscillations leading to larger nocebo responses, increases in alpha oscillations in nocebo-augmented pain
Pazzaglia	2016	9 & 9	NVS & Conditioning with NVS	Laser	50-60 trials (<1-second plateau)	EEG	Laser-evoked potentials that are responses to laser radiant heat pulses and reflect activation of Aδ nociceptors	N2/P2 amplitude reduction
Piedimonte	2017	17 & 17	NVS & Conditioning with NVS	Electric	20 trials in conditioning 4 trials in evocation (<1-second plateau)	EEG	Early and late contingent negative variation amplitudes that relate to sensory and motor components of pain, respectively	Contingent negative variation amplitudes showed higher early negativity in nocebo trials
Hird	2018	14	Conditioning	Laser & Electric	90 trials in conditioning /evocation (<1-second plateau)	EEG	Laser-evoked and electric evoked potentials (as above), specifically the stimulus-preceding negativity	Stimulus-preceding negativity at centroparietal electrodes was found to differentiate pain intensity expectation, with nocebo

							component, related to processing of imminent pain.	trials linked to lower amplitudes
Tu	2019	21	Conditioning & Observational learning	Thermal	20 trials in direct conditioning (2-second plateau)	MEG	Resting-state MEG, pre- and post- the nocebo manipulation	Decreased alpha connectivity between the left rostral anterior cingulate cortex and left middle temporal gyrus
Benedetti	2006	37	NVS	Ischemic	n/a	Pharmacology	Pharmacological manipulations and measurement of adrenocorticotropic hormone and cortisol plasma concentrations	Increased adrenocorticotropic hormone and cortisol plasma concentrations
Benedetti	2014	35	Socially induced NVS	Hypoxia-induced headache	n/a	Biochemical	Pharmacological manipulations and measurement of Salivary PG, TXA2, and cortisol	Prostaglandin, thromboxane A_2, and salivary cortisol increases
Scott	2008	5	NVS	Needle punctures	n/a	PET	Measurement of μ-opioid and dopamine (D2/D3 receptor) neurotransmission	Deactivation of μ-opioid and mesolimbic dopamine neurotransmission
Egorova	2015	30	Conditioning & Nonconscious evocation	Thermal	22 trials in conditioning (4-second plateau)	tDCS	Manipulation of brain activity via electrical currents that increase and decrease neuronal excitability in the DLPFC	Involvement of dorsolateral prefrontal cortex activation
Keltner	2006	13	Conditioning	Thermal	5 trials in preconditioning 10 trials in conditioning (30-second plateau)	fMRI	Differences in BOLD activations	Increased activation in caudal anterior cingulate cortex and cerebellum, and thalamic levels

Kong	2008	13	Conditioning with NVS	Thermal	6 trials in conditioning 2 trials in evocation (7-second plateau)	fMRI	Differences in BOLD activations and connectivity analyses	Decreased activations in dorsolateral prefrontal cortex and orbitofrontal cortex. Increased activations in mid temporal gyrus, insula, anterior cingulate, hippocampus
Kong	2013	46	Conditioning	Thermal	4 trials in conditioning 3 trials in evocation (12-second plateau)	fMRI	Differences in BOLD activations	Functional connectivity between frontoparietal regions and rostral anterior cingulate /medial prefrontal cortex was associated with nocebo
Rodriguez-Raecke	2010	38	NVS	Thermal	n/a	fMRI	Differences in BOLD activations	Increased activation of operculum.
Ellerbrock	2015	20	NVS	Thermal	n/a	fMRI	Differences in BOLD activations and connectivity analyses	Increased activation of the operculum. Deactivation of the periaqueductal grey.
Freeman	2015	24	Conditioning with NVS	Thermal	3 trials in conditioning 1 trial in evocation (7-second plateau)	fMRI	Differences in BOLD activations	Increased activations in insula, orbitofrontal cortex, and periaqueductal grey
Jensen	2015	24	Conditioning & Nonconscious evocation	Thermal	25 trials in conditioning (4-second plateau)	fMRI	Differences in BOLD activations	Increased activations in insula, anterior cingulate cortex, thalamus, brainstem, amygdala, /hippoc.
Egorova	2020		Conditioning with NVS	Thermal	3 trials in conditioning 1 trial in evocation (7-second plateau)	fMRI	Differences in BOLD activations and connectivity analyses	Increased amygdala-striatum connectivity correlated with magnitude of nocebo responses.

Schmid	2013	18	NVS	Visceral pressure	n/a	fMRI	Differences in BOLD activations	Increased insula and somatosensory cortex activation
Schmid	2015	22	NVS	Visceral pressure	n/a	fMRI	Differences in BOLD activations and connectivity analyses	Hyperactivation in somatosensory cortex, dorsolateral prefrontal cortex, midcingulate cortex, posterior cingulate cortex, insula, thalamus, and amygdala
Geuter	2013	23	Conditioning with NVS	Thermal	6 trials in preconditioning 6 trials in conditioning (17-second plateau)	Spinal fMRI	Differences in spinal BOLD activations	Increased activation in the ipsilateral dorsal horn of the spinal cord.
Tinnermann	2017	49	Conditioning with NVS	Thermal	16 trials in preconditioning 16 trials in conditioning (16-second plateau)	Spinal and brain fMRI	Differences in brain and spinal BOLD activations	Increased activation in spinal cord, slightly more caudal and medial than pain cluster. Increased activation in prefrontal areas, amygdala, periaqueductal gray. Deactivation of the rostral anterior cingulate cortex.

Note: These selected studies examined experimentally induced nocebo hyperalgesia in healthy humans. The studies are categorized as described in the main text, based on neurobiological outcome and subsequently based on their relevance to each other. In all studies, nocebo effects were evoked and assessed during a phase where all pain stimulations were equivalent intensity. The number of trials and length of pain stimulations at plateau are also listed. All studies employed diverse nocebo induction paradigms and conditioning paradigms employed a range of different numbers of stimuli and pain application methods. *The column listing nocebo acquisition trials also lists acquisition (i.e., reinforced) trials included within evocation phases when applicable. Abbreviations: NVS, Negative (Verbal) Suggestions; EEG, electroencephalography; MEG, magnetoencephalography; PET, Positron Emission Tomography; tDCS, transcranial Direct Current Stimulation; fMRI, functional Magnetic Resonance Imaging.

Electroencephalography (EEG) and magnetoencephalography (MEG) are non-invasive imaging techniques that either directly or indirectly, respectively, measure electrical activity in the brain through electrodes placed on the scalp [24,25]. Neuronal oscillations in the classical frequency bands as well as neuronal (de)activations in response to a specific stimulation have now been consistently related to sensory, cognitive, and affective processes [26–29]. EEG and MEG are thus valuable techniques for unravelling the neurophysiology underlying nocebo hyperalgesia. Six studies to date have used these methods to examine nocebo-related resting-state or event-specific alterations. Of these 6 studies, 1 used negative suggestion alone [5] and 5 used conditioning methods to induce nocebo hyperalgesia. Conditioning was used with [21] or without [30] negative suggestions, while 2 studies examined conditioning in separate groups either with or without negative suggestions [31,32] and in 1 (MEG) study conditioning was combined with observational learning [15].

Albu and Meagher (2016) investigated alterations in EEG activity in a study using negative verbal suggestions regarding inert nocebo and control creams. The nocebo manipulation resulted in a significant increase in thermal pain ratings. Moreover, a significant increase in low alpha EEG power (8-10Hz) was found in the nocebo group relative to the control group when comparing a 5-minute EEG recording during noxious heat stimulation pre- to post-acquisition of the nocebo effect. The exact topography of this finding was not specified. This change in low alpha activity, however, correlated to an increase in pain catastrophizing and not to an increase in pain ratings. Pain catastrophizing in this study was measured via the Pain Catastrophizing Scale [33] that assesses catastrophizing thoughts related to pain, or pain-related worrying [34]. The authors suggested that the increase in low alpha power reflects a negative cognitive-affective state in relation to pain, in a process parallel to, or potentially involved in, nocebo hyperalgesia.

Thomaidou and colleagues (2021a) studied electrophysiological processes underlying nocebo hyperalgesia, aiming to identify EEG biomarkers of nocebo-augmented pain. Nocebo effects on thermal pain were induced through conditioning and negative suggestions regarding the pain increasing effects of an inert gel. Nocebo hyperalgesia led to widespread pre- to post-acquisition increases in resting-state long-range temporal correlations of brain oscillations, which were negatively associated with nocebo magnitudes. Individuals with strong long-range temporal correlations of brain oscillations during pre-acquisition rest showed larger nocebo responses than those with weak long-range temporal correlations. Moreover, increases in alpha and decreases in beta and gamma oscillations were found during nocebo-augmented pain in the evocation phase. This study highlighted the role of increased cognitive processing of pain at the electrophysiological level, under nocebo-hyperalgesic conditions.

Pazzaglia and colleagues (2016) used laser pulses to measure laser-evoked potentials (LEPs), aiming to investigate potentials related to cognitive control, such as the N2 and P2 components. In two groups, either negative suggestions in combination with conditioning or negative suggestions alone about a nocebo cream were used to induce nocebo effects. A neutral cream was used as the control stimulus. Post-treatment pain ratings were compared to baseline and between the cream-treated hand and the untreated hand. The authors demonstrated reduced habituation to pain as a result of the nocebo manipulations. Diminished N2/P2 LEP amplitudes in central scalp regions paralleled the diminished habituation.

Piedimonte and colleagues (2017) aimed to differentiate between specific sensory-anticipatory and motor components of electrically induced nocebo hyperalgesia. To this end, the authors examined contingent negative variation (CNV) amplitudes. Early CNV is considered an event-related potential related to the anticipation of an

upcoming event, while late CNV is considered to be related to motor preparation for an event [35–37]. Early CNV component amplitudes showed significantly higher early negativity in nocebo trials, as demonstrated by a comparison between placebo cues (signaling a decrease in pain) and nocebo cues (signaling an increase in pain) during acquisition and evocation and in frontal, central, and parietal brain regions. Differences in late negativity were not found during nocebo evocation, suggesting that the motor reaction to pain may not be affected by nocebo hyperalgesia. The authors conclude that based on their results, expectation of hyperalgesia may affect the early, sensory component of pain, thereby producing a modulation of pain perception. The expectation of hyperalgesia under nocebo conditions seems to be related, based on this study, to the perception of increased pain, via an "early" cognitive mechanism that anticipates a painful stimulus.

Hird and colleagues (2018) used both electric and laser-evoked pain which allowed testing for time-sensitive EEG components, while also contrasting the effects of different types of pain on brain signals. Electric-evoked potentials (EEPs) and LEPs were recorded throughout the experiment while participants underwent the nocebo manipulation. An effect of the nocebo manipulation on pain ratings was found, suggesting that, compared to the placebo cue (signaling a 75% likelihood of low pain), the nocebo cue (signaling a 75% likelihood of high pain) increased ratings for stimuli of moderate intensity, in response to both laser and electric stimulation. Hird and colleagues (2018) investigated the stimulus-preceding negativity (SPN) component, which is thought to be a slow-wave EEG correlate of imminent pain anticipation. The SPN at centroparietal electrodes was found to differentiate pain intensity expectation (i.e., anticipation of high pain intensity versus low pain intensity) with nocebo trials linked to more negative amplitudes. This was found in response to laser-evoked pain, but not to electric pain stimuli, indicating morphological differences in brain activations between the two stimulation types. The topographical findings were

connected to previous studies indicating sources in the anterior insula and cingulate cortex (Brown et al., 2008).

Tu and colleagues (2019) aimed to study distinct learning processes of nocebo effects on thermal pain, using MEG. Classical conditioning and observational learning were compared and both conscious and unconscious visual cues were used. In the classical conditioning phase, participants were asked to learn the associations between neutral faces presented on a screen and the experience of low and high pain. In the observational learning phase, a different pair of faces were accompanied by observing a model experiencing and rating low and high pain. Resting-state MEG data were recorded twice for each subject, before and after conditioning. All nocebo manipulations significantly induced nocebo hyperalgesia and significant changes in brain connectivity were demonstrated after conditioning across all frequency bands. A decrease in alpha band connectivity between the left rostral Anterior Cingulate Cortex (rACC) and left middle temporal gyrus (MTG) was the most consistent predictor of the magnitude of induced nocebo effects across all manipulations. The authors discuss their finding in relation to earlier imaging research linking the rACC, a primary center for sensory-discriminative processing (Tinnermann et al., 2017), with the nocebo effect.

In sum, with electrophysiological studies in the nocebo field being limited, the few studies that have explored the electrophysiological correlates of nocebo hyperalgesia have focused on different aspects. None of the studies described in this review used similar behavioral or imaging analysis methods therefore challenging the comparison of results. What seems to be a recurrent pattern in these studies is the involvement of brain components in nocebo hyperalgesia that have previously been implicated in cognitive and affective processes. This is demonstrated by activations in (low) alpha band activity, the early CNV component, and SPN component, reductions in the N2/P2 LEP

component and alpha connectivity and with source regions for these diverse results identified in the anterior insula, cingulate gyrus, and middle temporal gyrus [5,15,31,32]. Moreover, learning has been implicated at an electrophysiological level, as shown by alterations in gamma band activity under nocebo hyperalgesic conditions as well as the involvement of long-range temporal correlations (Thomaidou et al., 2021a).

Neurochemical and biochemical correlates of nocebo hyperalgesia

Neurochemicals play a key role in nociception and in the cognitive and affective processes that modulate pain perception [38]. In nocebo hyperalgesia, where cognitive and emotional factors such as learning and anxiety have been shown to play a role [39–41], related neurochemicals may be involved. Next to neurochemicals, other biochemicals such as enzymes have been shown to modulate pain transmission [42] and may also be relevant in nocebo hyperalgesia. Three studies examined chemical processes involved in nocebo hyperalgesia, using negative suggestions [18,22,23].

Benedetti and colleagues (2006) studied cortisol and the hypothalamic-pituitary-adrenal (HPA) axis by using a neuropharmacological approach to examine neurochemical correlates of anxiety, a state that is related to fear and may thus, similarly to fear [43], also be linked to nocebo hyperalgesia [39]. Participants were subdivided into 4 groups and underwent ischemic pain inductions. One group received a sham hyperalgesic pill and intravenous proglumide, a non-selective antagonist of cholecystokinin (CCK) type-A/B receptors [44]. A second group received a sham hyperalgesic pill and intravenous diazepam, a benzodiazepine and potent anxiolytic agent that increases the effect of the inhibitory neurotransmitter gamma-aminobutyric acid [45]. A nocebo control group received a sham hyperalgesic pill and an inert saline solution, while the other control group was only administered a saline

solution and no nocebo manipulation. In the nocebo control group, significant nocebo hyperalgesia and HPA axis hyperactivity were observed, as shown by increased adrenocorticotropic hormone (ACTH) and cortisol plasma concentrations. Proglumide administration only blocked nocebo hyperalgesia reports but not the nocebo-induced hyperactivity of the HPA axis, while diazepam blocked both nocebo hyperalgesia and nocebo-induced HPA axis hyperactivity. Based on these results, the authors suggested that the CCK antagonist proglumide may act on anxiety-induced hyperalgesia, as proglumide affected pain but not the HPA axis. In relation to their findings that highlight two different anxiety pathways for HPA hyperactivity and hyperalgesia, they discuss that hyperalgesia may occur when anticipatory anxiety is about the pain itself [46].

In a later study, Benedetti and his colleagues (2014) studied nocebo effects on hypoxia-induced headache, a symptom experienced at high altitudes due to the altered synthesis of eicosanoid signaling molecules, such as prostaglandin (PG) and thromboxane A_2 (TXA_2), through the cyclooxygenase (COX) enzyme at height [47]. Blockade of PG synthesis with aspirin can prevent high altitude headache [48]. In a social nocebo manipulation, prior to a mountaineering trip, researchers provided negative suggestions to only one participant, who communicated the suggestions to 35 of his peers. Of the participants not reached by the nocebo suggestions, 38 were allocated to a control group. Participants visited a research location at an altitude of 3500 meters, where headache sufferers were further subdivided into groups that received aspirin (25mg/kg), placebo, or no treatment. Salivary PG, TXA_2, and cortisol were measured at sea level and at 3500 meters. Additionally, identical nocebo and control groups extracted from a separate participant sample went up to only 1500 meters, where no hypoxia is supposed to take place, and underwent the same procedures. At 3500 meters, headache occurrence and intensity were significantly higher in the nocebo group relative to the control group. Larger increases in PG and TXA_2 were

found in the nocebo group relative to the control group. Cortisol increase was found only in the nocebo group. Aspirin relieved the headache and blocked PG and TXA_2 increases in headache sufferers of the nocebo and control groups, while placebo administration had these effects only in the nocebo group. At 1500 meters there were no significant nocebo effects or increases in PG and TXA_2. The authors concluded that socially induced nocebo effects affected the biochemical pathway related to PG synthesis; however, negative expectations were insufficient in initiating pain and PG synthesis in the absence of hypoxia. This study indicates that nocebo hyperalgesia can affect peripheral biochemical pain mechanisms.

In a PET study, Scott and colleagues (2008) examined the contribution of the endogenous opioid and dopaminergic (DA) systems in the induction of placebo hypoalgesia. Participants underwent intramuscular pain inductions and four PET scans were obtained, two with and two without placebo administration. While this study aimed to induce placebo effects, five participants showed significant increases in pain reports during placebo administration who can be considered nocebo responders. The researchers found significant changes in μ-opioid and DA (D_2/D_3 receptor) neurotransmission between high placebo and nocebo responders. Compared to placebo responders, nocebo responders demonstrated a deactivation of μ-opioid and DA neurotransmission in specific brain regions: the right nucleus accumbens and left ventral putamen. For μ-opioids these regions additionally included the nucleus accumbens, subgenual ACC, orbitofrontal cortex (OFC), anterior insula, periaqueductal gray (PAG), and amygdala. Notably, the regions and neurotransmitter systems involved in placebo and nocebo effects overlapped. Collectively, these studies have contributed to an early understanding of biochemical variables that may be implicated in nocebo hyperalgesia.

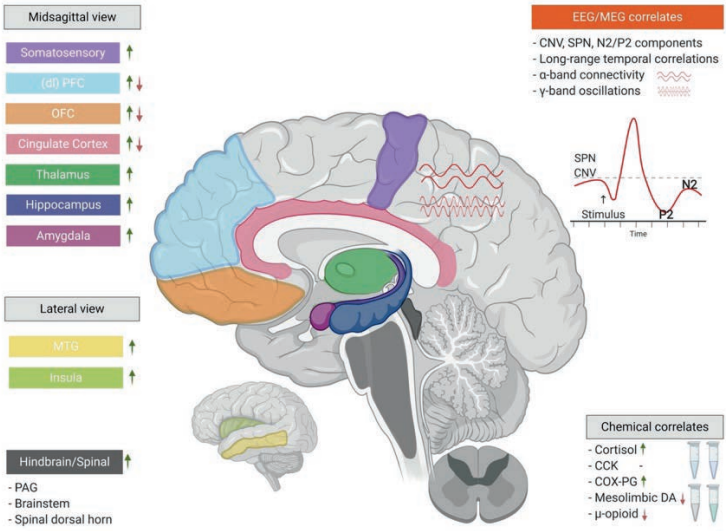

Figure 2. The neurobiological correlates of nocebo hyperalgesia. When classical conditioning and/or negative suggestions are used to experimentally induce nocebo hyperalgesia, a complex interplay of electrophysiology, neurochemistry, and central nervous system functionality come into play. These neurobiological factors involve a wide array of functions ranging from basic nociceptive to cognitive-affective. Green upward arrows indicate increases/activations of particular regions, components, or chemicals, while red downward arrows indicate decreases/deactivations (*CCK's role cannot be simplified by a red/green arrow). EEG, electroencephalography; MEG, magnetoencephalography; SPN, stimulus-preceding negativity; CNV, contingent negative variation; (dl)PFC, (dorsolateral) prefrontal cortex; OFC, orbitofrontal cortex; MTG, middle temporal gyrus; PAG, periaqueductal grey; CCK, cholecystokinin; COX-PG, cyclooxygenase-prostaglandin pathway; DA, dopamine. This figure was created using BioRender.com.

Functional and structural correlates of nocebo hyperalgesia

Transcranial Direct Current stimulation

Transcranial Direct Current Stimulation (tDCS) is a non-invasive neuromodulatory technique which delivers low electrical currents via scalp electrodes that can increase or decrease neuronal excitability [49,50]. When positive stimulation (anodal tDCS) is delivered, neuronal excitability is increased [50]. When negative stimulation (cathodal tDCS) is delivered, there is a decrease in neuronal excitability [50]. Among other uses, tDCS can help investigate whether a brain region of interest is implicated in a specific process, such as the acquisition or the evocation of nocebo hyperalgesic responses. One study looked at the influence of tDCS on nocebo hyperalgesia using a conditioning paradigm [41].

Egorova and colleagues (2015) aimed to modulate nocebo effects by altering the excitability of the right dorsolateral prefrontal cortex (rDLPFC) using tDCS. Thirty participants were randomized into two tDCS groups that received either anodal or cathodal rDLPFC stimulation. Bipolar tDCS was administered to the rDLPFC for 20 minutes during rest, after nocebo conditioning and before nocebo evocation. During the conditioning phase, geometric fractal shapes were used as visual cues, paired to high and low pain stimulations. A significant nocebo effect was induced only in the anodal tDCS group, indicating that conditioned nocebo effects can be elicited after anodal but not cathodal rDLPFC stimulation. This study was not able to unequivocally determine whether tDCS led to enhancement of nocebo hyperalgesia in the anodal condition and/or whether it caused a reduction in the cathodal condition. However, the authors speculated, based on earlier literature [51], that cathodal tDCS presumably reduced the effects of conditioning.

Functional imaging

Functional Magnetic Resonance Imaging (fMRI) is a technique that can map brain function by detecting blood-oxygen-level dependent (BOLD) changes and thereby indirectly measuring brain activity [52,53]. The measurement of BOLD activity in the brain has helped researchers explore functional brain correlates, from sensory perception to higher cognitive functions such as the formation of expectations [1,54–56]. When comparing the frequently used brain imaging techniques EEG and fMRI, fMRI provides higher spatial resolution whereas EEG provides greater temporal resolution. MRI also enables high resolution structural brain imaging. In the 12 studies reviewed below, fMRI has shed light onto some of the functional and anatomical similarities and differences between nocebo hyperalgesia and related effects such as placebo analgesia. These studies used various nocebo induction methods: 3 used conditioning [57–59], 4 used negative suggestions [8,20,60,61], and 5 used a combination of conditioning and negative suggestions [9,19,62–64]. Studies that described nocebo effects in somatic pain, visceral pain, or spinal pain are summarized separately because of the methodological differences in the study designs and their proposed underlying mechanisms.

Eight studies examined brain activation in response to nocebo hyperalgesia for somatic pain using fMRI. In a study by Keltner and colleagues (2006), participants were conditioned to associate two distinct visual cues (red or blue) with thermal pain stimuli of high and low intensity, respectively. The imaging data of the nocebo evocation phase revealed that during high intensity pain stimulation, high and low pain expectations produced differential brain activations. The caudal ACC, the cerebellum, and the dorsolateral pontomesencephalic region had increased BOLD activation when expectations were for higher pain intensity. During low intensity pain stimulation, the two expectation levels did however not yield significant differences in BOLD activations.

The authors stated that this difference indicated that negative expectations in combination with high pain stimulation elicited a sum of neural activity that enhanced activation of afferent pain circuitry. In the context of descending pain modulation, expectation and pain intensity may have acted in an additive manner on afferent pathways when these were activated by high pain stimulation.

Kong and colleagues (2008) informed their participants that a (sham) acupuncture treatment on the arm may increase their pain sensitivity while also conditioning them with surreptitiously increased thermal pain stimulations. During the fMRI session, participants underwent the nocebo manipulation again and then received all pain stimulations at moderate intensity. After administering the inert treatment, pain intensity ratings significantly increased for the nocebo sites compared to control sites on the arm. Pre- and post-treatment differences in brain activations revealed significant increases in activations during nocebo, as compared to control trials, in the dorsal ACC, insula, Superior Temporal Gyrus (STG), left frontal and parietal operculum, medial frontal gyrus, OFC, superior parietal lobule, hippocampus, right putamen, lateral PFC, and middle temporal gyrus (MTG). Furthermore, significant positive correlations were observed between nocebo magnitudes and activations in the bilateral insula and left primary motor cortex. Significant negative correlations were observed between nocebo magnitudes and activations in the dlPFC and left OFC. Activation differences in these brain regions suggest that nocebo hyperalgesia predominantly engaged the affective-cognitive pain circuit.

In a later study, Kong and colleagues (2013) studied pain expectations but now without employing a sham treatment. In this fMRI study, visual cues were associated with high thermal pain stimulations. When predictive cues were paired with moderate pain intensity, nocebo hyperalgesic responses were reported. fMRI data from the evocation of nocebo hyperalgesia did not yield major findings related to the

experience of pain following the high-pain visual cue. However, the researchers analyzed pretest resting state fMRI data and found that functional connectivity between frontoparietal regions and the rACC and medial PFC was positively associated with nocebo responses. These data suggested that a frontoparietal network controlling top-down regulation of pain and other incoming information [65] may also be involved in the processing of pain under nocebo hyperalgesic conditions.

Rodriguez-Raecke and colleagues (2010) implemented a novel nocebo paradigm by carrying out a longitudinal nocebo experiment, focusing on (lack of) reduced habituation. Participants in one group were told that over 8 testing sessions they would become more sensitive to repeated thermal pain stimulations. The control group was not given any information and thus was expected to exhibit typical habituation to thermal pain. Indeed, while the control group showed habituation, the nocebo group did not report significantly lower pain levels over time, indicating that the acquisition of a nocebo response was successful. fMRI data were collected on day 1 and day 8 of the experiment. These data showed predominantly significantly increased activation of the operculum in the nocebo, as compared to the control group. Differences in activation of the operculum indicated a potential involvement of early nociceptive processing in nocebo hyperalgesia [66].

In another longitudinal experiment, Ellerbrock and colleagues (2015) tested participants for 21 consecutive days, with fMRI scanning taking place on days 1, 8, 14, and 21. The nocebo manipulation consisted of a negative suggestion aiming to reduce habituation to pain. Participants in this nocebo group were told that in an earlier study, repetitive thermal pain stimuli were increasingly painful over days. Participants in the control group were not given suggestions. Negative suggestions resulted in activation of the operculum, such that it was increasingly activated over time in the nocebo group. Moreover, the nocebo induction largely

inhibited activation in the PAG for the nocebo group relative to the control group. Across both groups, the operculum exhibited a gradual decrease in connectivity with the basal ganglia and a gradual increase in connectivity with the STG. However, no differences in connectivity were identified between the nocebo and control groups. The authors suggested that nocebo suggestions may modulate the contribution of the operculum on a pain-transmitting process that involves basal ganglia–thalamocortical loops. Importantly, the nocebo-mediated inhibition of PAG activation suggested that nocebo effects may impede descending pain modulation.

Freeman and colleagues (2015) studied negative suggestions regarding a pain-increasing effect of an inert cream labeled "Capsaicin" which was delivered to participants over three experimental sessions. In the first session, pain calibrations were conducted. In the second and third session, baseline pain stimuli were administered at a moderate intensity and short conditioning procedures took place. In the final part of the third session, the evocation phase took place inside the MR scanner. Negative suggestions significantly increased subjective pain ratings. The fMRI results showed that the expectation of increased pain induced significant BOLD activations in the insula, OFC, and PAG. While an involvement of the PAG in nocebo hyperalgesia has been found in previous research (Ellerbrock et al., 2015), it is notable that unlike in Ellerbrock et al. (2015), in this study PAG activation was increased in response to the nocebo manipulation.

Jensen and colleagues (2015) aimed to investigate the neural correlates of specifically nonconscious conditioned nocebo hyperalgesia. Participants were told to focus on images presented on a screen that would accompany pain stimulations and to rate their pain following each stimulus. They were then conditioned by use of images that depicted neutral male facial expressions, presented supraliminally. During the subsequent evocation phase, supraliminal and subliminal presentations

of the conditioned faces were accompanied by moderate pain stimulations. Both conscious and nonconscious exposure to the conditioned cues led to significant nocebo responses. Nocebo hyperalgesia, irrespective of exposure type, revealed increased activation in several regions involved in nociceptive processing, such as the ACC, insula, thalamus, and brainstem. As compared to conscious nocebo, nonconscious nocebo led to increased activation of the thalamus, amygdala, and hippocampus. Involvement of these subcortical structures may reflect processing and encoding of a perceived threat [67], given the aversive nature of pain.

In a recent study, Egorova and collogues (2020), aimed to investigate the effects of administering an inert cream while providing neutral information regarding its effects on pain. This neutral condition was compared to a nocebo, in which negative suggestions were provided about the effects of a second cream. A conditioning paradigm was used in which increased pain was administered in the nocebo as compared to the neutral skin patches. The evocation phase took place inside the MRI scanner. While this study focused on how participants responded to pain following the administration of the neutral cream, connectivity analyses were conducted for the nocebo condition as well. These results showed increased connectivity between the left amygdala and the striatum and this increase was correlated with the magnitude of nocebo responses. With a particular focus on the amygdala, this study highlighted an involvement of the amygdala in modulating or reflecting the magnitude of nocebo responses.

Taken together, fMRI studies that explored brain correlates of nocebo effects on somatic pain have provided some consistent findings. As expected, sensory-discriminative and descending processing has been implicated in the presentation of nocebo hyperalgesic responses, with brain areas such as the ACC, operculum, PAG, and the PFC being consistently involved in nocebo hyperalgesia [8,9,62]. Studies consistently

show that nocebo responses also implicate other cognitive as well as affective processes, as evident by the involvement of areas such as the OFC and DLPFC, ACC, insula, amygdala, and hippocampus [18,19,59]. Interestingly, studies that only employed conditioning but did not use negative suggestions to induce negative treatment expectations, did not observe an involvement of brain areas responsible for affective processes such as fear [57,58].

Functional imaging in visceral models

Two studies investigated negative treatment expectations in an experimental model of visceral pain in which a pressure-controlled barostat system was used to inflated rectal balloons to an individualized designated pressure. Two studies used verbal suggestions and 1 used conditioning methods alone to induce nocebo effects. Schmid and colleagues (2013) told participants that they would experience increased pain as a result of receiving an opioid antagonist in one scanning session and saline solution in a control session. In reality, only saline was administered intravenously. Participants reported significantly higher pain levels during the expectation of a hyperalgesic treatment, as compared to the control sessions. The fMRI analyses indicated significantly increased pain-induced activation within the somatosensory cortex under nocebo conditions. Moreover, negative expectations in the nocebo group led to increased insula activation compared to neutral expectations.

In an fMRI study by this research group, Schmid and colleagues (2015) informed participants in a nocebo group that increased pain would occur over time due to sensitization, in response to repeated rectal distensions, while a control group did not receive any negative suggestions. In reality, previous work has revealed no evidence of sensitization [68]. The nocebo group reported higher pain levels in the evocation phase as compared to

the first session, indicating nocebo sensitization. When only nocebo responders (n=14) were contrasted to the control group, greater activations were found in the amygdala and secondary somatosensory cortex during pain anticipation. During the pain inductions, nocebo responders demonstrated significantly enhanced hyperactivation of the amygdala, thalamus, and insula. As a function of negative expectations, the insular cortex showed increased connectivity with the midcingulate cortex (MCC) extending to the posterior cingulate cortex (PCC) during pain stimulations. Schmid and colleagues stated that their findings highlighted an involvement of the MCC in visceral nocebo effects.

In sum, visceral and somatic experimental models of nocebo hyperalgesia show a consistent involvement of cognitive-affective brain regions such as the hippocampus and amygdala. A consistent finding that seems to be prominent in visceral pain studies but also in somatic pain studies although somewhat less consistently, is the involvement of the insula in nocebo hyperalgesia. The insula is thought to be crucial for neural functions such as sensory integration and pain-related decision making [69–72]. The insula may thus constitute a primary brain region for the cognitive modulation of visceral and somatic pain [73]. Visceral pain studies have also found a role of the MCC in nocebo hyperalgesia, which was not observed in somatic pain studies. The MCC has been implicated in pain-related processes, including cognitive modulation and fear responses related to pain [74], central sensitization to visceral pain and pain modulation in patients with chronic abdominal pain [75–79]. These findings suggest that nocebo effects on visceral pain show similarities to other types of pain. At the same time, these studies highlight a distinct implication of structures such as the MCC in visceral pain.

Spinal imaging

fMRI has been used to image the function of the entire central nervous system. Because of specialized requirements for spinal MR images, however [80,81], neurobiological nocebo research has predominantly focused on brain mechanisms. Relatively recently, the focus has expanded to the spinal cord, which plays a key role, not only in the afferent transmission of pain signals, but also in the descending modulation of pain [82]. In nocebo effects, hyperalgesia may be attributed to sensory and cognitive-emotional brain processes such as those described earlier. However, Benedetti and colleagues (2014) also showed that peripheral biochemical mechanisms may also play a role in nocebo effects. Whether there is an additional early or late source of increased pain perception in the spinal cord, is a question of high relevance and importance. Two studies examined spinal fMRI for nocebo hyperalgesia both induced by conditioning combined with verbal suggestions methods.

Geuter & Büchel (2013) investigated thermal conditioning combined with the suggestion that a (sham) capsaicin cream would enhance their perceived pain, while a control cream would have no effect on pain. Significant nocebo effects were reported. The fMRI results revealed that the nocebo manipulation led to increased BOLD signal in the ipsilateral dorsal horn of the spinal cord. Interestingly, the location of the nocebo-enhanced pain signal largely overlapped with the main effect of pain during heat stimulation. Moreover, response time to painful stimulation differed, with the signal increasing earlier when the nocebo treatment was applied compared to control. Overall, the findings demonstrate nocebo-induced increases in spinal pain signals, indicating that an early pain-facilitating mechanism takes place at the spinal level.

Tinnermann et al. (2017) integrated spinal and brain imaging, aiming to unravel whether nocebo hyperalgesia is mediated through cortico-subcortico-spinal network interactions, similarly to other forms of cognitive pain modulation. In this study, the impact of perceived value

of a nocebo treatment on the magnitude of nocebo responses was also explored. Participants were allocated to one of two groups and received negative suggestions regarding the hyperalgesic effect of inert creams, one labeled as expensive and one as cheap. An additional neutral cream was used as a control. Nocebo effects were successfully induced and participants who received the sham expensive cream reported a significantly higher nocebo effect than participants who received the sham cheap cream. Regions that displayed neural representations of nocebo hyperalgesia irrespective of medication value were identified in the spinal cord at the height of spinal segment C6, slightly more caudal and medial than the pain cluster. This location was almost identical to the results of Geuter & Büchel (2013). Furthermore, compared to the cheap cream group, the expensive cream group had greater activation differences between nocebo and control trials in prefrontal areas, the right amygdala, and the PAG. Moreover, the level of rACC deactivation predicted the strength of reported nocebo hyperalgesia and the spinal cord and rACC revealed coupling with the PAG that correlated with the nocebo magnitude.

Taken together, these initial spinal imaging studies on nocebo hyperalgesia showed that both ascending and descending pain modulation at the spinal cord level may be involved in the presentation of nocebo effects. Modulation of the rACC-PAG-spinal axis could represent a mechanism through which the descending pain pathway interacts with higher-cognitive information, such as learned information or negative suggestions, to modulate pain processing. With the pain signal being amplified already at the spinal level, however, interesting questions may be raised regarding the contributing role of the spinal cord in pain amplification under nocebo conditions. While the observed amplification of spinal pain signals suggests a key role for spinal modulatory processes in nocebo hyperalgesia, further modulations at later, cortical areas remain important.

Discussion

This review provides an overview of the neurobiological correlates of experimentally induced nocebo hyperalgesia. fMRI findings showed that activity might be amplified already in the spinal cord and further modulated by higher cognitive representations, such as cognitive and affective processes. Electrophysiological findings, though limited, also pointed towards involvement of cognitive-affective processes. Neurochemical findings were not consistent on whether cortisol may play a role in nocebo hyperalgesia, but pointed towards an involvement of specific endogenous neurotransmitter systems. Due to the multifaceted nature of nocebo hyperalgesia as a learned effect, physiological components remain difficult to disentangle from other variables, such as cognitive mechanisms related to sensory perception. Central issues arising from the compilation of neurobiological findings from the nocebo literature are the widespread inconsistency in methods used and results yielded, –albeit this being understandable given the youth of the nocebo field. This diversity in methods and reporting of findings seriously challenges the interpretation of these findings. In discussing the results of this review, we have attempted to broadly categorize findings into neurobiological processes. It should be noted that this compartmentalization adds clarity to the interpretation of the results, the boundaries between these categories are blurred and categories largely overlap.

Sensory discrimination

Sensory discrimination allows for the processing of details both within the sensory input and between distinct types of sensations. It is

unsurprising that this broad, primary type of pain processing is involved in nocebo hyperalgesia. Yet, findings that show increased involvement of sensory-discriminatory processes linked to nocebo, as compared to control pain, are very valuable. These findings reveal that typical perception of increased pain stimulation may involve very similar pain mechanisms as aggravated pain under hyperalgesic conditions (i.e., in the absence of increased pain stimulation). Electrophysiological findings showed the important involvement of sensory discrimination. Alpha activity has long been thought to reflect functional blocking of task-irrelevant pathways [83]. However, Albu and Meagher's (2016) findings may point towards an expectation-related inhibition of sensory processing or attention to somatic states, at least on a whole-brain level. Tu et al. (2019) also highlighted a role of alpha band activity, consistently with previous studies showing a clear link between sensory perception and alpha oscillations [84–88]. Moreover, Hird and colleagues (2018) found that SPN, an EEG correlate of imminent pain, was related to the nocebo responses, which points to a role of electrophysiological nociceptive processes under hyperalgesic conditions.

Biochemical correlates of nocebo hyperalgesia also reflect an involvement of primary sensory processing, although results appear less robust and generally have not been reproduced. Scott and colleagues (2008) demonstrated that nocebo hyperalgesia was characterized by a deactivation of the μ-opioid receptor system, in key nocebo-related brain areas such as the ACC, OFC, insula, amygdala, and PAG. This study further demonstrated that placebo analgesia was characterized by increased activations of the same systems in overlapping brain regions. However, these results should be interpreted with caution because the experimental paradigm did not purposely induce negative expectations, instead, findings are presented for those participants who showed nocebo responses upon a placebo manipulation. In an investigation of the contribution of biochemical correlates of nocebo hyperalgesia in the peripheral nervous system, Benedetti and colleagues (2014) found that

nocebo hyperalgesia affected a specific biochemical pain pathway related to PG synthesis; however, in the absence of hypoxia-related activation of the COX-PG pathway, negative expectations were insufficient in initiating pain and PG synthesis. While these results highlighted a role of peripheral biochemicals that are directly related to pain signaling in nocebo hyperalgesia, they also pinpoint that nocebo hyperalgesia may be dependent on the intensity of an underlying pain.

Functional imaging studies have also implicated sensory discrimination in nocebo hyperalgesia, evident through the involvement of brain areas such as the thalamus and somatosensory cortex [41,61]. Interestingly, pain transmission via the spinal cord under nocebo hyperalgesic conditions also reveals vast similarities between the typical perception of a high pain stimulus and the perception of high pain resulting from expectations under hyperalgesic conditions [62,63]. Future studies could integrate the measures used in the abovementioned studies to cross-validate their results and achieve a more specific characterization of the various components involved in nocebo hyperalgesia. For example, peripheral components such as those found in Benedetti et al. (2014) may interact with peripheral or spinal components such as those discussed by Tinnerman and colleagues (2017) and a targeted manipulation of these variables could increase the robustness and interpretability of the current literature.

Pain integration and modulation

While there is overlap between all pain processing components, a further possible categorization of neural mechanisms pertains to the central sensory modulation of pain. A consistent finding across the articles reviewed here is that nocebo hyperalgesia involves brain areas that are thought to be responsible for the modulation of pain signals [59,89–91]. Some of these key areas include the dlPFC, OFC, and PAG [8,9,19,62].

Egorova and colleagues (2015) discuss that the dlPFC involves downstream circuits to, amongst others, the anterior insula, hypothalamus, and PAG, which are known to be involved in pain modulation [92]. Each of these areas has been implicated in nocebo hyperalgesia in the studies included in this review. Moreover, previous research does indicate a specific involvement of the rDLPFC in pain perception and cognitive evaluation of incoming stimuli [58,93]. Importantly, in visceral pain studies, the involvement of the insula in nocebo hyperalgesia also marks mechanisms of sensory integration and cognitive pain evaluation [69–71]. Thus, cognitive processing in frontal areas may interact with primary control centers for descending pain inhibition such as the PAG [94] thereby modulating pain under hyperalgesic conditions.

Findings involving the ACC deserve special attention, as they are most consistent across studies and indicate that higher order cognitive controls also play an important role in efferent pain modulation. The ACC is highlighted as a key region involved in cognitive pain processing [19,57,59,63]. Kong et al. (2008) showed that nocebo hyperalgesia was predominantly produced through a modulatory pain pathway involving the bilateral ACC. Based on the findings by Tinnermann and colleagues (2017) a modulatory function of the rACC on the descending pain system under hyperalgesic conditions is also evident. Notably, studies that include elaborate suggestions with a heavy negative load and hyperalgesic treatments, such as that of Kong et al. (2008), found an extensive involvement of cognitive pain processing, mediated through the ACC. Studies that did not employ extensive negative suggestions or sham treatments, however [8,58,60], showed a main involvement of pain-modulatory processes, but not affective processes, with a notable lack of ACC involvement. A possibility to replicate nocebo findings and evaluate the role of the ACC in hyperalgesia may exist, for example in patient studies or subdural electrode techniques that have been found to be powerful in locating sources of deep brain activity [95].

Concurrently, the spinal cord has also been found to interact with higher-order areas such as the PAG in nocebo hyperalgesia [62,63]. Pain modulation may thus involve an interconnected and wide-spread circuit, with nocebo studies showing both afferent pain amplification under nocebo conditions and efferent pain modulation [62,63]. This highlights a role of the entire pain system, from physiological nociceptive signaling in the spinal cord all the way to cognitive modulatory processing in the brain in nocebo hyperalgesia.

Learning leading to expectations

Since the formation of negative expectations through learning lays at the core of nocebo hyperalgesia, it is unsurprising that cognitive modulation was found to be an important factor in nocebo hyperalgesia. MEG findings implicated alpha band connectivity between the rACC and MTG, which may reflect a process in which experience might be encoded through the dynamics of neural networks [96,97]. Concurrently, analyses of EEG biomarkers (Thomaidou et al., 2021a) indicated a main involvement of long-range temporal correlations of brain oscillations as well as gamma band activity, both of which have previously been linked to learning [98–100]. These electrophysiological findings connect nocebo hyperalgesia to learning processes that can be reflected through electrophysiological components. Time-sensitive responses to nocebo hyperalgesia were studied through ERPs, which also highlighted the role of pain expectation. The reduction in N2/P2 amplitudes that was found by Pazzaglia and colleagues (2016), as well as the involvement of SPN found in Hird and colleagues, are linked to the predictability and temporal expectations of nociceptive stimuli [101,102], supporting the notion that nocebo effects are reliant on pain expectations. On the other hand, the finding of CNV differentiation between placebo and nocebo effects also highlighted the role of expectations, but additionally

indicated that differential electrophysiological processes characterize learned expectation of analgesia and hyperalgesia. In sum, EEG and MEG studies provide support that learning is involved in nocebo hyperalgesia, while also highlighting the role of pain processing, at the electrophysiological level.

It is worth noting that the role of regions such as the amygdala and the hippocampus, that are perhaps best summarized in the context of (affective) learning, highlight the more refined and specific aspects of learning that underlie nocebo hyperalgesia. Many of the aforementioned relevant brain areas point towards integrative learning mechanisms being involved in nocebo hyperalgesia, including, for instance, the ACC and dlPFC. While it is generally accepted that learning plays a key role in nocebo hyperalgesia [4,103,104], unravelling the more exact learning correlates that contribute to the formation of nocebo effects is imperative. The amygdala and the hippocampus have specifically been implicated in aversive learning and conditions such as phobias, where fear learning plays a crucial role [105–107]. The role that the hippocampus plays in nocebo hyperalgesia [19,59] relates to previous findings that the hippocampus mediates aversive learning [108]. This may in turn highlight an involvement of aversive learning processes in nocebo hyperalgesia. Moreover, within brain networks that include the ACC [18,63], expectations and pain processing may be integrated in a way that involves the evaluation of sensory information based on learned negative expectations. In collaboration with the dlPFC, brain regions such as the ACC and the somatosensory cortex integrate information [109] and are reportedly involved in expectation, anticipation, and error processing [89,110], which are also essential elements of associative learning processes. At the same time, electrophysiological findings described in this review connect nocebo hyperalgesia to long-term learning processes [5,15] as well as brain plasticity, with subcortical alpha-band oscillations engaging in rhythmic activities that have a plasticity function [111]. These findings provide supporting physiological evidence of learning via association

and long-term potentiation being central mediating factors of nocebo effects [112–114].

Anxiety and fear

In the induction of nocebo hyperalgesia, anxiety and stress have long been thought to be modulatory factors. Benedetti and colleagues (2006), distinguished between different physiological anxiety pathways and pointed towards a potential distinction of HPA-mediated anxiety and anxiety related specifically to pain, with the latter being a potential contributor to nocebo hyperalgesia. However, the studies that investigated cortisol, a key chemical marker of stress states, found that, while cortisol seems to increase in response to pain or negative nocebo suggestions, there is no clear evidence in support of a modulatory role of the hormone on nocebo hyperalgesia (Benedetti 2006; 2014).

The role of affective processing in nocebo hyperalgesia is marked by findings implicating the amygdala [20,59,63], a primary region for fear processing and evaluation. While the amygdala has been implicated in nocebo effects by only a minority of studies, it is important to note that more threatening experimental contexts or verbal suggestions may potentially enhance the involvement of this brain region in nocebo effects. As such, it seems that fear, processed by amongst others the amygdala, may be a secondary modulatory factor in nocebo hyperalgesia. Moreover, the amygdala is extensively interconnected with areas that were consistently fount to be involved in nocebo hyperalgesia, such as the PFC, especially the OFC as well as the dlPFC [115]. These areas may thus play an additive role in the processing of pain under nocebo hyperalgesic conditions, especially due to their involvement in cognitive-affective processes [116]. The activation of the amygdala may not be essential, as frontal areas have also been shown to underlie nocebo hyperalgesia in the absence of an amygdala involvement [9,63].

Nevertheless, findings have linked the activation of the amygdala to the magnitude of nocebo responses (Egorova et al., 2020) such that higher fear or anxiety seem to be linked to higher nocebo hyperalgesia. Future fMRI research could shed light on the role of fear and related physiological processes in the presentation of nocebo hyperalgesia by manipulating and directly comparing the threatening nature of the nocebo context.

Limitations, future directions, and clinical implications

After summarizing the results reviewed here, it is important to note that the utilization of distinct learning methods for the induction of nocebo hyperalgesia may be influence the neurobiological findings of these experimental studies. In other fields of research, such as fundamental neuroscience in the domain of learning and memory, different types of learning have been shown to employ different brain processes and a complex architecture underlying distinct learning systems [117–120]. Concurrently, differences in the affective load or valence of negative suggestions [103,121] or even potentially the magnitude of induced nocebo hyperalgesic effects [122], may influence the physiological processes that are involved in the induction and evocation of nocebo responses. Few studies have systematically studied these methodological aspects of nocebo effects. The knowledge base on nocebo hyperalgesia could significantly benefit from future research focusing on replication, comparability between studies, and an examination of the influence that methodological aspects have on the neurobiological nocebo correlates. While the field of nocebo hyperalgesia is young, it is therefore also a contemporary field of science that could benefit by setting an example in replicating findings and compiling consistent and reliable results.

Overall, one important future aim for nocebo studies may involve the systematic examination of learning, as this seems to be a major factor

underlying nocebo hyperalgesia. This implication of learning is important, particularly in light of evidence-based theories that show how the social environment and interpersonal experiences shape the experience of pain in healthy and patient populations [123–125]. The specific learning correlates that are involved in nocebo effects have not been systematically manipulated and studied. Pharmacological and cognitive manipulations of learning are effective means in which learning has been studied in other fields of research [126,127]. Yet, several important biochemicals relevant for pain and/or cognitive processes have not yet been studied. What future nocebo studies could attempt, is a direct manipulation of learning via, for example, agents that affect the n-methyl-d-aspartate receptor system such as amino acids [128]. Alternatively, direct measures of learning ability (such as the Weschler Memory Scale;[129] may shed light onto the specific learning mechanisms that are involved, and how individual differences related to learning may facilitate the formation of nocebo effects. It is imperative for future research to focus on precise learning mechanisms and comparisons between learning mechanisms in order to better understand and potentially therapeutically target the fundamental mechanisms by which nocebo hyperalgesia is induced.

While some of the neurobiological correlates of nocebo effects are beginning to unravel, application of this knowledge in clinical contexts is also receiving increased attention. The studies reviewed in this article provide important insights into the key neurobiological mechanisms involved in nocebo effects. Notably, some of the neural correlates that have been linked to nocebo hyperalgesia are also implicated in chronic pain. For instance, data suggest that chronic pain involves brain structures such as the cingulate cortex and hippocampus may be associated with chronic stress and mesolimbic dopamine abnormalities that are involved in processing both nociceptive signaling and affective components of pain [20,74,130–132]. Such overlapping neural factors may be crucial in the search for biomarkers of nocebo effects and identifying

reliable risk factors for its formation. Considering the mechanisms of action of nocebo hyperalgesia may significantly aid the process of preventing or counteracting nocebo effects in pain patients.

We conducted a comprehensive review of the currently known neurobiological correlates of nocebo hyperalgesia. Functional studies showed that pain-related activity might be amplified already in the spinal cord and further modulated by higher cognitive representations. Electrophysiological findings, though limited, also pointed towards involvement of cognitive-affective processes. Neurochemical findings were not consistent on whether cortisol may play a role in nocebo hyperalgesia. These findings are an important step in identifying the neurobiological mechanisms through which nocebo effects may exacerbate pain. Nevertheless, one major limitation arising from the compilation of neurobiological findings from the nocebo literature is the inconsistency in methods and results. Future studies in this field should consider not only the pressing need for consistency and reproduction of findings, but also the need for transparency about what findings reflect. Traceable and consistent methods and results in neurobiological nocebo studies are necessary in order for a reliable picture to be drawn. A better understanding of nocebo effects on pain might eventually lead to the development of methods to identify, minimize or prevent nocebo effects on pain.

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Chapter 4.

Learning mechanisms in nocebo hyperalgesia: The role of conditioning and extinction processes.

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Abstract

Nocebo hyperalgesia is a clinically relevant phenomenon and may be formed as a result of associative learning, implemented by classical conditioning. This study explored distinct nocebo conditioning methods and their consequences for nocebo attenuation methods. Healthy participants (N = 140) were recruited and randomized to the following nocebo hyperalgesia induction groups: conditioning with continuous reinforcement (CRF), conditioning with partial reinforcement (PRF), and a sham-conditioning control group. In the attenuation phase, counterconditioning was compared to extinction. During induction, participants experienced increased thermal pain in 100% of nocebo trials in the CRF groups, while in only 70% of nocebo trials in the PRF groups. During evocation, pain stimulation was equivalent across all trials. During attenuation, pain stimulation was decreased on nocebo trials relative to control trials for the counterconditioning groups, while pain remained equivalent across all trials for the extinction groups. Results showed that both PRF and CRF significantly induced nocebo hyperalgesia, but CRF was a more potent nocebo induction method, as compared to PRF. Counterconditioning was more effective than extinction in attenuating nocebo hyperalgesia. Neither CRF nor PRF resulted in resistance to extinction. However, compared to CRF, conditioning with PRF resulted in more resistance to counterconditioning. These findings demonstrate that the more ambiguous learning method of PRF can induce nocebo hyperalgesia and may potentially explain the treatment resistance and chronification seen in clinical practice. Further research is required to establish whether attenuation with counterconditioning is generalizable to clinical settings.

Introduction

It has been demonstrated that negative expectations regarding treatment outcomes may aggravate pain symptoms [1–4], a phenomenon termed nocebo hyperalgesia [2,5]. In experimental research, nocebo hyperalgesia is defined as a significant increase in pain following a nocebo treatment, relative to no-treatment or a control treatment. Negative expectations may enhance aversive side-effects [6] or produce deleterious effects on pain recovery [7]. Classical conditioning is an important underlying mechanism of nocebo hyperalgesia [8–10]. In conditioning paradigms, the pairing of a conditioned stimulus (CS; e.g., an inert treatment) with an unconditioned stimulus (US; e.g., surreptitiously increased pain) leads to a learned association [11,12]. As a result of this learned association, an inert treatment can evoke increases in perceived pain [10].

The vast majority of nocebo studies induce hyperalgesia by use of conditioning with continuous reinforcement (100% pairing of CS and US). In a more ambiguous type of conditioning with partial reinforcement, stimuli are paired in less than 100% of trials, thus the contingency between pain and an inert nocebo treatment is more variable. Partial reinforcement is of particular clinical interest due to its variable nature, which resembles the more ambiguous and inconsistent learning that may occur in clinical settings [13]. Partial reinforcement conditioning has been successfully used in fear research [14] and was recently also implemented in nocebo research [15]. Colagiuri and colleagues compared continuous and partial reinforcement schedules and found that nocebo hyperalgesia can be induced through partial reinforcement [15]. Additionally, Colagiuri and colleagues [15] investigated the consistent finding from fear studies that partial reinforcement conditioning shows more resistance to extinction than continuous reinforcement [14,16,17]. In contrast to findings in other fields of research [14], extinction was unsuccessful in attenuating nocebo hyperalgesia

irrespective of the conditioning schedule [15]. This indicated that, once established, nocebo hyperalgesia may be especially resistant to extinction; a relevant finding for chronic pain conditions, where learned effects may persist and not become extinct.

If extinction is unsuccessful in attenuating these learned effects, a more active approach may be needed to attenuate nocebo hyperalgesia. A promising novel method is counterconditioning. Unlike in extinction, during counterconditioning the negative stimulus is replaced by a more positive stimulus [18]. Counterconditioning has recently been successful in different fields [19,20]. However, despite its potential as a basis for the treatment of nocebo-augmented pain [21], it remains unclear whether counterconditioning would be an effective intervention for the attenuation of learned nocebo responses.

In this study, we compared two nocebo induction methods, conditioning with partial and continuous reinforcement. Furthermore, we examined the consequences of partial versus continuous conditioning for the attenuation of nocebo hyperalgesia via counterconditioning or extinction. We expected to reproduce earlier findings that partial reinforcement would successfully induce nocebo hyperalgesia and that compared to continuous reinforcement, partial reinforcement conditioning would lead to more resistance to extinction. We furthermore examined counterconditioning as a potential attenuation method for nocebo hyperalgesia. The implementation of novel, clinically relevant learning-based methods for investigating nocebo hyperalgesia is an important step towards eventually diminishing nocebo effects in clinical settings.

Materials and Methods

Participants

One hundred and forty participants were enrolled in this study. The required sample size for the primary analysis was calculated based on a previous similar nocebo study [15]. The analysis was conducted in G*power 3.1 [22] for a mixed model ANOVA. The effect size was f = 0.26, alpha error probability was set at $\alpha = 0.05$, desired power was set at 0.95, and the correlation for repeated measures was set at 0.05 (because of the subjectivity and high variability expected in pain ratings). According to the total sample size indicated, we planned for 140 participants to be enrolled, of which a total of 122 participants were included in the study. This sample size for the primary hypothesis is similar to previous studies examining subtle between-groups differences such as conditioning with partial reinforcement or anxiety correlates [15,23]. The main groups were split in half for the purposes of some of the secondary analyses in this two-by-two design, resulting in subgroups of 24-25 participants, a sample size that has been used in previous nocebo studies that yielded significant results with good effect sizes [21,24,25].

Participants were required to be between 18 and 35 years old, have a good understanding of the Dutch language as well as (corrected to) normal vision and hearing. Exclusion criteria were serious medical or psychiatric conditions, pregnancy, painful health conditions experienced in the past 6 months, and pain or use of analgesic medication at the time of testing. Participants who were determined to have too high of a threshold for pain upon their visit to the department (i.e., when thermode maximum temperatures were not sufficient to induce at least moderate pain) were also excluded from the study. All participants were asked to refrain from alcohol and caffeine consumption, as well as the use of drugs and analgesic medication, in the 12 hours before the testing appointment. Participants were recruited via flyers, social media

advertisements, and the online recruitment website Sona (Sona Systems, Tallinn, Estonia). Study participation involved a 2-hour testing appointment at a research laboratory of the Faculty of Social and Behavioral Sciences of Leiden University, the Netherlands. All participants provided written informed consent prior to the start of the experiment. After completing the experiment, all participants were reimbursed by either cash or study credits for their participation. This study was approved by the Leiden University Psychology Research Ethics Committee (CEP18-0816/318) and pre-registered on ClinicalTrials.gov (NCT03793790).

Design

This study utilized a randomized, two-by-two design, with an additional control group (Figure 1). A randomization list was created by an independent researcher and participants were randomly allocated to one of 5 groups only after the calibration procedure was complete, so to reduce any risk of bias. All participants underwent a two-phase study design of which each phase consisted of two parts. The induction phase (phase 1) comprised an induction part in which associations were learned and an evocation part in which learned associations were tested. The attenuation phase (phase 2) comprised either counterconditioning or extinction to examine the attenuation of the learned responses and a second evocation part to test whether learned associations were still present. Group 1 received conditioning with partial reinforcement and counterconditioning. Group 2 received conditioning with partial reinforcement and extinction. Group 3 received conditioning with continuous reinforcement and counterconditioning. Group 4 received conditioning with continuous reinforcement and extinction. Group 5 (the sham control group) received sham conditioning and also

underwent 'extinction' in order to keep the length and procedures of the experiment identical for all.

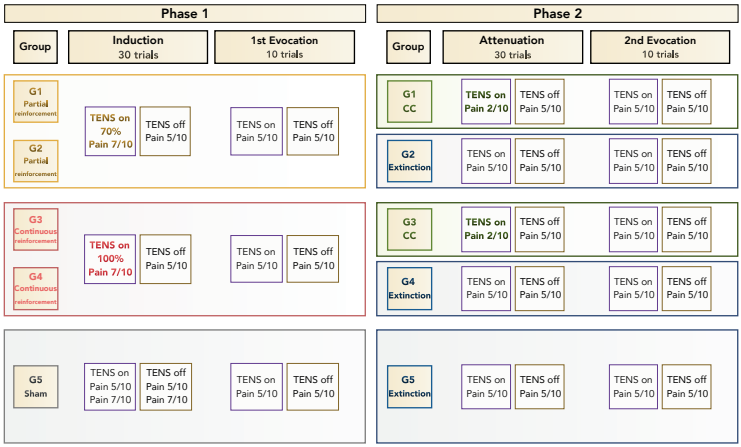

Figure 1. Illustration of the experimental design. During partial reinforcement (G1 and G2) participants received high pain in 70% of nocebo trials and moderate pain in 30% of nocebo trials. The sham group (G5) received high pain in 50% of nocebo trials and in 50% of control trials. In the attenuation phase, during counterconditioning (G1 and G3) participants received low pain in all nocebo trials, while during extinction (G2 and G4) participants received moderate pain for both the nocebo and the control trials. The sham group underwent extinction to keep the procedure equal in length. CC, Counterconditioning.

Thermal pain application

Thermal pain stimuli were delivered to the non-dominant volar forearm using 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 were rated on a pain numeric rating scale (NRS) ranging from 0 (no pain) to 10 (worst pain imaginable).

Sensory thresholds

To test warmth and pain threshold levels, heat stimuli were applied and participants were asked to indicate the first moment at which they perceived warmth and the first moment they perceived pain. The average of 3 warmth detection values and 3 heat pain detection values were determined as the threshold values for warmth and pain respectively. This method followed published standardized and protocolled procedures [26].

Pain calibration protocol and administered stimuli

Pain calibrations were conducted in order to select the temperatures that would be used to induce low, moderate, and high pain in phases 1 and 2. The calibrations were individually tailored, based on the NRS ratings of 42 heat stimuli of varying intensities, as well as participants' bodily and facial reactions to pain stimuli. For the calibration procedure as well as throughout the experiment, each stimulus was initiated from a 32°C baseline, increased to a target temperature, and presented for 4 seconds, excluding a ramp up rate of 8°C per second and a return rate of 8°C per second. The inter-stimulus interval was 8 seconds. Median temperatures consistently rated and experienced as NRS 2 to 3 were selected and used to induce low pain, median temperatures rated as NRS 4 to 6 were used to induce moderate pain, and median temperatures rated as NRS 7 to 8 were used to induce high pain. During induction and during attenuation, 15 nocebo and 15 control stimuli were administered in pseudorandom order, so that no three stimuli of the same type were administered in a row. During each of the two evocations, 5 nocebo and 5 control stimuli were administered in pseudorandom order. To reduce habituation to heat-pain, the thermode was moved twice (mid-way through phases 1 and 2) to a more proximal site on the same arm.

Nocebo treatment

A commercial Transcutaneous Electrical Nerve Stimulation (TENS) device (Beurer EM 80) was used to serve as the nocebo treatment in the procedure. Negative suggestions were used to create expectations regarding the pain enhancing effects of the device (Appendix 1). Two TENS electrodes were placed in a diagonal line on the ball of the hand and the inner elbow. Prior to the start of the induction phase, participants underwent a short mock calibration procedure during which they felt a light electrical pulse of the TENS. This pulse was delivered in order to increase the believability of the nocebo verbal suggestion. Participants were told that the device was called "ENS", to avoid that participants would recognize or associate any prior experience with this device. The device was not actually activated during the conditioning procedure, but messages displayed on a computer screen via E-Prime 2.0 (Psychology Software Tools, Pittsburgh, PA, USA) signaled the sham activation and deactivation of the TENS device during nocebo and control trials, respectively.

As part of the nocebo suggestions, participants read an information sheet (see Appendix 1), displayed on a tablet, containing (sham) information regarding the supposed effects of the TENS treatment. During nocebo induction, negative suggestions indicated to all participants that when the messages "ENS on" (in purple font; nocebo cue) and "ENS off" (in yellow font; control cue) were displayed, their pain would be aggravated or not altered, respectively.

Sham TENS activation was paired to surreptitiously increased pain stimulation during nocebo trials, while moderate pain was delivered during control trials during the induction phase. For the partial reinforcement groups, the activation of the TENS device was paired with high pain stimuli in only 70% of nocebo trials (un-paired trials were

pseudorandomized to achieve an approximately even distribution throughout the induction phase). The continuous reinforcement groups received high pain stimuli in 100% of nocebo trials. The control group received sham conditioning, where TENS activation was not consistently paired to the intensity of pain stimuli but rather, this group received high pain in 50% of nocebo trials and in 50% of control trials. In the first evocation phase, all pain stimuli were applied at moderate intensity, preceded by the nocebo and control cues, to evoke conditioned responses. Increased pain reports for the first nocebo trial as compared to the first control trial in this phase indicated nocebo hyperalgesia. During attenuation, the counterconditioning groups received surreptitiously decreased pain stimulations during TENS activation, while TENS deactivation was still paired to moderate pain inductions. During extinction, participants continued being exposed to pain stimuli at only moderate intensity preceded by the nocebo and control cues. During the second evocation phase, all pain stimuli were applied at moderate intensity, preceded by the nocebo and control visual cues, to test whether nocebo responses were diminished after attenuation.

Questionnaires

Four questionnaires were used to measure baseline differences in psychological characteristics. Total scores were used for all questionnaires. A short State Anxiety version of the State-Trait Anxiety Inventory, (STAI-S) [27,28] was used once before the start (STAI state pre) and once after the end of the experiment (STAI state post). Scores on this questionnaire range from 20 to 80, with higher scores indicating higher state anxiety. Cronbach's alpha in this study were 0.77 (pre) and 0.74 (post). The State-Trait Anxiety Inventory, Trait version (STAI-T) [27] was also used, with scores also ranging from 20 to 80 and higher scores

indicating higher trait anxiety. Cronbach's alpha was 0.83 in this study. The Pain Catastrophizing Scale (PCS) [29] was used to assess catastrophizing thoughts related to pain, with scores ranging from 0 to 52, where higher scores indicate more frequent catastrophizing thoughts. Cronbach's alpha was 0.87 in this study. The revised Life Orientation Test (LOT-R) [30] was used to measure dispositional optimism versus pessimism. Scores on this questionnaire range from 0 to 24, with higher scores indicating higher optimism. Cronbach's alpha was 0.69 in this study. Participants were also asked to rate their tiredness on a 0-10 NRS scale from "not at all" to "very much". Moreover, a screening questionnaire containing demographic and health questions was used to screen participants for inclusion in the study. At the end of the experiment, participants completed an exit questionnaire containing manipulation check questions assessing pain expectations (rated on the pain NRS), how much they trusted the experimenters, and how honest they thought the experimenters were (rated on a 0-10 NRS from "not at all" to "very much"). The exit questionnaire also assessed whether participants believed the cover story or were aware of the real purpose of the experiment (i.e., the manipulation of expectations or use of conditioning). All questionnaires, as well as a debriefing from, were displayed on a tablet via web-based survey software (Qualtrics, Provo, Utah, USA).

Experimental Procedure

On the day of the appointment, participants were first provided with information about the experiment and were asked to provide written informed-consent. Then, participants completed the screening and the psychological questionnaires. Following this, they read the information sheet about the (sham) pain enhancing effects of the TENS device. Warmth and pain threshold levels were then tested and individual pain

stimuli were calibrated. Participants then underwent nocebo induction through conditioning with partial reinforcement, continuous reinforcement, or sham conditioning. The first evocation phase where nocebo responses were tested then followed. Subsequently, participants underwent nocebo attenuation, through either counterconditioning or extinction. A second evocation phase then followed, where the presence of nocebo responses after attenuation was tested. After the end of the experiment participants were asked to complete the exit questionnaire. Then, a debriefing was conducted and participants were reimbursed for their participation.

Statistical Analyses

All data were analyzed by use of SPSS 23.0 (IBM Corp., Armonk, NY, USA). A one-way analysis of variance (ANOVA) was conducted between all groups for mean scores on each of the questionnaires and the tiredness rating, in order to determine whether any personal characteristics could have influenced the results. One-way ANOVAs were also used to assess between-groups differences in state anxiety, trust in the experimenter, and pain expectations, as assessed at the end of the experiment and in temperatures used to induce pain and the NRS pain scores throughout the experiment. As these analyses involved multiple between-groups comparisons, the threshold for significance was set at $P < 0.01$.

Primary and secondary outcome measures

The magnitude of reported nocebo hyperalgesia (primary outcome measure) was measured within-subjects, and was defined as the difference in pain ratings for the first nocebo trial compared to the first

control trial, during first evocation. The reduction of induced nocebo hyperalgesia after attenuation was measured as the change in reported pain for the first nocebo trials between the first and second evocation. The first trials of each testing phase were selected since previous studies indicate the effect to be clearest in those trials [15,31]. Difference scores between nocebo and control trials as mentioned above were only used for manipulation checks and descriptive purposes (Tables 1 and 2). To conduct mixed model analysis of variance (ANOVA), the assumptions of normality, independence and homogeneity of the variances were checked. Unless otherwise stated, the threshold for significance was set at $P < 0.05$. As an effect size measure, partial eta-squared (η_p^2) was calculated for analyses of primary and secondary outcomes, with η_p^2 of 0.01 considered small, 0.06 considered medium, and 0.14 large [32,33].

Nocebo hyperalgesia induction

First, to examine whether a significant nocebo response was present after nocebo induction, a 3x2 mixed model ANOVA was used, treating induction group as the between-subjects factor with 3 levels (partial reinforcement, continuous reinforcement, or sham) and magnitude of the nocebo response as a within-subjects factor with 2 levels (first nocebo trial, first control trial). A conservative Bonferroni correction was applied and the threshold for significance was set at $P < 0.01$. Where a significant interaction is detected, planned contrasts are analysed (2x2 mixed ANOVAs) between each of the pairs of experimental groups.

Nocebo hyperalgesia attenuation

In order to test the hypothesis that counterconditioning would be more effective than extinction in attenuating nocebo hyperalgesia, a 2x2 mixed

model ANOVA was performed with attenuation group as the between-subjects factor with two levels (counterconditioning, extinction) and the nocebo reduction as the within-subjects factor with 2 levels (first nocebo trial of the first evocation phase pre-attenuation, first nocebo trial of the second evocation phase post-attenuation).

Resistance to extinction

To test the hypothesis that conditioning with partial reinforcement would lead to a more durable nocebo effect as compared to conditioning with continuous reinforcement, we explored resistance to extinction. A 2x2 mixed model ANOVA was performed with the induction group as between-subjects factor with two levels (partial reinforcement-extinction, continuous reinforcement-extinction) and the nocebo response as the within-subjects factor with 2 levels (first nocebo trial of the first evocation phase pre-attenuation, first nocebo trial of the second evocation phase post-attenuation). Following this, two repeated measures ANOVAs were conducted with the nocebo response as the within-subjects factor with 2 levels (as described above), to test whether extinction significantly reduced the magnitude of nocebo hyperalgesia within the partial reinforcement group and within the continuous reinforcement group.

Manipulation-check for the time-course of extinction

Because of the unique attenuation paradigm in the experiment, we implemented a design that applied 10 evocation trials, that were essentially extinction trials, before the start of the 30 attenuation trials. In our paradigm, 30 induction trials were followed by 10 evocation trials, which in turn were followed by 30 extinction trials. Evocation trials,

however, are identical to extinction trials. This exposed participants to a longer extinction time (i.e., essentially 40 trials), as compared to the 30 induction trials. In order to verify that any extinction or resistance effects were not present after an equal number of induction and extinction trials, we analyzed the 30th trial after the start of evocation. A 2x2 mixed model ANOVA was performed with the induction group as between-subjects factor with two levels (partial reinforcement-extinction, continuous reinforcement-extinction) and the nocebo response during extinction as the within-subjects factor with 2 levels (20th nocebo extinction trial, 21st control extinction trial).

Resistance to counterconditioning

It was also assessed whether a resistance effect to counterconditioning was present. A 2x2 mixed model ANOVA was performed with induction group as the between-subjects factor with 2 levels (partial reinforcement-counterconditioning, continuous reinforcement-counterconditioning) and nocebo response as the within-subject factor with 2 levels (first nocebo trial of the first evocation phase pre-attenuation, first nocebo trial of the second evocation phase post-attenuation). Following this, two repeated measures ANOVAs were conducted with the nocebo response as the within-subjects factor with 2 levels (as described above), to test whether counterconditioning significantly reduced the magnitude of nocebo hyperalgesia within partial reinforcement and within the continuous reinforcement group.

Time-course of attenuation

To explore the time-course and slopes of attenuation, a line graph was plotted. Mean NRS pain ratings were plotted for the nocebo trials after

the end of nocebo induction, in the partial reinforcement-counterconditioning group, the partial reinforcement-extinction group, the continuous reinforcement-counterconditioning group, and the continuous reinforcement-extinction group.

Manipulation-check for control trials

We ran manipulation checks to examine any effect of changes in control trial ratings on the reduction of nocebo responses after attenuation. This was done to assure that the effects of attenuation were not driven by changes in the ratings of control trials (TENS off), which could confound the results, for example if between-groups differences were detected, or in the case that general sensitization or habituation to pain had occurred. First, an analysis of the control trial ratings in all groups was performed. A 5x2 mixed model ANOVA was performed with group as the between-subjects factor with 5 levels (groups 1, 2, 3, 4, and 5), and the first control trial rating of each evocation phase as the within-subjects factor with 2 levels (control pre-attenuation, control post-attenuation). As this analysis involved multiple between groups comparisons, a conservative Bonferroni correction was applied and the threshold for significance was set at $P < 0.01$. A non-significant result would indicate that the control trials did not yield significant changes, confirming that they did not affect the within-subjects results of the analyses. Furthermore, we conducted a 2x2 mixed model ANOVA between the attenuation groups (counterconditioning, extinction) and the pre- to post- attenuation difference score between nocebo and control trials. In this way, we examined the reduction in the magnitude of nocebo hyperalgesia from pre- to post- attenuation, by directly comparing control trials to nocebo trials.

Questionnaires

Finally, we studied the relationship of nocebo hyperalgesia and anxiety, pain catastrophizing, and optimism. Scores obtained through the four psychological questionnaires were analyzed using correlation analyses, to explore whether any of these psychological characteristics were associated with the magnitude of induced nocebo hyperalgesia.

Results

Participants, temperatures, and pain ratings

A total of 140 participants were enrolled in this study (118 females, 22 males). Six participants were excluded due to technical difficulties or noise disturbance in the lab, 4 participants were unable to complete the study due to sleepiness, intense anxiety, or inability to follow instructions, 3 participants were excluded due to exhibiting a too-high threshold for pain (i.e., not reaching a moderate pain rating during calibrations), 2 participants were excluded due to fulfilling one of the health-related exclusion criteria (namely, experiencing moderate head or neck pain at the time of testing), 2 participants were excluded due to knowing the purpose of the experiment as assessed in the post-assessment survey, and 1 participant was excluded due to insufficient understanding of Dutch. A total of 122 participants were included in the final analyses, 102 females and 20 males. Randomization across the five groups resulted in a total of 25 participants in group 1 (partial reinforcement-counterconditioning), 24 participants in group 2 (partial

reinforcement-extinction), 24 participants in group 3 (continuous reinforcement-counterconditioning), 24 participants in group 4 (continuous reinforcement-extinction) and 25 participants in group 5 (sham). Participants were stratified for gender, so that each group contained 5 male participants.

Descriptive data of the questionnaire scores, temperature levels, and pain ratings are listed in Table 1. One-way ANOVAs indicated that there were no significant between-groups differences in the mean scores on any of the psychological questionnaires. The mean warmth detection threshold across all participants was 33.5°C (standard deviation; SD = 0.5) and the mean pain threshold was 42.3°C (SD = 2.9). The results of a one-way ANOVA indicated that there were no significant group differences in the mean temperatures selected to induce low, moderate and high pain. A one-way ANOVA detected a significant group difference in the ratings of the control trials of the induction phase, however when the analysis was performed again with the sham group removed, it was revealed that this difference was merely driven by the sham group, where half of the control trials were purposely paired with high pain stimulation. Despite moving the thermode several times during the experiment in order to avoid habituation to the heat stimuli, an overall decrease in pain ratings over time was observed (see Table 1, Conditioning and Attenuation rows).

Table 1. Group means and standard deviations, as well as between-groups *P* values.

	1 PRF - CC		2 PRF - Extinction		3 CRF - CC		4 CRF - Extinction		5 Sham		All groups		*P* value
	Mean	SD	Mean	SD	Mean	SD	Mean	SD	Mean	SD	Mean	SD	
STAI Trait	40.2	8.0	37.2	5.7	37.6	8.1	36.8	7.4	38.8	6.6	38.1	7.2	0.45
STAI State pre	50.8	24.4	52.7	23.1	48.6	25.1	44.3	18.6	44.2	16.6	48.1	21.7	0.56
PCS	13.6	8.1	12.9	6.6	11.9	5.4	13.8	6.8	13.4	8.0	13.1	7.0	0.90
LOT-R optimism	16.9	3.3	14.8	3.5	17.2	2.6	16.1	2.9	15.1	3.4	16.0	3.3	0.33
Tiredness (NRS)	3.4	2.0	2.9	2.1	3.4	2.3	3.9	2.5	3.3	2.3	3.4	2.3	0.60
Low °C	45.0	0.8	45.1	1.1	45.5	1.1	45.0	0.9	45.0	0.5	45.1	0.9	0.31
Moderate °C	46.8	0.7	47.0	0.8	47.0	0.8	46.7	1.1	46.9	0.7	46.9	0.8	0.61
High °C	48.4	0.5	48.6	0.7	48.8	0.6	48.5	0.8	48.5	0.5	48.6	0.6	0.31
Nocebo trials NRS ^	6.6	1.7	6.2	1.7	7.3	1.5	6.6	1.6	5.8	1.6	6.5	1.7	0.03
Control trials NRS	4.6	1.8	4.4	1.8	4.8	1.6	3.7	1.5	5.7	1.7	4.6	1.8	0.002 *
Nocebo trials NRS	3.2	2.0	4.4	2.0	2.6	1.4	3.8	2.0	4.0	2.1	3.6	2.0	0.01**
Control trials NRS ^	4.5	2.0	3.9	2.1	4.1	1.7	3.2	2.0	3.9	1.9	3.9	2.0	0.31
STAI State post	32.3	8.7	29.8	7.5	31.6	10.6	31.3	8.3	31.1	5.8	31.2	8.2	0.87
Trust in researcher (NRS)	8.5	2.1	8.6	1.7	8.7	1.8	8.6	1.7	9.3	0.9	8.7	1.7	0.49
Honesty researcher (NRS)	7.4	2.3	7.6	2.2	7.5	2.2	6.7	2.3	7.2	2.6	7.3	2.3	0.65
Pain expect. Indu.	6.4	1.8	6.0	1.7	6.4	1.7	6.5	1.7	5.2	1.9	6.1	1.8	0.06
Pain expect. Atten.	4.4	1.9	4.9	2.1	3.6	2.2	4.3	1.9	4.1	1.8	4.2	2.0	0.22

Note: Alpha set at 0.01. The NRS was always 0-10.
^ Excluding 1 trial from each phase immediately after thermode was moved (trial 21 of each phase).

Normality checks

The ANOVA assumptions of normality, independence, and homogeneity of the variances were checked. A non-significant Shapiro-Wilk test and histograms of standardized residuals indicated a normal distribution of the data. Within- and between-groups independence was established by randomization into groups. Homogeneity of variances was tested via a Levene's test, which indicated non-significant results, thereby confirming homogeneity of variance in the data.

Nocebo hyperalgesia induction

The mean magnitudes of reported nocebo hyperalgesia after induction are listed in Table 2. A 3x2 mixed model ANOVA was conducted to establish whether there was a significant difference in the magnitude of induced nocebo hyperalgesia between partial reinforcement, continuous reinforcement, and sham. The analysis revealed a significant group by trial interaction between the 3 induction groups and the magnitude of nocebo responses ($F(2,119) = 20.75$, $P < 0.001$, $\eta_p^2 = 0.26$). Figure 2 illustrates the differences in pain ratings for the first nocebo trial and the first control trial of the first evocation, across all three groups. Three 2x2 mixed model ANOVA planned analyses revealed a significant interaction between the partial reinforcement and sham group and the magnitude of nocebo responses ($F(1,72) = 20.58$, $P < 0.001$, $\eta_p^2 = 0.22$), between the continuous reinforcement and sham group and the magnitude of nocebo responses ($F(1,71) = 45.22$, $P < 0.001$, $\eta_p^2 = 0.39$), and between the partial reinforcement and continuous reinforcement groups and the magnitude of nocebo responses ($F(1,95) = 7.28$, $P = 0.008$, $\eta_p^2 = 0.07$). These results indicated that conditioning with partial reinforcement and with continuous reinforcement were both effective in inducing significant nocebo responses, with continuous reinforcement producing a significantly larger nocebo response as compared to partial reinforcement.

Table 2. Group means and standard deviations for the magnitude of nocebo responses after induction and attenuation, as well as for the reduction of nocebo hyperalgesia after attenuation.

Phase 1								**Phase 2**				
Induction group	Nocebo magnitude		**Attenuation group**	Nocebo magnitude		Nocebo reduction		**Induction - Attenuation group**	Nocebo magnitude		Nocebo reduction	
	Mean	SD		Mean	SD	Mean	SD		Mean	SD	Mean	SD
PRF	0.9	1.0	**CC**	-0.6	1.2	1.8	1.7	**PRF - CC**	-0.2	1.1	1.2	1.8
PRF								**PRF - Ext**	0.3	0.6	0.6	1.0
CRF	1.5	1.1	**Extinction**	0.3	0.6	0.9	1.1	**CRF - CC**	-1.0	1.2	2.3	1.6
CRF								**CRF - Ext**	0.3	0.6	1.3	1.1
Sham	-0.02	0.2										

Note: Scores are reported on a 0-10 pain numeric rating scale.

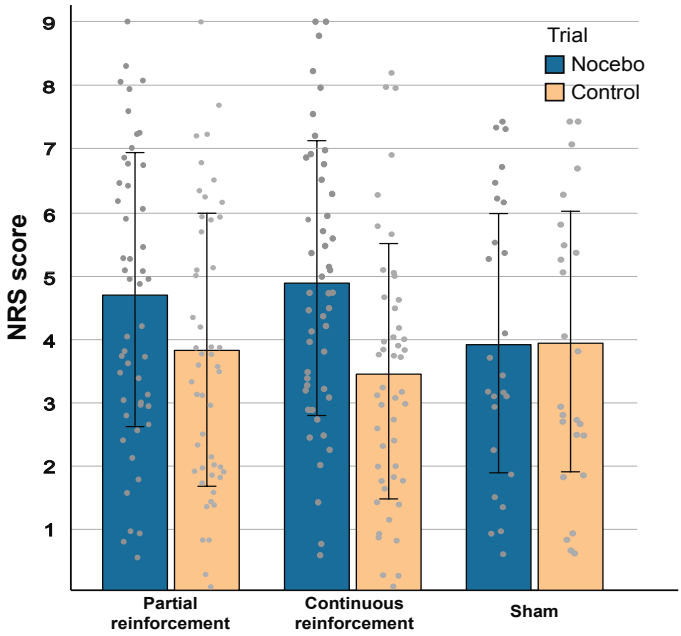

Figure 2. NRS pain ratings for the first nocebo and the first control trial of the first evocation. Mean Numeric Rating Scale (NRS) pain ratings and standard deviations are depicted across all groups (N = 122). Dots represent the (jittered) individual data points. In both the partial reinforcement and the continuous reinforcement groups, evocation pain reports during nocebo trials were significantly higher than pain reports during control trials. Sham conditioning, as expected, did not induce nocebo hyperalgesia. Conditioning with partial reinforcement yielded a significantly smaller nocebo effect than conditioning with continuous reinforcement.

Attenuated nocebo hyperalgesia

Counterconditioning vs extinction

The mean reduction and mean magnitudes of reported nocebo hyperalgesia after attenuation are listed in Table 2. To examine whether counterconditioning was more effective than extinction in attenuating

the induced nocebo responses, a 2x2 mixed model ANOVA was conducted. The analysis revealed a significant interaction between the counterconditioning and extinction groups and the reduction of nocebo responses ($F(1,95) = 6.51$, $P = 0.012$, $\eta_p^2 = 0.06$), indicating significantly higher efficacy of counterconditioning compared to extinction. Figure 3 illustrates the differences in pain ratings for the first nocebo trial of the first evocation and the first nocebo trial of the second evocation, between the counterconditioning and extinction groups.

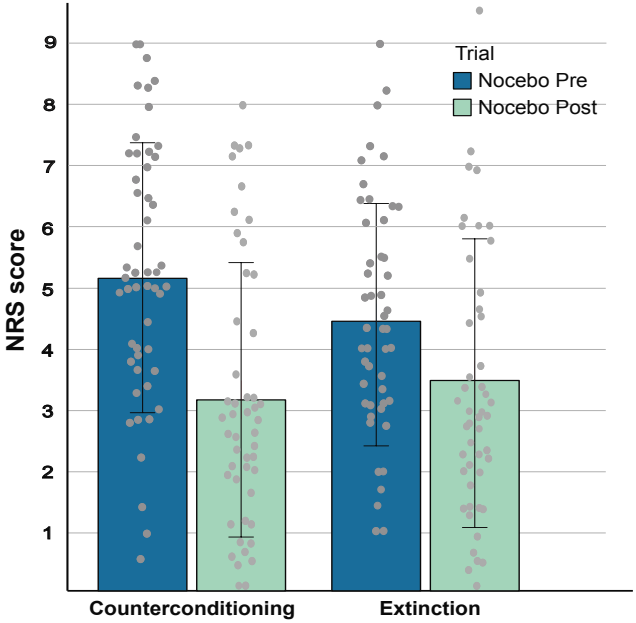

Figure 3. Comparison of nocebo magnitudes after counterconditioning and extinction. Differences in mean Numeric Rating Scale (NRS) pain ratings and standard deviations between the attenuation groups (N = 97) are depicted. Dots represent the (jittered) individual data points. Differences between the first nocebo trial of the first evocation (Nocebo Pre) and the first nocebo trial of the second evocation (Nocebo Post) illustrate the significant reduction of nocebo hyperalgesia achieved by both methods. Attenuation with counterconditioning was more effective in diminishing nocebo responses.

Resistance to extinction

The mean reduction and mean magnitudes of reported nocebo hyperalgesia after extinction are listed in Table 2. We conducted a 2x2 mixed model ANOVA to examine whether conditioning with partial reinforcement resulted in nocebo hyperalgesia that was more resistant to extinction, as compared to conditioning with continuous reinforcement. A non-significant interaction effect showed no significant difference in resistance to extinction between conditioning with partial reinforcement and continuous reinforcement ($F(1,46) = 0.63$, $P = 0.43$, $\eta_p^2 = 0.01$). Figure 4 illustrates differences in pain ratings for the first nocebo trial of the first evocation and the first nocebo trial of the second evocation, between the partial reinforcement-extinction group and the continuous reinforcement-extinction group. Furthermore, two repeated measures ANOVAs showed a significant effect of trial type (first nocebo evocation trial pre attenuation, first nocebo evocation trial post attenuation) in the partial reinforcement group ($F(1,23) = 5.26$, $P = 0.03$, $\eta_p^2 = 0.19$) and the continuous reinforcement group ($F(1,23) = 10.39$, $P = 0.004$, $\eta_p^2 = 0.31$), indicating that extinction significantly reduced nocebo responses in both groups.

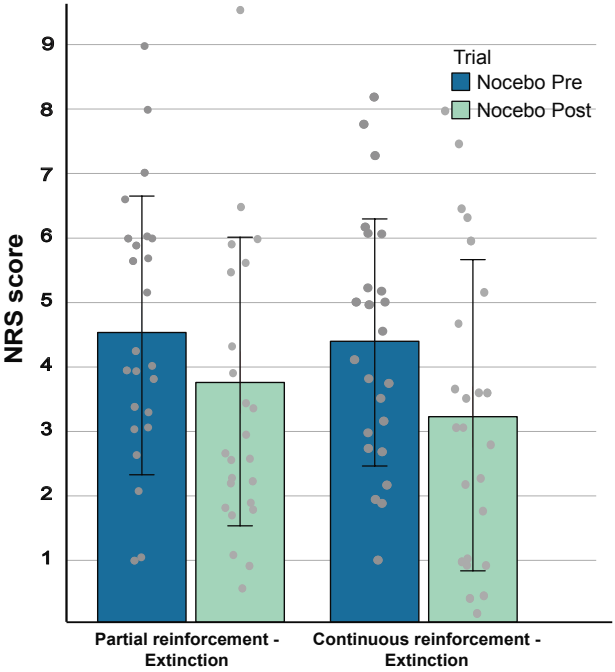

Figure 4. Comparison of nocebo magnitudes after extinction, between the partial reinforcement and continuous reinforcement. Differences in mean Numeric Rating Scale (NRS) pain ratings and standard deviations between the partial reinforcement-extinction group and the continuous reinforcement-extinction group (N = 48) are depicted. Dots represent the (jittered) individual data points. The direction of the difference between the first nocebo trial of the first evocation (Nocebo Pre) and the first nocebo trial of the second evocation (Nocebo Post) pointed towards partial reinforcement resulting in a more durable nocebo response, compared to continuous reinforcement, however this difference did not reach significance.

Manipulation-check for the time-course of extinction

In order to verify that resistance to extinction was not present at an earlier stage during attenuation, we analyzed the 20th attenuation trial, however again, no resistance to extinction was shown. When the 30th

trial after the first trial of evocation was used instead of the 30th trial after the start of attenuation, a 2x2 mixed model ANOVA showed no significant difference in resistance to extinction between conditioning with partial reinforcement versus continuous reinforcement ($F(1,46) = 0.61$, $P = 0.44$, $\eta_p^2 = 0.01$).

Resistance to counterconditioning

The mean reduction and mean magnitude of reported nocebo hyperalgesia after counterconditioning are listed in Table 2. We conducted a 2x2 mixed model ANOVA to examine whether conditioning with partial reinforcement resulted in nocebo hyperalgesia that was more resistant to counterconditioning, as compared to conditioning with continuous reinforcement. The analyses showed a significant difference in the resistance to counterconditioning between conditioning with partial reinforcement versus continuous reinforcement ($F(1,47) = 4.99$, $P = 0.03$, $\eta_p^2 = 0.09$). Figure 5 illustrates the differences in pain ratings for the first nocebo trial of the first evocation and the first nocebo trial of the second evocation, between the partial reinforcement-counterconditioning group and the continuous reinforcement-counterconditioning group. This finding indicated that partial reinforcement led to more resistance to counterconditioning than continuous reinforcement. Furthermore, two repeated measures ANOVAs showed a significant effect of trial type (first nocebo evocation trial pre attenuation, first nocebo evocation trial post attenuation) in the partial reinforcement group ($F(1,24) = 15.96$, $P = 0.001$, $\eta_p^2 = 0.39$) and the continuous reinforcement group ($F(1,23) = 27.65$, $P < 0.001$, $\eta_p^2 = 0.54$), indicating that counterconditioning significantly reduced nocebo responses in both groups.

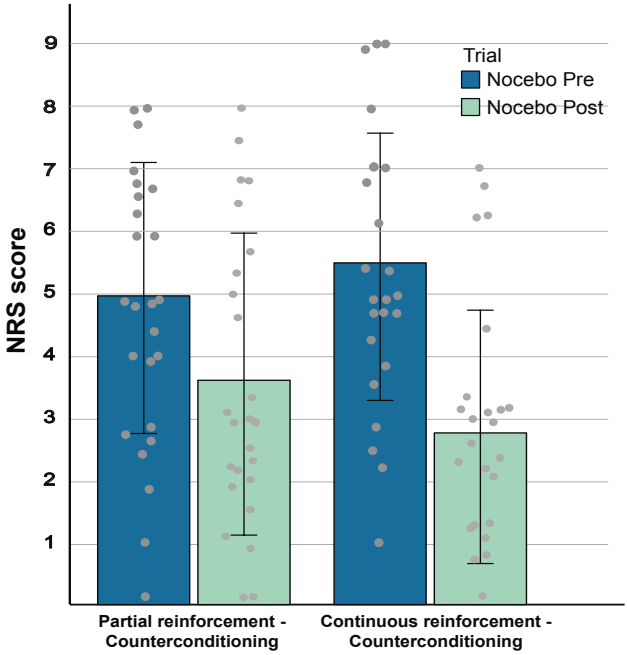

Figure 5. Comparison of nocebo magnitudes after counterconditioning, between the partial reinforcement and continuous reinforcement. Differences in mean Numeric Rating Scale (NRS) pain ratings and standard deviations between the partial reinforcement-counterconditioning group and the continuous reinforcement-counterconditioning group (N = 49) are depicted. Dots represent the (jittered) individual data points.

Time-course of attenuation

To explore the time-course and slopes of attenuation, a line graph was plotted. Figure 6 displays the time-course of attenuation between all four active groups, from the start of the first evocation and throughout the attenuation phase. During attenuation, due to the counterconditioning groups receiving lower pain stimulations than the extinction groups, the nocebo trial NRS scores of the counterconditioning groups were visibly

lower. It is also visible that this persisted as a learned effect, at the start of the second evocation, when all groups received moderate pain. This reduced nocebo response illustrates the higher effectiveness of counterconditioning in diminishing the previously induced nocebo hyperalgesia. Additionally, the attenuation slopes illustrate that participants in the partial reinforcement-extinction and partial reinforcement-counterconditioning groups consistently provided higher pain ratings than participants in the corresponding continuous reinforcement groups, despite the fact that they were receiving pain stimulations of the same intensity (low pain in the counterconditioning and moderate pain in the extinction groups). This points to a tendency for resistance to attenuation after partial reinforcement as compared to after continuous reinforcement, already during learning. However, during the second evocation, the difference between partial reinforcement and continuous reinforcement did not reach significance in the extinction groups.

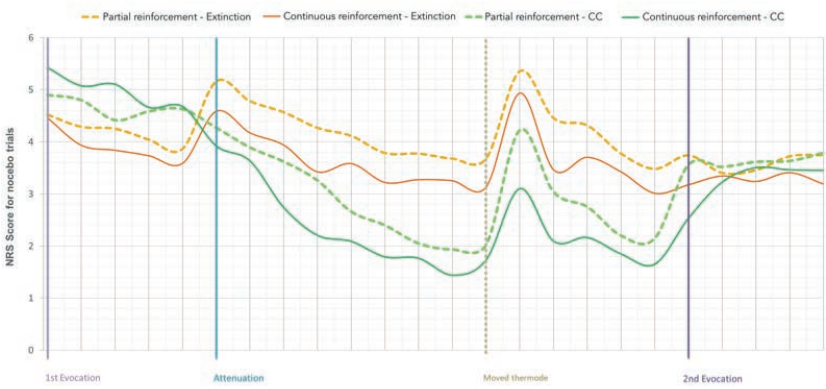

Figure 6. Pain ratings for only the nocebo trials after the end of induction, across all active conditioning groups. Numeric Rating Scale (NRS) pain ratings during nocebo trials illustrate the time-course of attenuation. The dotted vertical line indicates the thermode moving point. In evocations, all stimuli were administered at the same intensity.

Manipulation check for the control trials

Lastly, as a first manipulation check, it was assessed whether changes in the ratings of the control trials influenced the results of the attenuation phase. A 5x2 mixed model ANOVA revealed no significant differences in the NRS pain ratings for control trials before and after attenuation ($F(4,117) = 0.62$, $P = 0.64$, $\eta_p^2 = 0.02$). This result indicates that the control trials did not yield significant changes from pre- to post- attenuation and that the reduction in nocebo hyperalgesia was in fact driven by changes in nocebo responses before and after attenuation. To further examine whether control trials could have affected the attenuation results, a 2x2 mixed model ANOVA was conducted with the attenuation group as the between-subjects factor and the magnitude of nocebo hyperalgesia as the within-subjects factor with two levels (nocebo-control difference score pre-attenuation, nocebo-control difference score post-attenuation). The analysis revealed a significant interaction between the counterconditioning and extinction groups and the reduction of nocebo responses ($F(1,95) = 6.87$, $P = 0.009$, $\eta_p^2 = 0.07$), indicating significantly higher efficacy of counterconditioning compared to extinction (Figure 6), also when the control trials where included in the analysis. Figure 6 depicts time-series data for the evocation and attenuation phases and illustrates that control trials did not yield changes from pre- to post-attenuation that would impact nocebo trials.

Questionnaires

Spearman's Rank-Order Correlation analyses indicated that there was no significant relationship between the magnitude of nocebo hyperalgesia in the active groups and trait or state anxiety, pain catastrophizing, or optimism scores (STAI trait: $r = -0.07$, $P = 0.49$; STAI state pre: $r = -0.06$, $P = 0.55$; STAI state post: $r = -0.13$, $P = 0.19$; PCS: $r = -0.06$, $P = 0.54$; LOT-R: $r = -0.05$, $P = 0.59$).

Discussion

The current study compared distinct and novel methods for the induction and attenuation of nocebo hyperalgesia. We demonstrated that partial reinforcement conditioning was sufficient to induce nocebo hyperalgesia, as was continuous reinforcement conditioning. Furthermore, we showed that counterconditioning is a more potent method than extinction for the attenuation of nocebo hyperalgesia. Interestingly, our results also showed that, despite pain ratings remaining consistently higher in the partial reinforcement group compared to the continuous reinforcement group during extinction, this difference did not reach significance and resistance to extinction after conditioning with partial reinforcement was not observed. Importantly, we found that while counterconditioning was sufficient to attenuate nocebo responses irrespective of induction method, nocebo hyperalgesia was significantly more resistant to counterconditioning when induced via partial, as compared to continuous reinforcement. These findings have a number of implications related to experimental models and clinical practice.

The finding that conditioning with partial reinforcement is, albeit less potent than continuous reinforcement, sufficient to induce nocebo hyperalgesia, is in line with previous research by Colagiuri and colleagues [15]. Reproducing these results and reaffirming the potency of the more ambiguous partial learning method has important theoretical and clinical implications. Conditioning with learning schedules that provide more variable contingencies bears a closer resemblance to what nocebo theories postulate regarding the ambiguity of learning and negative suggestions in clinical contexts [4,15]. Employing a more ecologically valid paradigm can have a crucial impact on our understanding of how and why nocebo hyperalgesia may present in pain patients.

Studying the attenuation of nocebo hyperalgesia provides insights into the mechanisms that may contribute to the chronification of pain. While in the present study extinction was sufficient to attenuate nocebo hyperalgesia, counterconditioning was a more powerful intervention, reversing nocebo responses into an effect resembling placebo responses. Counterconditioning being more powerful than extinction can be explained by counterconditioning involving a paradigm that bears closer resemblance to successful exposure therapy techniques. For example, for the treatment of phobias [34,35] and anxiety [36], the initial association between the aversive stimulus and fear becomes attenuated through a procedure involving the removal of fear or threat [37]. However, in the current study, extinction entailed a reduction of pain to the levels of control (moderate) pain stimulations, rather than the entire removal of these aversive stimuli. In pain paradigms it is often impossible, both experimentally and clinically, to achieve the entire removal of the aversive stimulus during extinction. In counterconditioning however, the painful stimuli were reduced to a level that was perceived as less unpleasant in comparison even to control pain stimulations, leading to a significantly larger reduction of nocebo responses. This is in line with findings by Meulders and colleagues [20] who showed that changing the valence of aversive stimuli might improve fear reduction and potentially prevent relapse. In contrast to the frequently observed lack in effectivity and durability of extinction [34,38,39], this counterconditioning finding indicates that there may be a path to attempt more active ways of minimizing learned responses.

Nocebo hyperalgesia has consistently been found to be resistant to extinction [15,24,40], which may indicate an important mechanism of pain chronification. Moreover, research exploring the learning correlates and effectivity of conditioning with partial reinforcement has previously shown that ambiguous learning schedules produce durable conditioned effects [13,14,16,17], including previous partial reinforcement research on nocebo [15] and placebo effects [13]. In this study, we did not find a

statistically significantly larger resistance to extinction after partial, as compared to continuous reinforcement. Extinction trends pointed towards partial reinforcement resulting in more durable nocebo responses compared to continuous reinforcement, as illustrated in Figures 4 and 6, however this difference was not significant. Moreover, it was observed that during attenuation, pain reports in the partial reinforcement group did remain consistently higher than those in the continuous reinforcement group (Figure 6), despite the fact that after induction, partial reinforcement produced a significantly weaker nocebo response than continuous reinforcement. The effectivity of extinction even after partial reinforcement could be explained by the fact that exposure to extinction was longer than exposure to nocebo induction, when considering the first evocation phase. It is worth pointing out, however, that in real-word contexts, patients may be exposed to shorter periods during which nocebo hyperalgesia is acquired and longer periods of extinction. As such, the current model provides novel evidence that nocebo hyperalgesia can be extinguished over prolonged exposure to extinction, even after partial reinforcement learning.

Interestingly, a partial reinforcement resistance effect was found when attenuation involved counterconditioning. Counterconditioning was still successful in attenuating nocebo effects after conditioning with partial reinforcement, however, counterconditioning was observed to be substantially more effective after conditioning with continuous reinforcement (Table 2, Fig. 5). Importantly, this effect was observed despite the fact that partial reinforcement had resulted in a significantly weaker nocebo response. This counterconditioning-specific resistance effect could be attributed to negativity bias (i.e., the tendency to attend to or remember negative experiences over neutral or positive experiences [41–43]). According to this theory, when provided with inconsistent positive and negative information about the same stimulus, individuals are more likely to retain the negative information [44]. A negativity bias may have taken place following the ambiguous

information provided to participants in the partial reinforcement group and the formation of mixed expectations regarding the activation of the nocebo treatment. The partial reinforcement-counterconditioning group was exposed to a wider range of negative and positive suggestions and associations. It is possible that during the final evocation the negative treatment associations were retained over the positive ones. A resistance to the attenuation with counterconditioning may thus be in line with previous literature about this type of negativity bias [44]. This effect may be of important clinical relevance, as it could shed light on the etiology of pain chronification following exposure to inconsistent, mixed information and experiences. In turn, however, this means that the potency of counterconditioning following ambiguous and variable learning remains uncertain. Gaining a better understanding of the learning mechanisms underlying the process of re-writing negative associations can create great future value for counterconditioning.

One limitation of this study, as mentioned earlier, was the discrepancy in the length of induction and attenuation, which may explain why nocebo responses were not resistant to extinction. Colagiuri and colleagues [15] only applied extinction, allowed the paradigm to comprise an equal number of induction and attenuation trials. In the current study, due to our aim of comparing counterconditioning and extinction, a longer evocation phase was preferred before the start of attenuation. Participants were thus exposed to longer extinction, as compared to induction. Nevertheless, in clinical contexts and chronic pain, unequal lengths of exposure to suggestions, learning, and extinction may also exist. Future research should address the role that the time-course of induction and attenuation may play. Another limitation of this study and a common obstacle in nocebo studies, was related to the nocebo suggestions. In this novel counterconditioning approach, the suggestions had to indicate that the same treatment could increase but also decrease pain sensitivity. The suggestion that pain would be decreased by the same device that previously increased pain sensitivity

could have been confusing to participants. In future research, nocebo suggestions can be optimized by comparing different cover stories and instructions, as these are crucial for influencing expectations. A common limitation in the learning process of extinction is the return of the conditioned response, such as fear, following the passing of time [45]. The current study did not examine this effect, which is thought to result from competing learned effects and deficits in inhibitory learning and more specifically deficits in the neural regulation needed during extinction [45]. Future counterconditioning experiments could shed light on whether – and under which conditions– such a reinstatement could take place following counterconditioning. Further research into the effectivity and durability of counterconditioning is necessary to establish whether this method can provide a basis for clinical interventions targeting nocebo effects. Lastly, controlling for variables in our sample such as caffeine intake and age range, or limiting our sample to higher education students may have created potential confounding variables. In future studies, it would be important to allow for more variance in the participant sample and to collect and check data related to variables such as caffeine intake, age, and education. Overall, future studies should collect and analyze manipulation check data to see whether and how different variables can influence study outcomes.

The present study implemented a complete, clinically relevant model of nocebo hyperalgesia, from acquisition to attenuation. The findings reproduced prior evidence of ambiguous and variable learning being sufficient to induce nocebo hyperalgesia, and that this type of induction method may be more resistant to treatments. This study also provided evidence that counterconditioning is a powerful method for the attenuation of nocebo hyperalgesia. Counterconditioning, however, may be less potent in attenuating effects that have been induced by more ambiguous learning and should therefore undergo further assessment within ecologically valid experimental models.

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Chapter 5.

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

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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).

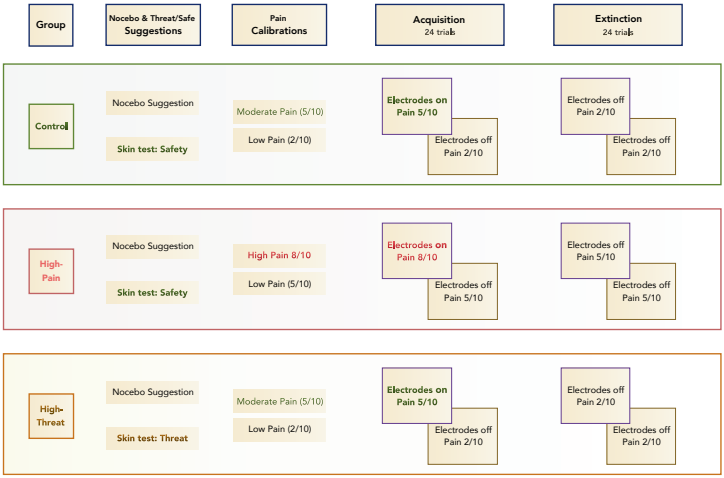

Figure 1. Illustration of the experimental design. Participants were randomly allocated to 1 of 3 groups: Control-nocebo, High-pain, High-threat. Participants in the Control group received lower pain levels during control and nocebo trials and no threat induction. Participants in the High-pain group 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.

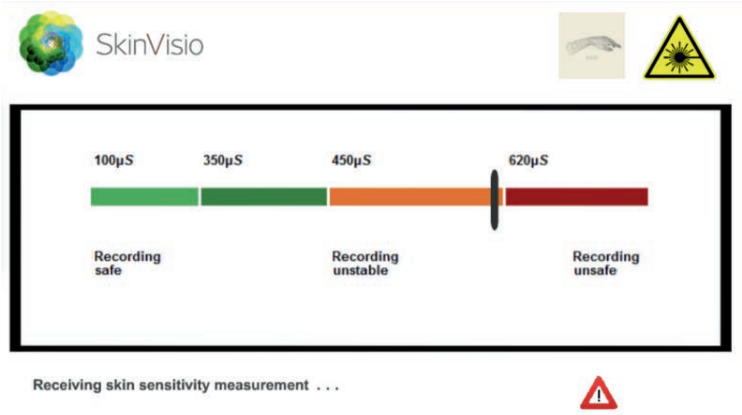

Figure 2. The mock skin sensitivity scale that participants viewed as part of the threat manipulation. The scale was displayed on a screen as an animation. For the High-threat group, the scale fluctuated within the orange and red zones. For the Control and High-pain groups, the scale fluctuated within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

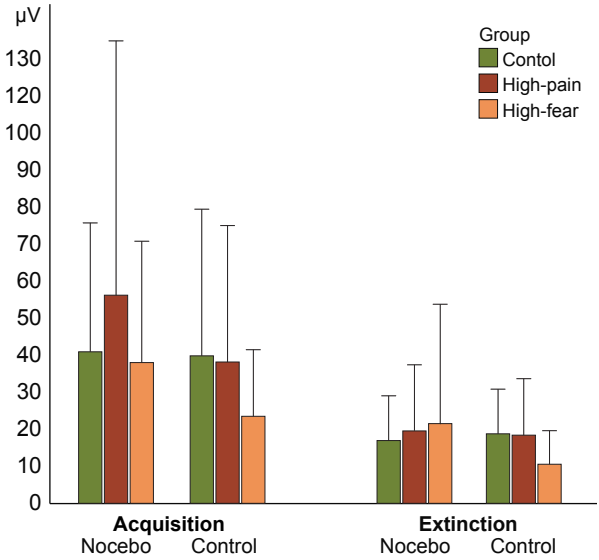

Figure 3. Means and standard deviations of startle responses as measured via electromyography. As compared to the Control group (N = 23), participants in the High-pain (N = 21) and High-threat group (N = 22) showed larger startle responses during nocebo trials as compared control trials of the acquisition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

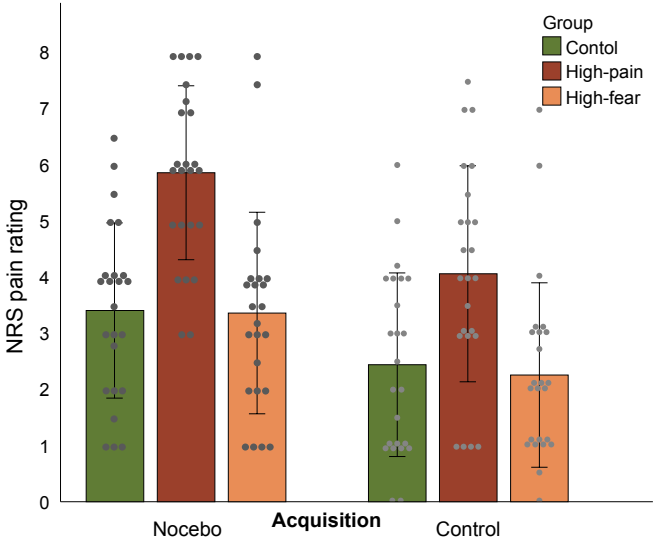

Figure 4. Acquisition of nocebo responses. Mean Numeric Rating Scale (NRS) pain ratings (sd) are depicted across all three groups (N = 72) for the first nocebo and the first control trial of extinction phase.

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**).

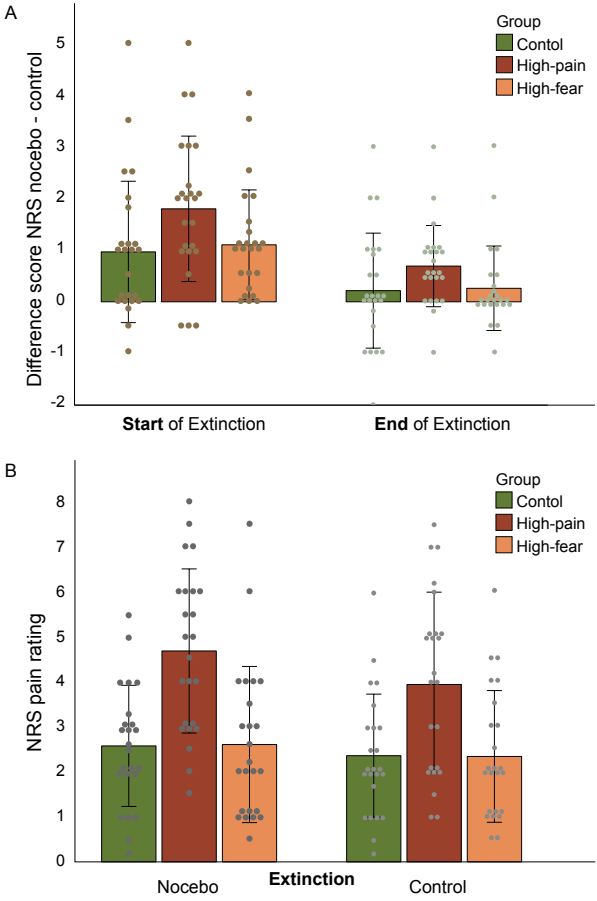

Figure 5. Extinction of nocebo hyperalgesia. A) Extinction of nocebo responses, from the start to the end of extinction. Nocebo magnitudes and standard deviations, based on Numeric Rating Scale (NRS) pain ratings, at the start and at the end extinction are depicted, between all three groups. In addition, 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.

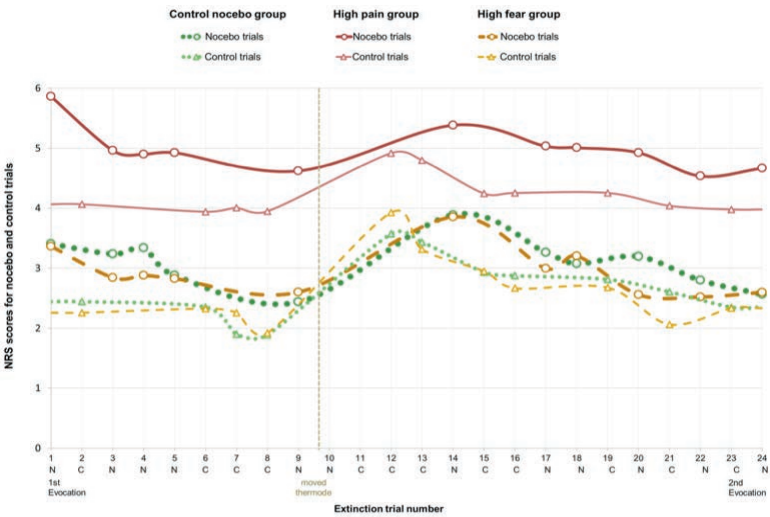

Figure 6. Pain ratings for the nocebo and control trials in the extinction phase, across all three groups. Numeric Rating Scale (NRS) pain ratings during nocebo and control trials illustrate the evocation of nocebo responses and time-course of extinction, for the Control-nocebo, High-pain, and High-threat 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]).

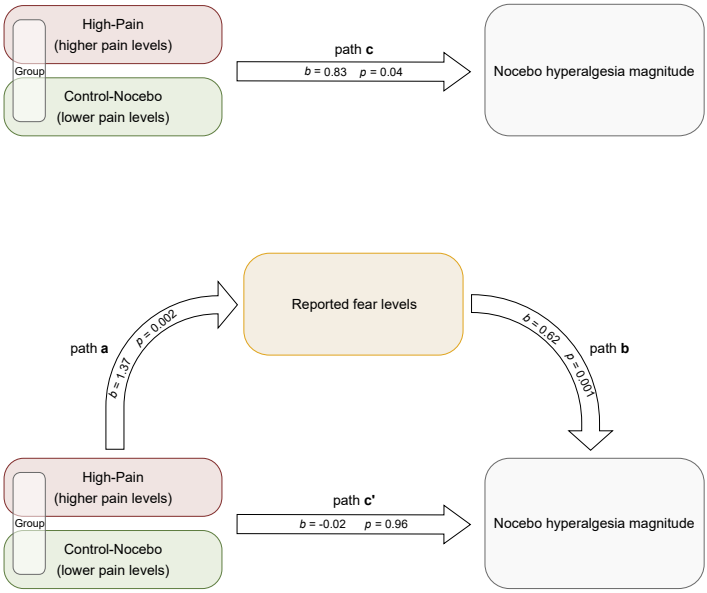

Figure 7. Diagram of the hypothesized mediation model and results. Administration of higher pain (High-pain group) compared to lower pain (Control group) significantly predicted the magnitude of nocebo responses, and this effect was mediated by reported fear levels.

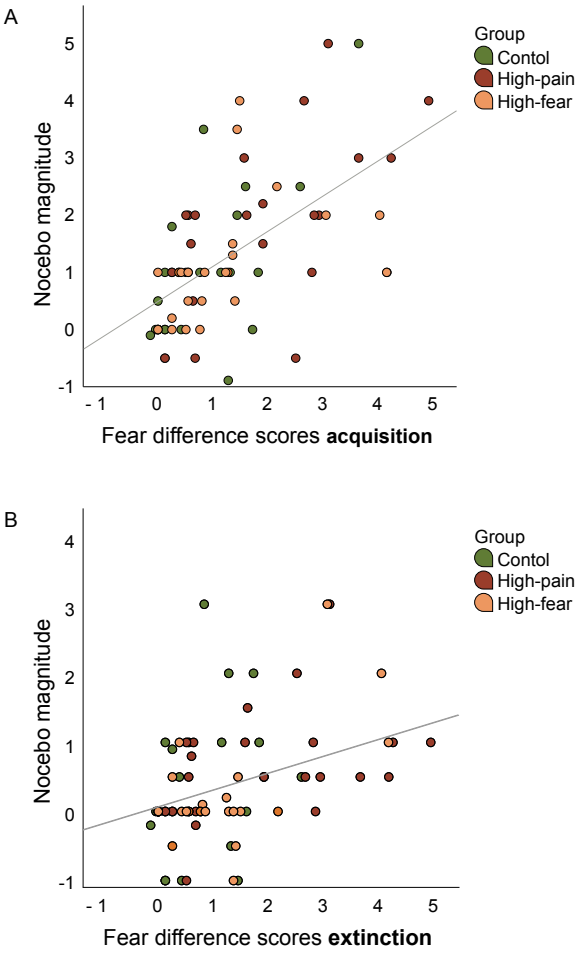

Figure 8. Correlations between the magnitude of nocebo responses and reported fear levels. A) In the acquisition phase, there was a significant high correlation across all groups between nocebo response magnitudes and reported fear levels. B) In the extinction phase, there was a significant moderate 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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Chapter 6.

Temporal structure of brain oscillations predicts learned nocebo responses to pain.

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Abstract

This study aimed to identify electrophysiological biomarkers of nocebo-augmented pain. Nocebo hyperalgesia (i.e., increases in perceived pain resulting from negative expectations) was induced in 36 healthy participants through classical conditioning and negative suggestions. In a baseline phase, participants received high thermal pain stimulations. During acquisition, participants learned to associate an inert gel applied to their forearm with high pain, relative to a moderate intensity control stimulus administered without gel. During evocation, nocebo and control stimuli were both accompanied by moderate pain to measure nocebo responses. Electroencephalography was recorded during rest (pre and post nocebo acquisition) and during pain stimulation (baseline, nocebo acquisition and evocation). Nocebo hyperalgesia led to pre- to post-acquisition increases in long-range temporal correlations (LRTC), with beta-band alterations being negatively associated with nocebo magnitudes. Moreover, individuals with strong LRTC at rest showed larger nocebo responses than those with weaker LRTC. Nocebo acquisition trials showed reduced alpha power. Alpha power was higher while LRTC were lower during nocebo-augmented pain, compared to baseline. By involving LRTC, these findings support nocebo learning theories and highlight a role of nocebo-induced cognitive processing. This study provides novel insights into neural underpinnings of nocebo hyperalgesia, a phenomenon that greatly impacts the experience of pain.

Introduction

The experience of pain varies widely between and within individuals and can be shaped by cognitive processes such as learning. Nocebo hyperalgesia, a worsening in perceived pain attributed to negative expectations, demonstrates that learning can be detrimental for the experience of pain [1–3]. Memories and negative expectations may directly impact pain processing [4,5], yet it remains unclear which specific processes are involved in cognitive pain reappraisal and how negative expectations may shape physiological characteristics of pain.

Electroencephalography (EEG) can be used to identify physiological markers of phenomena that include cognitive components [6,7] such as nocebo effects. EEG has been used in cognitive and pain research and has largely focused on spectral characteristics of brain oscillations, with evidence indicating that expectations [8,9] and cognitive pain regulation [10,11] are reflected through alterations in the alpha and beta power bands. Concurrently, EEG research has shown that gamma oscillations are involved in associative learning [12] and encoding of ongoing pain [13]. Alpha and gamma oscillations may also act in synergy during the cognitive stages of nociceptive processing [14]. How EEG measures within these frequency bands relate to pain and cognitive processing under hyperalgesic conditions remains unclear.

Electrophysiological research into nocebo effects has been scarce and has mainly focused on the power spectrum of oscillations [15–19]. However, in order to more precisely pinpoint cognitive processes involved in nocebo, it may be valuable to utilize sophisticated EEG biomarkers such as Detrended Fluctuation Analysis (DFA), a component that quantifies long-range temporal correlations (LRTC) between oscillating groups of neurons and determines how oscillation

amplitudes change over time [20]. Higher LRTC generally indicate higher complexity of neural activity and have accordingly been shown to play a role in cognitive processes such as attention and cognitive reappraisal [20–22]. Decreases in LRTC of oscillations have been found in schizophrenia [23] and Alzheimer's disease[24], with both disorders being characterized by cognitive deficiencies. Moreover, strong LRTC of beta and gamma oscillations have been associated with poor sustained attention performance [25]. Despite its evident and intricate relationship to cognitive processing, complexity of brain activity has never been tested under nocebo hyperalgesic conditions.

As described, we based this study on earlier findings relating to changes in (resting-state) oscillatory power in the alpha band. Additionally, we aimed to explore nocebo correlates relating specifically to LRTC of brain oscillations during active pain states throughout the experiment. We expected that the magnitude of induced nocebo hyperalgesia would be positively correlated to pre- to post-acquisition LRTC alterations in the alpha band, while we expected the opposite relationship in the beta and gamma bands. Furthermore, we expected that the experience of control versus nocebo trials during the acquisition and evocation phases would be characterized by divergent EEG biomarker values. Additionally, we expected that the experience of nocebo-augmented pain and baseline high-pain stimulations would be characterized by divergent EEG biomarker values. Finally, we explored the relationship between pain-related psychological characteristics and measures of EEG.

Materials and methods

Participants

Participants of either sex were enrolled in this study. The required sample size for the primary analysis was calculated based on a previous nocebo study [18] that induced nocebo hyperalgesia on thermal pain by use of conditioning, in an MEG paradigm. This study was used merely as an indicator of an appropriate sample size for this comparable study, in lack of a more fitting study to base a power analysis on. Tu et al. (2019) found that a decrease in alpha band connectivity predicted the magnitude of conditioned nocebo hyperalgesia ($r = 0.46$, $p = 0.04$). The power analysis was conducted in G*power 3.1 [26] for our primary hypothesis. Alpha error probability was set at $\alpha = 0.05$, and desired power was set at 0.80. With r of 0.46, the sample size indicated was 36 participants. A replacement protocol was used for excluded participants.

Inclusion criteria were: age between 18 and 35 years, a good understanding of the English language, and (corrected to) normal vision and hearing. Exclusion criteria were pregnancy or breastfeeding, any pain on the day of testing, having recent injuries on the arms, painful health conditions experienced in the past 6 months, ever having experienced chronic medical or psychiatric conditions, and having consumed psychotropic or analgesic medication, recreational drugs, or more than 3 units of alcohol, in the 24 hours prior to the study appointment. Testing of included participants was discontinued in the case that they would be determined to have too high of a pain threshold (i.e., when thermode maximum temperatures were not sufficient to induce at least moderate pain) or when they would not reliably report a difference (a mean of at least 1.5 on the NRS) between the administered temperatures for control and nocebo trials in the acquisition phase. Participants were recruited through the online website Sona (Sona Systems, Tallinn, Estonia). Study participation involved a 3-hour

recording session at a laboratory of the Faculty of Social and Behavioral Sciences of Leiden University, the Netherlands. All participants provided written informed consent prior to participation. After completing the experiment, all participants were reimbursed by either study credits or cash. The study was approved by the Leiden University Psychology Research Ethics Committee (CEP19-1031/532; all methods and procedures were performed in accordance with the relevant guidelines and regulations) and pre-registered on ClinicalTrials.gov (NCT04199858, 16/12/2019; planned analyses of frequency biomarkers were not conducted due to the scope of this paper).

Experimental design

This study utilized a within-subjects design. All participants underwent 1) a calibration phase, 2) a baseline phase, and a nocebo phase comprising 3) nocebo acquisition and 4) nocebo evocation (**Fig. 1a**). During the first phase, calibrations for warmth and pain perception were conducted. During the baseline phase, moderate- and high-pain stimuli were administered. During nocebo acquisition, a conditioning procedure took place, in which associations were learned between the nocebo treatment and higher pain. Participants were conditioned to associate a sham pain-increasing gel with high (increased) pain stimulations, and no gel (control) with moderate-pain stimulations. During nocebo evocation, these learned associations were tested.

Thermal pain application

Thermal pain stimuli were delivered to the volar forearm using a Thermal Sensory Analyzer with a 3×3 cm ATS thermode probe (TSA-

II; Medoc Advanced Medical Systems, Ramat Yishai, Israel). During the calibrations and baseline phases, both arms were used for pain stimulations. During the nocebo phase, only the right arm was used (**Fig. 1b**). Throughout the experiment, pain intensities were rated on a numeric rating scale (NRS) ranging from 0 (no pain) to 10 (worst pain imaginable in this context).

Sensory and pain thresholds

Before the start of the experimental phases, warmth and pain threshold levels were tested for each participant, heat stimuli were applied and participants were asked to indicate the first moment at which they perceived warmth and pain. After a practice trial for each, the average of 3 warmth detection values and 3 heat pain detection values determined the thresholds for warmth and pain, respectively. This method follows published standardized procedures [27].

Pain calibration protocol

Pain calibrations were conducted in order to determine the temperatures that would induce moderate and high pain during baseline and nocebo phases. The calibrations were individually tailored, based on the NRS ratings of 16 heat stimuli of varying intensities. Throughout the experiment, each pain stimulus was initiated from a 32°C baseline, increased to a target temperature, and presented for 10 seconds at plateau. The ramp up and return rates were 8°C per second. During calibrations the inter-stimulus interval (ISI) was 5 seconds, during which NRS pain ratings were given. Median temperatures rated as NRS 3 to 5

were used to induce moderate pain and median temperatures rated as NRS 6 to 8 were used to induce high pain.

Baseline, acquisition, and evocation phases

During baseline, 2 moderate and 6 high pain trials were administered on both arms, with an ISI of 5 seconds. During acquisition, 16 nocebo and 16 control stimuli were administered in alternating order. During evocation, 8 nocebo and 8 control stimuli were administered in alternating order. During nocebo acquisition and evocation, the ISI was 10 seconds. In all phases the thermode was moved to a more proximal site on the arm after each pain trial, in order to avoid habituation or sensitization to heat-pain.

Nocebo manipulation

A commercial moisturizing gel that was given the name "Trans-Dermal Aspartate" or "TDA" was used as the nocebo treatment in the procedure; participants were told it was a capsaicin gel used on the skin for research purposes only. Half of the participants received the gel from a blue jar and the other half from a brown jar, both featuring sham pharmaceutical labels. Negative suggestions were used to create expectations regarding the pain enhancing effects of the gel. Participants were told that the gel is a capsaicin-based gel that is known for its pain-increasing properties. Participants' arms were marked with medical tape to create four 3x3 cm thermode-placement sites on both arms. Prior to the start of the acquisition phase, the gel was rubbed into the two nocebo sites (the first and third most proximal sites on the right arm). Messages displayed on a computer screen via E-Prime 2.0 (Psychology Software

Tools, Pittsburgh, PA, USA) indicated whether a trial was on a gel site or on a control site. The messages read "Trans-Dermal Aspartate, pain-increasing capsaicin, gel form" or "Control trial, no gel".

During nocebo acquisition, the nocebo gel was paired to surreptitiously increased pain stimulations during nocebo trials, while moderate pain was delivered during control trials. During nocebo evocation, all pain stimuli during both nocebo and control trials were applied at moderate intensity, to study whether evoked conditioned responses were elicited. Increased pain reports for a nocebo trial as compared to its preceding control trial in this phase indicated nocebo hyperalgesia.

EEG materials

EEG recordings were conducted using the ActiveTwo BioSemi (Amsterdam, the Netherlands) electrode system from 32 scalp electrodes. As reference electrodes, BioSemi replaces the ground electrodes that are used in conventional systems with two additional electrodes. The Common Mode Sense active electrode and Driven Right Leg passive electrode form a feedback loop, which drives the average potential of the participant as close as possible to the reference voltage of the analog-to-digital converter, thus rendering them references. Data was acquired at a sampling rate of 1024 Hz, band-passed filtered online during acquisition from 0.1 to 100 Hz (with a 100 Hz low-pass and 0.01 Hz high-pass hardware filter). Electrodes were placed on the scalp according to the international 10–20 system and where possible, electrode impedances were kept below 20 kOhm.

Questionnaires

Three questionnaires were used to measure baseline differences in psychological characteristics. The questionnaires were completed by participants prior to their lab visit. Total scores were used for the following questionnaires: The Pain Catastrophizing Scale (PCS; Sullivan, Bishop, and Pivik, 1995), the Fear of Pain Questionnaire (FPQ-III; McNeil and Rainwater, 1998), and the Experience of Cognitive intrusions on Pain scale (ECIP; Attridge et al., 2015). At the end of the experiment, participants also completed an exit questionnaire containing manipulation check questions, assessing, for example, whether participants understood the instructions. All questionnaires, as well as a debriefing form, were displayed via web-based survey software (Qualtrics, Provo, Utah, USA).

Experimental procedure

Before the day of testing, participants completed a brief online screening as well as the psychological questionnaires. On the day of the testing session, participants received further information about the procedures and provided written informed consent. Then, participants completed a brief screening for inclusion and were provided with information about the EEG and the (sham) pain-enhancing effects of the nocebo gel. EEG caps were then mounted, electrolyte gel was applied (SignaGel, Parker laboratories Inc., Fairfield, New Jersey, USA) and the scalp electrodes were placed. Warmth and pain threshold levels were then tested and individual pain stimuli were calibrated. Thereafter, continuous EEG recording started and the baseline phase was completed. Participants then completed a 5-minute resting-state recording with their eyes closed. Then, participants underwent nocebo acquisition and evocation. Subsequently, participants completed a second 5-minute resting-state recording. After the end of the experiment, participants completed the

exit questionnaire. Finally, a debriefing was conducted and participants were reimbursed for their participation.

Data handling

Analyses of behavioral data were performed for descriptive purposes and to confirm that a significant nocebo effect was induced. Next, specific hypotheses were tested, starting with resting-state EEG data. For all hypotheses, we looked at the frequency bands of interest (alpha, beta, and gamma) and two EEG parameters of interest (oscillatory power and Detrended Fluctuation Analysis). Our primary hypothesis was that there would be pre- to post-acquisition decreases in LRTC in the alpha band, given the role of alpha oscillations in pain processing as well as previous findings regarding the role of oscillatory complexity in cognitive functions. To test this, we assessed how nocebo acquisition affected EEG parameters during rest by examining differences from before to after nocebo acquisition. We then examined whether direct links could be observed between nocebo-induced changes in resting-state brain activity (pre- to post-acquisition) and the magnitude of induced nocebo hyperalgesia, with the aim to identify resting-state biomarkers of nocebo hyperalgesia. For this purpose, we correlated any pre- to post-acquisition changes in EEG parameters with the magnitude of reported nocebo hyperalgesia. We then examined EEG parameters during the experience of pain stimulations. We first asked whether the experience of control and nocebo trials during the acquisition and evocation phases would be characterized by divergent EEG biomarker values. We then focused on potential differences in brain activity during the experience of high pain at baseline and the experience of heightened pain under nocebo hyperalgesic conditions (i.e., when lower pain stimulation is perceived as high pain, during nocebo evocation). We thus compared the experience of baseline high-pain stimulations and nocebo-

augmented pain to establish whether they are characterized by divergent EEG biomarker values. Finally, we explored the correlation between pain-related psychological questionnaires and measures of EEG.

Nocebo manipulation check

The magnitude of reported nocebo hyperalgesia was measured within-subjects, and was defined as the difference in pain ratings for the first nocebo trial compared to the first control trial, during evocation. The first evocation trials were selected to answer the manipulation-check question of whether significant nocebo hyperalgesia was induced, as previous studies indicate the effect to be clearest in those trials [31,32].

Behavioral data handling

Behavioral data were analyzed by use of SPSS 23.0 (IBM Corp., Armonk, NY, USA). The threshold for significance was set at $P < 0.05$ and partial eta-squared (η_p^2) was computed as a measure of effect size, with η_p^2 of 0.01 considered small, 0.06 considered medium, and 0.14 considered a large effect size [33,34]. To conduct repeated measures analysis of variance (ANOVA), the assumptions of normality and homogeneity of variances were checked.

Computation of EEG biomarkers

Spectral and temporal biomarkers were computed for all EEG recordings within three canonical frequency bands: alpha (8–13 Hz),

beta (13–30 Hz) and gamma (30–45 Hz). To quantify local neural dynamics associated with resting-state brain activity and pain responses in our nocebo paradigm, spectral power was computed for all EEG electrodes using the *Welch* method implemented in Matlab. Relative power was computed as the relative contribution of power within a narrow band to the integrated power within the range 1–45 Hz. To investigate whether temporal structure of the EEG changed at rest and during pain responses in our nocebo paradigm, the amplitude envelope was extracted using the Hilbert transform and DFA was computed to quantify LRTC of neuronal oscillations [20–22]. DFA quantifies the rate at which auto-correlations of amplitude modulations decay within a signal, with the power-law exponent ranging from 0.5 (uncorrelated) to 1.0 (strong auto-correlations). Signals were filtered using a FIR-filter with a Hamming window with a length corresponding to two f_1 Hz cycles for a given frequency band [f_1, f_2]. To minimize artificial auto-correlations introduced by the FIR-filter, DFA was fitted in the interval from 4 to 20 seconds for alpha band and 2 to 20 secs for beta and gamma bands [20].

EEG processing

MATLAB 2020a (The MathWorks Inc., Natick, MA, 2014) was used for EEG preprocessing and analysis. Continuous EEG recordings were imported and preprocessed using EEGLAB [35], and analyzed using custom-made scripts from a MATLAB toolbox developed at Vrije Universiteit Amsterdam (VU). All signals were visually inspected for artifacts in windows of 10 seconds. Noisy channels (e.g., with no or bad conductance to the scalp) and segments containing transient artifacts were removed. Next, recordings were re-referenced to the average BioSemi reference. Independent Component Analysis (ICA) was used to project signals to components that are maximally independent from each other [36,37]. Eye components were rejected. Continuous EEG

recordings were segmented into conditions by pasting together all epochs of a single condition. Segmentation was done using markers of the following conditions: baseline moderate-pain stimulations (10 seconds each), baseline high-pain stimulations (10 seconds each), first eyes-closed rest (ECR1; 5 minutes), control acquisition stimulus (10 seconds each), nocebo acquisition stimulus (10 seconds each), control evocation stimulus (10 seconds each), nocebo evocation stimulus (10 seconds each), and second eyes-closed rest (ECR2; 5 minutes). Exclusion of certain segments (for example, segments that were too short for DFA computation) resulted in a varying number of participants across analyses and figures.

Statistical analysis

EEG biomarkers were computed and tested per EEG-channel for all 32 channels. Non-parametric paired Wilcoxon signed-rank test was used to test for differences between each two conditions. Multiple-comparison corrections were performed using a False Discovery Rate procedure (FDR) with $q = 0.05$ [35,36]. For the Wilcoxon signed-rank test, we reported the median of the two conditions tested, the *Z*-value and the *P*-value. To test for associations between EEG biomarkers and behavioral outcome measures, we calculated Spearman's rank correlation coefficient (r_s). On all spatial topographies, open white circles reflect statistical significance at $P < 0.05$, whereas closed white circles indicate statistical significance after FDR correction. Since some statistical effects were widespread across the cortex and others were localized above specific brain areas, we report statistics of the whole-brain average and additionally report statistics of specific electrodes in case of localized effects.

Results

Participants and pain reports

Thirty-nine participants were enrolled in this study and underwent calibration, conditioning, and evocation of nocebo hyperalgesia **(Fig. 1a, b)**. Testing of three participants was discontinued: one due to technical difficulties, one for experiencing discomfort and headache during testing, and one for not reporting differences in experienced pain between acquisition control and nocebo trials. A total of 36 participants (25 female) were included in final analyses. Mean warmth detection threshold across participants was 33.7°C (standard deviation; SD = 0.7) and mean pain threshold was 41.9°C (SD = 3.2). Mean temperatures used to induce moderate and high pain were 46.6°C (SD = 0.8) and 48.1°C (SD = 0.5), respectively. At baseline, mean NRS pain rating for control trials was 4.4 (SD = 1.7), while mean pain rating for nocebo trials was 7.4 (SD = 1.2). During nocebo acquisition, mean pain rating for control trials was 3.9 (SD = 1.8) and mean pain rating for nocebo trials was 7.3 (SD = 1.4). Conditioning of pain during the acquisition phase successfully induced negative associations with the gel and evoked nocebo responses during evocation **(Fig. 1c)**. A repeated measures ANOVA was conducted with trial type as within-subjects factor with two levels (first evocation nocebo trial, first evocation control trial), to establish whether significant nocebo hyperalgesia was induced. There was a significant difference between NRS reports for the first nocebo and first control trial of the evocation phase (F (1,35) = 27.44, p = 0.000008, η_p^2 = 0.44) indicating the presence of nocebo hyperalgesia.

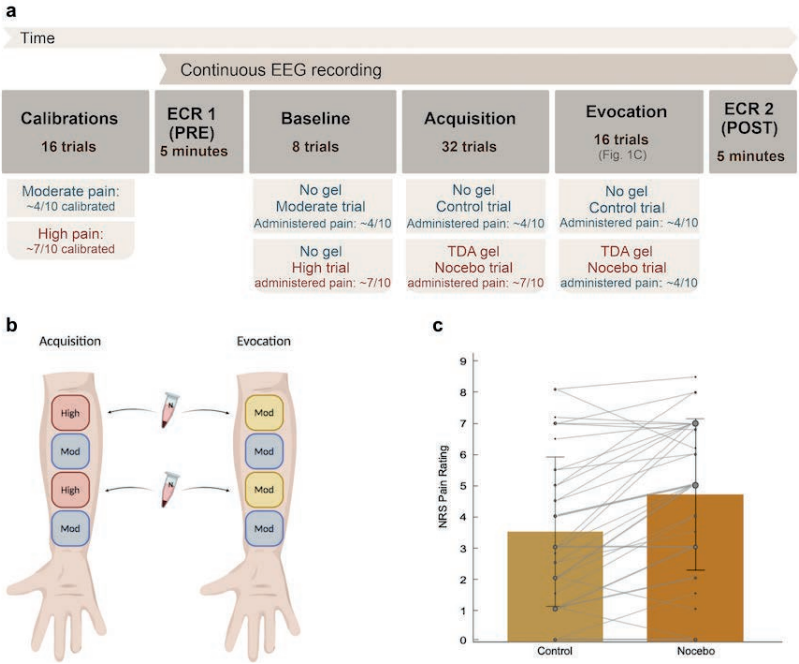

Figure 1 - Experimental protocol and induced nocebo effect. **(a)** At the start of the recording, participants completed a first (PRE) resting-state, received (baseline) moderate and high pain stimulations, and underwent nocebo acquisition via conditioning and verbal suggestions, nocebo evocation, and a second (POST) resting-state. Blue and red fonts indicate the lower and higher pain conditions, respectively. ECR, Eyes-Closed Rest; PRE, Pre-acquisition; POST, Post-acquisition; TDA, Trans-Dermal Aspartate (sham hyperalgesic gel). Approximate pain in **1a** represents the moderate and high pain stimulations administered during acquisition and evocation, while **1c** represents reported pain during moderate pain in evocation. (**b)** Application sites where either no gel or sham hyperalgesic gel "TDA" was applied. **(c)** Manipulation-check results showing the pain ratings (and SD) for first nocebo and first control trials of the evocation phase for all participants (n = 36).

Pre- to post-acquisition changes in LRTC are negatively associated with the magnitude of induced nocebo hyperalgesia

We asked whether differences in EEG due to nocebo conditioning were associated with magnitude of nocebo hyperalgesia **(Fig. 2)**. Our primary hypothesis was that pre- to post-acquisition differences in the alpha band would be associated with magnitudes of induced nocebo hyperalgesia. There was no significant association between change in resting-state alpha power from pre- to post-acquisition and magnitude of nocebo hyperalgesia (mean across electrodes, r_s = -0.04, p = 0.85). We then looked more broadly at spectral and temporal biomarkers in alpha, beta and gamma bands to test for associations with the magnitude of nocebo hyperalgesia. Long-range temporal correlations were negatively associated with induced nocebo hyperalgesia for beta band for one lead (Electrode PO4, r_s = -0.43, p = 0.02) **(Fig. 2b)**, but not for alpha and gamma bands (**Fig. 2a, c**).

LRTC of neuronal oscillations during rest predict pain response to nocebo treatment

We then asked whether resting-state EEG parameters can predict magnitude of nocebo hyperalgesia. There was no association between DFA and magnitude of nocebo hyperalgesia within the alpha band (r_s = 0.25, p = 0.18; **Fig. 2d**). Nocebo hyperalgesia was significantly positively correlated with DFA of beta (r_s = 0.51, p = 0.003) (**Fig. 2e**) and gamma band (r_s = 0.47, p = 0.008) (**Fig. 2f**). These results show that individuals with strong LRTC during rest have a larger nocebo effect than individuals with weak LRTC. Since stronger LRTC reflect more complex neural dynamics, these findings indicate that people with more complex brain activity are more susceptible to the acquisition of nocebo.

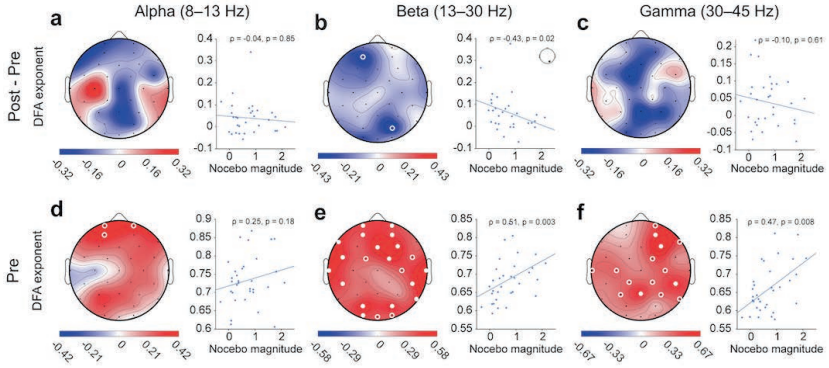

Figure 2. Complexity of neuronal oscillations at baseline predicts pain response to nocebo treatment. Spatial topographies show Spearman's rank correlation coefficient values (rho) of magnitude of nocebo hyperalgesia and EEG measures (n = 33). Magnitude of nocebo hyperalgesia was defined as the difference between mean pain response of all nocebo trials and all control trials during the evocation phase, per individual. Top row shows the association between magnitude of nocebo hyperalgesia and EEG parameters for the difference condition ECR_{POST} – ECR_{PRE}. Bottom row shows the correlation of nocebo hyperalgesia with EEG condition ECR_{PRE}. (**a-c**) Magnitude of nocebo hyperalgesia was negatively associated with DFA within beta and gamma bands. (**d-f**) Individuals with high DFA beta and gamma at baseline (ECR_{PRE}) show a larger nocebo effect during evocation. Red colors indicate positive correlations, whereas blue colors indicate negative correlations. Open white circles show statistical significance at $P < .05$. Closed white circles indicate significance after correcting for multiple comparisons using a False Discovery Rate procedure (FDR) with $q = 0.05$, per topography.

Nocebo conditioning suppresses power of alpha and beta oscillations

Next, we assessed whether parameters of resting-state EEG are altered during nocebo acquisition. To this end, non-parametric paired Wilcoxon signed-rank tests were conducted to compare differences in power and DFA between nocebo and control trials during the induction phase of the study (**Fig. 3a-c; Table 1**). Relative power of alpha oscillations was significantly lower during nocebo compared to control trials, in particular above parietal and occipital regions (Electrode PO3, $Z = 2.73$, $p = 0.0064$) (**Fig. 3d**). Relative power beta was significantly lower during nocebo than during control trials above central regions (Electrode Cz,

$Z = 3.05$, $p = 0.0023$) (**Fig. 3e**). There were no significant differences in relative power gamma between nocebo and control trials after multiple comparisons correction ($Z = -1.53$, $p = 0.13$) (**Fig. 3f, g**). There were no significant differences in LRTC between nocebo and control trials within alpha ($Z = -0.35$, $p = 0.73$), beta ($Z = -0.79$, $p = 0.43$) and gamma bands ($Z = -1.43$, $p = 0.15$) after multiple comparisons correction (**Fig. 3h-k**). We then asked whether neurophysiological changes in spectral power were also observed during the evocation phase of the study. No significant differences were observed between nocebo and control trials in the evocation phase (**supplementary** material).

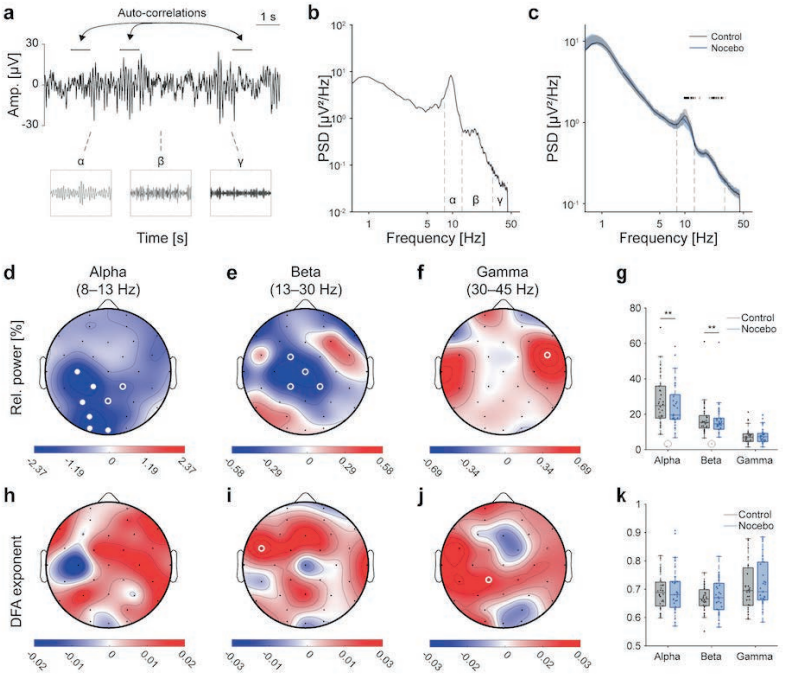

Figure 3. Oscillatory power of alpha and beta oscillations is suppressed during conditioning of nocebo hyperalgesia. **(a)** An EEG signal consists of brain oscillations with

power varying across frequency. Long-range temporal correlations were quantified using Detrended Fluctuation Analysis (DFA), which measures autocorrelations within a signal over time. **(b)** Power spectral density (PSD) was computed using Welch's method with a Hamming window and a frequency resolution of 0.125 Hz. Frequency bands were defined as alpha (8–13 Hz), beta (13–30 Hz) and gamma (30–45 Hz). **(c)** Mean PSD of control (grey line, $n = 34$) and nocebo trials (blue line, $n = 34$) plotted in log-log scale. Wilcoxon signed-rank test was used to test for statistical significance between control and nocebo trials, per frequency bin. Black bars indicate frequency bins with $P < .05$. Shaded areas indicate standard error of the mean. **(d-f)** Difference in relative power alpha (d), beta (e) and gamma band (f) for nocebo minus control trials, mean of all subjects. Open white circles show statistical significance at $P < .05$. Closed white circles indicate significance after correcting for multiple comparisons (FDR) with $q = 0.05$, per topography. **(g)** Boxplots for relative power alpha. (**h-j**) Difference in DFA (h), beta (i) and gamma band (j) for nocebo minus control, mean of all subjects. (**k**) Boxplots for DFA.

LRTC and alpha power differentiate nocebo pain from pain at baseline

Our next question was whether these differences in and associations with LRTC of beta and gamma oscillations were present only during rest or if they also reflected nocebo hyperalgesia. To this end, Wilcoxon signed-rank tests were used to compare power and DFA of high pain at baseline EEG measurement with nocebo trials during the evocation phase. (**Fig. 4, Table 2**). Compared to baseline high pain, relative power within the alpha band was significantly higher during nocebo pain ($Z = -3.5$, $p = 0.0004$) (**Fig. 4a**). Relative power of gamma oscillations was lower during nocebo pain than during baseline high pain ($Z = 3.3$, $p = 0.001$) (**Fig. 4c**). Relative power within the beta band was not significantly different between nocebo during evocation and baseline high pain ($Z = 0.5$, $p = 0.61$) (**Fig. 4b**). DFA was higher during nocebo pain than during baseline high pain for alpha oscillations above frontal and parietal regions, however, not significant after FDR-correction and not significant for the whole-brain average ($Z = -1.31$, $p = 0.19$) (**Fig. 4g**). DFA was lower during nocebo pain than during baseline high pain for beta ($Z = 3.14$, $p = 0.002$) and gamma band ($Z = 3.76$, $p = 0.0002$) (**Fig. 4h-i**). These results indicate that power within the alpha band was

higher during nocebo pain compared to baseline high pain. Interestingly, gamma power and LRTC were lower, suggesting that complexity of neuronal oscillations is lower, during nocebo-augmented pain compared to high pain at baseline. Indeed, based also on the results above, the complexity of neuronal oscillations seems to increase, from resting-state, to nocebo-augmented pain, to high administered pain.

No significant relationship between questionnaire scores and EEG biomarkers

Finally, we expected that there would be a relationship between scores on pain-related questionnaires and measures of EEG. Spearman's rank order correlations were conducted between total scores on each of the questionnaires (FPQ, PCS, and ECIP) and changes in resting-state EEG biomarker values from before to after nocebo induction. After correcting for multiple comparisons, no significant correlations were found between questionnaire scores and any of the biomarker values in any clusters of electrodes (**supplementary** material).

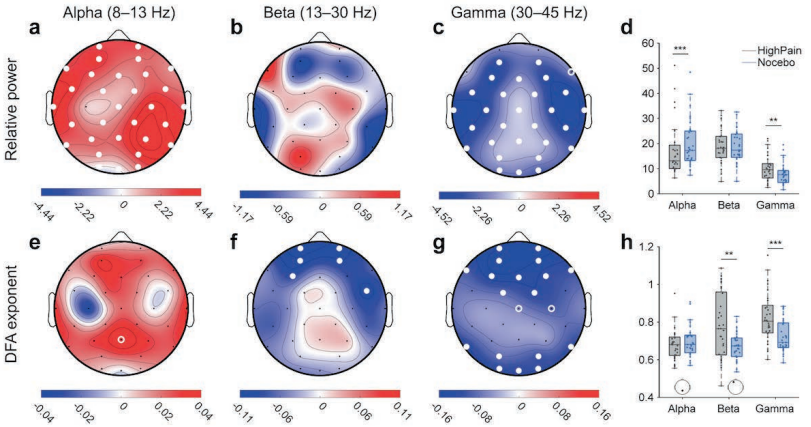

Figure 4. LRTC of beta and gamma oscillations differentiate nocebo pain from high pain at baseline. Spatial topographies show the mean difference NoceboEvocation - BaselineHighPain for all subjects (n = 33; **a-d**) Relative power of alpha oscillations was significantly higher, whereas relative power of gamma oscillations was significantly lower, during nocebo pain, compared to high pain at baseline. (**e-h**) DFA of alpha oscillations was significantly higher above frontal and parietal areas. DFA of beta and gamma oscillations – in particular above frontal regions – was significantly lower during nocebo pain compared to high pain at baseline. Open white circles show statistical significance at $P < .05$. Closed white circles indicate significance after correcting for multiple comparisons (FDR) with $q = 0.05$, per topography. All boxplots show the mean across all electrodes.

Table 1. Summary of statistics for differences in EEG parameters (nocebo/control) conditioning (Figure 2).

EEG parameter	Electrode	Mdn_{CONT}	Mdn_{NOC}	Z	p
Relative power alpha	WBA	17.2 ± 1.77	17.04 ± 1.58	1.75	0.08
	PO3	**24.88 ± 2.36**	**19.53 ± 2.12**	**2.73**	**0.0064**
Relative power beta	WBA	17.86 ± 1.13	18.55 ± 1.07	-0.20	0.84
	Cz	**15.42 ± 1.60**	**14.6 ± 1.56**	**3.05**	**0.0023**
Relative power gamma	WBA	6.99 ± 0.75	7.33 ± 0.70	-1.53	0.13
DFA alpha	WBA	0.69 ± 0.01	0.68 ± 0.01	-0.35	0.73
DFA beta	WBA	0.68 ± 0.01	0.69 ± 0.01	-0.79	0.43
DFA gamma	WBA	0.69 ± 0.01	0.69 ± 0.01	-1.43	0.15

Note: Rows show EEG parameters, columns show the median whole-brain average value across subjects for control and nocebo trials, Z- and P-value corresponding to the signed-rank test. The median EEG parameter value for each group is reported with the SE.

Table 2. Summary of statistics for differences in EEG parameters between nocebo during evocation and baseline high pain shown in Figure 4.

EEG parameter	Electrode	Mdn_{BHP}	Mdn_{NOC}	*Z*	*p*
Relative power alpha	**WBA**	**13.91 ± 1.84**	**19.11 ± 1.72**	**-3.51**	**0.0005**
Relative power beta	WBA	19.24 ± 1.19	17.83 ± 1.13	0.51	0.61
Relative power gamma	**WBA**	**9.37 ± 0.92**	**5.96 ± 0.79**	**3.28**	**0.001**
DFA alpha	WBA	0.68 ± 0.01	0.69 ± 0.01	-1.31	0.19
DFA beta	**WBA**	**0.73 ± 0.01**	**0.68 ± 0.01**	**3.14**	**0.0017**
DFA gamma	**WBA**	**0.81 ± 0.02**	**0.72 ± 0.01**	**3.76**	**0.0002**

Note: Wilcoxon signed-rank tests were performed on the whole-brain average per subject (computed as mean of all electrodes). Rows show EEG parameters, columns show the median whole-brain average value across subjects for control and nocebo trials, Z- and P-value corresponding to the signed-rank test. The median EEG parameter value for each group is reported with the standard error of the mean. BHP: Baseline high pain, NOC: Nocebo trials during evocation. Bold font weight indicates significance at $P < 0.05$.

Discussion

This study provides a novel characterization of the electrophysiological phenotype of nocebo hyperalgesia using EEG. Spectral and temporal dynamics of brain oscillations were studied at baseline, during resting-state pre- and post- measurements and during nocebo acquisition and evocation. The main findings of this study are (*i*) a negative correlation between LRTC of beta oscillations and the magnitude of nocebo hyperalgesia, (*ii*) a positive correlation between baseline LRTC and magnitude of nocebo hyperalgesia, (*iii*) alpha and beta power suppression during nocebo conditioning, and (*iv*) biomarker differences between the experience of high pain at baseline and the experience of nocebo-augmented pain.

In previous research, reduction in LRTC of oscillations has been reported in the alpha and beta bands in patients with cognitive disorders [23,24,38], while other studies link increased LRTC to reduced attention or cognitive performance [22,39–42]. LRTC characterize neuronal systems that require rapid reorganization and responsiveness to changing processing demands [25]. Previous research indicates that neuronal systems involved in sustained attention may be characterized by a less volatile state with decreased LRTC [25]. LRTC changes in the present study may thus be related to reduced attention or cognitive performance. In other words, effective conditioning required sustained attention with a relatively low cognitive load to result in stronger learning and thus larger nocebo hyperalgesia was characterized by reduced complexity of neural dynamics.

While on one hand sustained attention (characterized by decreased LRTC) was related to larger nocebo responses, on the other hand strong resting-state LRTC at baseline predict more effective conditioning of nocebo responses. We found that, during rest, before the start of the experimental phases, strong LRTC predicted higher nocebo responses. This finding relates to the above-mentioned studies, that pointed towards an involvement of LRTC in cognitive ability [23,24,38]. Stronger LRTC reflect more complex neural dynamics and therefore, it appears that people with more complex baseline brain activity may exhibit higher cognitive functioning (Montez et al., 2009) and are thus more susceptible to the acquisition of nocebo hyperalgesia through learning. Here, the implication of gamma band oscillations is in line with EEG research on (associative) learning, suggesting that memory encoding involves gamma oscillations [12,43,44] potentially in coordination with hippocampal function [45]. This links gamma oscillations, which were shown to be involved in nocebo in this study, to a role of the hippocampus in learning and nocebo hyperalgesia [46,47]. It is also noteworthy that emotional processes that may play a mediating role in nocebo hyperalgesia, such as fear [48], may engage patterns of gamma coupling in the amygdala [49], a structure

that has also been implicated in nocebo hyperalgesia [47,50,51]. Our finding of increased complexity of gamma-band oscillations in those more susceptible to nocebo hyperalgesia may thus provide electrophysiological evidence of specific underlying cognitive-emotional processes, such as associative learning ability as well as fear processing.

Alpha band oscillatory power has been shown to underlie the perceptual processing of incoming stimuli, including sensory perception [52]. Our study was methodologically different from the two previous studies on electrophysiological nocebo correlates [15,18] and our results do not show consistent support of previous findings relating alpha oscillations to nocebo hyperalgesia. While our findings indicate an involvement of alpha band oscillations during acquisition, we did not find pre- to post-acquisition changes in alpha oscillations. Methodologically, it possible that the time elapsed between the first and second resting state recordings was too long, resulting in a failure to capture electrophysiological changes in alpha oscillations related to nocebo processing.

Nevertheless, we found that nocebo trials during the acquisition phase were characterized by decreased power in the alpha band, as compared to control trials. Our finding may reflect the formation of pain expectations and an inhibitory function of alpha oscillations in pain perception. Moreover, alpha-band oscillations where involved when comparing the experience of baseline high-pain stimulations to the experience of increased pain under nocebo hyperalgesic conditions, in the evocation phase. We found that there was a significant increase in alpha-band power during nocebo responses, compared to baseline pain of a matched, high intensity pain stimulus. In line with the literature, these findings may reflect the role of alpha-band oscillations in expectations [8,9], and the cognitive regulation of pain [10,11].

We then aimed to differentiate the temporal electrophysiological profile of experiencing high pain at baseline from that of experiencing high pain as a result of induced nocebo hyperalgesia. We found that the complexity of neuronal oscillations was lower during nocebo-augmented pain compared to baseline pain of a matched, high intensity pain stimulus. Lower oscillatory complexity during nocebo-augmented pain may be in line with our finding that lower LRTC during acquisition were associated with higher nocebo magnitudes. This could mean that the evocation of nocebo hyperalgesia, due to a state of sustained attention, may be characterized by decreased LRTC [22,39–42]. Nocebo-augmented pain seems to rely on cognitive processes such as learning, memory recall, and pain modulation. Decreased LRTC may thus indicate increased attentional load or cognitive performance during nocebo-augmented pain responses. More specifically, the decreased LRTC of gamma oscillations during nocebo evocation, as compared to the baseline high pain, may alternatively or additionally indicate a learning process. It has previously been shown that while learning new information may lead to increased gamma power or synchronization [43,45,53], power of gamma oscillations may show a decrease after learning [54]. It is thus possible that in nocebo evocation, when learning is discontinued, gamma oscillations exhibit a decrease in power that reflects a previous active learning state. These results may thus highlight pronounced cognitive and learning-related differences between the neurophysiology of experiencing high pain and experiencing nocebo-evoked increased pain. Nevertheless, the LRTC findings in this study also highlight the intricacy of such complex biomarkers of temporal brain function and how they may characterize diverse cognitive functions and loads in different ways.

A number of limitations may have impacted the results of this study. First, aggregating trials of specific conditions into 10-second segments may have smeared out effects that could have been better captured using an event-related paradigm, in which the exact onset of each pain

stimulus or response could be used to epoch the data into segments locked to each trial. Furthermore, the generalizability of our findings may be limited by the recruitment of a healthy, young participant sample. Findings of this study may not be consistent with results derived from pain patients or individuals who have experienced severe or chronic pain in the past, as their electrophysiological phenotype may differ from that of healthy people [55].

With a number of novel aspects of the neurophysiological phenotype of nocebo hyperalgesia emerging through the present study, future directions are also coming into view. It is imperative for future research to focus on the generalizability and translation of experimental results into clinical practice. This study highlighted novel EEG biomarkers that are related to the experimental nocebo context. EEG is a practical and relatively cost-effective method that may provide a valuable means for the identification of nocebo-augmented pain as well as nocebo contexts. For these diagnostic potentials to be realized, a next step is for future studies to replicate our findings in clinical contexts and populations.

In sum, the present study yielded a number of novel findings regarding the electrophysiology that may underlie or mediate nocebo hyperalgesia. We identified both spectral and temporal parameters that are related to nocebo-augmented pain, with the latter presenting as the most important correlate of nocebo hyperalgesia in this study. The role of learning and attention at the electrophysiological level was highlighted through the involvement of LRTC as well as the extensive involvement of gamma oscillations under hyperalgesic conditions. These results are an important step towards identifying physiological biomarkers of nocebo hyperalgesia, a phenomenon that, to date, does not have any formal diagnostic criteria. The identification of biomarkers of nocebo hyperalgesia may thus prove imperative in the strive to identify and treat these effects.

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Chapter 7.

A pharmacological fMRI investigation of brain plasticity mechanisms in nocebo hyperalgesia.

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Thomaidou MA, Blythe JS, Veldhuijzen DS, Peerdeman KJ, van Lennep JPA, Giltay EJ, Cremers HR, Evers AWM. D-cycloserine and brain plasticity mechanisms in nocebo hyperalgesia: A pharmacological fMRI investigation.

Abstract

Negative outcome expectations can increase pain sensitivity, a phenomenon known as nocebo hyperalgesia. An important process thought to be involved in nocebo hyperalgesia is associative learning. In this study, we examined how a targeted pharmacological manipulation of learning would impact nocebo responses and their brain correlates. Participants (n = 50) received either a placebo or a single 80mg dose of D-cycloserine (a partial NMDA receptor agonist) and underwent fMRI. Behavioral conditioning and negative suggestions were used to induce nocebo responses. Participants underwent pre-conditioning outside the scanner. During scanning, we first delivered baseline pain stimulations, followed by nocebo acquisition and extinction phases. During acquisition, thermal pain stimulations of high intensity were paired with the supposed activation of sham electrical stimuli (nocebo trials), whereas moderate intensity pain was administered with inactive electrical stimulation (control trials). During extinction, moderate pain was administered across both nocebo and control trials. Nocebo hyperalgesia (reported pain difference between nocebo/control trials) was induced in both groups ($p < 0.001$). Nocebo magnitudes and brain activations did not show significant differences between D-cycloserine and placebo. In acquisition and extinction, there were significantly increased activations bilaterally in the amygdala, ACC, and insula, during nocebo compared to control trials. Nocebo acquisition trials also showed increased vlPFC activation. Increased opercular activation differentiated nocebo-augmented pain aggravation from baseline pain. These results support the involvement of integrative cognitive-emotional processes in nocebo hyperalgesia. We discuss our findings in relation to the role of particular learning mechanisms as well as fear in central pain modulation.

Introduction

Pain can arise as a debilitating symptom that is malleable and highly susceptible to an individual's internal and external environment [1,2]. Outcome expectations are shown to play a role in shaping pain responses to a given event or treatment [3–5]. While positive outcome expectations can produce beneficial effects from inert treatments (placebo effects), negative outcome expectations can blunt the effect of active interventions and even increase pain sensitivity in response to inert treatments, a phenomenon termed nocebo hyperalgesia [6–9].

An important process proposed to be involved in nocebo effects is associative learning [10–13]. Classical conditioning is used in experimental nocebo models to form expectations through associative learning [11]. In nocebo conditioning, negative associations form by pairing an inert nocebo stimulus (a sham treatment) to surreptitiously increased pain stimulations. After repeated trials, the nocebo stimulus evokes increases in perceived pain. Negative suggestions are commonly used to enhance conditioning [9,10,14]. Concurrently, conditioned nocebo effects have been shown to effectively reduce using extinction paradigms in which learned associations are discontinued [14–16].

One of the major neural components mediating associative learning processes are the N-methyl-D-aspartate (NMDA) receptors [17,18] whose agonism has been found to augment learning [19–22]. Enhanced NMDA receptor activity promotes local neuroplasticity, which in turn is believed to enhance the acquisition and consolidation of learned material in both animals [23,24] and humans [25,26]. Studies that used pharmacological agents such as D-cycloserine (DCS) to enhance NMDA-dependent learning support the implication of NMDA receptors in associative learning [27,28]. DCS is a compound that impacts NMDA-mediated neuroplasticity

differently in different doses. In lower doses (in most studies varying between 50-250 mg) it acts as a partial agonist at the glycine modulatory site of NMDA receptors [29]. To our knowledge, no studies have examined the role of NMDA-mediated learning in nocebo effects.

Recent findings on extinction-learning and exposure therapy indicates that DCS may be a promising agent for augmenting NMDA-dependent learning [27,30,31]. DCS has also been shown to enhance performance on declarative learning [32] and generalization of conditioned effects to novel contexts [33]. This evidence suggests that by agonizing NMDA receptors, DCS enhances specific learning processes, and can be used to manipulate and investigate the specific learning mechanisms involved in nocebo effects. By utilizing fMRI while pharmacologically agonizing NMDA-mediated learning during nocebo induction, precise neural processes involved in learned pain can be examined.

In the present study we aim to investigate for the first time the role of NMDA-receptor dependent learning in the acquisition and extinction of nocebo effects. As compared to placebo administration, we hypothesize that DCS will augment the acquisition of nocebo hyperalgesia and will induce nocebo effects that are more resistant to extinction. We further hypothesize that differential brain activation will be detected between the DCS and placebo groups during nocebo acquisition, evocation, and extinction, in a number of a priori regions of interest such as the prefrontal cortex, anterior cingulate cortex, amygdala, and hippocampus, that were implicated in previous nocebo studies [34]. We also hypothesize that neural activation will differ between the experience of nocebo-augmented pain and the experience of pain stimulations of the same high intensity.

Materials and Methods

Experimental design

This randomized clinical trial utilizes a placebo-controlled, double-blind design with respect to the pharmacological administration. A double-blind randomization list was created by the Leiden University Medical Center (LUMC) pharmacy. Participants were randomly allocated to one of two pharmacological groups: DCS or placebo. All participants underwent nocebo pre-conditioning outside the scanner and acquisition/extinction procedures in the MR scanner, by use of conditioning and negative verbal suggestions. The entire study consisted of two parts in the same testing day. The screening part lasted approximately 1 hour and took place at the department of Social and Behavioral Sciences, Leiden University, the Netherlands. The fMRI part lasted approximately 3 hours, of which approximately 1 hour took place in the 3 Tesla MRI scanner of the Leiden Institute of Brain and Cognition (LIBC) scanning facilities at the LUMC. This study was approved by the Medical Ethics Committee Leiden, The Hague, Delft (P19.003) and pre-registered on ClinicalTrials.gov (NCT04762836).

Participants

The required sample size for the primary analysis was calculated based on a previous imaging study that, similar to our primary study objective, investigated the effects of DCS in a learning task [32]. The analysis was conducted in G*power 3.1 [35] for a mixed model analysis of variance (ANOVA). In the experiment by Onur et al. [32] an ANOVA revealed a main effect of the pharmacological agent (DCS vs. placebo) [$F(1,27) = 5.454$; $P = .027$] on performance in a declarative learning task. We derived partial η^2 from the F statistic and degrees of freedom [36,37]. With

an effect size of $\eta^2 = 0.17$, alpha error probability set at $\alpha = 0.05$, and desired power set at 0.9, the sample size indicated 22 participants per pharmacological group. Given the potential for dropout and artefacts in imaging data, we recruited 25 participants per group in this study.

Inclusion criteria were: age between 18 and 35 years, a good command of the English language, and (corrected to) normal vision and hearing. Exclusion criteria were any history of chronic pain, serious medical or psychiatric conditions, experiencing pain on the day of the study or use of analgesic medication in the 24 hours prior to testing, use of psychotropic drugs in the month prior to testing, and being pregnant or breastfeeding. A physician performed a brief health screening based on our exclusion criteria and to assess vital signs. Participants also needed to be eligible to undergo MRI and were screened for standard MRI-compatibility exclusion criteria. Participants were recruited via the recruitment website Sona (Sona Systems, Tallinn, Estonia). All participants signed written informed consent and were reimbursed with a 90-euro payment.

Thermal pain stimulation

Thermal pain stimuli were delivered to participants' right volar forearm and pain intensities were rated on a numeric rating scale (NRS) ranging from 0 (no pain) to 10 (worst pain imaginable on the arm). In the screening part, pain stimuli were delivered via a Thermal Sensory Analyzer with a 3×3 cm thermode probe (TSA-II; Medoc Advanced Medical Systems, Ramat Yishai, Israel). In the MRI part, pain was delivered with an MR-compatible ATS 3x3 thermode attached to a Pathway device (Medoc Advanced Medical Systems, Israel).

Sensory thresholds

We followed a sensory-thresholds method that follows published standardized and protocolled procedures [38]. To test warmth and pain threshold levels, heat stimuli were applied from a baseline of 32°C on the forearm and participants were asked to indicate the first moment that they perceived warmth and the first moment that they perceived pain. After a practice trial for each, the average of 3 warmth and 3 pain detection values were calculated as thresholds for warmth and pain, respectively.

Pain calibration and administered stimuli

Throughout the experiment, each stimulus was initiated from a 32°C baseline, increased to a target temperature with ramp up and return rates of 8°C per second, and presented at peak temperature for 5 seconds. The maximum temperature that could be reached was 50°C. The inter-stimulus interval consisted of a pain rating screen with a 6 second duration followed by a fixation cross with a mean duration of 5 seconds, jittered around a normal distribution of ±2 seconds. Pain calibrations were conducted to select the temperatures that would induce moderate and high pain during nocebo conditioning. The calibrations were individually tailored, based on participants' NRS ratings of maximum 30 pain stimuli of varying intensities. We used the median temperatures that participants consistently rated as NRS 6 to 9 (high pain) for nocebo trials in the pre-conditioning and acquisition phases. We used median temperatures consistently rated as approximately NRS 3 to 5 (moderate pain) for all control trials as well as extinction nocebo trials.

After calibrations, a nocebo pre-conditioning took place and included 7 nocebo and 7 control trials, to increase the time of learning and ensure nocebo effects would be induced. At the start of the MRI session a

baseline phase took place during which 5 high and 5 moderate pain stimuli were administered. The acquisition and extinction phases each included 14 nocebo and 14 control trials. All trials were administered in pseudorandom order, so that no more than three trials of the same type were administered in a row. To reduce habituation or sensitization to heat-pain, we moved the thermode higher on the arm between functional scans; the thermode was moved to a more proximal site on the same arm after baseline and at one third and two thirds of the acquisition/extinction procedure).

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 procedure. Negative verbal suggestions were used to create expectations regarding the pain-enhancing effects of administering electrical stimuli in combination with thermal pain. Two electrodes were placed in a diagonal line on the base of the thumb and the inner elbow. Participants underwent a short mock calibration procedure during which they felt a light electrical pulse through the electrodes (ConMed MR-compatible Cleartrace ECG electrodes). This pulse was delivered in order to increase the believability of the nocebo manipulation. The device was not actually present during conditioning in the MRI scanner, but messages displayed on a computer monitor via E-Prime 3.0 (Psychology Software Tools, Pittsburgh, PA, USA) indicated the sham activation of the electrical stimulation during nocebo trials. Negative suggestions indicated to all participants that when the messages "on" (nocebo stimulus in either purple or yellow font, counterbalanced) and "off" (control stimulus in grey font) were displayed, their pain would be respectively aggravated (nocebo trials) or not altered (control trials). In the pre-conditioning and acquisition

phases, the activation of sham electrical stimulation was paired to covertly increased pain stimulation during nocebo trials.

Pharmacological manipulation

A single dose of DCS was administered at 80mg for all participants in the DCS group. The LUMC pharmacy prepared DCS (powder form) into capsules, as well as placebo capsules of identical appearance containing the inert agent microcrystalline cellulose. Because plasma concentrations were expected to peak between 1 and 3 hours after DCS administration [39,40], participants ingested the capsule 2 hours before entering the MRI scanner to undergo the main learning paradigm.

Measures

Pain

Throughout the experiment, participants were provided with a 6-second window to rate their pain on a sliding scale representing the pain NRS, following each pain stimulation. A message, presented on the computer monitor 2 seconds after the pain stimulus returned to skin temperature, prompted the pain rating to be given by use of a keyboard in the screening part and button boxes in the MRI session.

Learning

To assess learning rates before and after the administration of DCS, participants completed the Wechsler Memory Scale–Fourth Edition

(WMS–IV) subtest Verbal Paired Associates [41]. The test was performed twice, once before the administration of DCS and once at the end of the scanning session.

MRI

Data were collected at the Leiden Institute for Brain and Cognition imaging facilities at the Leiden University Medical Center, using a Phillips Achieva 3 Tesla scanner with a maximum gradient strength of 40 mT/m, bore diameter of 60cm, and field-of-view of 45cm (head-feet direction), and a 32-channel head coil. A structural MRI was made with a T1 weighted gradient echo sequence. Functional scans were taken utilizing T2* weighted gradient echo planar images (TR=2200ms, voxel size = 2.75 x 2.5 x 2.75mm, TE= 30ms, flip angle = 80°, matrix = 80 × 80, field of view = 220 × 220, slice thickness = 2.75mm, slice gap = 0.28mm, 40 slices per volume, sensitivity encoding factor = 2) with a 32-channel SENSE head coil. Functional scans began with two automatically discarded dummy volumes to allow for magnetic field stabilization. Heart rate (finger pulse oximeter) and respiration (respiratory belt transducer) were measured to correct for physiological artifacts during scanning.

Questionnaires

A questionnaire containing demographic and health questions was used to screen participants for inclusion. An MRI-compatibility questionnaire was also used, to ensure participants were eligible to enter the scanner. The Structured Clinical Interview for the Diagnostic and Statistical manual for Mental disorders (SCID-5-RV [42] was used to screen participants for psychiatric disorders. The following four questionnaires

were used to measure psychological characteristics: a short version of the State-Trait Anxiety Inventory, State version (STAI-S-s) [43,44], the State-Trait Anxiety Inventory, Trait version (STAI-T) [44], the Pain Catastrophizing Scale (PCS) [45] which assesses catastrophizing thoughts or worrying relating to pain [46], and the Body Vigilance Scale (BVS) [47] measuring vigilance about bodily sensations. Total scores were used.

Participants also completed an exit questionnaire at the end of the experiment, containing manipulation checks and questions about their participation and side effects. The questions were: "did you believe the information you received in this study", "how much did you worry about what the experimenter thought of you or changing your responses to please them", "were you focused on the pain stimulations during the study", and "did you notice the association between the electrical stimuli and pain aggravation". Questions were rated on a 0-10 NRS from "not at all" to "very much". All questionnaires were displayed on a computer monitor via web-based survey software (Qualtrics, Provo, Utah, USA).

Study procedures

During the screening part (see **Figure 1**), participants signed an informed consent form and completed the health, psychiatric, and MRI screening for inclusion in the study. Sensory and pain threshold levels were then tested, and pain stimuli were calibrated for each participant. The electrodes were then attached to the hand and arm and the short mock calibration of the sham electrical stimulation took place. Preconditioning was then completed. During the MRI part, participants first completed the WMS–IV and then received the oral pharmacological administration. During a 2-hour waiting time for DCS to take effect, participants had a small, standardized meal, completed the psychological questionnaires, and prepared to enter the MRI scanner. Then, participants entered the scanner, completed a structural scan, and

were then exposed to the baseline pain stimulations. Participants then underwent the nocebo acquisition and extinction procedures. After the end of the experiment, participants completed the second WMS–IV, were asked to answer the exit questions, and then were debriefed.

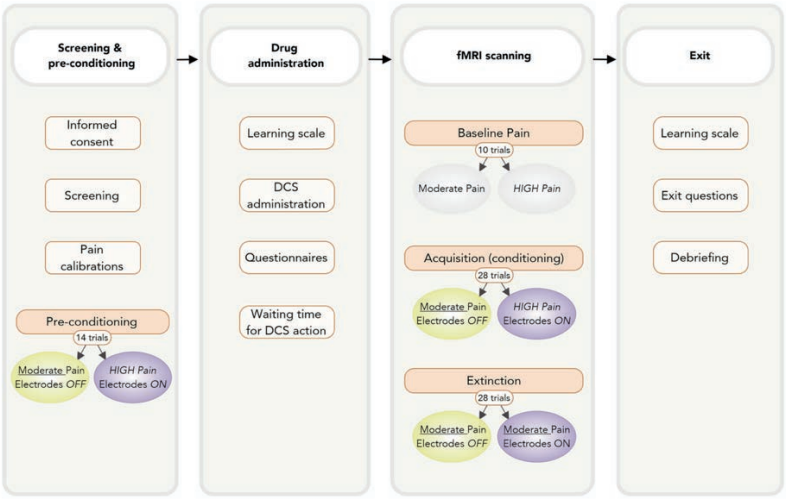

Figure 1. Timeline of the experimental procedure of this study**.** After screening for inclusion and pain calibrations, participants underwent a pre-conditioning phase. After the first learning task, D-cycloserine (DCS) or placebo was administered, and the psychological questionnaires were completed. In the fMRI part, participants completed structural scans and thereafter a first functional scan while receiving baseline moderate and high pain stimuli. Thereafter, three scans covered the acquisition and extinction of nocebo hyperalgesia. Finally, the second learning task, exit questionnaire, and a debriefing were completed.

Statistical analysis

Data screening and behavioral measures

Analyses of demographic, psychological, and behavioral measures were performed for descriptive purposes and as manipulation checks.

Behavioral data were analyzed and visualized by use of *R* programming software (version 4.1.2; R Core Team, 2019), including the MASS [48], stargazer [49], and ggplot2 [50] packages.

The magnitude of reported nocebo hyperalgesia was measured within-subjects and was defined as the difference in pain ratings for the first nocebo trial compared to the first control trial of the extinction phase. The first evocation trials were selected to check whether significant nocebo hyperalgesia was induced, as previous studies indicate the effect to be clearest in those trials [14,51]. We also compared the average of pain ratings of the first 5 pairs of extinction trials for a significant nocebo response, as these were used for brain imaging analysis where more trials are required. Repeated measures ANOVAs were conducted with *trial type* as within-subjects factor with two levels (nocebo trial, control trial), as a separate model, to test whether significant nocebo hyperalgesia was induced.

The first and last pairs of trials of the extinction phase were used to calculate the magnitude of extinction of nocebo responses. The reduction of nocebo responses was measured as the change in magnitude of nocebo responses (nocebo minus control trial difference score) between the start and the end of the extinction phase. A repeated-measures ANOVA was performed with *time of measurement* (pre to post) as within-subjects factor with two levels (nocebo magnitude before extinction, nocebo magnitude after extinction).

Pearson correlation analyses were performed between all questionnaire data (psychological questionnaires, exit questions) and the magnitude of nocebo responses (nocebo minus control trial difference score), to establish whether these factors impacted nocebo responding. We also conducted post-hoc exploratory mediation analyses, to explore potential between-groups mediating effects of the questionnaire scores on nocebo magnitudes.

For all behavioral analyses the threshold for significance was set at $p < 0.05$ and partial eta-squared (η_p^2) was computed as a measure of effect size, with η_p^2 of 0.01 considered small, 0.06 considered medium, and 0.14 considered a large effect size [52,53]. To conduct analysis of variance (ANOVA) and correlations, potential outliers and the assumptions of normality and homogeneity were checked.

Pharmacological manipulation

To test the first hypothesis, that DCS would lead to larger nocebo responses than a placebo, we examined whether nocebo hyperalgesia differed between the DCS and Control groups. A 2x2 mixed model ANOVA was performed, with *group* as the between-subjects factor and *trial type* as within-subjects factor (first extinction nocebo trial, first extinction control trial). We also examined the reduction of nocebo magnitudes after extinction. To compare extinction between the pharmacological groups, a 2x2 mixed model ANOVA was performed with *group* as the between-subjects factor and *time of measurement* (pre to post) as within-subjects factor with two levels (nocebo-control trial difference *before* extinction, nocebo-control trial difference *after* extinction).

fMRI analyses

In preprocessing the data anatomical scans were skull stripped with the Brain Extraction Toolbox (BET; [54]. Subsequent preprocessing was conducted in SPM12 (Wellcome Department of Cognitive Neurology, London; www.fil.ion.ucl.ac.uk/spm) running on MATLAB 2021A (MathWorks, Natick MA, USA; https://www.mathworks.com/products/matlab.htm. Functional scans

were realigned to correct for motion artifacts, low frequency drift, temporal autocorrelation, and spatial abnormalities. Structural scans were co-registered to the mean echo planar imaging space, and data was normalized and registered to Montreal Neurological Institute (MNI) space. A Gaussian spatial smoothing kernel of 6mm full width at half maximum was applied to the functional images. Data were visually inspected for successful co-registration. The four functional scans were preprocessed separately, then concatenated to one set of functional images per participant. Heart rate and respiratory data were preprocessed with the PhysIO toolbox [55].

For statistical analysis, functional images were modeled on a design matrix consisting of columns for 1. Baseline moderate pain trials, 2. Baseline high pain trials, 3. Acquisition control trials, 4. Acquisition nocebo trials, 5. Evocation control trials, 6. Evocation nocebo trials, 7. Extinction control trials, 8. Extinction nocebo trials (columns 1-8 of the design matrix, each modeled with the onset and duration of the approximately 7000ms pain stimulus), 9. Pain rating periods, 10. Control and nocebo anticipatory cues, 11. RETROICOR regressors for heart rate, respiration, and heart rate-respiration interaction [56]; 12. respiratory volume per time; 13. heart rate variability (HRV; 11-13 estimated with the PhysIO toolbox); and 14. six motion regressors for the rigid body transformation. All task regressors (1-10) were convolved with the hemodynamic response function. Data were additionally high-pass filtered with a cut-off of 128s and corrected for temporal autocorrelation with a first-order autoregressive model.

First level analyses pertaining to our hypotheses contrasted acquisition control trials with acquisition nocebo trials, evocation control trials with evocation nocebo trials, and baseline high pain trials with evocation nocebo trials. The evocation phase consisted of the first 10 extinction trials and exploratory analyses of the extinction phase included the remaining 18 extinction trials. Second level analyses compared these

contrasts between and across pharmacological groups. Masks pertaining to a priori regions of interest (ROI) including the ventrolateral prefrontal cortex (vlPFC), dorsolateral prefrontal cortex (dfPFC), amygdala, anterior cingulate cortex (ACC), operculum, and insula were drawn from the Harvard-Oxford Atlas (HOA; [57] in FSLeyes [58]. Masked ROI analyses were conducted separately per ROI. Statistical significance for all contrasts was corrected with a familywise error rate (FWE) correction to a p value of *pFWE* <.05. This was further adjusted with a Bonferroni correction per hypothesis to *pFWE* <.01 to correct for five ROI analyses per hypothesis, with a minimum cluster size of 10 voxels (2mm MNI space). Imaging data visualizations were carried out using FSL [59], ITK-SNAP (http://www.itksnap.org; [60], and ParaView (http://www.paraview.org; [61].

Results

Participants and pain reports

Fifty-three participants were enrolled in this study and 2 were excluded upon screening for inclusion, based on their medical history. The data of 1 participant that completed the study were excluded due to technical errors in the experiment. A total of 50 participants (39 women) were included in the final analyses. The mean age of participants was 23 years (SD = 3.3; **Table 1**). **Table 1** also displays mean warmth/pain detection thresholds, temperatures used, and reported pain differences during baseline as well as nocebo acquisition and extinction. Five participants that received DCS and two that received placebo self-reported noticing mild dizziness (*n*=2 in the DCS group), or sleepiness/tiredness (*n*=2 in the DCS group, *n*=1 in the placebo group).

On average, participants reported that they believed the information they received during the study (M = 7.3, SD = 2.3), they were not concerned about what the researcher thought of them or changing their responses out of compliance (M = 0.6, SD = 1.1), they were focused on the heat stimuli (M = 8.2, SD = 1.2), and they noticed the increased pain association with the nocebo electrical stimuli (Mean = 9.2, SD = 1.2). We ran Pearson's correlations between the magnitude of nocebo hyperalgesia and manipulation check exit questions and none of the responses to exit questions where significantly correlated with the magnitude of nocebo responses (all $p > 0.05$).

Table 1. Descriptive statistics of demographics, temperatures used, pain ratings, questionnaires, and learning rates.

	Mean	SD	Min.	Median	Max.
Age	22.9	3.3	18	22	35
Moderate pain used (°C)	46.78	0.56	45.00	46.9	48.00
High pain used (°C)	48.48	0.50	47.00	48.5	50.00
Warmth threshold (°C)	34.07	1.31	32.80	33.80	40.00
Pain threshold (°C)	44.47	2.56	34.80	45.10	47.20
Pre-conditioning pain difference	3.26	0.96	1.29	3.36	5.71
Baseline pain difference	2.40	1.33	-0.50	2.30	6.10
Acquisition pain difference	3.44	1.29	0.89	3.36	6.82
Extinction first trials pain difference *	1.77	1.47	-1.00	2.00	5.00
Extinction five trials pain difference *	1.14	1.05	-0.90	0.90	3.90
Extinction final trials pain difference *	0.73	0.63	-0.33	0.53	2.22
PCS score	25.12	6.82	13.00	24.00	45.00
BVS score	17.03	5.65	6.31	17.41	31.38
STAI state score	32.73	8.74	20.00	33.33	60.00
STAI trait score	37.46	7.385	25.00	37.00	58.00
Learning ± score total pre-DCS	5.59	1.49	2.00	5.75	8.00
Learning ± score total post-DCS	5.28	1.84	0.75	5.50	8.00
Learning ± change pre- to post-DCS	-0.31	1.59	-4.00	-0.50	5.00

Note: All temperatures are reported in degrees Celsius. * Pain differences represent the mean NRS difference between nocebo/high pain trials minus control/moderate pain trials for each phase. In extinction, the first pair (evocation), first 5 pairs (evocation), and final 9 pairs of nocebo and control trials are reported. ± Learning was measured as a manipulation check with the Wechsler Memory Scale. PCS, Pain Catastrophizing Scale; BVS, Body Vigilance Scale; STAI, Spielberger State-Trait Anxiety Inventory; DCS, D-cycloserine.

Behavioral results

The regression assumptions of linearity and homogeneity were met, and behavioral data were normally distributed. No outliers were present, determined by Mahalanobis distance. Correlation analyses of psychological questionnaire scores (**Table 1**) did not yield significant associations with nocebo magnitude or any other pain measures (all $p > 0.05$). Results on the between-groups mediating effect of questionnaire scores on nocebo magnitudes also yielded non-significant results (for all paths $p \geq 0.05$). Baseline and post-experimental learning rates are shown in **Table 1** and indicate that WMS learning rates remained stable from before to after DCS administration.

The nocebo manipulation successfully induced nocebo responses as measured during the first trials of extinction **(Figure 2A)**. Across both groups, there was a significant difference between pain reports for the first nocebo and first control trial of the extinction phase (F (1,49) = 73.03, $p < 0.001$, $\eta_p^2 = 0.19$) indicating the presence of nocebo hyperalgesia. We also found a significant nocebo effect in the first five pairs of extinction trials which were used as an evocation phase for fMRI analysis (F (1,49) = 59.73, $p < 0.001$, $\eta_p^2 = 0.08$; **Figure 2B**). Finally, there was significant extinction of nocebo responses, with the magnitude of nocebo responses being significantly lower in the last pair of (nocebo/control) extinction trials, as compared to the first pair of extinction trials (F (1,49) = 13.17, $p = 0.001$, $\eta_p^2 = 0.08$).

Pharmacological manipulation

A mixed ANOVA indicated that there was no significant interaction between drug group and the magnitude of nocebo responses based on trial type (F (1,48) = 0.002, $p = 0.97$, $\eta_p^2 < 0.001$; **Figure 2C**). This was aligned with no increases in learning rate from pre to post drug

administration in the DCS as compared to the placebo group (**Figure 2D, Figure 3**). We did not find an effect of DCS on the magnitude of extinction either, as there was no significant interaction between drug group and the reduction of nocebo responses at the end of extinction (F (1,48) = 0.11, p = 0.73, η_p^2 = 0.001).

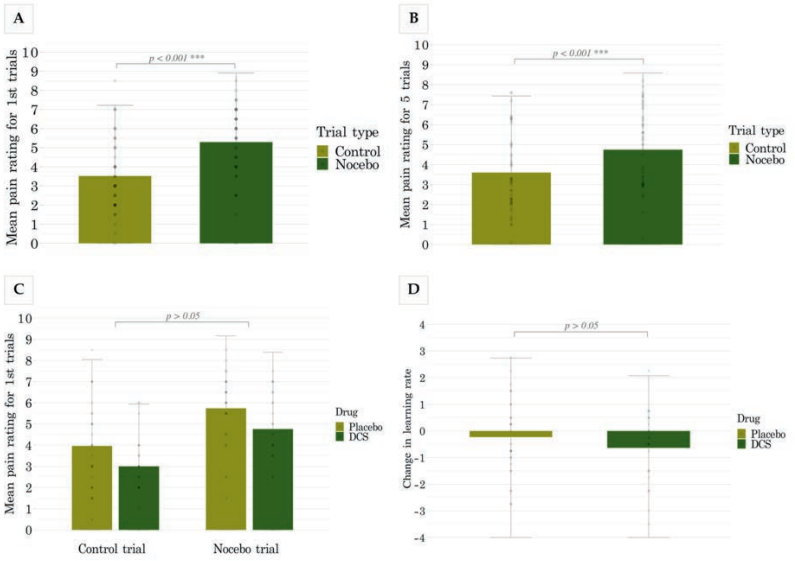

Figure 2. Behavioral results. Behavioral results represented as group means and standard deviations. There was a significant nocebo effect in the first pair (**A**) and first 5 pairs of extinction trials (**B**). Nocebo responses were not affected by D-cycloserine (DCS) compared to placebo (**C**). Learning rates were measured on the WMS-IV before and after DCS administration (**D**). There was only a slight non-significant reduction in learning rates of all participants, irrespective of drug group.

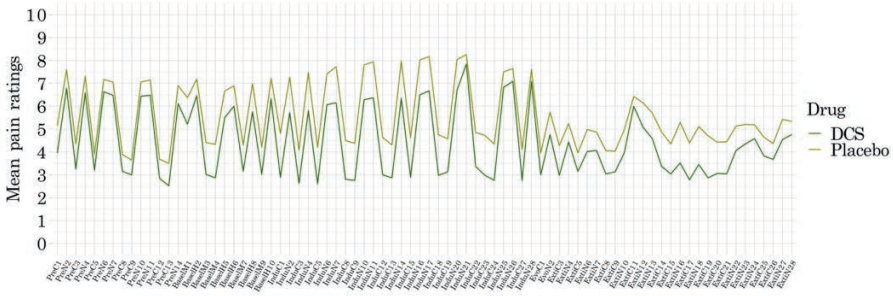

Figure 3. Mean pain ratings are shown in pre-conditioning (*Pre*) baseline (*Basel*), nocebo induction (*Indu*), and extinction (*Ext*) including the first two extinction trials (*Evocation*) where behavioral nocebo effects were measured. D-cycloserine (DCS) administration did not significantly affect the magnitude of nocebo responses. The slight group difference in ratings observed indicates a minor average difference in individually calibrated pain intensities and had no significant effects on outcomes.

fMRI results

Increased activity in evocation nocebo trials compared to baseline high pain was found in the right operculum (**Table 2**, **Figure 4A**). During the acquisition phase, we detected an increased BOLD response during nocebo trials in bilateral ACC, bilateral amygdala, bilateral insula, and bilateral vlPFC (all clusters from a priori analyses are presented in **Table 2**, **Figure 4B**, **Figure 5A-C**).

No clusters reached the threshold for significance in the evocation (first 10 extinction trials) control and nocebo contrast initially, but notably, in exploratory analyses of the evocation phase with no minimum cluster size, we detected an increased BOLD response during nocebo trials in the left insula (all clusters from exploratory findings in **Table 3**, **Figure 4C**), albeit this did not reach significance. Exploratory analysis of the remaining 18 extinction trials detected increased BOLD signal during

nocebo trials in bilateral amygdala and insula, as well as a small, below-threshold cluster of the ACC (**Table 3**, **Figure 4D**, **Figure 5A-C**). Parameter estimates were computed with MarsBaR [62] and are plotted for all clusters (**Figure 4E**).

No differences between pharmacological groups were detected in any a priori ROIs, or in exploratory whole brain analyses, for hypothesized contrasts between acquisition control/nocebo trials, evocation control/nocebo trials, or baseline high pain/evocation nocebo trials.

Table 2. Results of ROI analyses for acquisition nocebo > control, and evocation nocebo > baseline high pain contrasts.

	Region (HOA mask)	MNI-coordinates (peak voxel)				t value	*P*-value (peak voxel)	Voxels
Acquisition								
	ACC	LR	2	-6	42	9.87	<.001	1544
	Amygdala	L	-20	2	16	5.21	.002	110
	Amygdala	R	22	2	16	6.81	.002	115
	Insula	L	-36	16	14	8.29	<.001	1467
	Insula	R	36	4	8	7.05	<.001	1453
	vlPFC	L	-46	16	10	6.49	.002	68
	vlPFC	R	52	6	2	7.39	<.001	337
	ACC	LR	2	-6	42	9.87	<.001	1544
Baseline-evocation								
	Operculum	R	42	-12	14	6.04	<.001	173

Note. Coordinates given in x, y, z for MNI space. T statistics calculated with df=48, $p<.05_{FWE}$. HOA, Harvard Oxford Atlas; MNI, Montreal Neurological Institute; FWE, familywise error; vlPFC, ventrolateral prefrontal cortex; ACC, anterior cingulate cortex.

Table 3. Results of exploratory ROI analyses for evocation (first 10 extinction trials) nocebo > control, and extinction (remaining 18 extinction trials) nocebo > control contrasts.

	Region (HOA mask)	MNI coordinates (peak voxel)				t value	P value (peak voxel)	Voxels
Evocation								
	Insula	R	36	-22	10	4.33	.031	4
Extinction								
	ACC	R	12	-26	48	-4.54	.027	3
	Amygdala	L	-24	-8	-8	-5.93	.005	40
	Amygdala	R	24	-2	-12	-4.32	.016	14
	Insula	L	-38	-10	18	-6.31	.001	56
	Insula	R	36	-4	18	-4.63	.01	14

Note. Coordinates given in x, y, z for MNI space. T statistics calculated with df=48, $p<.05_{FWE}$. HOA, Harvard Oxford Atlas; MNI, Montreal Neurological Institute. FWE, familywise error.

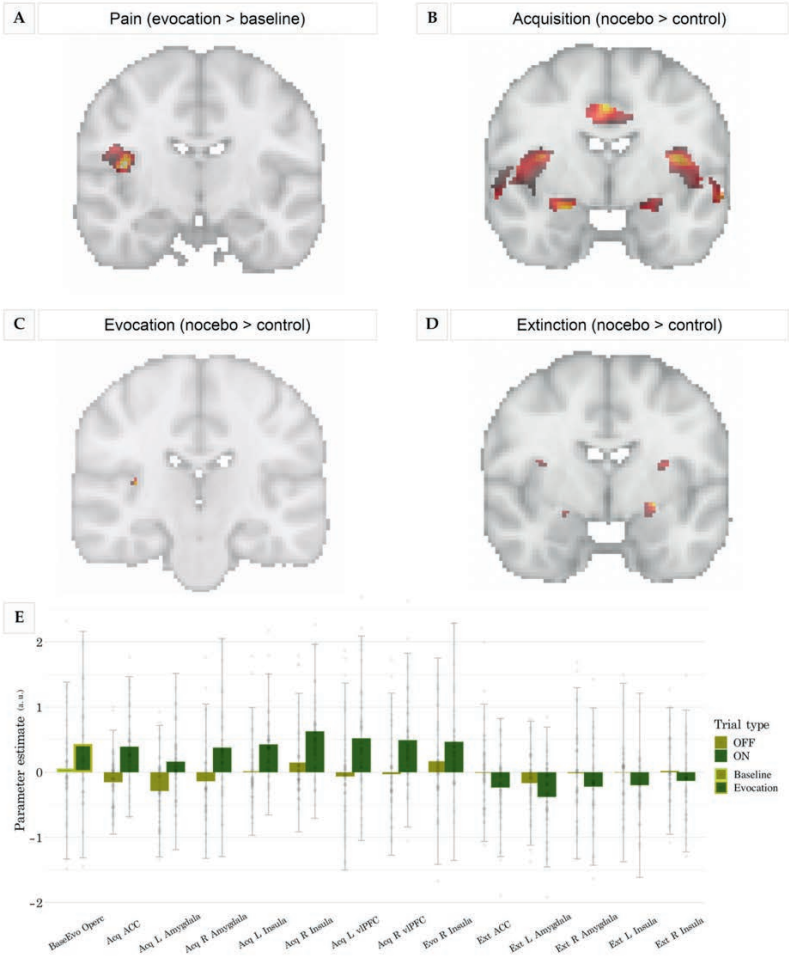

Figure 4. Results of the fMRI analysis. Differences in BOLD activations between baseline pain and nocebo-augmented increased pain responses were found in the operculum (**A**). Contrasting nocebo and control trials resulted in differential BOLD activations during nocebo acquisition (**B**), evocation (first 10 extinction trials; **C**), and extinction (last 18 extinction trials; **D**). Parameter estimates are plotted for all clusters and contrasts (**E**). Brain images are in neurological display convention.

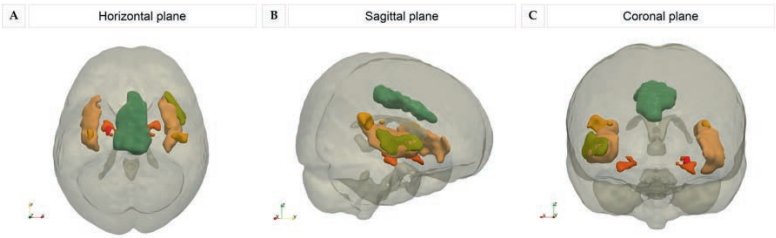

Figure 5. The loci of all significant fMRI results represented in a 3D model. Horizontal (**A**), sagittal (**B**), and coronal (**C**) views are displayed. As compared to control, nocebo trials and nocebo-augmented pain were characterized by differential BOLD activations in the vlPFC (light green), ACC (dark green), amygdala (red), and operculum/insula (orange).

Discussion

This study investigated the role of DCS on the acquisition and extinction of nocebo hyperalgesia in an fMRI study. Significant nocebo effects were induced but DCS did not influence nocebo magnitudes or brain activation, suggesting that the pharmacological manipulation did not influence learning in this nocebo paradigm. Results of the fMRI analyses indicated that in acquisition and extinction phases, there were significantly increased BOLD activations bilaterally in the amygdala, ACC, and insula, during nocebo compared to control trials. Nocebo acquisition trials also showed increased vlPFC activation. Increased opercular activation further differentiated nocebo-augmented pain aggravation from baseline high pain. These results are in line with previous nocebo studies and provide support for the involvement of specific cognitive processes in nocebo hyperalgesia.

The learning paradigm induced significant nocebo responses across both groups, as was anticipated. The pharmacological manipulation in this study did not affect learning of verbal pairs or nocebo associations. Although DCS is known to impact neuroplasticity [29] previous findings are mixed. Many studies show effects of DCS on phobia and symptoms that are known to result from aversive learning [29,63–65,65,66]. Yet, other studies have shown differential effects of DCS, for example facilitating procedural but not declarative learning [67], and extinction or memory consolidation, but not necessarily acquisition of learned responses [63,68,69]. These differential findings could theoretically be related to the dosage used, with doses in the relevant studies mentioned above varying from 50 to 250mg and fixed, rather than measured based on body weight. We choose a moderate dose of 80mg. Generally, there does not seem to be an apparent dose-related efficacy of DCS, with one review of the literature reporting that neither the dose nor the time of administration had an effect on the learning outcomes [65].

Interestingly, DCS augmentation effects have mainly been studied in phobic stimuli and for fear memory [29]. These results suggest that DCS is effective in modulating limbic NMDA circuits engaged in paradigms with a heavy fear load [70]. We did find increased amygdala activation for nocebo trials over control trials during acquisition and extinction of the nocebo effect irrespective of pharmacological group, and there seems to be some involvement of fear in nocebo (Schmid et al., 2015; Thomaidou, Veldhuijzen, et al., 2021; Tinnermann et al., 2017b). Speculatively, the type of pain-learning task employed in our nocebo experiment may potentially not primarily rely on the same fear-learning circuits that DCS has been found to affect in previous studies. DCS not affecting nocebo responding may point to a potential differentiation between the specific mechanisms involved in pain-learning versus fear-learning. In other words, we speculate that learning a negative nocebo association may not involve the NMDA-mediated learning that DCS may be able to augment in more fear-specific contexts.

The amygdala has been consistently implicated in fear-learning [32,74,75], but amygdala involvement may not be an essential feature or necessary prerequisite for nocebo induction. Other brain areas are shown to underlie nocebo hyperalgesia in the absence of an amygdala involvement [76–78]. Interestingly, the amygdala seems to be involved when experimental contexts or suggestions are especially negative or frightening, such as in visceral pain studies (Schmid et al., 2015) or studies of a higher threat-load that include extensive conditioning and negative suggestions [73]. In line with this, our study with pre-conditioning and negative suggestions showed increased activation of the amygdala on nocebo compared to control trials. Involvement of the amygdala in the more negative experimental contexts could suggest that fear may be a secondary modulatory factor in nocebo hyperalgesia [72,79]. Pain-related learning thus seems to potentially take place on two conceptual levels. On one hand, cortical-level associative learning mechanisms may be at the core of acquiring learned pain effects. On the other hand, fear-related learning, that may take place in subcortical loops involving the amygdala, mediates pain worsening, and may be a secondary modulatory factor in pain chronification [72,80].

Distinct learning mechanisms mediated via the vlPFC may also have engaged during nocebo acquisition in our study. The vlPFC is linked to learning, belief formation, and stimulus-response associations [81–85]. Neural circuits involving the vlPFC are thought to communicate through oscillations in gamma-band (60–160 Hz) frequency channels. This relates to previous studies implicating gamma-band oscillations as a marker of learning in nocebo acquisition [16,86,87]. The vlPFC, as the present study also suggests, may be implicated in sensory stimuli whose properties are processed bottom-up [88]. This corresponds to participants engaging in this type of bottom-up processing of nocebo versus control stimuli, only in the acquisition phase of our experiment, before top-down processing based on learned information of nocebo associations begins taking place.

The insula and more broadly the operculum are also thought to be central cognitive features of sensory perception [89–91]. Opercular involvement is consistently found in nocebo hyperalgesia and marks mechanisms of sensory discrimination and cognitive pain modulation [78,92–94]. We also found differences in insular and opercular activations between nocebo and control stimuli. It is perhaps unsurprising that sensory modulation is involved in the acquisition of nocebo hyperalgesia. Crucially, however, we found a persistence of insular activations even when all heat administrations were equal in intensity, during extinction, albeit this was only a small cluster of activations. This may indicate that the brain continues engaging in cognitive pain discrimination during nocebo responding, when nocebo stimuli were generally perceived as more painful while all heat intensities were actually identical. Indeed, this is in line with findings of the present study indicating that nocebo responses were not completely attenuated.

We also found further differences in opercular activations between evoked nocebo responses and baseline pain. Before learning took place, we administered participants with baseline high pain stimuli. Our results show increased activation of the operculum during nocebo-augmented high pain in the evocation phase, as compared to the baseline high pain stimuli. The operculum was significantly less engaged in experiencing high pain before learning took place, while increased cognitive sensory processing seems to take place when pain sensitivity is increased under nocebo hyperalgesic conditions. The consistent involvement of subregions of the operculum, ACC, and PFC in nocebo responding may underscore a primary role of cognitive and sensory integration and modulation in nocebo hyperalgesia.

A limitation of this study was in analyzing the small number of initial nocebo evocation trials, before prolonged extinction, which underpowered the evocation results. Given that extinction begins soon after the pairing of the cues and varying stimulus intensities is

discontinued, only the first few extinction trials can be considered to distinctly represent nocebo evocation. One solution that would allow future studies to examine brain activity during evocation, before extinction occurs, is to reinforce nocebo associations throughout extinction. Some nocebo studies employed such continued reinforcement paradigms and achieved persistent nocebo responding that may have been less contaminated by extinction effects [92,94]. Additionally, while we conducted our power analysis for this study based on the behavioral-pharmacological primary outcome, a sample of 25 participants per group is considered a minimum required sample for fMRI analyses, and this may have led to some of the imaging results for smaller brain clusters being underpowered [95,96].

This study also had other limitations that future research should address. It is important to note that the generalizability of our results may be limited, due to the recruitment of a healthy, young participant sample. Results of this study may not represent pain processing correlates in patients or individuals who have experienced severe or chronic pain, as their neurophysiology may differ from that of healthy people [97]. It is imperative for future research to replicate our findings both in patient populations and in more realistic clinical contexts.

Albeit the pharmacological manipulation using a partial NMDA receptor agonist did not affect nocebo responses, this study provided important support for the integration of specific cognitive-emotional and sensory processes in nocebo hyperalgesia. The acquisition of nocebo hyperalgesia was primarily characterized by increased activation in brain regions that cognitively integrate and modulate pain inputs. We showed that cognitive-emotional processing of pain signals in the operculum and ACC may integrate prior negative experiences. Understanding the intricate relationship of learning and sensory modulation in the formation of negative pain associations is highly relevant for the effective management of pain.

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Chapter 8.

Summary and general discussion

Summary

This thesis adds to a growing literature that has been challenging antiquated understandings of pain as a bottom-up process. In this project, we conducted a series of biobehavioral studies to further our understanding of how bottom-up pain signaling can be influenced by the top-down processing that may often be involved in pain. We employed diverse methodologies, such as a large-scale meta-analysis, a comprehensive review, behavioral experimental studies, as well as experiments utilizing imaging techniques such as fMRI, EEG, and EMG. We examined the types of experiences, such as receiving negative information or experiencing a negative effect first-hand, that may lead to stronger or more persistent nocebo effects on pain. We furthermore aimed to unravel underlying biobehavioral components of such learned pain responses. Behavioral paradigms were used to model real-life pain experiences, through validated experimental pain induction methods, novel experimental learning manipulations, as well as a close examination of emotional correlates such as fear. Concurrently, diverse, innovative neuroscientific methods –including a pharmacological manipulation– were used to examine the biobehavioral underpinnings of nocebo hyperalgesic responses. Our findings add to the growing knowledgebase from the field of nocebo hyperalgesia, demonstrating that learning by experience can decisively influence the processing and perception of noxious stimuli.

In **chapter 2**, a systematic review and meta-analysis indicated that learning by experience is a potent mechanism that can influence the perception and persistence of pain. Building upon the past two decades of proliferation in nocebo research, this comprehensive meta-analysis delivers novel insights into the currently known behavioral correlates and pain outcomes under nocebo hyperalgesic conditions. Classical

conditioning was found to be more powerful than verbally delivered negative information, showing that experienced adversity may be more powerful in inducing negative expectations, as compared to verbal suggestions. In examining what moderates effect sizes of nocebo responses between different studies, however, we found no significant moderating factors within our data. It should be noted that several factors were not systematically measured, such as fear of the pain stimulations or specific contextual factors, which may potentially account for some of the variability in nocebo magnitudes. More systematic and comparable studies on these aspects are needed. **Chapter 2** thus leaves little doubt regarding the potency of learned effects on pain perception, but raises a number of questions and points to knowledge-gaps regarding the potential cognitive-emotional and biobehavioral moderating and mediating factors in nocebo hyperalgesia.

Chapter 2 also highlighted nocebo effects as being present across the different sensations and types of pain, which led us, in **chapter 3**, to dive deeper into the diverse literature on the neurobiological correlates of nocebo hyperalgesia. To summarize and further utilize current knowledge, a comprehensive review of the neurobiological nocebo literature on pain was conducted. Twenty-two studies were included based on exhaustive database searches. A narrative review of these experiments highlighted the nocebo effect as a top-down phenomenon based on learned effects. Nocebo effects were shown to be influenced by basic nociceptive signal conduction in the spinal cord, as well as by higher cognitive functions such as emotional processing and expectations. Importantly, a marked inconsistency in methods used and results yielded between nocebo studies, led to a motivation for using consistent and comparable methods in the experimental work of this PhD project. We suggest that the field as whole attempts reproduction and replication of experimental methods, in order to reach a robust and reliable knowledge base for nocebo effects. Finally, with this review of the literature, the central question emerged which exact learning

mechanisms may give rise to nocebo responses and how this relates to pain outside of the laboratory and in real-world settings.

Chapter 4 presents a first experimental study that aimed to demonstrate whether nocebo effects can be induced –and how they may persist– when based on inconsistent and variable learning, more akin to what patients may experience within clinical settings. We compared a typical conditioning paradigm to one with variable reinforcement of the nocebo association between pain and an inert treatment. We also attempted to attenuate the induced nocebo effect to examine the dynamics of different learning schedules over time. While it was unsurprising to find that a more ambiguous learning method led to smaller –albeit significant– nocebo effects, we observed that, interestingly, these smaller effects were more persistent over time, and resisted counterconditioning. This study addressed treatment resistance and chronification of pain relevant to potential experiences in clinical settings and highlighted a role of different types of learning in nocebo hyperalgesia, thereby addressing some of the questions left open in chapters 2 and 3. However, this study did not address the impact of important emotional correlates such as fear, a factor that was not consistently reported in the studies analyzed in chapter 2, but may be implicated in nocebo hyperalgesia.

Therefore, in **chapter 5**, we designed a follow-up experimental study to examine the role of fear in learned pain responses. Despite its known involvement in pain and other clinical outcomes, fear was mostly overlooked by the nocebo field, and our study was the first to manipulate and measure the involvement of different types of fear in nocebo hyperalgesia. Here, we also imaged fear responses, by measuring startle reactions via EMG during a nocebo paradigm. While we retained a typical nocebo induction group as a control, we additionally created one group which would receive higher pain stimulations overall, and another group that received frightful information regarding a potential

bad outcome. These two groups, as expected, reported overall higher levels of fear of pain, and the higher-pain group also responded with significantly larger nocebo hyperalgesia. The results further indicated that more research is needed to unravel the intricacies of central pain integration with cognitive-emotional factors. The value of utilizing known imaging markers to measure fear of pain on a biobehavioral level, led to the novel approach of using electrophysiological biomarkers to further understand pain integration and processing under nocebo hyperalgesic conditions. In the next chapter, we thus applied imaging of the brain by use of EEG, in order to better understand how neurocognitive processing affects pain experiences under hyperalgesic conditions.

Chapter 6 examines the electrophysiology of learned pain responses through the lens of the currently known markers of pain and of emotional processing. We utilized sophisticated EEG biomarkers to characterize complex electrophysiological patterns during baseline pain perception, learning, and then evocation of nocebo responses. We additionally measured and computed brain electrophysiology at rest, before and after the experimental paradigm, to explore baseline characteristics that may modulate the acquisition of hyperalgesic effects and to examine the changes from before to after nocebo acquisition. Indeed, we found that individuals who exhibit higher complexity of neuronal patterns of oscillations at baseline showed larger nocebo responses. At the same time, differences were also found in how the brain processes increased pain stimulation at baseline versus a nocebo-augmented perceived pain increase. EEG provided several novel insights into the neurophysiological phenotype of nocebo hyperalgesia, enabling us to paint an initial broad picture of a complex neural signature of nocebo hyperalgesia. Questions were also raised by this study, as EEG methods encounter limitations in terms of localizing effects in the brain, as well as in measuring specific functional contributions of

different brain structures. A follow-up study utilizing fMRI, in chapter 7, attempts to address such limitations.

In **chapter 7** a novel pharmacological fMRI study examined closely the specific contribution of distinct brain regions and the NMDA receptors that occupy them and facilitate learning. While still utilizing consistent experimental nocebo induction methods for purposes of comparability and reliability within the field, in this study we attempted a pharmacological manipulation of learning during nocebo induction in the MR scanner. We used D-cycloserine, a medication known for its potential to enhance learning through NMDA receptor agonism, to examine whether a group with augmented learning ability would show a larger nocebo response than a group of participants receiving placebo. We found that, despite the pharmacological manipulation not showing any significant behavioral effects, brain regions previously implicated in associative types of learning differentiate nocebo stimuli from control trials. This final neuroimaging study also confirmed results found in chapters 3, 5, and 6 on the emotional correlates of nocebo hyperalgesia, thereby opening the door for future research to focus on district brain plasticity mechanisms as potential driving factors of learned effects on pain.

In the general discussion of this thesis, we integrate and interpret the findings of this PhD project in relation to each other and to the broader literature on learned effects on experimental and clinical pain. There are two central findings that arise from the work of this dissertation, both related to the intricate dynamics between nociceptive processing and cognitive-emotional experiential factors. The most central finding, that specific modes of learning shape pain processing in the brain, is discussed as the chief cognitive driver of nocebo hyperalgesia. We discuss how learning is able to alter future pain experiences based on past experience and negative expectations. The second critical finding of this project, that fear-learning may play a mediating role in nocebo

induction and persistence, is discussed in relation to gaps in the literature and our general understanding of negativity bias and emotional memory. We further discuss the limitations of this work and of this model-based scientific field as a whole, and we propose future directions in nocebo research and for clinical practice. We conclude that nocebo hyperalgesia decidedly influences pain, and that such learned effects rely on the brain's tendency to learn, adapt, and integrate cognitive and emotional information, especially in relation to prior negative experiences.

General discussion

Nocebo hyperalgesia has been researched as a negative pain outcome for over three decades. The work on reviewing this literature, conducted as part of this PhD project, resynthesized current knowledge and investigated common themes such as the central role of behavioral conditioning, as well as a focus of the field on emotions such as anxiety and stress. In the sections that follow, we start by discussing *lessons from the literature and the impact of methodological focus in understanding nocebo hyperalgesia*. Next, we discuss this project in relation to the overarching concepts and wider implications of two central conclusions derived from our findings. First, we *identify cognitive mechanisms under the umbrella of associative learning*, beyond the more general established correlate of associative learning. Second, we discuss *a potential cooperation of cognitive and fear-specific learning mechanisms*. Limitations in the research are addressed and theoretical considerations as well as future directions for the field are also discussed.

Lessons from the literature: impact of methodological focus in understanding nocebo hyperalgesia

On the consistent measurement of relevant covariates

As **chapter 2** concluded, learning by experience, for example via classical conditioning, influences how pain is ultimately perceived, but which biobehavioral processes underlie this indirect outcome remained an open question. Our primary findings indicated that classical conditioning was more powerful and reliable in inducing nocebo effects, as compared to mere verbal suggestion of a negative outcome. As corroborated by our study in **chapter 5**, this indicates that when a negative effect is practically experienced, nocebo effects are stronger than when a negative outcome is only verbally communicated. While this may seem intuitive, it is valuable to produce an evidence-based verification, from studies across the board, that associative learning (the cognitive mechanism underlying classical conditioning [2–4]) is the most powerful means for inducing nocebo effects on pain. In **chapter 2** we also highlighted how multiple types of pain are influenced by negative learned associations, indicating that under nocebo hyperalgesic conditions, pain processing can lead to amplified pain responses regardless of the nature of the noxious stimulus. The finding that across different experimental paradigms, contexts, and types of pain, nocebo effects are consistently induced, is in line with novel perspectives of pain as a subjective and ever-changing experience. Nevertheless, our meta-analysis was unable to fully rely on current published research to address some crucial questions of interest on nocebo hyperalgesia, mainly due to methodological and logistical limitations. For example, the nocebo literature may face research challenges such as publication bias for significant findings, or the content and ecological validity of experimental models built to induce nocebo effects. Further on, we discuss the limitations posed by unpublished null or underwhelming results that are inaccessible to our literature review efforts, and we

expand on concerns emerging from **chapter 2** regarding experimental modeling approaches.

Other wider methodological considerations arising from this project concern the choice of measures and paradigms in pain research. In our meta-analysis, overall magnitudes of nocebo responding could not be explained based on any of the measures that we collected from the experimental studies included. It appears that, no matter the number of learning trials, the type of sensation, or any other obtainable factor, nocebo effects up to 2.5 points magnitude (out of 10) can be obtained, with no one factor moderating this variability. This finding opened questions for future research relating to the variables that we were not able to obtain from previous studies. For example, while some important factors that influence nocebo have only incidentally been studied (see for example a study by Tinnerman and colleagues [5]), using more consistent methods in experimental models, as well as consistent in- and exclusion criteria, may provide a more stable platform on which nocebo magnitudes can be assessed and compared between studies. Additionally, measuring fear levels and reporting in detail the intensities of administered pain may point us towards potentially stronger moderators of nocebo magnitudes.

Implications for biobehavioral nocebo research

Methodological challenges may be of particular importance in biobehavioral and neuroimaging research into nocebo hyperalgesia. The neurobiological foundations of nocebo hyperalgesia are characterized by an apparent intricacy and consistency as well as replicability are central in understanding and tackling negative learned effects. **Chapter 3** presents a comprehensive review of the neurobiological underpinnings of nocebo hyperalgesia, with a focus on neuroimaging. Much of what we know about pain perception is based on self-reported pain levels.

Complex sensory phenomena such as nocebo hyperalgesia, that may implicate diverse cognitive processes, are thus very difficult to investigate reliably based on influenceable and volatile scores obtained through self-report. While self-report is the most accurate measure of subjective pain experiences that we currently have, in order to gain a comprehensive picture of learned effects on pain, there is a need for directly measuring biobehavioral factors under nocebo hyperalgesic conditions. This closer look into the neurobiology of nocebo effects is of high importance given the convolution, subjectivity, and potential inter- and intra-individual variability of experienced pain.

Despite the important takeaways provided by our comprehensive summary of the neurobiological nocebo literature, widespread inconsistencies in findings are also shown and we discuss this as a worrying trend to be addressed in future research. The utilization of distinct learning paradigms for inducing nocebo hyperalgesia may influence neurobiological findings. In other fields of research, such as in the domains of learning and memory, different types of learning have been shown to employ different brain processes, with complex architectures underlying distinct learning systems [6–9]. Concurrently, differences in emotional load, frightfulness of negative suggestions [10,11], or even the magnitude of induced hyperalgesia [12], may influence the neurobiological processes that are involved in nocebo responses. For these reasons, it is important for the nocebo field to begin employing more consistent methods and pursue replication of studies, in order to achieve reliable and meaningful findings. In the experimental parts of this project, we conformed with this recommendation, using validated and consistent experimental models for nocebo induction, while also implementing novel aspects.

Identifying cognitive mechanisms under the umbrella of associative learning

Learning as a non-unitary phenomenon

While it is apparent that nocebo effects involve a vast array of brain structures and processes [13,14], upon a systematic and detailed inspection of research to date, in **chapter 3**, we were able to synthesize a complete summary of those reproducible findings that paint a more concise and accurate picture of nocebo neurobiology. Our comprehensive review of the neuroscientific nocebo literature highlights a small number of consistent neuroimaging findings that tend to implicate specific cognitive correlates in the processing of nocebo pain. When discounting for known pain processing and sensory discrimination areas such as the somatosensory cortices and thalamus, the brain structures consistently implicated in nocebo hyperalgesia indicate a central role of learning by experience and cognitive pain modulation. When different types of learning and pain integration become involved in this process, evident by imaging findings –including our own– nocebo hyperalgesia can broadly be seen as a complex cognitive-sensory mechanism that arises through the integration of negative association learning and nociception.

While learning was shown to broadly underlie nocebo responses on pain in **chapter 3**, learning is not a unitary phenomenon, but rather it is shown to rely on distinct and often competing mechanisms [8,15,16]. For instance, even in basic non-conscious systems such as polymer networks and magnetic spins in solids, learning networks have been shown to memorize associative patterns from their environment based on specific learning modes that depend on particular contextual and stimulus-specific factors [17]. Higher order systems such as the human brain have been shown to learn and retrieve information based on distinct and often cooperating neural systems [18,19]. In our experimental studies we set out to examine specific learning mechanisms and their unique

contributions to learned nocebo effects. As discussed below, our findings add some level of detail to the existing literature, by focusing in on specific cognitive and emotional mechanisms, beyond the usual focus on the broader concept of associative learning.

To understand some of the features of nocebo hyperalgesia that the current behavioral and neuroimaging literature does not tackle, we designed and carried out a series of experimental studies on learned nocebo effects. **Chapter 4** indicates that, when replicating a clinically relevant context on ambiguous and inconsistent learning, nocebo effects can still be induced. In the continuous reinforcement group of this study we used a typical nocebo paradigm, comparable to many previous studies [109–111][20–24]. But using a second group, we also set out to reproduce results from a prior study [24] that utilized partially reinforced learning. Our objective was achieved; we showed that next to a typical nocebo paradigm (that is shown to dependably induce a nocebo effect in **chapter 2**), a more ambiguous and ecologically valid learning method is still able to induce a hyperalgesic effect, at least to some extent.

Not only is this realistic type of learning sufficient to alter the experience of pain, but ambiguity may add strength to learning so that nocebo effects can withstand attenuation over time. This was an important building block in our understanding of pain chronification from the lens of nocebo hyperalgesia. Our **chapter 4** results were in line with some initial studies that have indicated that nocebo effects may rely upon especially durable learned associations that resist attenuation [23–25]. When attempting to attenuate the induced effects, we observed that continuously reinforced, reliable nocebo associations were easier to reverse, whereas ambiguous, partially reinforced learning led to significant resistance to attenuation. We confirmed that ambiguous and variable learning can lead to hyperalgesic effects, and additionally showed that these variable associations persist over time, even after active countering of such a negative association. It appears that negative

and aversive experience prevails over newly learned positive information, and the uncertainty that comes from this variability of possible outcomes seems to reinforce negative pain expectations [24]. This serves an important realistic indicator for learning under specific real-world conditions, where, according to nocebo research, patients are thought to acquire hyperalgesic effects on their symptoms due to a variable mixture of contextual, communicative, and experiential factors [1,26–29].

In attenuating nocebo effects in **Chapter 4**, we compared a typical extinction paradigm, where learning of nocebo associations is simply discontinued, to counterconditioning. In counterconditioning, we reversed the learned associations by pairing the nocebo treatment with a positive, instead of a negative pain outcome. During both attenuation methods, new learning takes place. But our novel counterconditioning method taught participants that instead of increased pain, they would experience reduced pain when a nocebo treatment was applied. Essentially representing a placebo paradigm [21], this attenuation method showed for the first time that counterconditioning is a more potent method than extinction for the attenuation of nocebo hyperalgesia. This finding indicated that new, positive learning may effectively overwrite negative pain expectations, which may open new directions for behavioral treatments for pain symptoms that may be aggravated as a result of prior negative experiences [11].

Neuroimaging evidence of multifaceted learning processes

Building on this research and on the few existing nocebo neuroimaging studies summarized in **chapter 3**, in **chapter 6** we report an EEG experiment that expands our knowledge of the neurophysiological characteristics of learning in nocebo hyperalgesia. In **chapter 3** we described results from EEG studies that are not yet replicated, with each

study using vastly diverging methods. Our study partly overlapped with two previous nocebo experiments [4,20], but additionally to the resting state measurements we were reproducing from those existing studies, we endeavored for the first time to image the brain's electrophysiology during the learning and evocation of negative pain associations. Thanks to the rigorous analytical power of established EEG biomarkers, we were able to image complex neurophysiological patterns that are markers of specific learning patterns that engage complex cortical and subcortical learning processes.

Our most important findings in **chapter 6** were based on detrended fluctuation analysis, a sophisticated analytical method that reveals the patterns of long-range temporal correlations in the brain, during rest or within a specific task, such as nocebo induction. Our findings added to what we saw in **chapter 4**: complex learning dynamics –translated in **chapter 6** into enhanced complexity in neural dynamics– were associated with larger nocebo magnitudes. Long-range neural networks have been associated with integrative processes in the brain and when thought of in relation to a pain learning task, may mark a process of consolidating information via cooperating memory and sensory processing systems in the brain. In line with this interpretation, connectivity findings in fMRI and also EEG results in **chapter 3** provided evidence of cognitive-sensory integration in nocebo hyperalgesia, for instance by highlighting a role of connectivity between memory regions and the ACC. Taken together, these findings suggest that individuals whose neural patterns of activation are characterized by complex dynamics at rest may engage in increased cognitive integration between past and current pain experiences, in turn being potentially more susceptible to learning nocebo associations.

Past pain experiences have been shown to form differential expectations that influence pain processing [4,20,24,26,30]. In **chapter 6** we reported significant increases in alpha-band power in nocebo responders during

nocebo-augmented pain compared to a baseline pain stimulus. In line with the literature, this finding reflects the role of alpha-band oscillations in the formation of expectations [31,32] and in the cognitive regulation of pain through the integration of past experiences in pain processing [32,33]. Taken together, our EEG findings went beyond merely implicating associative learning in nocebo, by providing a more detailed neurophysiological characterization of a potential cortical integration between learned effects and the processing of noxious stimuli. Findings that point towards long-range temporal correlations in neural dynamics as feature of learning negative associations are crucial because they suggest a potential involvement of integrative learning in nocebo hyperalgesia.

In **chapter 7** we reported an fMRI study designed to examine more precise implications of brain plasticity in pain processing, utilizing a targeted pharmacological manipulation of NMDA-dependent learning. We used induction methods consistent with our previous experiments in **chapters 4** to **6** and comparable to some existing fundamental nocebo fMRI studies [5,34–36]. The results supported findings of an integration of learned associations with sensory inputs under nocebo hyperalgesic conditions. Particularly, results that implicated regions such as the ACC and insula in learning nocebo associations, which are generally in line with the literature as reviewed in **chapter 3**, suggest that the most prominent difference between nocebo and control cues can be seen in brain areas that are thought to synthesize sensory perception based on beliefs and expectations [37]. Activity in the ACC has been related to the graded encoding of pain based on the magnitude of expected pain [37,38]. Brain mechanisms that involve the insula and ACC may thus reflect the meaning of learned negative cues [39]. This type of meaning-related processing of pain through learned expectations could be critical for preparing the sensory system to optimally process noxious information. [37]".

Facilitatory mechanisms are able to amplify the pain experience [39] through a long-range integrative process involving specific aspects of learning that encode and consolidate beliefs and expectations about previously experienced stimuli. Yet, different forms of biobehavioral modulation can influence pain via distinct systems [40] and many variables related to cognitive and emotional factors may further influence nocebo effects. It is noteworthy that in our nocebo meta-analysis presented in **chapter 2**, the studies examined did not generally report exact measures of certain key learning characteristics. For example, measures of baseline learning ability in distinct domains, such as the verbal or visual learning measures we obtained in **chapter 7**, can be helpful in pinpointing sub-processes of learning that are crucial for nocebo responding. Accordingly, direct physiological and behavioral measures of fear, when measured across studies, may hold the potential of better explaining under which conditions learned nocebo responses are augmented. While experimental studies most often measure anxiety levels, in **chapter 3** we showed that anxiety cannot reliably be shown to impact nocebo responses, as measured neurochemically and via imaging techniques. It is thus possible that, in accordance with our results in **chapters 5**, **6**, and **7**, integrative cognitive learning mechanisms function in collaboration with affective learning, despite these latter emotional factors being somewhat neglected in nocebo studies. More precise measures of learning and memory could indeed show a moderating effect on nocebo magnitudes and help explain these effects across the nocebo literature – an important objective for future research.

A potential cooperation of cognitive and fear-specific learning mechanisms

Fear seems to play a significant role in nocebo hyperalgesia, and our work has added to the understanding of how affective learning may

influence the formation of negative associations. In **chapter 3**, limbic structures such as the hippocampus and amygdala point towards a processing of fear in the brain under nocebo conditions [34,35,41]. Our threat manipulations in **chapter 5** support the notion that fear can amplify nocebo responses. At the same time, our EEG results in the gamma-band lead us to speculate that nocebo hyperalgesia potentially involves emotional processes such as fear, that have been shown to engage similar patterns of gamma coupling in the amygdala [42]. This aligned with our fMRI results that also implicated the amygdala in nocebo hyperalgesia. Both behavioral and brain imaging evidence thus suggests that fear is involved in nocebo, and our project attempted to pinpoint precise mechanisms by which fear of pain may affect pain endurance and chronification.

Nocebo attenuation and the challenge of negativity bias

Our behavioral study presented in **chapter 4** was one of the first studies to show an endurance effect of nocebo, and such a resistance to attenuation aligns well with earlier literature in fear conditioning [9,10]. In line with this literature, the resistance effects observed in **chapter 4** may be at least partly attributable to negativity bias (i.e., the tendency to attend to and remember negative experiences over neutral or positive ones [45–47]). A long line of research indicates that negativity bias is a potent attentional effect that can significantly impact our perception [45–47]. When provided with mixed positive and negative information regarding a given stimulus, individuals are more likely to retain negative knowledge [48]. In our study, such a negativity bias may have taken place in the ambiguous learning group that was exposed to a wider range of negative and positive suggestions and associations. In line with previous literature about this type of negativity bias [48], this effect may be of important clinical relevance in pain chronification after exposure to

inconsistent, mixed information and experiences in the clinical setting. Studies indicate that the amygdala is directly involved in coding not only fear but also ambiguity and uncertainty, and amygdala reactivity has previously been linked to classical conditioning under uncertain conditions [49]. Moreover, what we observed in our **chapter 2** meta-analysis was that, when compared to meta-analyses on placebo effects, learned effect on pain that rely on negative rather than positive associations appear to be larger in magnitude –albeit we were not able to systematically compare nocebo and placebo effects in the same set of studies. A potential stronger potency of negative, as compared to positive associations may in part be explained by enhanced learning under negative conditions, such as in experiments where participants learn to expect pain worsening rather than pain relief. We thus observe that during negative pain experiences a potent process of associative learning may interact with fear processing subcortically in the limbic system to create negative expectations and exert an important and enduring effect of the brain and its processing of pain.

Increased negativity: the role of fear

A long line of research has indicated that negative emotions, experiences, and negatively framed information are given more importance and learned more firmly by the brain [50–54], something thought to have an evolutionary explanation in the significance of negative information in avoiding threat [55]. In line with earlier work on fear [3,50,55], our experiment in **chapter 5** indicated that during conditioning, fear resulting from intense pain experiences adds to negative learning, but when the higher pain is never experienced but only anticipated, learning remains mostly unaffected. **Chapter 5** thus in part suggests that a concrete negative experience such as increased pain leads to worse pain responses than a mere anticipated negative experience,

and this effect was fully mediated by pain-related fear. For the first time in a nocebo study, we manipulated and measured fear levels directly and precisely (see also **chapter 2**), by obtaining self-reported levels as well as imaging startle responses via EMG. Startle responses are thought to represent a more direct biobehavioral fear response, as compared to self-reported fear [56,57]. Our results may thus add to a more complete picture of nocebo responses, that may be shaped through a process of learning pain associations by experience, in combination with the cooccurrence of adverse emotional factors such as fear.

This involvement of emotional factors in pain perception highlights the top-down features of pain processing. However, our research shows that nocebo effects do not always involve fear processing and the amygdala. Rather, it seems that only when a stimulus such as pain is identified as emotive to some level, meaning that it may be especially negative or frightening, brain regions concerned with the emotional and cognitive components of pain, such as the amygdala, hippocampus, insula, and ACC become involved [39]. Indeed, this seems to be the case in patients with chronic pain, who may have formed emotive associations with pain and for whom often it is fear of pain that is particularly disabling [58]. A recent study comparing young chronic pain patients and healthy peers indicated that in patients only, increased pain catastrophizing was associated with enhanced threat-safety learning and found resting-state functional connectivity alterations between the amygdala and the inferior parietal lobe, including the insula [59]. These findings are aligned with our fMRI results implicating the amygdala and insula in pain that is aggravated through learning. Insular activity is indeed not only involved in subjective pain experiences, but is also associated with fear processing, and conditions such as irritable bowel syndrome, chronic fatigue, and persisting or insufficiently explained pain symptoms [39,60,61]. Thus, based on our current understanding of the physiological underpinnings of emotional elements that can influence pain processing, learning often seems to take place on two levels. On one hand, a cortical-

level associative learning mechanism may be at the core of acquiring learned effects on pain. On the other hand, it appears that fear-related learning, that may take place in subcortical loops, mediates pain worsening, and may be associated to pain chronification.

In our research, overall, learning through the integration of experiences and pain processing may be differentiated from fear-learning under nocebo hyperalgesic conditions. In **chapters 3**, **4**, and **5** we discussed a mediating role of uncertainty and fear in nocebo hyperalgesia. However, in **chapter 7**, D-cycloserine not having any detectable effect on nocebo hyperalgesia is discussed from the perspective of subcortical NMDA receptor modulation. Because D-cycloserine seems to sometimes yield results in research on learned fear responses [62–66] but not always on other types of non-affective learning [67]. It is thus possible to speculate that D-cycloserine may be more effective in modulating subcortical NMDA circuits engaged in paradigms with a heavier fear load [68]. As such, our pharmacological experiment led us to speculate that the fear component reflected through findings in **chapters 3**, **5**, and **7**, could potentially be a secondary affective component that could modulate –but may not primarily underlie– nocebo hyperalgesic responses. While this is merely one speculation, further research specifically measuring fear levels is needed in order to understand the role of NMDA-dependent learning in nocebo hyperalgesia. Understanding the exact vulnerabilities caused by cooccurring affective and sensory processing is highly relevant for unravelling the etiology of persisting pain symptoms that are not fully explained by physical damage [59,61,69].

The challenge of persisting pain symptoms lies in the multidimensional character of pain processing, influenced by previous experiences, beliefs, pain cognitions, as well as emotional factors, additionally to neurobiological factors directly related to sensory input [70,71]. And while the cognitive and emotional literature on pain has yielded abundant evidence for their role in pain aggravation and chronification [61,72–75], the

current understanding of the precise mechanisms that underlie established biobehavioral correlates of nocebo hyperalgesia is still in its infancy. But as growing evidence, discussed in the current dissertation, builds on an explanatory framework for pain aggravation and chronification, the cooperation between negative experiences, cognitive and emotional learning, and sensory integration becomes increasingly relevant for experimental and clinical pain research. Maladaptive learning and emotional factors provide a clinical relevance to the currently known biobehavioral correlates of nocebo hyperalgesia, and have led some to hypothesize that targeted treatments could influence and even reverse the relevant neurobiological aberrances, by addressing learning and emotional dynamics [61]. Targeting central components such as aversive learning and fear of pain in patients may help normalize specific brain alterations that underlie learned pain responses. Still, issues of generalizability and ecological validity, as well as a lack in replication of findings within the field, may pose limitations in nocebo research and interpretation.

Limitations in the project and the field

A central limitation in the neuroscientific nocebo literature, as initially found in **chapters 2** and **3**, is the widespread inconsistency in methods used and results yielded by experimental research. In this project in particular, while **chapter 6** generally confirmed the involvement of intricate learning dynamics in the top-down, cognitive processing of pain signals, it did not replicate specific results of two previous EEG nocebo studies [4,20]. Three studies to date, including our own, that have examined the involvement of alpha oscillations in nocebo effects, have found divergent results. Alpha-band neuronal activity has long been implicated in internal cognitive states with low external informational loads [76–79]. It is thus likely that different phases and contexts of nocebo

experiments engage internal cognitive processing differentially and should be examined with precision within and between studies. We underscore a limitation within the nocebo field to replicate precise findings, as also discussed in detail in **chapter 3**, which can be overcome by sharing study protocols between researchers and a collaborative consideration of experimental study designs –an important objective for open and reliable science.

A lack of consistency and specificity in the research and reproduction of findings in the nocebo literature is an unsurprising feature of a young field of research. Biobehavioral nocebo studies have been striving to contribute novel findings to the knowledge base of learned pain responses, attempting new experimental models, methods, and manipulations each time. Our work in **chapters 2** and **3**, however, suggests that as the literature is growing, there is a pressing need for confirmatory research, of the kind that will at least keep one eye on comparability and replication of existing studies in the field. Through our systematic and comprehensive reviews in this dissertation, we found many novel paradigms and results, with novelty supposed as a golden standard in scientific publishing, as though an objective in itself. In pain research, however, novelty is not inherently equated to the successful furthering of our understanding of nocebo effects and pain. Yet, grant subsidies for research are mostly awarded for novel research and ethical dilemmas may arise when focusing on replication alone, which complicates the issue of replicating previous findings. While we strived, in this PhD project, to maintain consistent methods throughout our experiments and the existing literature, we also fell short of conducting direct and precise study replications.

Indeed, in a field of science that is still in its infancy, **chapters 4** to **7** contributed a mixture of reproduced findings (such as that of partial reinforcement in **chapter 4**), cutting-edge novel methods (such as the application of EEG biomarkers on the imaged experience of nocebo-

augmented pain), and novel biobehavioral manipulations and results. There is a known bias in publishing unique ideas that create novel scientific work. What our reviews (**chapters 2** and **3**) have inherently and inevitably discounted, is the unsuccessful attempts to induce or manipulate nocebo effects on pain. Our knowledge base for nocebo hyperalgesia thus has a blind spot, in that we cannot factor in those variables and outcomes that were never published in peer-reviewed scientific journals –an explicit inclusion criterion in **chapter 2**. With vast estimated numbers of "unexciting" unpublished scientific research in the social sciences, it is imperative for our scientific community to make an active effort in creating fairer and more accessible publication routes for those null results that add to our genuine understanding of complex and potentially detrimental biobehavioral effects on pain.

Further limitations relate to the experimental work of this thesis and concern the methods used, as well as the reproducibility and clinical significance of findings. What is the significance of findings in young, educated, healthy participants that experienced short-lived experimentally induced pain, fear, and nocebo effects? Ours is not the only field of biobehavioral science that largely relies on psychophysiological modelling approaches [80] in order to induce and quantify phenomena such as nocebo hyperalgesia. But in the construction of experimental models of nocebo hyperalgesia, less attention is paid to their clinical validity and more to creating the strongest, most reliable, or most reproducible laboratory models. Some studies have paid particular attention to the accuracy of modeling clinical pain, by inducing realistic visceral pain symptoms [29,81,82], which is an important step towards the real-world applicability of experimental conclusions. In our studies, we carefully considered the different types of experimental models that we could possibly build to represent the putative clinical phenomenon of nocebo hyperalgesia. We opted for idealized and exploratory models [83], in which a deliberate simplification of hypothesized mechanisms and processes was able to keep other

variables constant while exploring specific learned effects on pain. In **chapter 3** we discussed in detail that these types of models are necessary in studying nocebo effects, due to the multifaceted and convoluted nature of pain. But in utilizing experimental models, we and much of the field at large neglect to scrutinize the imbalances between epistemic accessibility to specific variables, and the ability to draw conclusions regarding a realistic and clinically relevant target system or process [83] such as nocebo hyperalgesia. There are lessons to be learned from decades of academic research into the modeling of hypothetical phenomena [83] for every branch of biobehavioral science. Such lessons may indicate that the field of nocebo research should progressively shift away from fundamental science –notwithstanding the invaluable scientific contribution of early fundamental research in any given field– and graduate to more ecologically valid research with a focus on clinical nocebo phenomena.

Future directions and recommendations

Considerations of the nature and content of our experimental models open new avenues for nocebo research as a model-based science. Currently, nocebo experiments typically induce hyperalgesia in healthy individuals, often building representational, idealized models of nocebo hyperalgesic effects, from acquisition to extinction. A vast array of scientific models are representational, in that they represent a selected aspect of the world, which is thus the model's target system [83]. Examples include the Bohr model of the atom, models of predator–prey interaction, the scale model of a bridge, and learned nocebo effects on experimentally induced pain. Different types of models could represent different aspects of the target system, or even distort the system or processes itself, raising the question what it means for an experimental model to represent a select part of a real or hypothetical phenomenon.

These are important questions for the field of pain and nocebo research to address and bear in mind while building experimental nocebo models. Idealized and exploratory models are a crucial means for pain research to cope with systems that are as difficult to study in their full complexity as pain [84]. But the shortcomings of experimental modeling need to be moved to the foreground if we are to attempt improving the ecological validity and representational powers of experimental nocebo research. For example, a consensus could be achieved between researchers and clinicians regarding which models best and most accurately represent nocebo hyperalgesia, and these models can provide a basis on which nocebo effects are researched, as is largely the case for example for animal models of schizophrenia [85,86].

Another solution for the distance between experimental nocebo models and real-world pain phenomena could be to utilize validation and calibration techniques based on clinical knowledge. Models play an important role in science, as vehicles for learning about the phenomena observed in the world that are out of reach or intensely convoluted, such as chronic pain. Experimental models of nocebo effects allow for 'surrogative reasoning', a mode of scientific investigation in which features and outcomes of a system are examined by studying a model, rather than reality itself [87]. But this type of model-based reasoning, with its limitations as discussed above, should be based on active evaluation and adaptation of models [88,89] if we are to best represent real phenomena in patient populations. Bach and colleagues have proposed a valuable method to assess face validity and the fit of an experimental model, called retrodictive validity since the aim is to 'retroactively predict' the experimentally induced value of a given biobehavioral attribute [80,90] such as a nocebo effect on pain. In experimental research on such attributes, hypothetical true scores can be influenced by experimental manipulations, and this allows us to apply metrological calibrations. Bach and colleagues propose that an influenced value representing the true score in such a calibration experiment can provide a retrodictive

validity criterion to assess the accuracy of a model [90]. A comprehensive validation of an experimental nocebo model should thus rely on some understanding of "true" or clinical nocebo scores and their correlates within their natural systems, such as in clinical practice and based on, for example, specific clusters and characteristics of chronic pain symptoms.

For experimental models to evolve and improve, and for the consistency that we missed in **chapters 2** and **3** to be achieved in the field, there is thus a need to obtain clinical markers that can provide a basis for model validations. This is not to say that nocebo experiments should necessarily be performed on clinical populations, but rather, clinical pain scores and nocebo markers can serve to optimize the valuable models on which we can examine nocebo effects with accuracy and precision [89,91]. In other words, focusing on the symptomatology of pain patients that are thought to present with negative learned effects on their pain may provide researchers with more accurate representations of potential nocebo magnitudes and impacts outside the laboratory. Potentially unrealistic assumptions are inevitable features of experimental modeling [91], especially so of hypothetical biobehavioral phenomena, but clinical measurements can provide promising avenues forward for building more clinically applicable representations of nocebo hyperalgesia. While few studies have attempted to measure nocebo susceptibility and responding in patient populations [28,29,92], it is imperative to start building on this work. Validating the assumptions of our idealized experimental models in a way that can be applicable across experimental settings holds the potential of combating the inconsistencies and lack of replicability in results, while concurrently encouraging a more valid and accurate platform for understanding nocebo effects and their biobehavioral moderators.

Clinical practice is proposed here as a powerful reflective tool for experimental research, a tool to inspect and improve our modeling and understanding of nocebo effects, learned associations, and top-down

pain processing. Yet, three decades of research into nocebo hyperalgesia have also provided us with important and clinically relevant insights into the detrimental effect of learning on pain experiences. The early knowledge that nocebo research has generated for clinical practice should not be underestimated. It is consistently shown that contextual experiences and communication of negative outcomes can shape the way in which individuals experience pain [12,92–96]. In this dissertation, we additionally showed that fear of pain as well as individuals' physiological learning patterns and baseline brain dynamics can further facilitate negative pain associations resulting in increased pain sensitivity. The phenomenon of pain can thus be seen as a system that, prior to conscious pain perception, engages in the top-down cognitive and often emotional processing of ascending noxious stimuli, giving rise to an inherently subjective pain experience. This PhD project supported and expanded upon previous work on nocebo effects, showing that learning and fear play key roles in this top-down processing of pain. In the clinic, these findings could be applied to defuse those factors that we now know to reinforce negative associations, such as negative suggestions by healthcare professionals, contextual triggers of negative associations, traumatic pain experiences, uncertainty, and fear. From the clinical perspective our finding that counterconditioning was more effective than extinction in minimizing nocebo responses may also open avenues for behavioral treatments for pain symptoms that may be aggravated by learning.

Conclusions

Negative experiences influence the brain and can decidedly alter the experience of pain. Past experience appears to shape future experience. This PhD dissertation focused on enriching our understanding of negative learned effects on pain by investigating nocebo hyperalgesic

effects and the factors that characterize biobehavioral aspects of pain processing. We carried out systematic and comprehensive reviews and a meta-analysis of existing studies, as well as a series of experimental studies utilizing a resourceful mixture of classic and innovative biobehavioral methods, including classical conditioning, EMG, EEG, and fMRI. Our findings emerging from this work support the understanding of learning as an intricate, multifaceted, and powerful process, able to detrimentally influence sensory perception, altering the way in which individuals perceive pain. Our knowledge from nocebo research highlights the vast variability of sensory perception and conscious experience in humans. The results of the present PhD project further support the notion that negative inputs from the environment become encoded in our plastic brains, producing measurable adverse effects on pain. If we are to utilize research to improve pain management and outcomes, there is a pressing need for scientific research to translate this growing understanding of learned pain responses beyond the laboratory and into clinical practice.

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Curriculum Vitae

Brief CV

Mia Athina Thomaidou was born on 23 September 1989 in Greece and grew up in a small suburb on the coast of Athens. She attended High School in Anavissos and Kalyvia between 2004 and 2007. In 2011, Mia moved to England and commenced her university studies a year later. During her undergraduate studies at the university of Westminster, London, she attended the University of Oxford for one year, where she completed and published a neuropharmacological research study. She also volunteered as an assistant clinical neuropsychologist at Chelsea and Westminster Hospital in London. In 2016 Mia received her bachelor's degree in Cognitive Neuroscience with honours. Pursuing her interest in science, she moved to the Netherlands and started a Master's in Clinical Neuropsychology at Leiden University. During that master's, she did a research internship at the Center of Neurogenomics and Cognitive Research, Vrije Universiteit Amsterdam, where she completed an independent research project based on brain imaging data mining. In late 2017, Mia started her PhD-project in the research group of Prof. Andrea Evers at Leiden University. Mia worked on a project sponsored by a Vici grant awarded to Prof. Evers by the Netherlands Organization for Scientific Research and investigated the biobehavioral underpinnings of learned pain. In addition to the research activities, she supervised and taught bachelor's and master's students and presented her work at numerous conferences. During her PhD, Mia also completed a second master's degree in Comparative Criminal Justice at Leiden Law School, with the aim of combining biobehavioral and legal research in her future career. In 2022, Mia is completing her PhD while also working on several research projects in collaboration with Rutgers University, New Jersey, that investigate how biobehavioral science is translated and used in criminal justice systems.

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Dutch Summary

Samenvatting

Dit proefschrift draagt bij aan een groeiend onderzoeksveld die verouderde opvattingen over pijn als een bottom-up proces ter discussie stelt. In dit project hebben we een reeks psychobiologische gedragsstudies uitgevoerd om onze kennis uit te breiden over hoe bottom-up pijnsignalering kan worden beïnvloed door de top-down verwerking, die tevens bij pijn betrokken is. We hebben hierbij gebruikt gemaakt van verschillende methodologieën, zoals een grootschalige meta-analyse, een omvangrijke review, verschillende gedrags-experimentele studies, evenals experimenten met behulp van beeldvormende technieken zoals fMRI, EEG en EMG. We onderzochten daarbij verschillende soorten ervaringen, zoals het ontvangen van negatieve informatie of het direct ervaren van een negatief effect, die kunnen leiden tot sterkere of meer aanhoudende nocebo-effecten op pijn. We wilden bovendien de onderliggende biologische gedragscomponenten van dergelijke aangeleerde pijnreacties ontrafelen. Gedragsparadigma's werden gebruikt om pijnervaringen uit het dagelijks leven te modelleren, door middel van gevalideerde experimentele pijninductiemethoden, nieuwe experimentele leermanipulaties, evenals een nauwkeurig onderzoek van emotionele correlaten zoals angst. Tegelijkertijd werden diverse, innovatieve neurowetenschappelijke methoden - waaronder een farmacologische manipulatie - gebruikt om de psychobiologische gedragsmatige onderbouwing van nocebo hyperalgetische reacties te onderzoeken. Onze bevindingen dragen bij aan de groeiende kennis op het gebied van nocebo hyperalgesie, welke aantoont dat leren door ervaring een bepalende invloed kan hebben op de verwerking en perceptie van pijnlijke stimuli.

In **hoofdstuk 2** werd door een systematische review en meta-analyse aangetoond, dat leren door ervaring een krachtig mechanisme is dat de perceptie en persistentie van pijn kan beïnvloeden. Voortbouwend op

de afgelopen twee decennia van toenemende focus op nocebo-onderzoek, geeft deze uitgebreide meta-analyse nieuwe inzichten in de huidige kennis van bekende gedragscorrelaten en pijnresultaten onder nocebo-hyperalgetische condities. Klassieke conditionering bleek krachtiger te zijn dan het geven van verbale negatieve informatie, wat aantoont dat het ervaren van ongemak bepalender kan zijn in het creëren van negatieve verwachtingen, in vergelijking met verbale methoden. Bij het onderzoeken van wat de effect grootte van nocebo-responsen tussen verschillende onderzoeken modereert, vonden we echter geen significante modererende factoren in onze data. Hierbij moet wel worden opgemerkt dat verschillende factoren niet systematisch werden gemeten, zoals angst voor de pijnstimulaties of specifieke contextuele factoren, die mogelijk een deel van de variabiliteit in nocebo reacties kunnen verklaren. Meer systematische en vergelijkbare studies over deze aspecten zijn nodig. **Hoofdstuk 2** laat dus weinig twijfel over de mogelijkheden van aangeleerde effecten op pijnperceptie, maar roept een aantal vragen op en identificeert kennishiaten met betrekking tot de mogelijke cognitief-emotionele en biologische gedragsmatige en mediërende factoren bij nocebo hyperalgesie.

In hoofdstuk 2 werd aangetoond dat nocebo-effecten aanwezig zijn bij verschillende sensaties en soorten pijn, dit bracht ons ertoe om ons in **hoofdstuk 3** te verdiepen in de diverse literatuur over de neurobiologische correlaten van nocebo-hyperalgesie. Om de huidige kennis samen te vatten en te kunnen benutten, werd een omvangrijke review gedaan van de neurobiologische nocebo-literatuur over pijn. Tweeëntwintig studies werden geïncludeerd op basis van uitgebreide database zoekopdrachten. Een review van deze experimenten benadrukte dat nocebo-effecten als een top-down fenomeen, gebaseerd is op aangeleerde effecten. Het is aangetoond dat nocebo-effecten worden beïnvloed door basale nociceptieve signaalgeleiding in het ruggenmerg, evenals door hogere cognitieve functies zoals emotionele verwerking en verwachtingen. Een belangrijke bevinding hierbij was de

duidelijke inconsistentie in gebruikte methoden en resultaten tussen nocebo-onderzoeken; dit zorgde voor de motivatie om consistente en vergelijkbare methoden te gebruiken in het experimentele werk van dit proefschrift. We adviseren dat het veld als geheel reproductie en replicatie van experimentele methoden moet nastreven, om een robuuste en betrouwbare kennisbasis voor nocebo-effecten te bewerkstelligen. Ten slotte kwam met dit literatuuronderzoek de centrale vraag naar voren welke exacte leermechanismen aanleiding kunnen geven tot nocebo-effecten en hoe dit zich verhoudt tot pijn buiten het laboratorium en in het dagelijks leven.

Hoofdstuk 4 beschrijft een eerste experimentele studie met als doel aan te tonen of nocebo-effecten kunnen worden geïnduceerd – en hoe ze kunnen aanhouden – wanneer ze gebaseerd zijn op inconsistent en variabel leren, vergelijkbaar met wat patiënten kunnen ervaren in klinische settings. We vergeleken een typisch conditioneringsparadigma met een paradigma met variabele versterking van de nocebo-associatie tussen pijn en een inerte behandeling. Daarnaast trachten we het geïnduceerde nocebo-effect te verminderen om de dynamiek van verschillende leerschema's in de loop van de tijd te onderzoeken. Hoewel niet verrassend, ontdekten we dat een meer ambigue leermethode leidde tot kleinere – zij het significante – nocebo-effecten. Bovendien merkten we op dat deze kleinere effecten, interessant genoeg, persistenter waren in de tijd en weerstand boden aan counterconditionering. Deze studie richtte zich op behandelingsresistentie en chronificatie van pijn wat relevant is voor mogelijke ervaringen in de klinische setting en benadrukte de rol van verschillende soorten leerprocessen bij nocebo-hyperalgesie, en beantwoordde daarmee enkele openstaande vragen uit de hoofdstukken 2 en 3. Deze studie ging echter niet in op de impact van belangrijke emotionele correlaten zoals angst, een factor die niet consequent werd gerapporteerd in de onderzoeken die in hoofdstuk 2 zijn geanalyseerd, maar mogelijk een rol speelt bij nocebo-hyperalgesie.

Daarom hebben we in **hoofdstuk 5** een experimentele vervolgstudie ontworpen om de rol van angst in aangeleerde pijnreacties te onderzoeken. Ondanks de aangetoonde betrokkenheid bij pijn en andere klinische uitkomsten, werd angst meestal over het hoofd gezien door het nocebo-veld, en onze studie was de eerste die de betrokkenheid van verschillende soorten angst bij nocebo-hyperalgesie heeft gemanipuleerd en gemeten. Hierbij hebben we ook angstreacties in beeld gebracht, door schrikreacties te meten via EMG tijdens een nocebo-paradigma. Naast de typische nocebo-inductiegroep als controle, creëerden we additioneel een groep die over het algemeen hogere pijnstimulaties zou krijgen, en een andere groep die angst-opwekkende informatie ontving over een mogelijk slecht resultaat. Deze twee groepen rapporteerden, zoals verwacht, over het algemeen hogere niveaus van angst voor pijn, en de groep met meer pijn reageerde ook met significant grotere nocebo-hyperalgesie. De resultaten gaven verder aan dat er meer onderzoek nodig is om de specifieke aspecten van centrale pijnintegratie met cognitief-emotionele factoren te ontrafelen. De meerwaarde van het gebruik van bekende beeldvormende instrumenten om angst voor pijn op biologisch gedragsniveau te meten, leidde tot de nieuwe benadering van het gebruik van elektrofysiologische biomarkers om pijnintegratie en pijnverwerking onder nocebo-hyperalgetische omstandigheden beter te begrijpen. In het volgende hoofdstuk hebben we daarom beeldvorming van de hersenen toegepast met behulp van EEG, om beter te begrijpen hoe neurocognitieve verwerking de pijnervaring beïnvloedt.

Hoofdstuk 6 onderzoekt de elektrofysiologie van aangeleerde pijnreacties in perspectief tot de momenteel bekende markers van pijn en van emotionele verwerking. We gebruikten geavanceerde EEG-biomarkers om complexe elektrofysiologische patronen te karakteriseren tijdens baseline pijnperceptie, en de acquisitie en vervolgens evocatie van nocebo-reacties. We hebben bovendien hersenelektrofysiologie in rust gemeten en berekend, voor en na het

experimentele paradigma, om baseline kenmerken te onderzoeken die de acquisitie van hyperalgetische effecten kunnen moduleren en om de veranderingen van voor tot na nocebo-acquisitie te onderzoeken. We ontdekten dat individuen die een hogere complexiteit van neuronale oscillatiepatronen hadden bij baseline, inderdaad grotere nocebo-responsen vertoonden. Tegelijkertijd werden ook verschillen gevonden in hoe het brein de verhoogde pijnstimulatie verwerkt bij baseline versus een nocebo-versterkte waargenomen pijntoename. EEG leverde verschillende nieuwe inzichten op in het neurofysiologische fenotype van nocebo-hyperalgesie, waardoor we een eerste globaal beeld konden schetsen van de complexe neurale signatuur van nocebo-hyperalgesie. Deze studie roept ook vragen op, aangezien EEG-methoden beperkingen hebben voor wat betreft de lokalisatie van effecten in de hersenen, evenals in het meten van specifieke functionele bijdragen van verschillende hersenstructuren. Een vervolgonderzoek met fMRI, in hoofdstuk 7, richt zich op deze beperkingen.

In **hoofdstuk 7** werd in een innovatieve farmacologische fMRI-studie, de specifieke bijdrage van verschillende hersengebieden met betrekking tot het leren van nocebo effecten en de betrokkenheid van NMDA-receptoren in deze hersengebieden die het leren vergemakkelijken, nauwkeurig onderzocht. Hoewel we nog steeds gebruik maakten van consistente experimentele nocebo-inductiemethoden met het oog op de vergelijkbaarheid en betrouwbaarheid in het veld, hebben we in deze studie getracht een farmacologische manipulatie van leren tijdens nocebo-inductie in de MR-scanner uit te voeren. We gebruikten hiervoor D-cycloserine, een medicijn dat bekend staat om zijn vermogen leren te verbeteren door middel van NMDA-receptor agonisme, om te onderzoeken of een groep met een verbeterd leervermogen een grotere nocebo-respons zou vertonen dan een groep deelnemers die placebo kregen. We ontdekten dat, ondanks de farmacologische manipulatie die geen significante gedragseffecten vertoonde, hersengebieden die eerder betrokken waren bij associatieve vormen van leren, nocebo-stimuli

onderscheiden van controlestimuli. Deze neuroimaging-studie bevestigde ook de resultaten gevonden in hoofdstukken 3, 5 en 6 over de emotionele correlaten van nocebo-hyperalgesie, en opent daarmee mogelijkheden voor toekomstig onderzoek naar specifieke hersen mechanismen als bepalende factoren van aangeleerde effecten.

In de discussie van dit proefschrift integreren en interpreteren we de bevindingen van dit proefschrift in relatie tot elkaar en tot de bredere literatuur over geleerde effecten op experimentele en klinische pijn. Er zijn twee centrale bevindingen die voortkomen uit het werk van dit proefschrift, beide gerelateerd aan de ingewikkelde dynamiek tussen nociceptieve verwerking en cognitief-emotionele experiëntiële factoren. De meest belangrijke bevinding, dat specifieke leermethoden pijnverwerking in de hersenen bepalen, wordt besproken als de belangrijkste cognitieve drijfveer van nocebo-hyperalgesie. We bespreken hoe leren toekomstige pijnervaringen kan veranderen op basis van ervaringen uit het verleden en negatieve verwachtingen. De tweede kritische bevinding van dit project, dat het leren van angst een mediërende rol kan spelen bij nocebo-inductie en persistentie, wordt besproken in relatie tot hiaten in de literatuur en ons algemene begrip van negativiteitsbias en emotioneel geheugen. We bespreken verder de beperkingen van dit werk en van dit modelgebaseerde wetenschappelijke veld als geheel, en we stellen toekomstige richtingen voor met betrekking tot nocebo-onderzoek en voor de klinische praktijk. We concluderen dat nocebo-effecten onmiskenbaar pijn beïnvloedt, en dat dergelijke aangeleerde effecten afhankelijk zijn van de neiging van de hersenen om cognitieve en emotionele informatie te leren, aan te passen en te integreren, vooral in relatie tot eerdere negatieve ervaringen.