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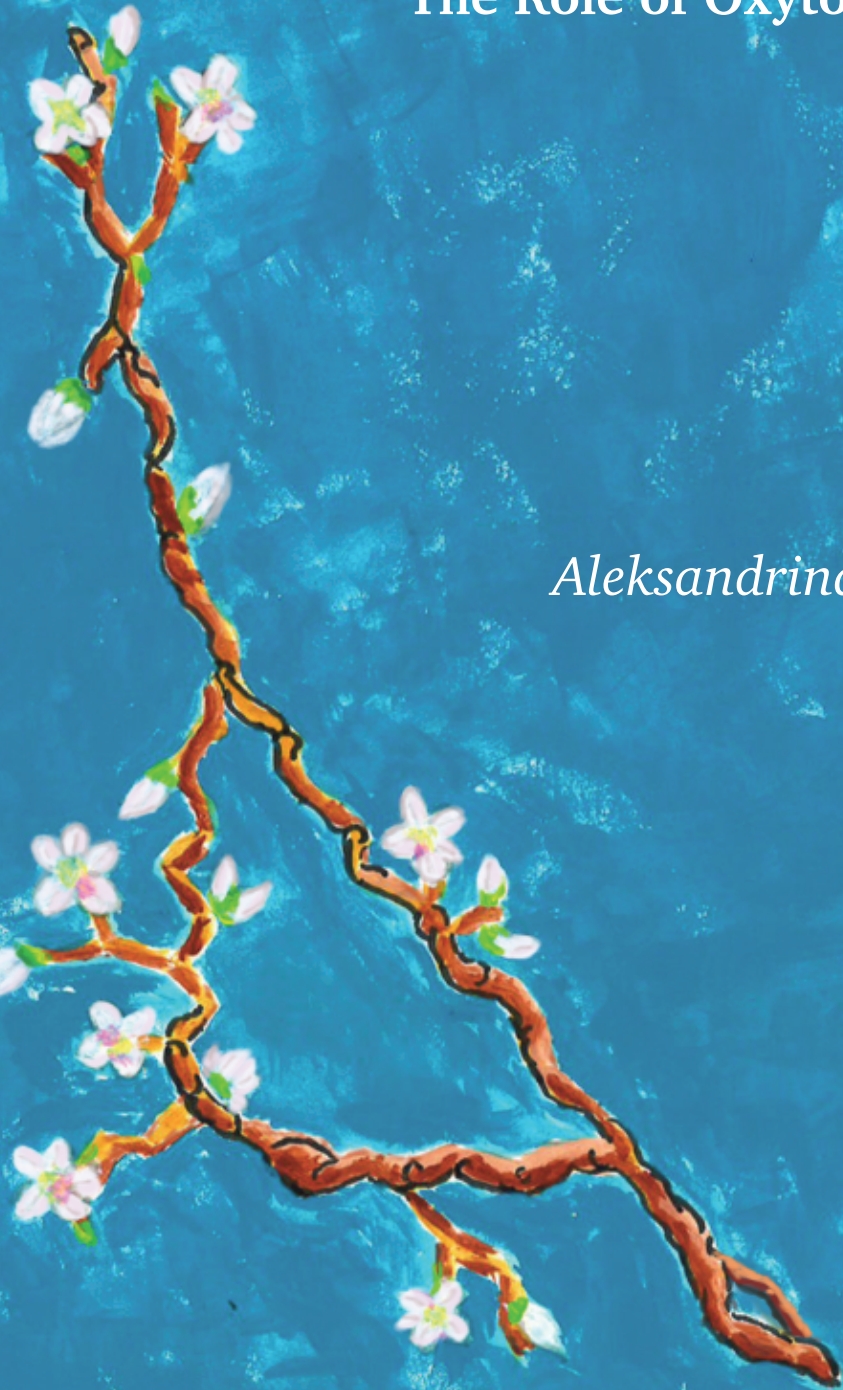
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Placebo Effects and The Endocrine System: The Role of Oxytocin

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Placebo Effects and the Endocrine System: the Role of Oxytocin

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Table of Contents:

Chapter 1	Introduction	7
Part I	Conditioning of oxytocin and other endocrine responses	25
Chapter 2	Conditioned hormonal responses: a systematic review in animals and humans	27
Chapter 3	Placebo effects in the neuroendocrine system: conditioning of the oxytocin responses	63
Chapter 4	Effects of oxytocin administration and conditioned oxytocin on brain activity: an fMRI study	87
Part II	Influencing placebo effects with oxytocin	121
Chapter 5	Enhancing placebo effects in somatic symptoms through oxytocin	123
Chapter 6	Effects of oxytocin on placebo and nocebo effects in a pain conditioning paradigm	145
Chapter 7	Summary and General discussion	163
	Nederlandse Samenvatting	185
	Curriculum Vitae	191
	Acknowledgements	195

Chapter 1

Introduction

The golden scientific standard to check treatment efficacy is to compare it to a control treatment that is similar to the initial treatment but does not have its active ingredients. We term this control treatment the placebo treatment. Applying this design enables scientists to distinguish the effects of the treatment from the nonspecific effects, i.e. the placebo effects. An important reason for this design is that a significant improvement is often also found in the placebo groups without an active treatment component. Placebo effects are defined as positive treatment outcomes due to mechanisms such as expectancies of the patients that cannot be attributed to the treatment's actions (1). Nocebo effects, to the contrary, are negative treatment outcomes that cannot be explained by treatment's specific mechanisms (2). Multiple meta-analyses demonstrated that a large body of evidence exists that the placebo effect improves chronic physical complaints such as pain (3), symptoms of chronic dermatological conditions, such as itch and skin lesions (4, 5), symptoms of mental disorders such as major depression (6) and alcohol use disorders (7), Parkinson's disease (8), irritable bowel syndrome (9), and many more. Previous research also focused on the link between placebo effects and the immune system and demonstrated that placebo effects can affect immune responses of the body (10). To the contrary, far less is known about the link between placebo effects and the endocrine system (11).

The endocrine system is an important regulatory system that maintains a wide range of physiological functions. The main function of the endocrine system is producing hormones, which are chemical elements that are released into the circularly system of the body and regulate functions of organs. Importantly, hormones produced in the periphery are able to reach the brain by penetrating the brain blood barrier or via regions that lack a brain blood barrier such as regions around the ventricles (12). At the same time, hormones that get released in the brain are spread through the whole body via the circulatory system (13). Being a part of a very complex system, hormones regulate various aspects of homeostasis: from blood pressure, digestion, sexual functions (14) to complex social behaviors (15). A dysfunction of the endocrine system is present in approximately 5% of the population (16). These disorders are mostly due to the underproduction of a certain hormones, such as insulin in diabetes type-1, or in contrast the excessive release of hormones, such as thyroid hormone in hyperthyroidism. Moreover,

Chapter 1

Introduction

hormones influence symptoms of mental health disorders, such as mood disorders (17), schizophrenia (18), borderline personality disorder (19) and post-traumatic stress disorder (20). Considering this major role of hormones in all aspects of human health, a better understanding of the physiological mechanisms that influence the production of hormones, and on the other hand, the effects of hormones on different health-related processes, is essential for finding new ways to treat various diseases.

However, despite the accumulating evidence on the importance of placebo effects for health outcomes and the major role of hormones in maintaining health, the interrelationship between these factors has not been studied extensively. Almost no studies have been done on how placebo effects can affect the endocrine system. Moreover, not much is known about possible effects of hormones on the placebo and nocebo effect. In this dissertation, we address this gap in knowledge and consider the bidirectional link between placebo effects and the endocrine system with a focus on the hormone oxytocin. The theoretical model that connects the aims of this dissertation is presented in Figure 1. As reflected in Figure 1, we distinguish two perspectives in this approach: 1) the possibility to trigger placebo effects in the endocrine system and specifically in oxytocin (upper part of the figure), and 2) the potential moderating role of oxytocin in placebo and nocebo effects (bottom part of the figure).

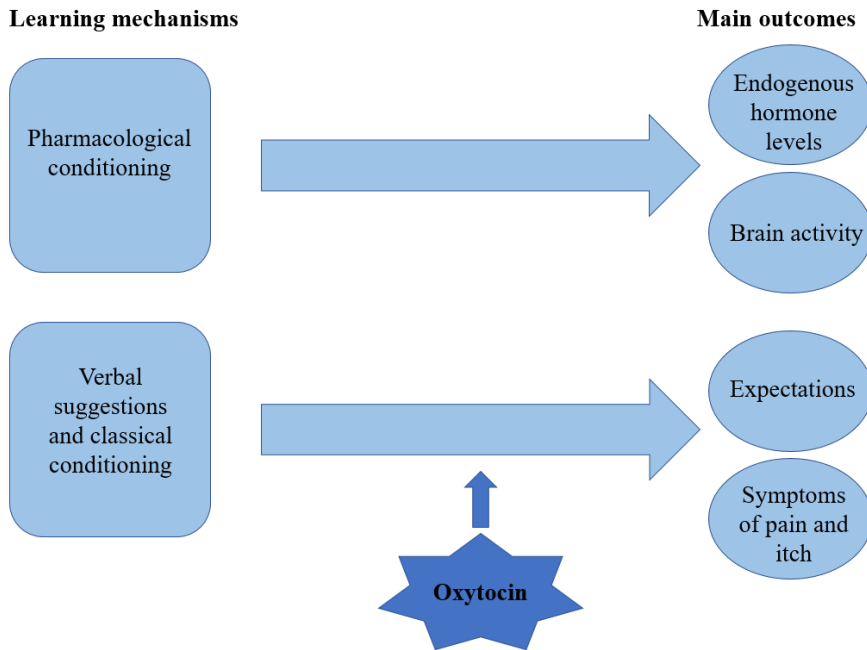


Figure 1. The working model of the dissertation.

Learning mechanisms of the placebo effect

The placebo effect is most commonly described from a learning perspective (21-23). Colloca and Miller (21) have proposed a learning theory of the placebo effect that states that placebo effects are triggered by expectations that in turn are being formed on the basis of how the brain interprets stimuli coming from the environment. The authors have described three mechanisms that link environmental stimuli with expectations: 1) classical conditioning (associative learning), 2) communication or verbal suggestions (instructional learning), and 3) social observational learning. In the following sections we will discuss the first two mechanisms and their role in eliciting placebo responses in the hormonal system, as the link between observational learning and the endocrine system has not been studied so far.

Classical conditioning

Chapter 1

Introduction

Classical or Pavlovian conditioning is a learning process in which an association is established between an initially neutral stimulus and a physiologically relevant unconditioned stimulus (US) leading to an unconditioned response (UR). After repeated pairings, the neutral stimulus becomes a conditioned stimulus (CS) and triggers a response similar to the US; the conditioned response (CR). Two broad but separate areas can be identified in which the principle of classical conditioning of placebo effects is applied: 1) pharmacological conditioning (conditioning of drug effects) and 2) conditioning by means of manipulation of stimulus intensities (conditioning of somatosensory sensations such as pain).

Pharmacological conditioning is frequently investigated by applying a two-phase conditioning design consisting of an acquisition phase (in which learning takes place) and an evocation phase (in which the learned association is tested). In the acquisition phase, an active pharmacological drug (US) is coupled with an initially neutral stimulus (e.g., a drink, CS). In the evocation phase, the drug is replaced with placebo and presented together with the CS. Physiological or psychological responses to the placebo are usually measured in the evocation phase (e.g., 24, 25, 26). In case of successful conditioning, the CS triggers a conditioned response similar to the drug effect even if the active medication is replaced by placebo. The placebo effect thus mimics the response to the medication in this paradigm. Convincing evidence exists that pharmacological conditioning can affect several immune parameters (10), such as interleukin-2 (27, 28), histamine (29), T-cell proliferative capacity (30), and interferon-gamma (28, 31). Placebo effects triggered by classical conditioning in the endocrine system are far less investigated. A number of animal studies have demonstrated that secretion of corticosterone (32, 33), insulin (34), and sex hormones (35), among others, can be classically conditioned. Far less human research focused on this topic, with only a few studies that investigated pharmacological conditioning of cortisol (36, 37), growth hormone (36) and insulin (38, 39). Because of the important role of hormonal responses in the majority of physiological functions and the prevalence of endocrine diseases, the possibility to induce placebo effects in the endocrine system (i.e., by means of pharmacological conditioning) holds great promise for clinical purposes.

Another frequently used application of conditioning is the manipulation of stimulus intensities in order to induce placebo (or nocebo) effects. Most of the research with the manipulation of stimulus intensities has been done in the field of pain (e.g., 40, 41-44). In such studies, placebo analgesia (i.e., placebo-induced pain relief) and nocebo hyperalgesia (i.e., nocebo-induced worsening of pain) are investigated by employing the same two-phase conditioning design as described above. However, in this type of research, the unconditioned response is triggered not by a pharmacological drug but by manipulating the intensities of the stimuli presented to a participant. In case of pain conditioning, in the acquisition phase, different levels of pain stimulation are coupled with the presentation of different visual stimuli (CS). For instance, low intensity pain stimuli are coupled with a green computer screen, and high pain intensity stimuli are coupled with a red computer screen. During this phase, an association between the color and the pain is established. In the evocation phase, participants receive the same levels of medium pain intensity with green and red cues. A decrease in reported pain in response to green trials in the evocation phase is considered indicative of placebo analgesia, while the increase in pain in response to red trials is considered indicative of nocebo hyperalgesia. Various conditioned stimuli have been used in these paradigm such as colors (40, 45), geometrical shapes (46), and photos of faces (47). Overall, a large body of evidence demonstrates that this conditioning procedure is effective in inducing placebo (see meta-analyses 3, 48) and nocebo effects (49, 50) for pain.

Verbal suggestions

Verbal suggestions are another well-investigated method to induce placebo effects in a laboratory setting. Giving information about positive effects of the treatment triggers response expectancies that are defined as anticipations of nonvolitional, subjective, and behavioral responses to particular cues (22). These expectancies are directly linked to the placebo response: if a patient is told that a sugar pill is a strong analgesic that should decrease his/her pain, most probably he/she will feel an actual pain decrease. A large body of evidence exists that giving mere verbal suggestions regarding symptom-reducing effects of a sham treatment can significantly reduce symptoms of, among others, pain (3), fatigue (51), headache

(52), and itch (53). At the same time, suggestions that a sham treatment would exacerbate symptoms have been shown to increase pain (50), nausea (54), and itch (53), and worsen physical performance (55).

Almost no research has been done on the possibility to induce placebo effects in the endocrine system by giving verbal suggestions. To our knowledge, only one study attempted to elicit placebo cortisol and growth hormone release and decrease by influencing conscious expectations of participants by giving verbal suggestions (36). Participants in this study were told that the medication they would receive (a placebo) would decrease or increase, depending on the group allocation, their cortisol and growth hormone levels. No effects of verbal suggestions on these hormones were found. Possibly, as changes in hormonal levels are automatic processes that are mostly unnoticeable by people, changing their conscious expectations might not be enough to induce physiological placebo effects. The existing evidence indicates that conditioning is necessary to trigger physiological changes. However, more research employing various types of verbal suggestions and focusing on different hormonal systems is needed to draw any conclusions about the effects of verbal suggestions on hormonal secretion.

Hormonal mechanisms of the placebo effect

The suggestion that the endocrine system may possibly be involved in the placebo effect as a mediator and/or a moderator is intriguing. A lot of attention in placebo research has been given to neurotransmitters and peptide hormones of the endogenous opioid and cannabinoid systems as possible mediators of placebo. It has been demonstrated that positive verbal suggestions and conditioning activate the release of endogenous opioids and cannabinoids, which in turn induce analgesic effects (56, 57). Moreover, it was shown in a series of studies that naloxone, a blocker of opioid receptors, prevents the development of the placebo effect for pain (58-60) and the opioids released during placebo treatment cause side effects similar to the opioid medication (61).

Some studies looked at possible effects of baseline hormonal levels on the response to placebo manipulations. Evidence from these studies remains mixed. For example, Johansen and colleagues (62) did not find a correlation of baseline cortisol and beta-endorphin levels with placebo and nocebo pain responses induced by verbal suggestions. However, baseline cortisol levels have been shown to be related

to the responsiveness to classical conditioning of cortisol (24): participants with higher baseline cortisol levels demonstrated a conditioned cortisol release, whereas participants with lower baseline cortisol levels did not. Furthermore, Ober and colleagues (63) showed that conditioned placebo immunosuppression was related to baseline noradrenaline levels. Baseline hormonal levels might indicate how successful pharmacological conditioning can be, however, this question needs to be further investigated. In general, more research is needed to identify the hormonal factors that can influence the placebo and nocebo effects and the pathways of their action.

Finally, administration of hormones and hormone-stimulating medication can be used to enhance or inhibit placebo and nocebo effects. Again, this area remains underexplored and only a few hormones were investigated in this context. Colloca and colleagues (64) found that administration of vasopressin, a peptide hormone that also regulates social and stress behaviors, enhances placebo analgesia induced by verbal suggestions, but only in women and not men. These findings correspond to behavioral effects of vasopressin, which has shown to increase cooperation and friendliness in women only (65, 66). Benedetti and colleagues (67) found that nocebo effects can be influenced by cholecystokinin, a peptide hormone of the gastrointestinal system that also has anxiogenic effects. The nocebo effect, induced by verbal suggestions, was abolished when patients received proglumide, the cholecystokinin antagonist (67).

A hormone that has both anxiogenic and social effects and might have a potential for influencing placebo and nocebo effects is oxytocin. Oxytocin has been shown to reduce stress (68) and increase trust (69, 70) and both of these factors might play a role in the placebo effect. Additionally, there is some evidence that oxytocin facilitates learning in a classical conditioning paradigms (71) and accelerates extinction of the conditioned response (72). Despite this potential, oxytocin has not been sufficiently investigated in the context of enhancing placebo and decreasing nocebo effects, with the exception of two studies that demonstrated conflicting results (64, 73). Colloca and colleagues (64) have used oxytocin to boost placebo analgesia induced by verbal suggestions and found no effect of this hormone on the placebo effect neither in men nor in women. However, Kessner and colleagues (73) have demonstrated that oxytocin increases placebo analgesia also induced by verbal suggestions. At this moment there is no clear

evidence about whether oxytocin can increase the placebo effect induced by verbal suggestions, and moreover, no studies looked at the possible effects of oxytocin on placebo and nocebo effects induced by classical conditioning.

Oxytocin

Oxytocin is a hormone and neuropeptide produced primarily in the hypothalamus. The first oxytocin research was focused primarily on its functions in labor regulation (74), lactation (75) and mother-infant bonding (76). Prosocial effects of oxytocin recently have drawn a lot of attention in social psychology (77). Oxytocin has been demonstrated to regulate emotion recognition (78) and emotional contact (79), decrease stress (68), and increase trust (69), empathy (80), and generosity (81). Neuroimaging studies have demonstrated that oxytocin affects the areas of the brain mostly linked to the perception of social stimuli, the amygdala, the area underlying stress responses and recognition of emotions (82, 83); the insula, the area that plays a role in emotional responses and empathy (84, 85); and the superior temporal gyrus, the area involved in the perception of emotions in faces (86). A two-part model has described the effects of oxytocin on the human brain: bottom-up effects of oxytocin reduce anxiety and facilitate approach behavior; and top-down effects of oxytocin increase reward from social interactions (86). Because of its prosocial effects, oxytocin has been intensively investigated as a treatment for mental disorders related to emotional deficits. Positive effects of oxytocin were found on symptoms of autism (87), schizophrenia (88), and borderline personality disorder (89). Moreover, oxytocin has beneficial metabolic and immune effects. Treatment with oxytocin, for example, increases insulin sensitivity and decreases weight in obese adults (90), reduces inflammation (91, 92), and increases healing processes (93) in animals.

Considering the potential beneficial effects of oxytocin for somatic and mental disorders, the possibility to induce placebo oxytocin release might have various clinical implications. However, no studies so far looked at whether it is possible to classically condition oxytocin release. Very little research has been done on classical conditioning of endocrine responses in general. On the other hand, oxytocin seems to

influence core mechanisms of placebo responding: trust (70), stress reactivity (68), and learning (71), which makes oxytocin interesting as a hormone that potentially can enhance the placebo effect.

Factors influencing the placebo and nocebo effect

In addition to the mechanisms mentioned above that are predominantly based on learning theory, there are numerous other factors that can influence placebo and nocebo effects. For example, the affective state, such as anxiety seems to play a possible role in placebo and nocebo effects. Colloca and colleagues for example showed that baseline dispositional anxiety levels are negatively related to placebo analgesia (64). Moreover, Benedetti and colleagues (94) demonstrated that diazepam, an anxiety-reducing drug, blocks nocebo hyperalgesia. Next to affective states, other factors, such as personality characteristics of extraversion (95), neuroticism (96), and suggestibility (97) have been incidentally found to affect the effects of placebo and nocebo responses. Interestingly enough, Pecina and colleagues (96) found that personality traits were related to a placebo-induced decrease of cortisol levels. This points at a possible role of stress hormones in the relationship between personality and the placebo effect.

Also, a link between genetic factors and placebo effects has been described in the literature (23, 98). Genetic polymorphisms in the catechol-O-methyltransferase (COMT) gene, rs4680, has been repeatedly shown to be associated with predicting placebo responses in chronic pain (99, 100), irritable bowel syndrome (99), and depression (101). Moreover, it has been demonstrated to be related to nocebo effects (102). The COMT gene is responsible for creating an enzyme that metabolizes catecholamines. This enzyme helps to control, among other things, the levels of the hormones adrenaline and noradrenaline. Possibly, therefore, these hormones might play a role in the placebo effect. However, this link has not been systematically investigated in the literature yet.

The current dissertation

In this dissertation, we address the link between endocrine parameters with a specific focus on oxytocin and placebo effects from two perspectives: eliciting endocrine/oxytocin responses by means of classical conditioning (Part I, Chapters 2, 3, 4), and influencing placebo effects with oxytocin (Part II, Chapters 5

and 6). Additionally, we explore the role of other factors, such as expectations, personality factors, affect and anxiety in these two main research questions.

Part I is dedicated to exploring the possibility to pharmacologically condition endocrine responses, in particular oxytocin. In **Chapter 2** we introduce the topic of classical conditioning of endocrine responses and systematically review studies on this topic done in animals and humans. This is the first systematic review on the topic of endocrine conditioning that includes both animal and human research. We summarize evidence from this field, give an elaborate overview of similarities and differences between animal and human research, describe the study designs used to condition hormonal responses and discuss possible benefits of applying classical conditioning in clinical practice.

In **Chapter 3** we address the topic introduced in Chapter 2 from an experimental perspective and present the results of a randomized controlled trial in which we investigated the possibility to elicit classically conditioned oxytocin responses. This is the first experiment on classical conditioning of oxytocin in humans and one of the few studies on classical conditioning of endocrine responses in general. We investigate oxytocin conditioning using a commonly used two-phase conditioning paradigm with three acquisition days and three evocation days, and explore conditioned oxytocin responses and their extinction process. Next to the measurement of conditioned changes in endogenous oxytocin levels, we also study possible effects of conditioning of oxytocin on a social task and pain sensitivity. Moreover, we explore the role of personality and affect in the classical conditioning of oxytocin responses.

Chapter 4 presents the results of an fMRI experiment that was a part of the trial described in Chapter 3. In this chapter, we compare the effects of endogenous conditioned oxytocin responses and exogenous oxytocin administration on brain activity in response to several tasks commonly used in oxytocin research. In this chapter, we aim to unravel possible brain mechanisms underlying oxytocin conditioning in relation to oxytocin administration.

Part II is dedicated to exploring the influence of oxytocin on the placebo effect. In **Chapter 5**, we describe the results of a randomized controlled trial in which we investigate whether oxytocin influences expectations of participants and enhances placebo effects for pain and itch. In this experiment, we aimed

to induce placebo effects by giving positive verbal suggestions in healthy women and use a standard dose (24 IU) of oxytocin to enhance these effects.

Chapter 6 presents the results of another randomized controlled trial that was performed as a follow-up for the trial described in Chapter 5 and addresses this question from a slightly different perspective. In this trial, we explore whether oxytocin could enhance placebo analgesia, reduce nocebo hyperalgesia, and influence the extinction of both. To induce placebo and nocebo effects, we used a classical conditioning procedure together with verbal suggestions. Moreover, we included a male sample and use a higher dose of oxytocin (40 IU) than in the study described in Chapter 5. Overall, these two experiments investigate the effect of oxytocin on placebo effect in both female and male samples, with different dosages of oxytocin and different methods of placebo induction.

Chapter 7 is the general discussion of the dissertation. This chapter summarizes the results of the conducted studies, connects them to the aims and hypotheses that we initially had and discusses them in the light of possible clinical implications.

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Chapter 1

Introduction

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Part I

Conditioning of Oxytocin and Other Endocrine Responses

Chapter 2

Conditioned hormonal responses: a systematic review in animals and humans

Published as

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Abstract

In contrast to classical conditioning of physiological responses such as immune responses and drug effects, only a limited number of studies investigated classical conditioning of endocrine responses. The present paper is the first systematic review that integrates evidence from animal and human trials regarding the possibility to condition the endocrine responses. Twenty-six animal and eight human studies were included in the review. We demonstrated that there is accumulating evidence that classical conditioning processes are able to influence specific endocrine responses, such as cortocosterone/cortisol and insulin, while more limited evidence exists for other hormones. Animal and human studies were generally consistent in their findings; however, the limited number of human studies makes it difficult to generalize and translate the results of animal research to humans. Next to methodological recommendations for future studies, we suggest several ways how classically conditioned endocrine responses can be used in clinical practice.

Keywords: classical conditioning; hormones; endocrine; associative learning

Introduction

Classical conditioning is a learning and memory phenomenon that serves as a regulatory adaptive mechanism, helping to prepare the organism for recurrent changes in homeostasis, for example due to food intake (1). Substantial research has been done on this topic looking in various areas: from fear conditioning (e.g., 2, 3) to conditioning of immune responses (e.g., 4) and drug tolerance effects (5). Classical conditioning is a learning process where an association is formed between a physiologically relevant stimulus (unconditioned stimulus, US; e.g., food) and a neutral stimulus (conditioned stimulus, CS; e.g., a sound of a bell). Initially, the biologically relevant stimulus elicits a physiological reaction (unconditioned response, UR, e.g., salivation), while the conditioned stimulus is biologically neutral and elicits no response. However, after repeated contingent pairing of both stimuli, the neutral stimulus will become a conditioned stimulus (CS) and evoke a physiological response (conditioned response, CR; e.g., salivation) in the absence of the US.

Animal, and to a lesser extend human studies, have looked at the effects of classical conditioning on the endocrine system. Hormone secretion can be conceptualized as an UR to various US; for example, drug intake. Stimuli occurring together with the US, for example the process or environment of a drug administration, can be associated with the hormonal responses and can become a CS.

Endocrine responses are involved in a variety of physiological processes, from blood pressure regulation to digestion and stress responsiveness. Moreover, endocrine over- or underproduction underlie various diseases, such as diabetes, thyroid disease, adrenal insufficiency, polycystic ovary syndrome and so on.

Therefore, utilizing our understanding of endocrine conditioning to control hormone levels through behavioral manipulation might have widespread clinical implications. Future practice might benefit from enhancing certain endocrine reactions by classical conditioning. Classical conditioning of endocrine parameters may be one of the underlying mechanisms of placebo-controlled dose reduction - a procedure in which a part of the pharmacological treatment is replaced by placebo while maintaining the efficacy of the treatment (6). Another possible implication of conditioned endocrine responses is reduction of the nocebo effects of medicines, unwanted treatment outcomes that are not due to the treatment mechanism

Chapter 2

Conditioned hormonal responses: a systematic review

itself, as classical conditioning is hypothesized to trigger this phenomenon (7). It might be possible to prevent forming unwanted conditioned responses in the endocrine system. For example, prior exposure to the conditioned cue or adding a salient additional cue at the time of conditioning can prevent the development of conditioned responses (8, 9). In summary, there are several ways how classical conditioning can be used in practice, however, a comprehensive summary of the knowledge in this field is necessary to draw any clinical conclusions.

Although a few studies have summarized the findings of conditioned endocrine responses, no systematic review covers both animal and human studies on this topic. Several older reviews focus on animal studies in a non-systematic manner (10, 11); and two papers described human studies, with one non-systematic report from more than a decade ago (12) and one recent systematic review only incorporating human studies (13). It currently remains unknown whether the results of animal research can be translated to humans. The aims of the current review are to systematically summarize the available knowledge on conditioned endocrine responses in both animal and human studies, to compare the results of animal and human trials, to present an overview of the research designs used in previous studies, to describe methodological caveats of conditioning research, and finally to propose possible implications of using classical conditioning paradigms in modifying hormonal responses.

Methods

Protocol registration

The systematic review was done following the guidelines of the Preferred Reporting Items for Systematic Reviews and Meta-Analyses (PRISMA) statement (14). The review protocol was registered on PROSPERO (registration number CRD42017058783).

Inclusion criteria

Original experimental studies were included in which classically conditioned hormonal responses were reported. Classically conditioned hormonal responses were defined as hormonal changes in response to a CS that was previously coupled with a US. The studies had to be written in English, had to be published in a peer-reviewed journal, and had to include a minimum of one control group. Single case studies and

conference abstracts were excluded. Furthermore, studies on anticipatory hormone release (incidental learning as a response to naturally occurring stimuli, such as time of the day) were excluded for this review. The current review did not include the studies done on glucose conditioning (e.g., 15, 16), as glucose is not a hormone. Even though it was sometimes speculated in these studies that conditioned changes in glucose were triggered by conditioned insulin responses, insulin was not measured directly. Also, studies on fear conditioning were not included in this review even if they measured corticosterone or cortisol. These studies are aimed at conditioning of fear and hormonal responses are measured as indicators of fear and not as a primary CR. No limitations regarding the year of publication were set.

Data search and study selection

The electronic bibliographic databases PubMed, EMBASE, PsycINFO, and CINAHL were searched from the inception until January, 2017, using the key words and connectors *endocrine* OR *hormonal* in combination with *conditioning* OR *associative learning* OR *anticipatory release* and specific names of the hormones such as for example *insulin*, *cortisol*, *testosterone*. The full search terms per database are presented in Supplementary material. The search included both human and animal trials that measured hormonal responses to conditioned stimuli. In addition, the search included trials on anticipatory hormone release that will be published separately.

The search was done by two authors (A.S. and I.K.) independently. The two authors screened the search results for eligibility based on the titles, abstracts, and finally full texts of the reports. The results of the search process were compared between the two authors and the final list of the included studies was made. Classification of the studies into intentional conditioning studies or anticipatory incidental learning studies were done by two authors (A.S. and I.K.). Studies that employed a two-phase experimental design with acquisition and evocation phases were labeled as intentional conditioning studies and included in the current review. Studies that measured anticipatory hormone release by incidental learning as a response to naturally occurring stimuli (such as time of the day), will be included in another review and discussed elsewhere. The inconsistencies were resolved by consulting with the second author (D.V.).

Data extraction and risk of bias assessment

Chapter 2

Conditioned hormonal responses: a systematic review

The data from the studies were extracted using a standardized data extraction form. The following data were extracted from each eligible study: first author's name, year of publication, journal name, study design, sample characteristics, number of acquisition sessions, number of evocation sessions, US, UR, CS, CR, groups, hormonal outcome, timing of outcome assessment, and main study results.

The two authors (A.S. and I.K.) assessed for risk of bias using the Cochrane Collaboration's tool for assessing risk of bias in human trials (17). For assessing risk of bias in animal studies, the guidelines from O'Connor and Sargeant (18) and the SYstematic Review Centre for Laboratory animal Experimentation (SYRCLE) (19) were used. Selection bias (randomization process and allocation concealment), performance bias (blinding of participants and research personnel), detection bias (blinding of the outcome assessment), attrition bias (reasons for withdrawal in all conditions), and reporting bias (handling of missing data) were assessed by using these guidelines. In case of inconsistencies in the assessment of the two reviewers, the second author (D.V) was consulted. In case no information was provided about a certain bias, unclear risk of bias was chosen. In case the study protocol was not pre-registered, the reporting bias was selected to be unclear as well.

Results

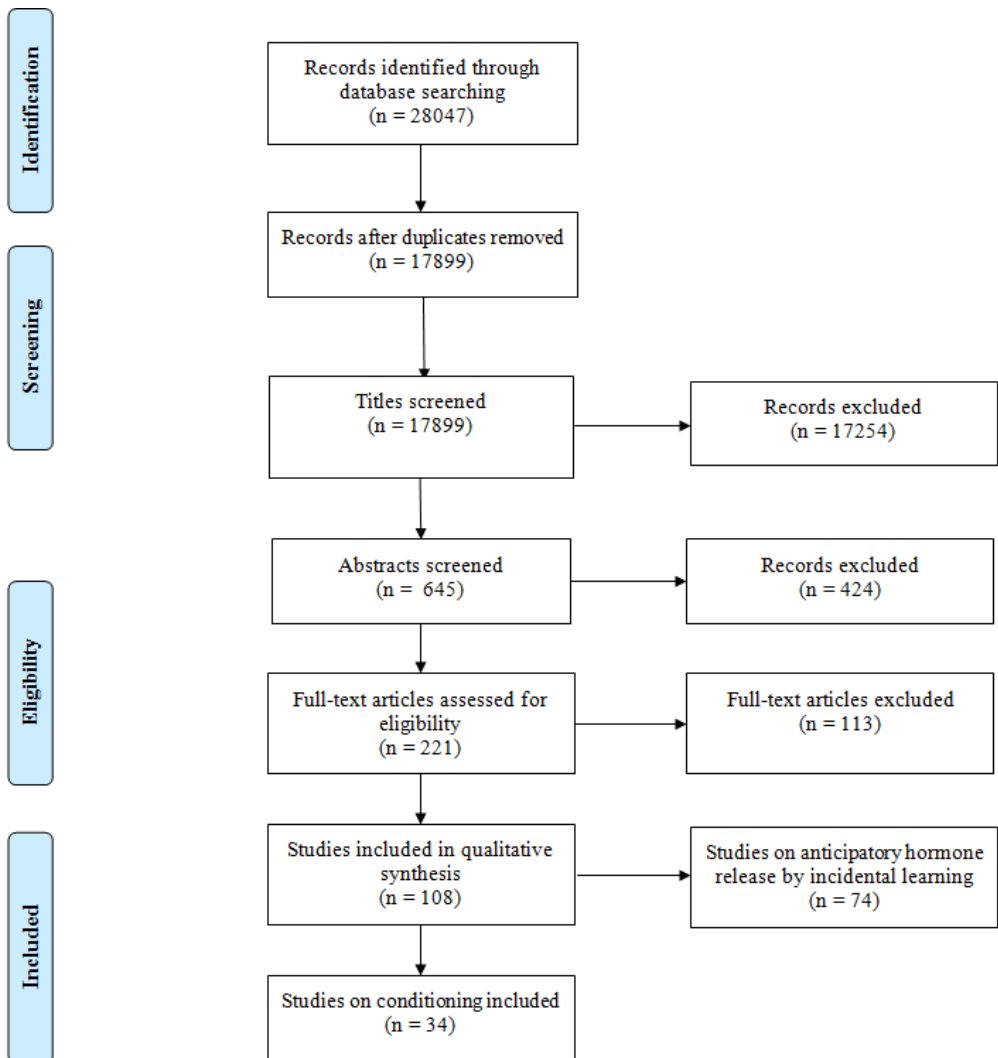
Search results and studies' characteristics

The number of articles found on each step of the systematic search are presented in the Flow Diagram (Figure 1). In total 108 eligible studies were identified of the 17,899 initially identified unique articles that matched the search criteria; 34 of these were found eligible for inclusion in the current review, whereas 74 studies will be included in a separate review on anticipatory endocrine responses by incidental learning. The overview of the study characteristics and study findings of all included studies is presented in Tables 1 and 2, for animal and human studies separately.

The vast majority of animal studies were performed in males (20 out of 26), two studies- in females, two- in mixed groups and other two studies did not report the gender of the animals. Half of the human studies included only males as participants, two studies included both males and females, and two studies only females. Three (out of 8) human studies were performed in the same lab and written by the same first

author (20-22). The far majority of both the animal and human studies (31 out of 34) were published before 2008.

Figure 1. Flow diagram with the number of included and excluded studies.



Endocrine parameters

Most commonly measured hormones in the studies involving animals were corticosterone (in rodents) and cortisol (in other species). Over half of animal studies (15 out of 26) measured conditioned changes in either corticosterone or cortisol using various US. Thirteen of these studies found significant changes in the corticosterone or cortisol levels after conditioning (23-35), while 2 studies could not demonstrate conditioned corticosterone and cortisol alterations (36, 37). The studies reported variable conditioned responses: increased or decreased responses were found depending on various factors, including the US used. For example, cyclophosphamide injection as a US (23) led to conditioned corticosterone increase, while food as a US (27) led to a conditioned decrease of corticosterone. Six animal studies investigated conditioned insulin release: five of them found significant increases in insulin levels (38-42), while one found null results (43). Oxytocin was investigated in two of animal trials (44, 45) of which both found a significant increase in conditioned oxytocin release. There were also single studies that demonstrated conditioned release of adrenaline, noradrenaline, and dopamine (46), testosterone and luteinizing hormone (47) and melatonin (48).

Human trials measured conditioned responses of several hormonal systems and almost all of them included measurements of several hormones at the same time. Consistent with animal trials, most of the human trials looked at conditioned responses of cortisol (5 out of 8). Four of these studies found significant conditioned decreased or increased cortisol levels (7, 21, 49, 50) and one study had null results (20). Insulin conditioning was the subject of interest in four the human studies: 2 of these studies demonstrated conditioned insulin increases (21, 22), while 2 found no conditioned changes in insulin (20, 51). Two human studies measured noradrenaline: one of these demonstrated a significant conditioned increase in noradrenaline (21), while the other reported null results (20). Significant increases in growth hormone was reported in two the studies (7, 21). Two human studies measured glucagon and both of them failed to find conditioned glucagon release (20, 21). One human study demonstrated significant conditioned decreases in adrenaline (epinephrine) (22), and one study failed to find conditioned changes

in endocrine indicators of nausea (adrenocorticotrophic hormone, antidiuretic hormone, pancreatic polypeptide) (52). In general, there were more null results found in human studies than in animal studies.

Study designs

The two-phase conditioning design of the studies included acquisition and evocation trials. In acquisition trials, a US that triggered certain hormonal changes (UR) was associated with an initially neutral stimulus (becoming the CS). In the evocation trial(s), only the CS was presented and conditioned hormonal responses (CR) were measured.

The number of acquisition and evocation trials varied across the studies. Animal trials in general had many acquisition trials, varying from 1 (e.g., 23, 33) up to 28 (38), while human studies had between 1 (52) and 6 (51, 53) acquisition trials. In contrast, the majority of studies included only 1 evocation trial: 19 animal studies (e.g., 24, 25) and 7 human studies (e.g., 7, 49). The maximum number of evocation trials in animal studies was 8 (38) and in human studies 6 (22).

Most of the studies employed a between-subject design: 21 of animal studies (e.g., 23, 24) and 7 human studies (e.g., 7, 51, 52). In general, animal studies included multiple control groups, including an undisturbed control (no manipulations done) (24), a CS only (24), a US only (36), an unpaired (US and CS are presented in an uncoupled manner) (31), a conditioned not re-exposed (CS is not presented during the evocation) (33), or a placebo control group (a placebo was used instead of a US) (29). Most human studies (6 out of 8) included a placebo control group (7, 20-22, 50, 51). One other study included a control group in which the CS was given an hour before the US (52) and one study employed a within-group comparison (49).

The US used to elicit hormonal changes varied between the studies. For example, food was often used as a US in order to elicit insulin release (27, 37), while administration of various agents including cyclophosphamide (23), nicotine bitartrate (25, 28), and corticotrophin-releasing factor (32) were used for the conditioning of hormone release (e.g., corticosterone).

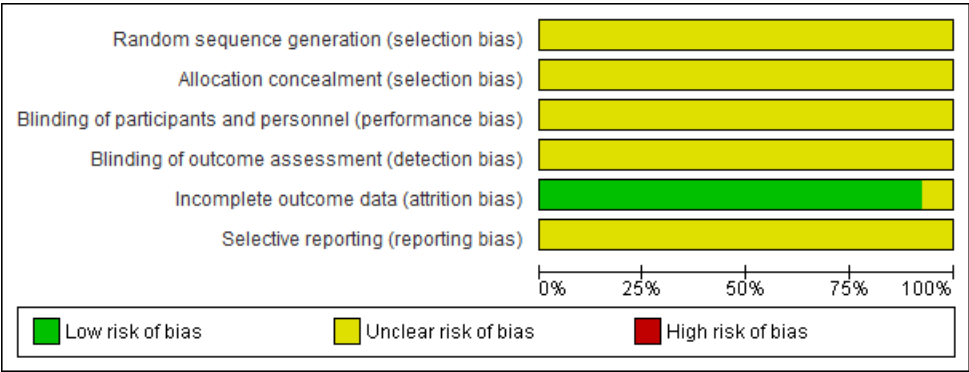
The CS also varied across the studies. Most of the animal studies used taste (10 out of 26) (e.g., 23, 29), smell (6 out of 26) (e.g., 25, 29), or sound (6 out of 26) (e.g., 27, 38) as a CS. In two animal studies,

distinctive surroundings, such as placing animal in an unknown room, were used as a CS (28) (28, 45). Single animal studies used other CS such as restricting water availability (48), switching off the aeration in the fish tank (24) and intra-arterial injection of sodium saccharin solution (32). Among human studies, also smell (3 out of 8) (20-22) and taste (2 out of 8) (50, 52) were the most commonly used CS. Single human studies used other CS: both smell and taste (51), an injection procedure (7) and an experimental context (54).

Risk of bias assessment

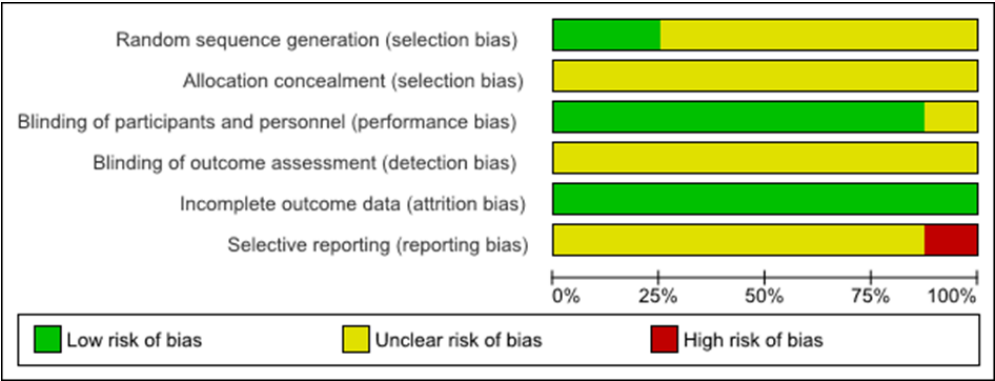
The results of the risk of bias assessment are presented in Figure 2 and 3, for animal and human studies separately. Most animal studies lack important information to evaluate risk of bias and were assigned an unclear risk of bias. Human studies provide more details regarding the procedures and, therefore, in general have lower risk of bias. The results of the studies with the higher risk of bias did not differ from the results of the studies with the lower risk of bias: several studies that demonstrated null results (20, 36, 37, 39, 51, 52) had the same risk of bias as other studies that reported significant findings.

Figure 2. Risk of bias for animal studies. Risk of bias is presented as a percentage across all included studies and for each separate study.



	Random sequence generation (selection bias)	Allocation concealment (selection bias)	Blinding of participants and personnel (performance bias)	Blinding of outcome assessment (detection bias)	Incomplete outcome data (attrition bias)	Selective reporting (reporting bias)
Ader, 1976	?	?	?	?	+	?
Baretto, 2007	?	?	?	?	?	?
Buske-Kirschbaum, 1996	?	?	?	?	+	?
Coover, 1977	?	?	?	?	+	?
Coover, 1980	?	?	?	?	+	?
Davis, 2005	?	?	?	?	+	?
Detke, 1989	?	?	?	?	+	?
Dyck, 1990	?	?	?	?	+	?
Exton, 1995	?	?	?	?	+	?
Golombek, 1994	?	?	?	?	+	?
Graham, 1980	?	?	?	?	+	?
Janz, 1991	?	?	?	?	+	?
Janz, 1996	?	?	?	?	+	?
Kassil, 1998	?	?	?	?	+	?
Kreutz, 1992	?	?	?	?	+	?
Morell, 1988	?	?	?	?	+	?
Natelson, 1984	?	?	?	?	+	?
Onaka, 1998	?	?	?	?	+	?
Pacheco-Lopez, 2004	?	?	?	?	+	?
Rozendaal, 1990	?	?	?	?	?	?
Smotherman, 1980	?	?	?	?	+	?
Smotherman & Levene, 1980	?	?	?	?	+	?
Surwit, 1985	?	?	?	?	+	?
Tancin, 2001	?	?	?	?	+	?
Woods, 1972	?	?	?	?	+	?
Woods, 1977	?	?	?	?	+	?

Figure 3. Risk of bias for human studies. Risk of bias is presented as a percentage across all included studies and for each separate study.



	Random sequence generation (selection bias)	Allocation concealment (selection bias)	Blinding of participants and personnel (performance bias)	Blinding of outcome assessment (detection bias)	Incomplete outcome data (attrition bias)	Selective reporting (reporting bias)
Benedetti, 2003	?	?	+	?	+	?
Hall, 2016	?	?	?	?	+	?
Klosterhalfen, 2000	?	?	+	?	+	?
Overduin, 1997	?	?	+	?	+	?
Sabbioni, 1997	+	?	+	?	+	?
Stockhorst, 1999	?	?	+	?	+	?
Stockhorst, 2004	?	?	+	?	+	?
Stockhorst, 2011	+	?	+	?	+	-

Table 1. Animal studies (n = 26)

	Subjects	Study design	Conditioned stimulus	Unconditioned stimulus	Conditioning protocol			Endocrine conditioned responses	Result
	Species (N, sex) #			Nature, Dose, Administration route	Acquisition trials	Rest days	Evocation trials		
Ader, 1976; experiment 1	rats (9-12 per group, M)	between-subject, randomized; 10 groups: control (nondeprived, deprived, saccharine, cyclophosphamide), conditioned (saccharine, H ₂ O, deprived), nonconditioned (saccharine, H ₂ O, deprived)	0.1% saccharine solution	50 mg/kg cyclophosphamide injection	1	3	2	corticosterone in group	↑
Ader, 1976; experiment 2	rats (55, M)	between-subject, randomized; 4 groups: conditioned (saccharine, H ₂ O, deprived), nonconditioned (saccharine)	0.1% saccharine solution	50 mg/kg cyclophosphamide injection	1	3	1	corticosterone in conditioned group	↑
Baretto & Volpato, 2007	Nile tilapia fish (75, mixed sex)	between-subject, randomized; 5 groups: conditioned, undisturbed control, CS only, US control (daily US except on the final day), US last day (daily US including the final day)	aeration off for 30 seconds	air emersion	10	1	1	cortisol in conditioned group	↑
Buske-Kirschbaum et al., 1996	rats (26, M)	between-subject, randomized; 3 groups: conditioned,	peppermint odor	0.1 mg/kg nicotine bitartrate injection	4	2	1	corticosterone	↑

Chapter 2

Conditioned hormonal responses: a systematic review

unpaired, control
(saline)

Coover et al., 1977; experiment 1	rats (60, M)	between-subject, randomized; 2x5 design: food (fed, not fed) x sample time (0, 5, 10, 20, 50 min after CS)	experimenter entering the room	food	14	1	1	corticosterone in not fed group 50 min after CS	↓
Coover et al., 1977; experiment 2	rats (270, M)	between-subject, randomized; 3x4x3 design: food (conditioned fed, conditioned not fed, pseudoconditioned not fed) x trials number (1, 6, 14, 24) x sample time (0, 10, 20 min after the CS)	placing the animal in sound attenuating chamber	food	1, 6, 14, 24	1	1	corticosterone in conditioned not fed 1 trial	↑
								corticosterone in conditioned not fed 6 trials	n.s.
								corticosterone in conditioned not fed 14, 24 trials	↓
Coover et al., 1977; experiment 3	rats (120, M)	between-subject, randomized; 2x4x3 design: food (conditioned fed, conditioned not fed, pseudoconditioned not fed) x trials (1, 14) x sample time (0, 10, 20 min after the CS)	placing the animal in sound attenuating chamber	food	1, 14	1	1	corticosterone in conditioned not fed 1 trial	n.s.
								corticosterone in conditioned not fed 14 trials	↓
Coover et al., 1980; experiment 1	rats (68, M)	between-subject, randomized; 2 groups: fed, unfed	experimenter entering the room	food	14	1	1	corticosterone in conditioned unfed 10 min after CS	↓
								corticosterone in conditioned	↑

Chapter 2

Conditioned hormonal responses: a systematic review

								unfed 20 and 40 min after CS	
Coover et al., 1980; experiment 2	rats (92, M)	between-subject, randomized; 3x3 design: surgery (operated with ventromedial hypothalamus lesions, control operated, not operated) x group (decapitated immediately, fed, unfed)	placing the animal in sound attenuating chamber	food	14	1	1	corticosterone in operated unfed	n.s.
								corticosterone in unfed 10 min after CS	↓
Davis et al., 2005	rats (140, M)	between-subject, randomized; 2x2x2 design: sessions (5, 10) x CS (distinctive context, home cage) X test (nicotine test, saline test)	distinctive context room	1.0 ml/kg of body weight nicotine bitartrate	5, 10	1	1	corticosterone in distinctive context group	↑
Detke et al., 1989;	rats (8, M)	within-subject, pseudo-randomized order of trials.	long CS (A or B)- 35 sec noise; short CS (x)- 10 sec noise. The presentation of the US took place on Ax but not Bx trials	1 ml of 12,5% glucose solution	28 trials per session, several sessions		8 in replication 1 and 7 in replication 2	insulin in Ax condition	↑
Dyck et al., 1990; experiment 1	mice (30, F)	between-subject, randomized; 5 groups: IL-1, IL-1 conditioned, IL-1 conditioned (no cues, not re-exposed),	0.35% saccharine solution + injection of LiCl	0.2-0.5 mg of recombinant interleukin 1-beta injection	2	2	1	corticosterone in conditioned group	↑

Chapter 2

Conditioned hormonal responses: a systematic review

		unconditioned, negative control							
Dyck et al., 1990; experiment 2	mice (50, F)	between-subject, randomized; 5 groups: IL-1, IL-1 conditioned (saccharine+ LiCl); IL-1 conditioned (saccharine), IL-1 conditioned (no cues, not re- exposed), cue control unconditioned	0.35% saccharine solution + injection of LiCl	0.5 mg of recombinant interleukin 1-beta injection	1	1	1	corticosterone in conditioned group	↑
Dyck et al., 1990; experiment 3	mice (27, F)	between-subject, randomized; 3 groups: IL-1 conditioned, IL-1 conditioned (no cues, not re- exposed), negative control	peppermint odor	0.2 mg of recombinant interleukin 1-beta injection	4	7	1	corticosterone in conditioned group	↑
Exton et al., 1995	rats (58 first analysis, 28 control study, M)	between-subject, randomized; 4 groups: conditioned with saccharine, conditioned with water, saccharine only, water only	1% saccharine solution	1 ml intraperitoneal injection of lipopolysaccharide	1	7	1	corticosterone in conditioned group with saccharine	↓
Golombek et al., 1994	rats (60, M)	between-subject, randomized; 6 groups: US for training & no treatment at the day of trial, US for training & US for trial, CS+US for training & CS for trial, CS+US for training & no treatment on day of trial, CS+US for	restricted water availability	lights off	7	1	1	pineal melatonin in CS+US for training & CS for trial	↑

training, CS+US
for trial, CS+US for
treatment & US for
trial

Graham & Desjardins, 1980; experiment 1	rats (M)	between-subject, randomized; 5 groups: CS followed immediately by US , CS alone, CS followed by a 6-hour delay US, US alone, removal from the home cage and being handled	vapors of methyl salicylate	placing in a cage with a sexually receptive female	14	1	1	luteinizing hormone in conditioned group	↑
Graham & Desjardins, 1980; experiment 2	rats (M)	between-subject, randomized; 5 groups: CS followed immediately by US , CS alone, CS followed by a 6-hour delay US, US alone, removal from the home cage and being handled	vapors of methyl salicylate	placing in a cage with a sexually receptive female	14	1	1	testosterone in conditioned group	↑
Janz et al., 1991	rats (72, M)	between-subject, randomized; 5 groups: conditioned reexposed to saccharin, conditioned not reexposed, water followed by IL-1 on the training day, negative control (saline injection,	0,1% saccharine solution	5 ng interleukin	1	1	1	corticosterone in conditioned group	n.s.

Chapter 2

Conditioned hormonal responses: a systematic review

and saccharin), US
only group (drug on
the test)

Janz et al., 1996	rats (38, M)	between-subject, randomized; 4 groups: paired reexposed to saccharin, paired group not reexposed, the unpaired group received plain water followed by an LPS injection, the injection, cue, and water deprivation control group received saccharin followed by a saline injection	0,15% saccharine solution	ip injection of 50 µg lipopolysaccharide	1	5	1	corticosterone in conditioned group	↑
Kassil et al., 1998	rats (58, MF)	between-subject, randomized; 2 groups: experimental (conditioning: saccharine + rotation; test: saccharine); control (conditioning: water + rotation; test: saccharine)	2 ml of 0.2 % saccharine solution	angular acceleration (rotation)	1	5, 10 and 15 days	3	adrenaline, noradrenaline, dopamine in conditioned group	↑
Kreutz et al., 1992	rats (11, M)	within-subject comparison: 4 conditioning trials, 4 control trials (injection of saline and injection of Ringer solution), 2 test trials (injection of sodium	an intraarterial injection of 0.25 ml sodium saccharin solution	injection of 0.154 pmol corticotrophin- releasing factor dissolved in Ringer solution	4	2	2	corticosterone during test trials	↑

		saccharine and Ringer solution)							
Morell et al., 1988	mice (M)	between-subject, randomized; 4 groups: lean conditioned, lean control (US and CS not contingent), obese conditioned, obese control (US and CS not contingent)	odor of mentholatum for 5 min prior to feeding and 3 min during the feeding	food	21 days and 4 additional days	1 day and 2 weeks	2	insulin in the obese conditioned group	↑
Natelson et al., 1984	rhesus monkeys (6, M)	within-subject; test sessions (presentation of CS) and control sessions (no CS)	30-min tone	food	at least 1 month daily			cortisol during test trials	n.s.
Onaka & Yagi, 1998	rats (32, M)	between-subject, randomized; 3 groups: conditioned, control 1 (vehicle +US), control 2 (US+ CS 2 h after)	sucrose solution (0.75–2.0 M) or NaCl	cholecystokinin octapeptide 20 mg/kg	1	3 hours	1	oxytocin in conditioned group	↑
Pacheco-Lopez et al., 2004	rats (32, M)	between-subject, randomized; 4 groups: conditioned, conditioned not re-exposed, placebo, unconditioned	0.2% saccharin solution	2.0 mg/kg staphylococcal enterotoxin B, injection	1	6	1	corticosterone	↑
Rooszendaal et al., 1990	rats (34, M)	within-subject, time points comparison; groups: control, central amygdala lesioned, sham-lesioned	sound of the door opening	food	5	2	1	insulin after the CS in sham-operated group and control groups	↑

Chapter 2

Conditioned hormonal responses: a systematic review

Smotherman & Levine, 1980	rats (48, M)	between-subject; randomized; 6 groups: conditioned ACTH + milk, conditioned ACTH + water, conditioned saline + milk, conditioned saline +water, conditioned not injected + milk, not injected not conditioned + milk	sweetened milk solution	ip injections of lithium chloride (.40 M, 7.5 ml/kg)	1		1	corticosterone in milk conditioned groups	↑
Smotherman et al., 1980; experiment 1	rats (37, M)	between-subject, randomized; 2 groups: conditioned and saline	sweetened milk solution	ip injections of lithium chloride (.40 M, 7.5 ml/kg)	1		1	corticosterone in conditioned group	↑
Smotherman et al., 1980; experiment 2	rats (60, M)	between-subject; randomized; 2x3 design: treatments (conditioned, saline) x preexposures to CS (2, 5, 10)	sweetened milk solution	ip injections of lithium chloride (.40 M, 7.5 ml/kg)	1		1	corticosterone in conditioned 2 and 5 preexposures groups	↑
Smotherman et al., 1980, experiment 4	rats (60, M)	between-subject; randomized; 6 groups: control, conditioned (with 5, 6, 7, 8, 9 or 10 preexposures)	sweetened milk solution	ip injections of lithium chloride (.40 M, 7.5 ml/kg)	1		1	corticosterone in conditioned 5 and 6 preexposures groups	↑
Surwit et al., 1985	mice (24)	between-subject design, randomized; 2x3 design: group (obese, control) x conditioning (conditioned, CS without US, noncontingent CS and US)	metronome sound	shaking	7	1	1	insulin in conditioned group	n.s.

Tancin et al., 2001	cows (20, F)	within-subject comparison: milking and suckling in known and unknown surrounding	known surroundings	milking and suckling				oxytocin in unknown surroundings	↓
Woods et al., 1972	rats	between-subject, randomized; 3 groups: conditioned, control 1 (water on test trial), control 2 (US on test trial)	smell of menthol	300 mg/kg of tolbutamide	6		1	insulin in conditioned group	↑
Woods et al., 1977	rats (48, M)	between-subject, 6 groups: CS & US at 11:30, US at 11:30 & CS at random, CS at 11:25 & US at random, US & CS together but at a random time; ad libitum food & CS at random; ad libitum food & CS the random interval prior to the time the appropriate meal-fed rats received their food.	5 min of odor of mentholatum	food	21	1	1	insulin in meal fed groups	↑

number of species and sex reported if this information is available; M- males; F- females; US- unconditioned stimulus, CS- conditioned stimulus

Table 2. Human studies (n = 8).

Authors, year of publication	Subjects N (sex)#	Study design	Conditioned stimulus	Unconditioned stimulus Nature, Dose, Administration route	Conditioning protocol			Endocrine conditioned responses	Result
					Acquisition trials	Rest days	Evocation trials		

Chapter 2

Conditioned hormonal responses: a systematic review

Benedetti et al., 2003	95 (47 M, 48F)	between-subject, randomized; 9 groups: conditioned and control groups with various suggestions	injection procedure	sumatriptan injection	2	1	1	growth hormone levels in the conditioned groups cortisol levels in the conditioned groups	↑ ↓
Hall et al., 2016	32 (F)	within-group comparison for cortisol, 4 conditioned groups: latent inhibition with overshadowing, latent inhibition, overshadowing, control	context of rotation	rotation	2	1 day	1	cortisol after the CS presentation in all groups	↑
Klosterhalfen et al., 2000	90 (42M, 48F)	between-subject, randomized; 2 groups: conditioned, control (CS 1 hour before US)	100 ml elder-berry juice	rotation procedure	1	7	1	adrenocorticotrophic hormone, antidiuretic hormone, pancreatic polypeptide in conditioned group	n.s.
Overduin & Jansen, 1997	20 (F)	between-group, randomized; 2 groups: conditioned, placebo	peppermint flavor	50 g ad lib glucose	6	2	1	insulin levels in conditioned group	n.s.
Sabbioni et al., 1997	25 (M)	between-subject, randomized; 2 groups: conditioned, placebo	lemon-lime with bitter tonic beverage	5 ml dexamethasone ad lib	3	7	1	plasma cortisol levels in conditioned group	↑
Stockhorst et al., 1999	20 (M)	between-subject, randomized; 2 groups: conditioned, placebo	rosewood-peppermint oil smell	intravenous insulin injection (0.035 units/kg)	4	1	1	insulin in conditioned group glucagon in conditioned group	n.s. n.s.

								noradrenaline in conditioned group	n.s.
								cortisol in conditioned group	n.s.
Stockhorst et al., 2004	30 (M)	between-subject, randomized; 3 groups: insulin conditioned, glucose conditioned, placebo	rosewood-peppermint oil smell	intravenous insulin injection (0.05 IU/kg) or intravenous glucose injection (15%, 0.5 g/kg)	4	2	1	cortisol, insulin in the glucose conditioned group	↑
								glucagon in the glucose conditioned group	n.s.
								noradrenaline, growth hormone in the insulin conditioned group	↑
Stockhorst et al., 2011	32 (M)	between-subject, randomized; 2 groups: conditioned, placebo	smell of meta-cresol	soluble H-insulin intranasal spray (20U[0.2ml])	6	1	6	peripheral insulin in conditioned group	↑
								epinephrine in conditioned group	↓

number of species and sex reported if this information is available; M- males; F- females; US- unconditioned stimulus, CS- conditioned stimulus

Discussion

This is the first review systematically summarizing findings of studies on intentional conditioning of the endocrine system in both animals and humans. Findings of classical conditioning studies using a two-phase design incorporating acquisition and evocation trials were systematically reviewed. This review demonstrates that there is an accumulating evidence from both animal and human studies that classical

Chapter 2

Conditioned hormonal responses: a systematic review

conditioning processes are able to influence specific endocrine responses. Some endocrine responses have been more thoroughly studied, such as corticosterone/cortisol and insulin, while fewer studies looked at other hormones, such as testosterone, luteinizing hormone, oxytocin, growth hormone, glucagon and melatonin, adrenaline, and noradrenaline. Most notably, animal and human studies were generally consistent in their findings: conditioned responses were found in the same endocrine systems in both animal and human trials. Nevertheless, some methodological caveats exist that need attention in future research.

The present review demonstrated that not all endocrine systems were investigated equally in the context of classical conditioning. The majority of the studies focused on hypothalamus-pituitary-adrenal axis and on insulin responses, while evidence for other hormones is very limited or absent. Future studies should focus on other endocrine parameters, especially those that play a possible role in the treatment of diseases, such as thyroid hormone, growth hormone, prolactin, adrenocorticotrophic hormone etc.

Additionally, the majority of the studies were performed in males and none of them looked at the possible moderation of the conditioned response by gender. So far there is no evidence that gender moderates placebo effects (55). Nevertheless, considering the gender specificity of the endocrine responses, future research should examine possible differences in endocrine conditioning between males and females.

We have also documented that the results of the animal studies are more extensive than the results of the human studies and most of the animal studies found coherent conditioned endocrine responses. The human studies vary more in this respect. Only two human studies reviewed here found conditioned cortisol changes and since the review search was done, one newly appeared study failed to condition the cortisol response in humans (56). Also conditioning of insulin responses was less successful in human studies compared to animal studies. Possibly, conditionability of insulin responses in humans depends more on the US Stockhorst and colleagues (22) demonstrated that it is possible to condition insulin release using intranasal insulin spray as a US, but not when using an intravenous insulin injection (20). Another difference between animal and human research that might lead to different findings, is the number of acquisition trials. Animal studies in general have more acquisition trials than human studies.

Possibly, a low number of acquisition trials in human research might lead to more contradictory results, as a small number of acquisition trials might not always be enough to establish the association between the US and the CS.

In general, the limited number of human studies makes it difficult to generalize the results of the animal research to humans. Several methodological limitations exist that complicate human endocrine conditioning experiments. First of all, application of a two-phase design is time consuming, since it implies several sessions with the administration of pharmacological agents and acquiring biological samples, which is done easier in animals than in humans. Moreover, the environment in animal studies is more controllable in comparison to human studies. Various factors that are difficult to strictly control in humans over a period of time, such as food consumption, physical activity, and psychogenic stressors, can confound human research, potentially creating noise in the outcomes. Finally, cognitions and expectations might play an additional role in the conditioned hormone release in humans. Although Benedetti and colleagues (7) showed that giving verbal suggestions regarding the change of cortisol and growth hormone levels did not influence the secretion of these hormones, other studies with an experimental classical conditioning design did not take the expectations of the participants in consideration. Presentation of the CS can trigger cognitive expectations that have to be taken into account while interpreting the results. For future research, it is important to further identify the role of expectancies and cognitions in the classically conditioned hormone release, because these can be possibly manipulated in clinical practice.

It is also important to point out that there was no consistency in the reviewed studies regarding the exact study design or procedures that were used to condition endocrine responses. Different number of acquisition and evocation trials were used in different studies. To date it is unclear what number of acquisition trials is optimal to develop conditioned responses in the endocrine system, and whether this is dependent on the species that is being examined, the conditioned drug of interest, or other factors such as the context of conditioning. Only two studies included in this review compared the effectiveness of different numbers of acquisition trials in eliciting conditioned endocrine responses and both of them

concerned conditioned corticosterone. Coover et al. (27) showed that conditioned corticosterone decreased in response to a CS associated with feeding after 14 acquisition trials, but that 6 trials were not enough to demonstrate this response. Davis et al. (57) demonstrated that conditioned corticosterone release already developed after 5 pairing of nicotine bitartrate with a distinctive context and this release increased marginally after 10 acquisition trials. These results are generally in line with the research on conditioned immune responses that demonstrated that more acquisition trials may lead to a stronger conditioned response (58), however more research in the endocrine system is needed to confirm this proposition in relation to the nature and intensity of the US. The number of evocation trials was significantly lower than the number of acquisition trials: the majority of studies used only 1 evocation trial to test whether conditioning took place. This general use of only 1 evocation trial makes it unfortunately impossible to study the extinction process in the endocrine system, since more evocation trials are needed to see how fast the classically conditioned endocrine responses disappear.

A wide variety was found regarding the different types of the CS that were used in the different studies.

Most of the animal and human trials used either gustatory (e.g., 50, 59) or olfactory (e.g., 21, 51, 60) conditioned stimuli. It was previously reported that gustatory and olfactory CS associate with visceral US stronger than other types of CS (61, 62). This can be explained by the naturalistic association and biological importance of such stimuli, because they are associated with food intake and are important for survival. However, other types of CS were also employed in a few studies and were successfully associated with endocrine changes. For example, Benedetti et al. (7) used an injection procedure as a CS to condition changes in cortisol. Speculatively, the nature of the CS seems of marginal significance since conditioned effects were found with both biologically relevant and other types of CS, but future studies may investigate this further and compare the efficiency of various CSs.

Finally, only one of the reviewed studies looked into the neuronal mechanisms of the conditioned endocrine responses. Roozendaal et al. (40) demonstrated that the central nucleus of the amygdala was involved in the conditioned insulin release and its lesioning abolished this conditioned response in rats. The most well-investigated neural pathways of conditioning are the mechanisms involved in the

conditioning of pain responses: it was demonstrated that the descending pain control system gets activated during placebo analgesia (63). Possibly, these findings can be extended to endocrine conditioning in that brain regions responsible for endocrine regulation are also responsible for the release of hormones during conditioning. Nevertheless, this remains only a speculation and future research should focus on further identifying the neural pathways that underlie conditioned endocrine responses in both animals and humans.

The main limitation of this review is heterogeneity of the included studies that makes it difficult to compare them and make definitive conclusions. There is no standard conditioning design established and, therefore, every experiment recruited a different procedure for the conditioning of endocrine responses. In case of null results, such variability in procedures makes it difficult to conclude whether the failure to find conditioned responses was caused by an unappropriated experimental design or by the fact that the certain endocrine system is not malleable to conditioning.

Several methodological aspects of the present review have to be mentioned. The review has not included studies on anticipatory hormone release induced by incidental learning; that is considered to be a conditioned response to naturally occurring conditioned stimuli such as a particular time of the day, called “Zeitgeber” (64). Incidental classical conditioning in this case is established naturally already before the start of the experiment, as for example, increase in insulin levels in response to a food taste without the food ingestion (65). Instead, in this review we focused on intentional conditioning studies that included acquisition and evocation phases and established a connection between initially neutral stimulus and an US. Additionally, the present review did not include glucose conditioning studies (e.g., 15, 16) even though conditioned changes in glucose levels might indicate conditioned insulin response. This link between conditioned insulin and glucose responses not always seem to exist, as for example, Stockhorst et al. (20) found a conditioned glucose decrease during conditioning with intravenous insulin but no conditioned changes in insulin levels. As it is not possible to prove that conditioned changes in glucose are triggered by conditioned insulin response, studies that measured only glucose - and therefore no hormonal outcomes - were not included in this review.

This review shows promising results for applying mechanisms of classical conditioning in clinical practice. For instance, it was demonstrated that cortisol levels can be increased (50) using the classical conditioning procedures. Administration of cortisone (a prodrug of cortisol) can have fear-reducing effects in phobic patients (66) and might prevent the occurrence or reduce the severity of post-traumatic stress disorder after a traumatic event (67). Therefore, speculatively, using the classical conditioning procedure to elicit endogenous cortisol release might be beneficial for these groups of patients. Another clinically relevant example could be applying classical conditioning of insulin responses as demonstrated by Stockhorst et al. (22) in healthy control subjects to patients with diabetes type-2 who suffer from dysfunctional insulin system and heightened glucose levels.

Also, placebo-controlled dose reduction is based on the principles of classical conditioning (6). It was demonstrated that placebo-controlled dose reduction can be as efficient as full treatment for ADHD and psoriasis (68, 69). Although these two studies did not measure endocrine parameters and the mechanisms of the symptoms reduction remain therefore unclear, it might be possible that the classical conditioning procedure triggered endocrine changes that lead to the reduction of symptoms.

Finally, unwanted side effects of medications, nocebo effects, can be triggered by classically conditioned endocrine and other pharmacological responses (7). For example, Hall et al. (49) showed that nausea as well as cortisol release can be simultaneously classically conditioned and that their effects can be diminished using overshadowing (presentation of a salient additional cue at the time of conditioning).

This principle could be applied to other unwanted side effects, for example, nausea caused by chemotherapy in cancer patients, that has been proposed to be triggered by classical conditioning and shown to be related to heightened cortisol levels (70). Presenting a salient cue such as a distinctive drink along with chemotherapy, might decrease the conditioned nausea and cortisol levels. It would be also worthwhile for future studies to look into possibilities of using overshadowing for the modification of other endocrine conditioned responses.

Overall, despite the heterogeneity of the described studies, this systematic review delivers supportive evidence that endocrine levels can be influenced by classical conditioning mechanisms, at least for certain

endocrine parameters (e.g., insulin and cortisol) in animal studies and to a lesser extent in human studies. Nevertheless, to be able to use classical conditioning in clinical practice, several concrete questions still have to be answered by future research. First, it is important to investigate if all hormones can be influenced by conditioning and how conditioned hormone responses generalize to the other hormonal and immune parameters. Second, more insight is needed in the extinction of the conditioned hormonal responses and the optimal way to reinforce them. Moreover, individual differences and possible predictors of endocrine responses remain underexplored. Finally, more knowledge is needed about how the conditioned responses influence various health outcomes and behavior. It is important that laboratory studies focus on investigating classical conditioning phenomena in various hormonal systems and use controlled designs of high methodological quality. Clinical trials may further explore the possibilities of applying the conditioning paradigms in clinical settings for dose reductions, enhancements of treatment favorable endocrine parameters, and reduction of unfavorable conditioned endocrine responses.

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Chapter 2

Conditioned hormonal responses: a systematic review

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Supplementary material. Search terms for the electronic search in PubMed, PsychInfo/CINAHL and Embase.

Pubmed	PsychInfo/ CINAHL	Embase
"Hormones"[Mesh] OR "Corticosterone"[Mesh] OR "Hydrocortisone"[Mesh] OR "Adrenocorticotrophic Hormone"[Mesh] OR "Corticotropin-Releasing Hormone"[Mesh] OR "Adrenocorticotrophic Hormone"[Mesh] OR "Luteinizing Hormone"[Mesh] OR "Testosterone"[Mesh] OR "Estrogens"[Mesh] OR "Gonadotropin- Releasing Hormone"[Mesh] OR "Follicle Stimulating Hormone"[Mesh] OR "Oxytocin"[Mesh] OR "Prolactin"[Mesh] OR "Dehydroepiandrosterone"[Mesh] OR "Progesterone"[Mesh] OR "Thyrotropin- Releasing Hormone"[Mesh] OR "Thyrotropin"[Mesh] OR "Glucagon"[Mesh] OR "Glucagon-Like Peptide 1"[Mesh] OR "Ghrelin"[Mesh] OR "C-Peptide"[Mesh] OR "Insulin"[Mesh] OR "Pancreatic Polypeptide"[Mesh] OR "Leptin"[Mesh] OR "Incretins"[Mesh] OR "Renin"[Mesh] OR "Angiotensins"[Mesh] OR "Aldosterone"[Mesh] OR "Vasopressins"[Mesh] OR "Epinephrine"[Mesh] OR "Norepinephrine"[Mesh] OR "Melatonin"[Mesh] OR "Cholecystokinin"[Mesh] OR "Human Growth Hormone"[Mesh] OR "Insulin- Like Growth Factor I"[Mesh] OR "Melanocyte-Stimulating Hormones"[Mesh] OR "Blood Glucose"[Mesh] OR "Glucose"[Mesh] OR	(DE "Classical Conditioning" OR DE "Conditioned Emotional Responses" OR DE "Conditioned Responses" OR DE "Higher Order Conditioning" OR DE "Unconditioned Responses" OR DE "Serial Anticipation (Learning)" OR TI Conditioning OR TI Conditioned OR TI "Food-anticipatory activity" OR TI "Meal-anticipatory" OR TI Anticipatory OR TI Anticipation OR TI "Cephalic phase" OR TI "Cephalic insulin" OR TI "Schedule-induced" OR AB Conditioning OR AB Conditioned OR AB "Food- anticipatory activity" OR AB "Meal-anticipatory" OR AB Anticipatory OR AB Anticipation OR AB "Cephalic phase" OR AB "Cephalic insulin" OR AB "Schedule-induced") AND (DE "Hormones" OR DE "Adrenal Cortex Hormones" OR DE "Adrenal Medulla Hormones" OR DE "Cholecystokinin" OR DE "Corticotropin Releasing Factor" OR DE "Epinephrine" OR DE "Ghrelin" OR DE "Glucagon" OR DE	((endocrine or hormon* or neuroendocrine or cortisol or corticosterone or hydrocortisone or adrenocorticotrophic hormone or adrenocorticotropin or corticotropin-releasing hormone or estrogen or testosterone or gonadotropin- releasing hormone or luteinizing hormone or follicle-stimulating hormone or progesterone or dehydroepiandrosterone or oxytocin or prolactin or thyrotropin-releasing hormone or thyroid-stimulating hormone or thyrotropin or glucagon or gut peptide* or ghrelin or glucagon-like peptide or insulin or c-peptide or pancreatic polypeptide or obestatin or leptin or incretin or renin or angiotensin or aldosterone or antidiuretic hormone or vasopressin or epinephrine or adrenaline or norepinephrine or noradrenaline or melatonin or cholecystokinin or growth hormone or insulin-like growth factor 1 or melanocyte-stimulating hormone or glucose or blood

"Glucocorticoids"[Mesh] OR endocrine[Title] OR hormon*[Title] OR neuroendocrine[Title] OR cortisol[Title/Abstract] OR corticosterone[Title/Abstract] OR hydrocortisone[Title/Abstract] OR "adrenocorticotrop hormone"[Title/Abstract] OR adrenocorticotropin[Title/Abstract] OR "corticotropin-releasing hormone"[Title/Abstract] OR estrogen[Title/Abstract] OR testosterone[Title/Abstract] OR "gonadotropin-releasing hormone"[Title/Abstract] OR "luteinizing hormone"[Title/Abstract] OR "follicle- stimulating hormone"[Title/Abstract] OR progesterone[Title/Abstract] OR dehydroepiandrosterone[Title/Abstract] OR oxytocin[Title/Abstract] OR prolactin[Title/Abstract] OR "thyrotropin- releasing hormone"[Title/Abstract] OR "thyroid-stimulating hormone"[Title/Abstract] OR thyrotropin[Title/Abstract] OR glucagon[Title/Abstract] OR "gut peptide*" [Title/Abstract] OR ghrelin[Title/Abstract] OR "glucagon-like peptide"[Title/Abstract] OR insulin[Title/Abstract] OR c- peptide[Title/Abstract] OR "pancreatic polypeptide"[Title/Abstract] OR obestatin[Title/Abstract] OR leptin[Title/Abstract] OR incretin[Title/Abstract] OR renin[Title/Abstract] OR angiotensin[Title/Abstract] OR aldosterone[Title/Abstract] OR "antidiuretic hormone"[Title/Abstract] OR vasopressin[Title/Abstract] OR epinephrine[Title/Abstract] OR adrenaline[Title/Abstract] OR norepinephrine[Title/Abstract] OR noradrenaline[Title/Abstract] OR melatonin[Title/Abstract] OR cholecystokinin[Title/Abstract] OR "growth hormone"[Title/Abstract] OR "insulin-like growth factor	"Gonadotropic Hormones" OR DE "Insulin" OR DE "Leptin" OR DE "Melatonin" OR DE "Orexin" OR DE "Parathyroid Hormone" OR DE "Pituitary Hormones" OR DE "Progestational Hormones" OR DE "Sex Hormones" OR DE "Thyroid Hormones" OR DE "Corticosterone" OR DE "Cortisone" OR DE "Hydrocortisone" OR DE "Corticotropin" OR DE "Estrogens" OR DE "Testosterone" OR DE "Luteinizing Hormone" OR DE "Progesterone" OR DE "Oxytocin" OR DE "Prolactin" OR DE "Thyrotropin" OR DE "Glucagon" OR DE "Ghrelin" OR DE "Insulin" OR DE "Leptin" OR DE "Angiotensin" OR DE "Aldosterone" OR DE "Epinephrine" OR DE "Norepinephrine" OR DE "Melatonin" OR DE "Cholecystokinin" OR DE "Somatropin" OR DE "Vasopressin" OR DE "Melanocyte Stimulating Hormone" OR DE "Glucose" OR DE "Blood Sugar" OR TI "Endocrine" OR TI "Hormon*" OR TI "Neuroendocrine" OR TI "Cortisol" OR TI "Corticosterone" OR TI "Hydrocortisone" OR TI "Adrenocorticotrop hormone" OR TI "Adrenocorticotropin" OR TI "Corticotropin-releasing hormone" OR TI "Estrogen" OR TI "Testosterone" OR TI "Gonadotropin-releasing hormone" OR TI "Luteinizing hormone" OR TI "Follicle- stimulating hormone" OR TI "Progesterone" OR TI "Dehydroepiandrosterone" OR TI	sugar or glucoregulatory).ti. or (cortisol or corticosterone or hydrocortisone or adrenocorticotrop hormone or adrenocorticotropin or corticotropin-releasing hormone or estrogen or testosterone or gonadotropin- releasing hormone or luteinizing hormone or follicle-stimulating hormone or progesterone or dehydroepiandrosterone or oxytocin or prolactin or thyrotropin-releasing hormone or thyroid-stimulating hormone or thyrotropin or glucagon or gut peptide* or ghrelin or glucagon-like peptide or insulin or c-peptide or pancreatic polypeptide or obestatin or leptin or incretin or renin or angiotensin or aldosterone or antidiuretic hormone or vasopressin or epinephrine or adrenaline or norepinephrine or noradrenaline or melatonin or cholecystokinin or growth hormone or insulin-like growth factor 1 or melanocyte-stimulating hormone or glucose or blood sugar or glucoregulatory).ab.) AND ((Conditioning or Conditioned or Food-anticipatory activity or Meal-anticipatory or Anticipatory or Anticipation or Schedule-induced or Cephalic phase or Cephalic insulin or Oral sensory stimulation).ti. or (Conditioning or Conditioned or Food-anticipatory activity or Meal-anticipatory or Anticipatory or Anticipation
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Chapter 3

Placebo effects in the neuroendocrine system:
conditioning of the oxytocin responses.

Published as

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Abstract

Objective: Evidence exists that placebo effects may potentially influence hormone secretion. However, only few studies examined placebo effects in the endocrine system, including oxytocin placebo responses, which may have several beneficial effects. We studied whether it is possible to trigger oxytocin placebo responses using a classical conditioning paradigm. **Methods:** Ninety-nine healthy females were assigned to conditioned, placebo or drug-control group. In the two-phase conditioning paradigm, participants in the conditioned and drug-control groups received an oxytocin nasal spray combined with a distinctive smell (conditioned stimulus, CS) during three acquisition days, while the placebo group received placebo nasal spray. Subsequently, the conditioned and placebo groups received placebo spray with the CS and the drug-control group- oxytocin spray during three evocation days. Salivary oxytocin was measured at baseline and at different points after the spray administration. Pain sensitivity and facial evaluation tests previously used in oxytocin research were also administered. **Results:** A significant increase of oxytocin levels in the conditioned group at 5, 20, and 50 minutes after the CS on evocation day 1 was demonstrated. On evocation day 2, a trend for increased oxytocin levels was found at 5 and 20 minutes. No placebo responses were found on evocation day 3. Neither exogenous nor conditioned oxytocin affected pain or facial tasks. **Conclusions:** Results indicate that oxytocin release can be conditioned and that this response extinguishes over time. Triggering hormonal release by placebo manipulation offers various clinical possibilities, e.g., enhancing effects of pharmacological treatments or reducing dosages of medications.

Trial Registration: The study was registered as a clinical trial on www.trialregister.nl (number NTR5596).

Keywords: classical conditioning; pharmacological conditioning; oxytocin; endocrine system; placebo effect

Introduction

Extensive research has demonstrated that placebo effects can significantly alleviate subjective symptoms of pain (1), fatigue (2), depression (3). There are also studies that indicated that placebo affects not only subjective symptoms, but physiological and neurological processes underlying these symptoms. For example, placebo analgesia has been demonstrated to be triggered by the endogenous opioid (4) and cannabinoid systems (5). Animal research repeatedly showed that hormones, for example insulin and corticosterone can be also affected by placebo effect (6). Classical or Pavlovian conditioning is proposed to underlie these placebo endocrine responses. Conditioning is a learning process in which an association is established between an initially neutral stimulus and a physiologically relevant unconditioned stimulus (US) so that after repeated pairings, the neutral stimulus becomes a conditioned stimulus (CS) and triggers a physiological response similar (or opposite, in case of paradoxical conditioning) to the US; the conditioned response (CR).

Human research on conditioning of placebo endocrine responses is more limited. It was shown that it is possible to elicit placebo insulin release by pairing a distinct smell (CS) with intranasal insulin spray (US) (7). There is also some evidence showing that cortisol increase (8) and decrease (9) can be conditioned, even though a recent study (10) did not find a conditioned cortisol response. Other hormonal systems have not been sufficiently investigated in humans and it is not known if these findings can be generalized to other endocrine parameters and, moreover, the duration of conditioning placebo effects have not been examined in human studies so far. Being able to alter hormonal responses by a rather simple behavioral manipulation (e.g., by an exposure to a particular smell or taste), has however widespread clinical implications. For instance, classical conditioning mechanisms lie at the basis of placebo-controlled dose reduction schedules, in which a part of the active medication is replaced by placebos while maintaining the efficacy of the treatment (11). It is thus important to also explore which hormonal responses can be altered by applying the principles of classical conditioning.

The aim of the current study was to investigate whether it is possible to elicit conditioned placebo oxytocin release in humans. Oxytocin is a hormone and neuropeptide produced primarily in the

hypothalamus and it was initially investigated in the context of the labor regulation (12) and mother-infant bonding (13). More recently, oxytocin has attracted a lot of attention for its prosocial effects. It has been proposed to play an important role in emotion recognition (14), emotional contact (15) and stress responsiveness (16). Disruption of oxytocin responses has been found in the mental disorders such as autism (17), schizophrenia (18), borderline disorder (19) and post-traumatic stress disorder (20) and currently oxytocin is extensively investigated as a treatment for these conditions (21-23). Being able to influence oxytocin release with placebo manipulation could, therefore, open additional perspectives for the treatment of conditions related to emotional deficits.

So far only one study demonstrated that oxytocin levels can be manipulated by classical conditioning in rats (24). In this randomized controlled trial, we investigated whether it is possible to trigger placebo oxytocin release by employing a classical conditioning paradigm. We hypothesized that after the repeated coupling of oxytocin nasal spray with a distinctive smell, the smell alone would trigger endogenous oxytocin release. Moreover, we expected to find the strongest conditioned oxytocin release during the first evocation day, and possible extinction pattern during the next non-reinforced evocation trials.

Methods

Participants

99 healthy females participated in the study. Only females who were taking oral contraceptives were included as they have stable levels of oxytocin during the cycle (25). All females were tested while in their pill weeks and not in their stop weeks. Exclusion criteria were: psychiatric (DSM-V) conditions, somatic conditions that might interfere with the participant's safety and/or the study protocol, Raynaud's phenomenon, severe neurological or neurosurgical conditions, pregnancy or breast feeding, and heavy use of alcohol or drugs. Participants were asked to refrain from taking analgesic and anti-inflammatory medication and recreational drugs during the two weeks of testing, drinking alcohol and doing physical exercise 24 hour before each session, and drinking caffeinated drinks and eating a meal two hours before

each session. During the screening, participants were asked to sign an informed consent form and at the end of each session they received a monetary reward.

Sample size was calculated on the basis of a pilot study performed in our lab aimed at conditioning of cortisol with a similar study design (26). The effect size of the pilot was $d = 0.527$ and the sample size was estimated to be 33 participants per group.

Study design

The study was a randomized controlled trial. Participants were randomly assigned to one of three groups: 1) conditioned group, 2) placebo group, and 3) drug-control group. The study had a single-blind design. Participants did not know whether they would receive oxytocin or placebo. Researchers knew when participants were included in the drug-control group (due to the absence of the CS in the evocation phase) but were blinded regarding the conditioned and placebo group.

In line with previous conditioning studies (7, 10), a randomized placebo-controlled conditioning paradigm consisting of 2 phases (acquisition and evocation phases) was applied (Figure 1). Both acquisition and evocation phases lasted for three consecutive days with a four-day break between the last acquisition and the first evocation day to avoid potential drug residual effects from the last acquisition day interfering with the first evocation day. For the experimental group, the procedure was the following: In the acquisition phase, an association between a US (24IU of oxytocin nasal spray) and a CS (smell of rosewood oil) was established. Participants were asked to smell the odor with a custom-made olfactometer (27) for a minute before and a minute immediately after the oxytocin spray administration. In the evocation phase, participants were administered a placebo spray paired with the same smell as in the acquisition phase. A similar procedure was used for participants in the placebo group, but instead of oxytocin spray they received a placebo spray during both phases. Participants in the drug-control group were administered the oxytocin spray during both the acquisition and evocation phases, but did not receive a CS and were tested in a different lab during the evocation phase in order to eliminate possible conditioning effects triggered by the CS and the environment of the CS administration. This was done in order to avoid a conditioned response in the drug-control group.

The study was approved by Medical Ethical Committee of Leiden University Medical Centre (NL52683.058.15). The randomization was performed by the department of Clinical Pharmacy of the Leiden University Medical Center. The block randomization was used with a size of a block of 9 participants per block.

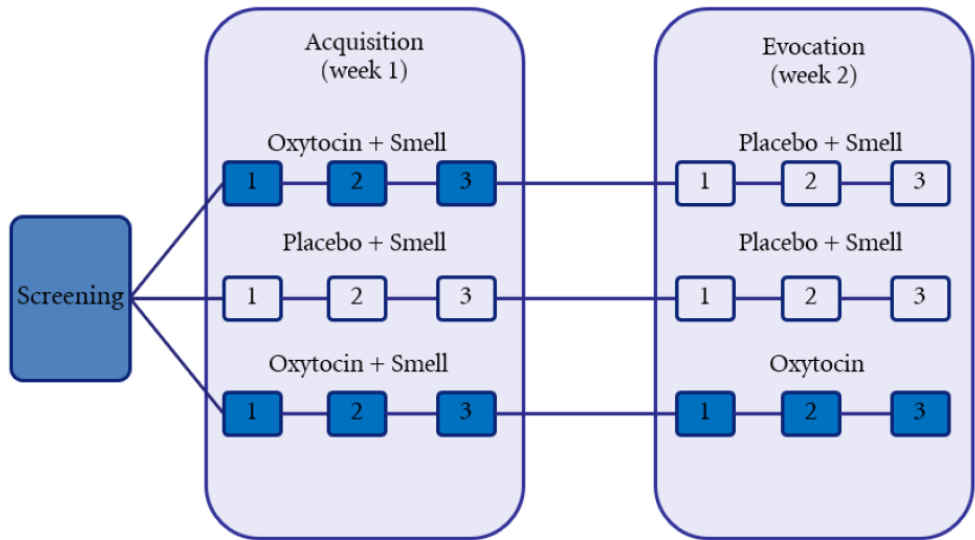


Figure 1. Study design

Procedure

Screening. Upon arrival at the lab, participants were asked several questions about their somatic and mental health to check the inclusion criteria. Next, participants were asked to fill in several questionnaires including questionnaires on demographics and psychological characteristics. A saliva sample was taken to establish baseline oxytocin levels. Afterwards, two pain tasks were performed to measure the baseline pain sensitivity levels: a Cold Pressor Task (CPT) and a task with heat pain stimulation. The aim of the heat pain stimulation was to determine the heat pain thresholds that were used in a MRI part of this experiment, which will be reported on separately. After this, participants were informed about their eligibility to participate in the study.

Acquisition phase. The acquisition phase consisted of three consecutive days that lasted 15 minutes each and started at fixed times between 2 p.m. and 6 p.m. Upon arrival to the lab, a baseline saliva sample was taken and the participants were asked questions about their health and food and alcohol consumption. Afterwards, participants were exposed to a CS (distinctive smell) for a minute. Immediately after the CS, they were administered 24 IU of oxytocin or placebo, depending on the group allocation, and were presented with the CS for another minute. The interval between the smell administration was no longer than 1.5 minutes.

Evocation phase. All evocation sessions started at the same time as acquisition sessions between 2 p.m. and 6 p.m. Identical to the acquisition days, upon arrival at the lab, a baseline saliva sample was taken. Then depending on group allocation, participants were administered the CS and a placebo spray (in the conditioned and placebo groups) or oxytocin without the CS (in the drug-control group). Three saliva measurements were completed on evocation days 1 and 2: at 5, 20 and 50 minutes after the nasal spray administration. In addition, 30 minutes after the spray administration, participants performed a computer task in which facial trustworthiness and attractiveness was evaluated, and 40 minutes after the nasal spray administration, they were exposed to CPT. Evocation day 3 started similar to the previous evocation days but after the second saliva measurement (5 minutes after the spray administration), participants were brought to the MRI facilities of Leiden University Medical Centre. Details of the (f)MRI part of this experiment will be reported separately. After the end of the last evocation day, participants were fully debriefed.

The experiment took place in the laboratory facilities of the Social Science department of Leiden University. The data collection took place between February, 2016 and August, 2017.

Intervention

Unconditioned stimulus. The unconditioned stimulus was a 24 IU of oxytocin (Syntocinon Spray) or a placebo nasal spray. The placebo spray looked and tasted identically to oxytocin and was prepared by the department of Clinical Pharmacy of the Leiden University Medical Center. The nasal spray was

administered by the experimenter with two puffs (one puff per nostril) using a MAD Nasal mucosal atomization device (Teleflex, Inc., Research Triangle Park).

Conditioned stimulus. The conditioned stimulus was the administration of a smell of rosewood oil for one minute immediately before and one minute directly after the spray administration. The smell was administered via a custom-made olfactometer, a device that delivered medicinal air with the airflow of 4 litres per minute through a jar with 5 drops of the Rosewood oil (Etherische olie Rozenhoud, www.aromaolie.nl) into nasal goggles that participants wore. During the smell administration, participants sat under a cylinder connected to the air-conditioning system of the building, in order to prevent the smell from spreading into the room.

Materials and measurements

Saliva samples. Participants were asked to collect between 1.5 and 2 ml of saliva in a cryotube using a passive-drool method. Samples were immediately frozen first on dry ice and then in a -80°C freezer. Salivary oxytocin was assayed using commercial ELISA kits with extraction (Enzo Life Sciences, Farmingdale, NY) purchased in November 2017 and April 2018. The addition of the extraction procedure, which reduces matrix interference and concentrates the sample, has been described previously (28). This method is consistent with currently recommended best practices (29). Lower level of detection for oxytocin was 0.5 pg/ml after extraction; extraction efficiency was 99%; intra- and inter-assay coefficients of variation were 10.2% and 11.8%, respectively.

The Cold Pressor Task was used to assess pain sensitivity. The waterbath consisted of a 2.7 liter styrofoam tank with cold water, which was maintained at a fixed temperature of 4°C. Participants were asked to hold their dominant hand in the water for 1 minute while every 15 seconds their pain levels were assessed on a numerical rating scale with a question “How much pain do you have now?”. The participant verbally gave an answer on a 0-10 scale with decimals (0 = no pain at all, 10 = worst pain ever experienced). The pain intensity scores in response to the baseline and post-intervention CPT were calculated as the mean scores of the four pain rating measurement points during each CPT.

The facial attractiveness and trustworthiness task was used to measure how trustworthy and attractive participants find faces of strangers. Participants were asked to rate neutral male and female faces on their attractiveness and on trustworthiness using a 7-point Likert scale (1 = not attractive/trustworthy; 7 = extremely attractive/trustworthy). In total 32 pictures from the Radboud Faces Database (30) were presented in a fixed order and different faces were used on evocation days 1 and 2.

Extraversion and neuroticism were measured with the short version of the Eysenck Personality Questionnaire (31). The total score for neuroticism and extraversion ranges from 0 to 12, with higher scores indicating higher neuroticism and higher extraversion, respectively.

Optimism was measured with the revised Life Orientation Test (32). The total score ranges from 0 to 24, with higher score indicating higher optimism.

Depression and trait anxiety were measured with the Hospital anxiety and depression scale (HADS) (33). The HADS is divided into 2 subscales: the depression subscale and the anxiety subscale, both containing 8 items. The score per scale ranges from 0 to 8, with higher scores indicating higher depression and anxiety.

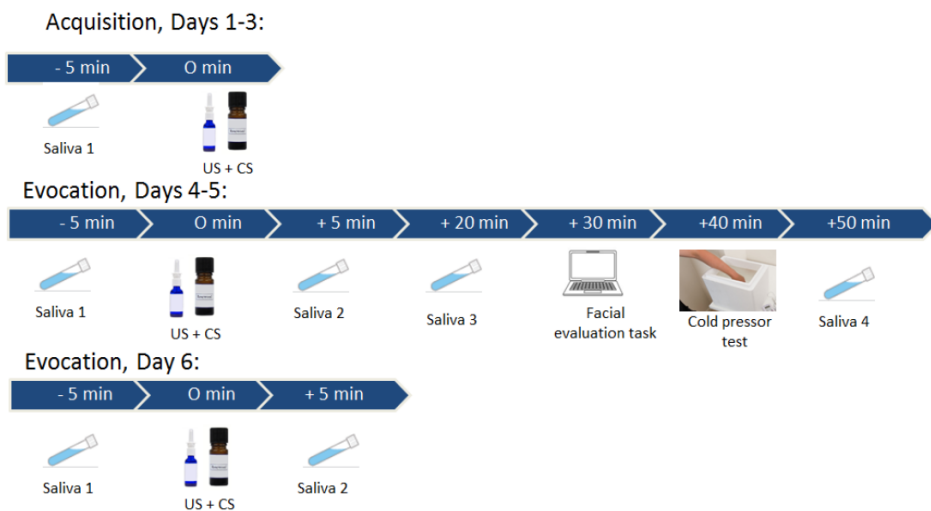


Figure 2. Timeline of the experimental days.

Statistical Analysis

To compare the groups on baseline characteristics such as baseline oxytocin levels, age, BMI, baseline pain sensitivity in response to the CPT, and psychological characteristics an analysis of variance (ANOVA) was performed.

To examine the differences in oxytocin levels between the groups during the evocation days, we used a linear-mixed effects model approach to account for the dependencies between repeated measurements of the same subject. We used the `lmer` function of the `lme4` package (34) in R (R Core Team, 2013) for the mixed-models analysis. The multilevel structure of the data was defined by measurements (level 1) nested in subjects (level 2). Parameters were estimated using the full maximum likelihood procedure.

Measurement moments were dummy-coded such that the slope of each dummy represents the change from one measurement moment to the next, and these dummies were added to the regression as separate predictors. Besides a random intercept, the models included a random effect for the slope of each dummy variable. Separate models were tested per evocation day, first for the two main groups (conditioned and placebo) and then the drug-control group was added to examine the changes within this group. We first examined the slopes of the oxytocin change between the measurement moments in the three groups separately.

To confirm the results of the multilevel within-group analysis and look at the between-group differences, we performed sensitivity analyses with repeated measures analysis of covariance (ANCOVA) in which we compared the oxytocin salivary levels after the spray administration between the conditioned oxytocin and the placebo groups with the baseline levels as a covariate. We ran these analyses separately for evocation days 4, 5 and 6.

In line with the other conditioning studies (35), we determined conditioning responders and non-responders on basis of the conditioned oxytocin release after the CS administration. If the increase of oxytocin levels from baseline to 5 minutes after the CS administration of a participant from the

conditioned group exceeded 1 SD of the change in the control group, the participant was marked at a responder (35). This was separately done for evocation sessions 1, 2 and 3. Responders and non-responders from the conditioned group were compared on the basis of their baseline characteristics (oxytocin levels, age, BMI, baseline sensitivity in response to the CPT, extraversion, neuroticism, optimism, pessimism, depression, and anxiety) with t-tests.

To investigate the effects of the manipulations on the pain sensitivity in response to the CPT, a repeated measures analysis of covariance (ANCOVA) was performed with the evocation day number as a within-subject factor, group as a between-subject factor, and baseline pain sensitivity as a covariate. To explore the effect of conditioning on the perceived facial attractiveness and trustworthiness, a repeated measures ANOVA with the evocation day number as a within-subject factor and group as a between-subject factor was performed.

Results

Baseline characteristics

One participant did not complete the baseline cold pressor task due to technical problems; the rest of the baseline measurements were completed by all participants. There were no significant differences between the three groups in age ($F(2, 96)=0.76, p=.47$), Body Mass Index ($F(2, 96)=0.85, p=.43$), baseline pain sensitivity in response to the cold pressor task ($F(2, 95)=0.30, p=.74$), and questionnaires measuring constructs such as extraversion ($F(2, 96)=0.14, p=.87$), neuroticism ($F(2, 96)=0.85, p=.43$), optimism ($F(2, 96)=0.03, p=.97$), depression ($F(2, 96)=0.70, p=.50$), and trait anxiety ($F(2, 96)=0.64, p=.53$) (Table 1).

Table 1. Baseline characteristics across the groups with standard deviations.

Conditioned group	Placebo group	Drug-control group (n = 33)

	(n = 33)	(n = 33)	M (SD)
	M (SD)	M (SD)	
Age	21.2 (2.8)	21.7 (1.9)	21.9 (2.5)
Body mass index	22.4 (2.5)	21.8 (2.1)	22.5 (2.4)
Screening pain sensitivity	4.5 (1.6)	4.5 (2.3)	4.7 (2.2)
Extraversion	10.2 (2.9)	10.2 (1.9)	9.9 (2.7)
Neuroticism	3.3 (2)	2.8 (1.7)	2.8 (1.9)
Optimism	8 (1.5)	8 (1.7)	8 (1.4)
Depression	1.1 (1.2)	0.8 (1.0)	0.9 (0.8)
Trait anxiety	2.9 (1.8)	2.5 (2.2)	2.9 (1.8)

Endogenous oxytocin release

Due to sample clogging or contamination, 52 samples from 10 participants could not be analyzed; all other samples (1235) were included in the analyses. We first tested salivary oxytocin levels at baseline during the three acquisition days. The baseline levels of oxytocin did not differ between the groups on the first ($F(2,92)=2.16$, $p=.12$), second ($F(2,93)=0.32$, $p=.73$) or third ($F(2,93)=0.04$, $p=.96$) acquisition day, indicating that there were no differences in endogenous oxytocin levels at baseline and no pharmacological carry-over effects of the oxytocin spray administration from one day to the next day during this phase (Table 2).

The mean oxytocin levels for each evocation day are presented in Figure 3 and Table 2. To examine a conditioned oxytocin release in response to the CS in the conditioned group during the evocation days, we applied a mixed-models approach. We examined the slopes of the oxytocin change between the

measurement moments in the three groups, separately. On evocation day 1 in the conditioned group, a significant increase in oxytocin levels from the baseline to 5 minutes after the CS administration was found ($b=19.55$, $t(187)=3.33$, $p=.001$), followed by a trend to decrease from 5 to 20 minutes ($b=-10.42$, $t(187)=-1.79$, $p=.071$) and non-significant changes 20 minutes to 50 minutes ($b=-0.70$, $t(187)=-0.21$, $p=.84$) after the placebo spray with CS administration. In the placebo group, the changes from the baseline to 5 minutes after the placebo spray with CS administration ($b=-1.82$, $t(187)=-0.31$, $p=.75$), 5 minutes to 20 minutes ($b=-1.21$, $t(187)=-0.21$, $p=.83$) and 20 minutes to 50 minutes ($b=-3.45$, $t(187)=-1.03$, $p=.31$) were all not significant. This pattern indicated that there was a significant increase of endogenous oxytocin levels that remained for 50 minutes in the conditioned group while no such increase was found in the placebo group. The oxytocin levels in the drug-control group greatly increased from baseline to 5 minutes after oxytocin administration ($b=1686$, $t(272)=7.12$, $p<.001$) and then significantly decreased from 5 to 20 minutes ($b=-736.15$, $t(272)=-4.6$, $p<.001$), and from 20 to 50 minutes ($b=-212.41$, $t(272)=-3.18$, $p<.01$) after the spray administration.

On evocation day 2, a trend towards a significant increase of oxytocin levels from baseline to 5 minutes after the CS administration was found ($b=15.22$, $t(187)=1.87$, $p=0.062$) followed by no change of oxytocin levels from 5 minutes to 20 minutes ($b=-12.05$, $t(187)=-1.54$, $p=0.12$) and a significant drop of oxytocin levels from 20 to 50 minutes ($b=-5.98$, $t(187)=-3.21$, $p<0.01$) in the conditioned group. In the placebo group, the changes from the baseline to 5 minutes ($b=0.47$, $t(187)=0.06$, $p=0.95$), 5 minutes to 20 minutes ($b=-0.61$, $t(187)=-0.08$, $p=0.94$), and 20 minutes to 50 minutes ($b=-1.76$, $t(187)=1.84$, $p=0.34$) were all not significant. This pattern indicated a trend for increased conditioned oxytocin levels in the conditioned group that remained present from 5 minutes until 20 minutes after the CS administration. In the drug-control group, the oxytocin levels increased significantly from baseline to 5 minutes after the spray administration ($b=1480.26$, $t(272)=6.57$, $p<0.001$) followed by a significant decrease from 5 to 20 minutes ($b=-536.16$, $t(272)=-3.76$, $p<0.001$), and from 20 to 50 minutes ($b=-242.38$, $t(272)=-3.36$, $p<0.001$).

Finally, on the evocation day 3, that included two measurement moments (baseline and 5 minutes), no significant changes from baseline to 5 minutes after the CS administration were found, neither in the conditioned ($b = 3.57$, $t(59) = 1.10$, $p = 0.28$) nor in the placebo group ($b = 3.02$, $t(59) = 0.96$, $p = 0.34$). In the drug-control group, there was a significant increase in oxytocin levels from the baseline to 5 minutes after the spray administration ($b = 1552.25$, $t(87) = 5.94$, $p < 0.001$).

The sensitivity analysis using repeated measures ANCOVA comparing the conditioned and control groups on the evocation day 1 showed that the salivary oxytocin levels in the conditioned group were higher comparison to the control group ($F(1, 61) = 7.84$, $p = .007$, $\eta^2 = 0.114$) after controlling for the baseline levels. No effect of time ($F(2, 61) = 0.642$, $p = 0.53$, $\eta^2 = 0.01$) or a time x group interaction ($F(2, 61) = 1.10$, $p = 0.56$, $\eta^2 = 0.01$) on oxytocin levels during the evocation session 1 was found. Salivary levels of the conditioned and control group did not significantly differ neither on evocation day 2 ($F(1, 61) = 1.76$, $p = .19$, $\eta^2 = 0.028$), No significant effect of time ($F(2, 61) = 0.86$, $p = .43$, $\eta^2 = 0.014$;) or a time x group interaction ($F(1, 61) = 1.53$, $p = .22$, $\eta^2 = 0.024$) on the salivary levels on the evocation day 2 was found. Finally, the two groups did not differ in the salivary oxytocin levels on the evocation day 3 ($F(1, 61) = 1.27$, $p = .27$, $\eta^2 = 0.021$) after controlling for the baseline levels.

Participants in the conditioned group could be divided into responders and non-responders. Ten responders were found on the evocation day 1, five responders on the evocation day 2 (the same as on evocation day 1) and one responder on evocation day 3. No significant differences were found between responders and non-responders on baseline characteristics and questionnaires: age, Body Mass Index, baseline oxytocin levels, baseline pain sensitivity in response to the CPT, extraversion, neuroticism, optimism, pessimism, depression, and trait anxiety (all $ps > .10$).

Table 2. Mean salivary oxytocin levels (pg/ml) across the groups and measurements with standard deviations.

Session	Measurement	Conditioned group (n = 33)	Placebo group (n = 33)	Drug-control group (n = 33)
Acquisition 1	Baseline	21.4 (35.7)	12.2 (12.1)	10.4 (6.6)
Acquisition 2	Baseline	13.8 (8.0)	16.2 (18.8)	14.2 (9.6)
Acquisition 3	Baseline	16.1 (14.6)	14.8 (11.0)	15.7 (24.8)
Evocation 1	Baseline	11.9 (8.4)	16.4 (18.8)	11.7 (6.8)
	5 minutes	31.5 (46)	14.6 (7.5)	1805.7 (2275.3)
	20 minutes	21.1(27.6)	13.4 (8.3)	1118.8 (1835.3)
	50 minutes	20.4 (23.9)	9.9 (5.1)	857.8 (1522.2)
Evocation 2	Baseline	13.4 (6.7)	12.8 (7.9)	13.6 (8.7)
	5 minutes	28.6 (64.4)	13.7 (6.5)	1595.7 (2139.6)
	20 minutes	16.5 (14.3)	12.6 (5.6)	1069.3 (1622.6)
	50 minutes	10.6 (7.3)	10.9 (5.5)	818.3 (1598.2)
Evocation 3	Baseline	12.4 (12.2)	16.1 (20.7)	15.1 (12.7)
	5 minutes	12.4 (7.3)	19.4 (42.6)	1762.9 (2519)

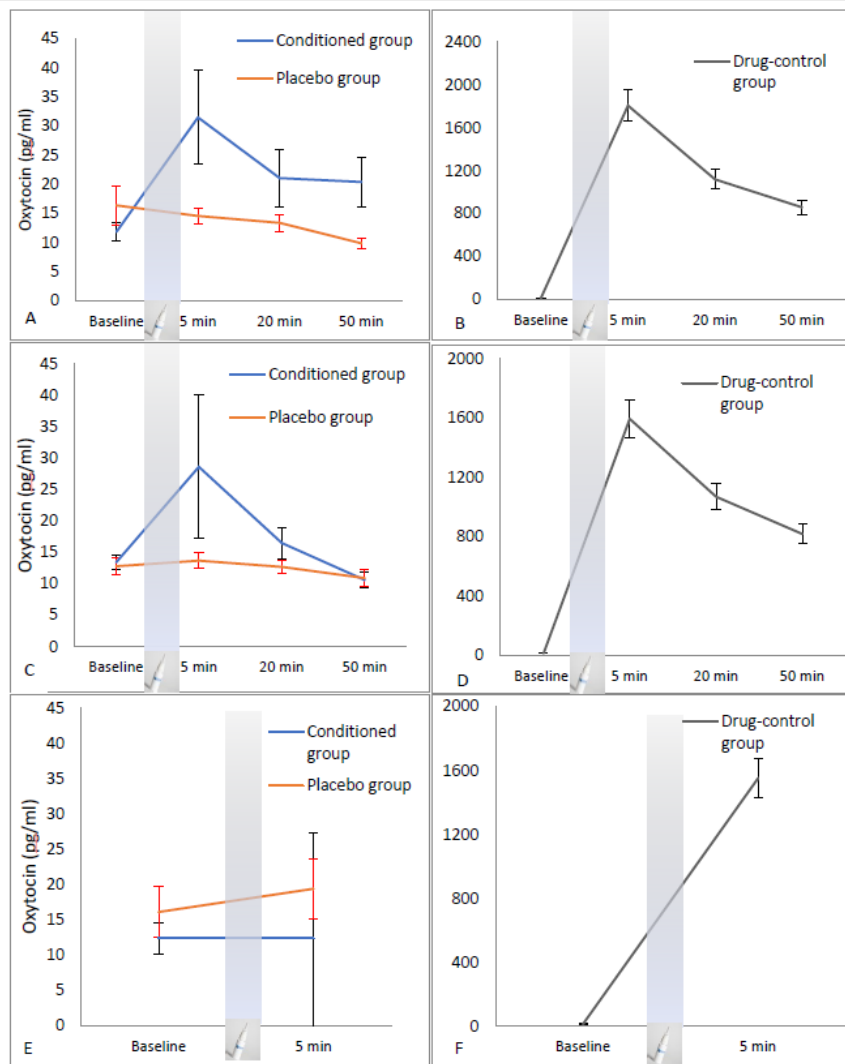


Figure 3. Salivary oxytocin levels (pg/ml) on evocation days 1 (A, B), 2 (C, D) and 3 (E, F) separately for each group (n= 99). Error bars indicate standard errors.

Pain perception

Repeated measures ANCOVA demonstrated that there was no effect of the group ($F(2,94)=0.13$, $p=.88$), the day ($F(1, 94)=0.51$, $p=.48$) and the group-by-day interaction ($F(2,94)=0.53$, $p=.70$) on the pain

sensitivity ratings, indicating that neither endogenous conditioned oxytocin release, nor exogenous oxytocin administration influenced pain sensitivity.

Perceived facial trustworthiness and attractiveness

Data of 94 participants were available for this analysis. Repeated measures ANOVA demonstrated that participants in all groups rated faces as less trustworthy on the second evocation day in comparison to the first evocation day ($F(1,92)=57.27, p<.001$). However, the three groups did not differ in their trustworthiness ratings ($F(2,92)=0.01, p=.99$) nor was the group-by-day interaction significant ($F(2,93)=2.99, p=.062$). There was no effect of the group ($F(2,92)=0.38, p=.38$), the day ($F(1,92)=3, p=.092$) or group-by-day interaction ($F(2,92)=0.22, p=.80$) on the perceived facial attractiveness ratings (Table 3).

Table 3. Mean task scores across the groups and evocation days with standard deviations.

Task	Session	Conditioned group	Placebo group	Drug-control group
Pain sensitivity	Evocation 1	4.8 (1.9)	5.0 (2.2)	4.9 (2.2)
	Evocation 2	4.7 (1.8)	4.8 (2.3)	4.9 (2.2)
Attractiveness score	Evocation 1	2.8 (0.5)	3.0 (0.6)	3.0 (0.6)
	Evocation 2	2.8 (0.6)	2.9 (0.6)	3.0 (0.6)
Trustworthiness score	Evocation 1	4.0 (0.7)	4.0 (0.8)	4.1 (0.6)
	Evocation 2	3.7 (0.8)	3.8 (0.9)	3.6 (0.5)

Discussion

The present study demonstrated for the first time that placebo effects can trigger endogenous oxytocin release. After three days of receiving oxytocin nasal spray with a distinctive rosewood oil smell, participants exhibited a conditioned increase of salivary oxytocin levels in response to the smell combined

with a placebo. Moreover, this study demonstrated that this conditioned oxytocin response followed an extinction pattern during the not-reinforced trials of the evocation phase.

The present experiment supports the existing evidence that hormonal responses can be modulated by classical conditioning in humans. Previous studies showed that insulin (7), cortisol (8, 9), and growth hormone levels (9) can be altered by conditioning in humans. However, no such evidence existed for oxytocin in humans. We found the strongest placebo response on the first evocation day as hypothesized. A trend towards an increase of oxytocin release over baseline was found on the second evocation day. No increase was found anymore on the third evocation day. These results are the first to demonstrate that endogenous placebo oxytocin release decreases when not reinforced upon subsequent trials. These findings correspond with the theory of extinction of conditioned responses (36). No previous data on extinction of hormonal placebo responses in humans are available, since human studies on conditioning of hormonal responses investigated conditioned responses for only one evocation day. Future research should replicate these findings and investigate whether conditioned responses of other hormones follow the same extinction pattern.

Literature proposes that intranasal administration of oxytocin triggers endogenous oxytocin release by feed-forward mechanisms, so that circulating oxytocin stimulates further oxytocin release (37-39). In our study, intranasal oxytocin was used as the US and endogenous oxytocin release was expected to be a conditioned response. The results supported this hypothesis: we found a conditioned increase of endogenous oxytocin levels in response to a CS.

The salivary oxytocin levels found in the drug-control group are consistent with the results found in previous research (38, 39): the oxytocin levels increased 100 times (10000%) from baseline after the oxytocin administration. The conditioned response was much smaller: salivary oxytocin levels increased two times (200%) compared to baseline at the highest peak. Noticeably, the conditioned response was nevertheless still higher than the magnitude of the endogenous oxytocin release in response to behavioral manipulation measured in blood: a 47% increase of endogenous oxytocin was found in response to watching an emotional video (40) and a 17% increase was found in response to massage (41). These

results show that classical conditioning can be an efficient and effective way to induce oxytocin release on demand. Moreover, the large standard errors in the conditioned group indicate the presence of responders and non-responders to the conditioning manipulation amongst the participants. These results add to the accumulative evidence on responders and non-responders to pharmacological conditioning (10, 35). Several factors may influence the response to conditioning. For example, baseline interleukin-2, noradrenaline and anxiety have been shown to predict the conditioned interleukin-2 response (35). Furthermore, baseline cortisol levels have been linked to the responsiveness to cortisol conditioning (10). In the present study, we were unable to find possible predictors of the conditioning response. More knowledge regarding what factors predict a conditioned response is needed.

Finally, we found no effect of the conditioned endogenous oxytocin release on pain sensitivity and perceived facial attractiveness and trustworthiness, even though these parameters were found to be influenced by exogenous oxytocin administration in previous studies (42, 43). By subsequently assessing the effects of exogenous oxytocin administration versus the conditioned and placebo-controlled groups, we were able to demonstrate that oxytocin did not affect pain sensitivity or perceived facial attractiveness or trustworthiness in the current dose and method of administration. Previous study on the perceived facial attractiveness and trustworthiness included larger groups (43) and as the power calculation of this study was not aimed to these secondary outcome parameters, the sample might have been too small to demonstrate these effects. Moreover, our findings regarding oxytocin effects on pain sensitivity are partly in line with recent findings that also show no effects of exogenous oxytocin on pain sensitivity (44, 45). Several limitations of the current study have to be addressed. First of all, the results cannot be generated to men and women who do not take hormonal contraceptives. As this was a first proof-of-concept study, we limited our sample to female participants who take hormonal contraception as they have been shown to have stable levels of oxytocin during the cycle (25). Future research should expand our findings and examine whether endogenous oxytocin levels are conditionable in men and women who are not using contraception. Secondly, the conditioned responses we found were very short-lasting and seemed to start extinguishing already after the first evocation day. In order to apply classical conditioning mechanisms to

clinical practice, it is important to find possible ways to prolong these effects, such as including more acquisition trials, partial reinforcement (46) or subtherapeutic conditioning (47). Another limitation concerns the reported levels of oxytocin measured in the drug control group. The found increase of oxytocin in the drug control group may potentially be partly explained by the contamination of the saliva by the intranasally administered oxytocin. Realizing this limitation, we did not directly compare the experimental group with the drug control group. Finally, there is an important limitation of the findings on the behavioral effects of intranasal oxytocin in general. There is a debate in the literature about the replicability and reliability of the studies that showed behavioral effects of intranasal oxytocin. Several reviews (48, 49) report failures to replicate the results of the studies on behavioral effects of oxytocin, and discuss the potential role of low statistical power of the published studies and possible publication bias. Additionally, an often-cited study on the trust enhancing effects of oxytocin (50), have been criticized for not-standard interpretation of the statistics (51). Leng (52) discusses that according to animal studies that measured oxytocin levels in cerebrospinal fluid after the exogenous oxytocin administration, only a small fraction of oxytocin reaches cerebrospinal fluid and it is unclear how these small amounts might cause behavioral changes. Additionally, the timing of behavioral effects (e.g., 53, 54) that are usually found at 45 minutes after intranasal oxytocin administration, does not correspond to the increase in cerebrospinal fluid oxytocin levels that occurs 75 minutes after the oxytocin administration (55). In this study we have also failed to find any behavioral effects of oxytocin and our results add to the contradictory evidence on the possible pain-reducing and trust-enhancing effects of oxytocin.

Regarding the clinical implications of the current study findings, there is some tentative evidence that oxytocin might have potential to improve social cognitions in autism (57), borderline personality disorder (23) and schizophrenia (22). Moreover, oxytocin has beneficial metabolic and immune effects. Treatment with oxytocin increases insulin sensitivity and decreases weight in obese adults (48), reduces inflammation (59, 60) and increases healing processes (61) in animals. Placebo-controlled dose reduction is one of the possible ways to use placebo effects directly in clinical practice. It is a drug-schedule in which patients first undergo a standard treatment and afterwards a part of their regular medication is

replaced with placebo, following for example a partial reinforcement schedule (62). Due to pharmacological conditioning, the response to placebo is hypothesized to mimic the drug response leading to maintenance of the treatment effectiveness with a potential reduction of associated side effects. Only a few trials so far investigated the efficiency of placebo-controlled dose reduction and first evidence demonstrated that it can be as efficient as a standard treatment for attention deficit and hyperactivity disorder and psoriasis (63, 64). Alternatively, conditioning-triggered placebo effects can be used in the enhancement of treatment effects of already existing therapies without the increase in the medication dose: it was demonstrated that classically conditioned immunosuppression enhances the effects of the routine treatment of renal transplant patients (65). Based on our findings indicating the possibility to condition hormonal responses, new placebo-controlled dose reduction trials or trials aimed at enhancement of treatment effects could be developed especially for symptoms requiring hormonal treatments.

In sum, the present study is the first proof of principle that salivary oxytocin levels can be conditioned by coupling intranasal oxytocin administration with a distinctive smell, and that the conditioned oxytocin extinguishes when not reinforced. This finding supports the limited evidence in animals and humans that hormonal responses can be influenced by the placebo effect. Considering the beneficial effects of oxytocin on a variety of mental health conditions, it is important that future studies look into possible clinical implications of oxytocin conditioning. Employing the placebo effect in clinical practice might be an easy and efficient way to enhance the effects of pharmacological treatments and reduce the dosages of medications, reducing costs and side effects of standard treatments.

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Chapter 3

Conditioning of the oxytocin responses

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Chapter 4

Effects of oxytocin administration and conditioned oxytocin on brain activity: an fMRI study

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Abstract

It has been demonstrated that secretion of several hormones can be classically conditioned, however, the underlying brain responses of such conditioning have never been investigated before. In this study we aimed to investigate how oxytocin administration and classically conditioned oxytocin influence brain activity. In total, 88 females were allocated to one of three groups: oxytocin administration, conditioned oxytocin, or placebo, and underwent an experiment consisting of three acquisition and three evocation days. Participants in the conditioned group received 24 IU of oxytocin together with a conditioned stimulus (CS) during three acquisition days and placebo with the CS on three evocation days. The oxytocin administration group received 24 IU of oxytocin and the placebo group received placebo during all days. On the last evocation day, fMRI scanning was performed for all participants during three tasks previously shown to be affected by oxytocin: the perception of emotional faces, crying baby sounds and heat pain. Region of interest analysis revealed that there was significantly lower activation in the right amygdala and in two clusters in the left superior temporal gyrus in the oxytocin administration group compared to the placebo group in response to observing fearful faces. The activation in the conditioned oxytocin group was in between the other two groups for these significant clusters but did not significantly differ from either group. No group differences were found in the other tasks. The findings carefully suggest that a conditioned response in brain activity was observed, however the conditioned group did not significantly differ from the other groups. Future research should therefore investigate the optimal timing of conditioned endocrine responses and study whether the findings generalize to other hormones as well.

Introduction

It has been shown that after repeated administration of medication that triggers a physiological change (unconditioned response), with an initially neutral conditioned stimulus (CS; such as, the taste or smell of the medication or the medication administration procedure), the CS alone can cause this physiological change (1). This principle is known as pharmacological conditioning. It has been proposed that physiological responses to a CS help organisms to adapt their state in preparation for an upcoming change and in this way maintain homeostasis (2). The principle of pharmacological conditioning has been demonstrated for various hormonal and immune parameters. For example, some evidence indicates that cortisol levels can be decreased by presenting participants with a distinctive drink previously coupled with a sumatriptan injection (3) and increased by giving a placebo injection that was previously coupled with dexamethasone (4). Evidence of the effects of pharmacological conditioning also exists for other hormones, such as growth hormone (4) and insulin (5), and for immune parameters, such as interleukin-2 (6), natural cell killer activity (7) and histamine (8).

Despite extensive research in the field of pharmacological conditioning, no studies so far investigated neural mechanisms underlying this phenomenon. From the pain conditioning literature, it is known that conditioned analgesia decreases brain activation in pain-sensitive brain regions, such as the thalamus, insula, and dorsal anterior cingulate cortex (9). It can be hypothesized that, similarly to the pain conditioning findings, pharmacological conditioned responses might trigger similar brain areas that are activated by the unconditioned stimulus (i.e., the medication).

In the present study we examined for the first time the neural underpinnings of pharmacological conditioning with oxytocin. Oxytocin is a peptide hormone produced in the hypothalamus and is found to have a wide range of effects on brain activity. Its receptors are densely situated in the hypothalamus, amygdala, olfactory bulbs, and cingulate cortex (10), areas that are also associated with maternal care, social attachment and emotional processing (11). It has been repeatedly demonstrated that exogenously administered oxytocin modifies brain responses to emotional (12, 13) and aversive visual stimuli (14), motivational tasks, involving trust (15), empathy (16), reward (17), and pain (18-20). Particularly,

oxytocin has been shown to reduce amygdala activation in response to aversive (14) and painful stimuli (20), which can be an underlying mechanism of stress reducing (21) and analgesic (22) effects of oxytocin described in previous research. Considering these positive physiological and psychological effects of oxytocin, pharmacological conditioning of oxytocin might have important clinical implications both in somatic and mental health. In this randomized placebo-controlled trial, we investigated the effects of oxytocin administration and conditioned oxytocin in comparison to a placebo-control group on brain activation in response to fMRI tasks that have previously shown to be affected by exogenous oxytocin administration: presentation of emotional faces (12), presentation of crying baby sounds (23) and thermal pain stimulation (20). We expected that the conditioned oxytocin group would demonstrate similar brain activation patterns as the group that received exogenous oxytocin.

Materials and Methods

Participants

This study is part of a study on the effects of pharmacological conditioning of oxytocin effects in which in total 99 healthy female volunteers were included (24). Of this initial sample, 88 participants took part in the MRI part of this study (11 participants did not continue with the last part due to health and planning reasons). Participants were randomly (based on a 1:1:1 ratio with a block randomization and a block size of 8) assigned to three groups: an oxytocin administration group (29 participants), a conditioned oxytocin group (29 participants), and a placebo control group (30 participants). Participants were screened for the following exclusion criteria: intake of analgesic and anti-inflammatory medication at the moment of the experiment, psychiatric, somatic, severe neurological or neurosurgical conditions that could interfere with the participant's safety or the study protocol, left-handedness, non-removable metal parts in the body, claustrophobia, (intended) pregnancy or breast feeding, and heavy use of alcohol or drugs. Only participants who used oral contraceptives during active use weeks were included in the trial to have a better control of menstrual cycle related hormonal changes (25). Participants were asked to refrain from drinking alcohol and doing intense physical exercise 24 hours before the sessions and drinking caffeinated

drinks two hours before the sessions. All participants gave informed consent to participate in the experiment and were debriefed and financially compensated afterwards.

Sample size

One participant was not able to perform the faces task due to a technical problem with the computer. The data of one participant from the conditioned group was excluded from the analysis due to excessive head motion (frame displacement > 1 mm on 50 slices) leaving data of 86 participants that were included in the analysis of the faces task (29 participants in the oxytocin administration group, 28 participants in the oxytocin conditioned group, 29 participants in the placebo group).

Due to a technical problem with the audio system, 5 participants were not able to perform the crying baby sounds task. Additionally, data of 5 participants were excluded due to excessive head motion (frame displacement > 1 mm on 75- 322 slices). Data of 78 participants in total were therefore included into the analysis of the crying baby sounds task (26 oxytocin administration group, 23 conditioned oxytocin group, 29 placebo group).

Due to technical problems with the thermode, 74 of 88 participants could take part in the pain task. Data of all of them were included in the analysis of the pain task (24 oxytocin administration group, 25 conditioned oxytocin, 25 placebo group).

Study design

The study had a single-blind design. Participants were randomly allocated to one of the three groups and did not know whether they received the oxytocin or the placebo spray. Researchers knew which participants were included in the oxytocin administration group (due to the absence of the CS in the evocation phase). However, researchers stayed blinded regarding the conditioned oxytocin and placebo groups. The trial was preregistered as a clinical trial on www.trialregister.nl (number NTR5596).

Procedures

The detailed procedures of the trial have been described elsewhere (24). Briefly, after an initial screening, participants were randomly allocated to an oxytocin administration group, a conditioned oxytocin group, or a placebo control group. In line with previous conditioning studies (26, 27), a two-phase conditioning

paradigm with an acquisition phase and an evocation phase was used. Both phases lasted for three consecutive days with a four-day break in between to allow wash-out of potential residual oxytocin effects from the previous phase. In the conditioned oxytocin group, the procedure was the following: in the acquisition phase, an association between a US (24 IU of oxytocin nasal spray) and a CS (a smell of rosewood oil) was established. Participants were administered the oxytocin spray which was immediately preceded and immediately followed by an odor of rosewood oil that was presented via a custom-made olfactometer (Wiff Online). In the evocation session, participants were administered a placebo spray paired with the same odor as in the acquisition phase. Participants in the placebo group underwent the same procedures but instead of the oxytocin spray they received a placebo spray during both phases. Participants in the oxytocin administration group received the oxytocin spray during both acquisition and evocation phases, however, they did not receive a CS during the evocation phase in order to avoid the occurrence of a conditioned response. The MRI experiment described in this study was performed on the third (last) evocation day in order to keep the conditioning context stable through the first experimental sessions.

On this third evocation day, upon arrival to the lab, participants were asked to provide a baseline saliva sample to measure their baseline oxytocin levels. Afterwards, placebo nasal spray with the odor of rosewood oil or oxytocin spray without the odor was administered, depending on group allocation. Five minutes after the spray administration, participants gave a second saliva sample and went to another lab (5-minutes walk) to participate in the MRI part of the experiment.

The MRI scanning started approximately 50 minutes after the spray administration. First, an anatomical scan was performed. This was followed by several functional scans in a fixed order: the emotional faces task, the crying sounds task and finally the pain task. In total, scanning lasted for around 50 minutes.

Faces task

Color photographs of males and females with four different emotional facial expressions (neutral, fearful, happy, and angry) from the Radboud Faces Database (28) were used. The pictures were grouped in blocks of 5 male and 5 female pictures of each particular valence. Each picture was presented for 1300

milliseconds with an interstimulus interval of 100 milliseconds and a block duration of 13.9 seconds. A total number of 16 blocks (4 blocks of each valence) were presented in randomized order with inter-block intervals of 10 seconds (total time of the stimulation: 7.56 minutes or 454 seconds). Stimulus presentation and response registration were controlled using E-Prime 2.0 software (Psychology Software Tools, Pittsburgh, PA).

During this task, participants were asked to focus on the screen and observe blocks of photos with faces. They were subsequently asked to rate the emotional arousal of each block on a scale from 1 (not arousing at all) to 4 (very arousing). The rating was done during the between block pause of 10 seconds. Participants could provide their ratings using button boxes placed on their thighs that were within easy reach.

Crying Baby Sounds task

The crying baby sounds task used in the current study was similar to the one that has been extensively described in previous studies (23, 29). The crying sounds were recorded from a 2-day old child. The control sounds were digitally created identical to the crying sounds in terms of duration, intensity, spectral content, and amplitude envelope but lacking an emotional meaning (29). The sounds were presented in 48 blocks (24 crying sounds and 24 control sounds) of 10 seconds with 6 seconds in between in randomized order. The order of the blocks was randomized within each participant. Participants were asked to focus on the sounds during the task.

Pain task

Pain stimuli were delivered with a standardized heat pain application device (fMRI-compatible ATS thermode attached to a Pathway device, Medoc Advanced Medical Systems, Ramat Yishai, Israel). The ATS thermode was applied to the dorsal site of the left arm of the participants when they were lying in the scanner. Before performing the functional scan, but within the scanner room, the temperature that elicited a medium pain intensity of 6 on a 0 to 10 numeric rating scale (0- no pain at all; 10- the worst pain imaginable) was identified for each participant. For this purpose, participants received a sequence of ascending temperatures with a peak temperature lasting for 5 seconds and an inter-stimulus interval of 15

seconds. Participants were asked to rate each stimulus using a 0 (no pain) -10 (worst pain imaginable) Numerical Rating Scale (NRS). The temperature that was rated as eliciting pain of 6 was used subsequently during the following functional MRI.

The pain task immediately followed the individual calibration phase and consisted of alternating 7 heat pain stimuli with a peak temperature lasting for 15 seconds, and 6 baseline stimuli of neutral to slightly warm (32 degrees Celsius) temperatures lasting between 13 and 17 seconds (on average 15 seconds) each. The purpose of the variable inter-stimulus times was to avoid anticipatory pain responses. Participants were instructed to focus on the sensation they experienced and were given the opportunity to stop the task at any moment by pressing an alarm bell. No participants pressed the alarm bell during the experiment.

Image acquisition

The MRI data were acquired on a Philips 3T MR-system (Best, The Netherlands) in the Leiden University Medical Centre. First, a T1 weighted high resolution anatomical scan was acquired (repetition time (TR) = 9.8 milliseconds, echo time (TE) = 4.6 milliseconds, flip angle = 8°; voxel size 0.875 x 0.875 x 1.2; 140 slices). Functional data were acquired with echoplanar images (EPI) using a T2*-weighted gradient echo sequence (TR = 2200 milliseconds; TE = 30 milliseconds; flip angle = 80°; voxel size 2.75 × 2.75 × 2.75 millimetres +10% slice gap, 38 transverse slices). Images were scanned parallel to the anterior–posterior commissure plane.

Statistical analysis of demographic and psychological variables

Group differences in age, BMI, and baseline oxytocin salivary levels from three evocation days were examined using one-way analysis of variance (ANOVA). As determined prior to the study, to check whether there was a significant conditioned oxytocin release, the oxytocin salivary after the spray administration on were compared between the conditioned oxytocin and the placebo groups with analysis of covariance (ANCOVA) in which baseline oxytocin levels served as a covariate for each evocation day separately. Next, the oxytocin administration group was added to the analysis and the three groups were compared on salivary oxytocin levels after the spray administration with analysis of covariance in which

baseline oxytocin levels served as a covariate. Finally, an ANOVA was used to compare the groups on the temperatures that elicited a pain of 6 and were used during the Pain task.

Image preprocessing and analyses

The data were pre-processed and analysed with FSL software Version 5.0.10 (FMRIB's Software Library, www.fmrib.ox.ac.uk/fsl (30)). Brain extraction from the anatomical scans was done using the Brain Extraction Tool as implemented in FSL (31). Motion correction of functional scans was done using MCFLIRT (32). Spatial smoothing was applied using a Gaussian kernel of full-width-at-half-maximum = 5 mm. High-pass temporal filtering was applied to the data (for faces task with high pass filter cut-off = 60 seconds; for crying baby sounds task filter = 50 seconds; for pain task filter = 90 seconds). Functional scans were registered to T1 weighted images, using Boundary-Based Registration, and then registered to a MNI-152 standard space image (Montreal Neurological Institute, Montreal, QC, Canada) using non-linear registration with a warp resolution of 10 millimetres.

The analysis consisted of three levels. The first level analysis was performed in the native space using general linear models. Blocks (for the faces task: angry, happy, fearful, neutral; for the crying baby sounds task: crying sounds, control sounds; for the pain task: painful stimuli) were used as predictors and convolved with a double-gamma hemodynamic response function and its temporal derivatives.

Regression coefficients were estimated in FSL. For the faces task, six contrasts were estimated: angry >/< neutral, happy >/< neutral, fearful >/< neutral. For the crying baby sounds task, two contrasts were estimated: cry >/< control. For the pain task, two contrasts were estimated: pain >/< control. These first level contrasts were submitted to the second level analysis that was separately run per group. Within the second level analysis, the effects of the tasks on the brain activation were investigated in each group separately. Finally, to investigate the differences between the three groups, the third level analysis was performed. The three groups were compared with each other using analysis of variance on the contrasts. The Randomise tool of FSL was used to perform voxel-wise permutation-based non-parametric testing and generate statistical inference for the analysis of variance. 5000 permutations per contrast were done.

The statistical tests were corrected for multiple comparisons with the threshold-free cluster enhancement (TFCE). A TFCE corrected statistical threshold of $p < 0.05$ was chosen.

For exploratory purposes, these analyses were first performed on the whole brain (as in Domes, Lischke (33) and Riem, Bakermans-Kranenburg (29)). Second, a region of interest analysis was run. The regions of interest were chosen based on previous literature. Based on a recent meta-analysis on the effects of oxytocin on the brain activity in response to emotion processing tasks (34), the following regions of interests were chosen for the faces task: the bilateral amygdala, the bilateral insula, the bilateral occipital fusiform gyrus and bilateral superior temporal gyrus. For the crying baby sounds task, the bilateral amygdala, bilateral insula and the bilateral inferior frontal gyrus pars triangularis were chosen as regions of interest (29). For the pain task, left and right amygdala separately were chosen as areas of interest (20). The masks for the regions of interest were taken from the Harvard-Oxford Cortical and Subcortical Structural Atlases (<https://fsl.fmrib.ox.ac.uk/fsl/fslwiki/data/atlas-descriptions.html>).

Results

Baseline characteristics

There were no significant differences between the three groups on age ($F(2, 87) = 0.495$, $p = 0.611$) and body mass index ($F(2, 87) = 1.083$, $p = 0.343$); the average age across the groups was 21.5 years ($SD = 2.4$) and mean BMI was 22.38 ($SD = 2.4$).

Salivary oxytocin levels

The salivary levels of the whole sample (99 participants) during all experimental days are presented elsewhere (24). Here we present the data of the sample included in the MRI part of the experiment. The mean salivary oxytocin levels across the groups and the measurements are presented in Table 1. Due to clogging of the saliva samples, 48 samples could not be analysed while 832 samples were included in the analysis. There were no significant differences between the three groups on their baseline salivary oxytocin levels on evocation day 1 ($F(2, 83) = 1.47$, $p = 0.234$), 2 ($F(2, 84) = 0.37$, $p = 0.964$) and 3 ($F(2, 83) = 0.53$, $p = 0.588$). There was a significant difference between the conditioned oxytocin and placebo groups in the levels of oxytocin after the CS administration on the evocation day1: the

conditioned oxytocin group had higher salivary oxytocin levels in comparison to the placebo group ($F(1, 55) = 5.98, p = 0.02$) after controlling for the baseline levels. No differences between conditioned oxytocin and placebo groups was found on the salivary oxytocin level on the evocation day 2 ($F(1, 55) = 1.84, p = 0.18$) and evocation day 3 ($F(1, 55) = 1, p = 0.32$). When the oxytocin administration group was added to the analysis, a significant main effect of group was found on evocation day 1 ($F(2, 79) = 14.92, p < 0.001$), evocation day 2 ($F(2, 79) = 15.29, p < 0.001$) and evocation day 3 ($F(2, 80) = 11.68, p < 0.001$). Post hoc Bonferroni comparison demonstrated that the oxytocin administration group had significantly higher salivary oxytocin levels than the placebo and the conditioned oxytocin group after controlling for the baseline levels during all evocation days (all p 's $< .001$).

Table 1. Mean salivary oxytocin levels (pg/ml) across the groups and measurement moments.

Evocation day	Measurement	Placebo group	Oxytocin administration group	Conditioned oxytocin group
Evocation day 1	Baseline	16.94 (19.64)	12.21 (7.05)	11.68 (8.97)
	+ 5 minutes	14.85 (7.65)	1912.79 (2429.66)	28.34 (44.63)
	+ 20 minutes	13.77 (8.47)	1020.81 (1860.49)	20.17 (28.76)
	+ 50 minutes	10.18 (5.1)	848.15 (1569.4)	21.17 (25.99)
Evocation day 2	Baseline	13.23 (8)	13.01 (9.32)	13.24 (7.12)
	+ 5 minutes	13.97 (6.6)	1727.86 (2272.05)	30.89 (70)
	+ 20 minutes	13.14 (5.62)	1102.87 (1650.52)	17.36 (15.37)

	+ 50 minutes	11.08 (5.66)	826.88 (1666.11)	10.14 (7.55)
Evocation day 3	Baseline	16.85 (21.56)	14.84 (13.6)	12.14 (12.91)
	+ 5 minutes	12.19 (7.49)	1719.83 (2639.64)	61.23 (43.93)

Faces task

First, to examine the effects of the emotional faces stimuli on brain activation, we looked at the second level analysis, with a specific focus on the placebo group to see the effects of the task on the brain activity regardless of the effects of oxytocin. The results of the second level analysis are presented in Table 2 (for all three groups separately). Full brain analysis in the placebo group (first column in Table 2) revealed a significant activation in two clusters in the right and left occipital fusiform gyrus, one cluster in the right superior temporal gyrus and one cluster in the right inferior temporal gyrus for the contrast fearful > neutral faces. The ROI analysis additionally showed clusters in the right amygdala, the right insula, the bilateral occipital fusiform gyrus and the bilateral superior temporal gyrus that were significantly active on the contrast fearful > neutral faces. Additionally, heightened activation in the left occipital fusiform gyrus was found on the contrast happy > neutral faces in the ROI analysis.

In the third level brain analysis we compared the three groups with each other. The whole brain analysis of variance with all three groups (oxytocin administration, conditioned oxytocin and placebo) demonstrated no significant differences in any of the three contrasts. The ROI analysis revealed that there was significantly higher activation in the right amygdala in the placebo group in comparison to the oxytocin administration group on the contrast fearful > neutral. To explore this result in detail, we plotted z-values and standard deviations of the three groups for this significant cluster (Figure 1). The z-values of the conditioned oxytocin group were in between the values of the oxytocin administration and placebo groups but did not significantly differ from either of these groups. Additionally, ROI analysis yielded a significant difference between placebo and oxytocin administration group in the left superior temporal

gyrus: the activation in the oxytocin administration group was significantly lower than in the placebo group on the contrast fearful > neutral (Figure 2). The Z-values of the conditioned oxytocin were, again, in between the values of the oxytocin administration and placebo groups but did not significantly differ from either of these groups. No significant activation was found in other regions of interest.

Crying baby sounds task

The results of the second level analysis of the Sounds task are presented in Table 3. First, we again explored the effects of the sounds on the brain activation in the placebo group alone (first column of Table 3). The whole brain analysis showed higher activation in one large cluster in the right superior temporal gyrus with extension to the planum polare and one cluster in the left planum polare on the cry > control sounds contrasts. Subsequent ROI analysis showed that the cry > control sounds contrast caused significant activation in the right and left amygdala, the right and left insula and the left inferior frontal gyrus pars triangularis. The third level analysis with the comparison between the three groups revealed neither significant differences in the whole brain level nor in the ROI analyses.

Pain task

The results of the second level analysis of the Pain task are presented in the Table 4. The effects of pain stimulation on brain activity were again first examined in the placebo group alone (first column of Table 4). The whole brain analysis revealed significant activation in 12 clusters across the brain on the contrast pain > control and in 1 cluster on the contrast control > pain (for the details see Table 4). Significantly increased activation in both the left and right amygdala were found on the ROI analysis on the contrast pain > control. The third level analysis, comparing the three groups, revealed no significant effect of oxytocin administration or conditioning with oxytocin on brain activity neither in the whole brain analysis nor in the ROI analysis with left and right amygdala.

Finally, there were no significant differences between the three groups on the temperature that was used during the Pain task (oxytocin administration group: $M = 46.2$, $SD = 1.1$; conditioned oxytocin group: $M = 46.4$, $SD = 1.2$; placebo group: $M = 45.3$, $SD = 1.6$; $F(2, 73) = 0.537$, $p = 0.587$).

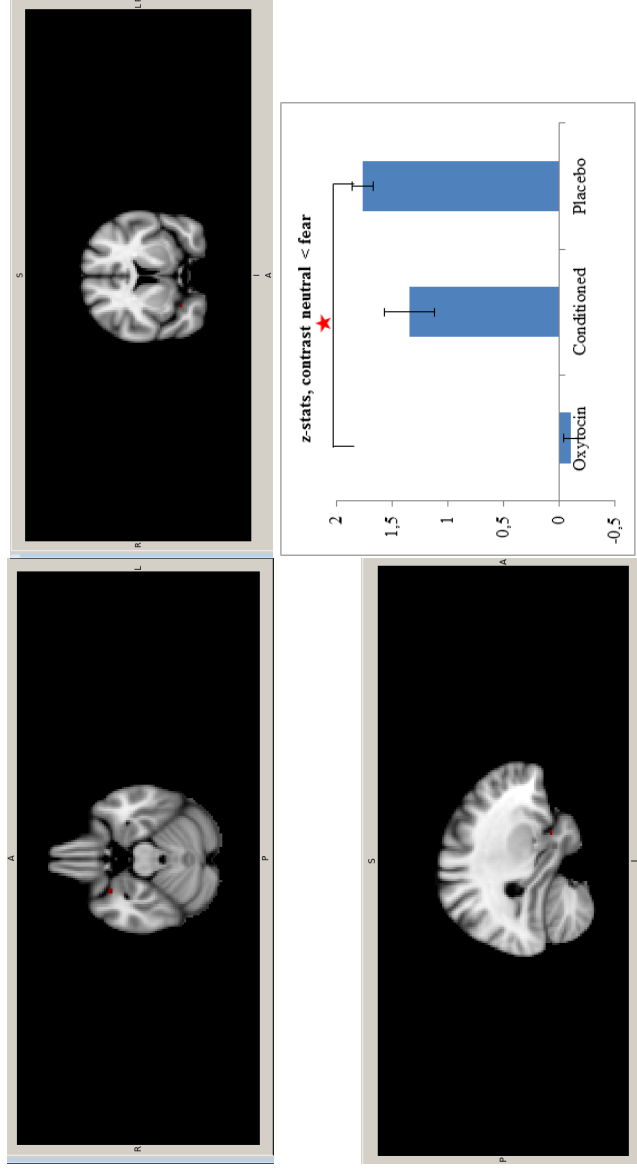


Figure 1. Cluster (28, 8, -24; t max = 3.81, cluster size = 4) with the significantly lower activation in the oxytocin group in comparison to the placebo group on the contrast neutral < fear and Z statistics with standard deviations from this cluster.

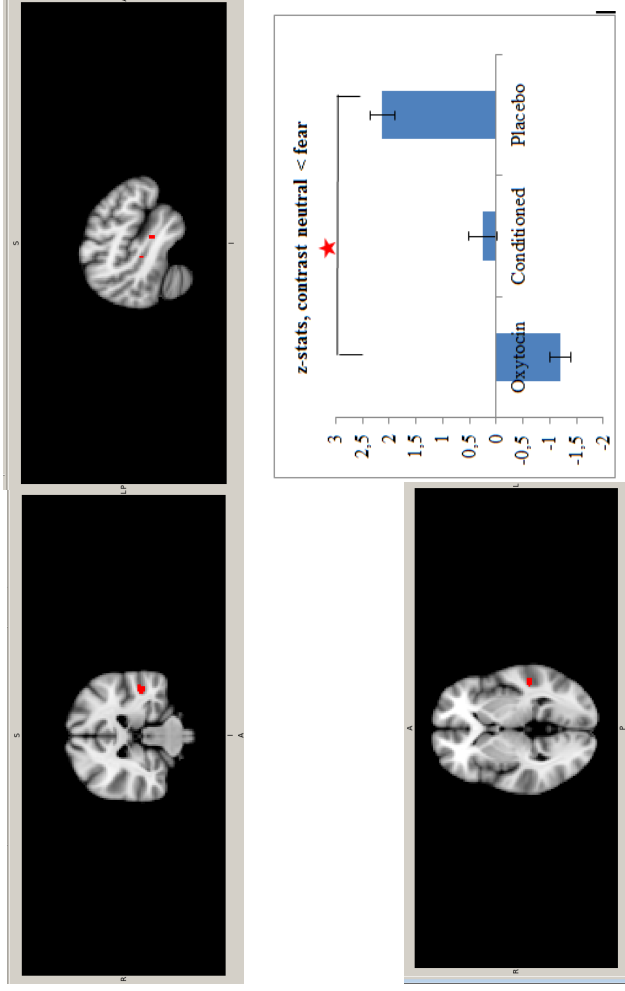


Figure 2. Clusters in the left superior temporal gyrus (cluster 1: -50, -32, 0; t max = 4.23, cluster size = 41; cluster 2: -46, -10, -12; t max = 4.84; cluster size = 14) with the significantly lower activation in the oxytocin group in comparison to the placebo group on the contrast neutral < fear and mean Z statistics with standard deviations from these clusters.

<i>WB</i> left occipital pole	5	4.71	0	-92	-6
<i>WB</i> left occipital pole superior deviation	50	5.61	-16	-98	4
<i>WB</i> right occipital pole	34	5.27	12	-102	6
	16	6.41	16	-102	-2
<i>ROI</i> left ofg extending to occipital pole	746	4.91	-6	-92	-10
3.neutral < fear					
<i>WB</i> bilateral OFG	4573	8.18	22	-90	-6
<i>WB</i> left OFG extending to	591	6.01	-28	-86	-12
	16	4.79	-36	-84	-10

lateral occipital cortex						200	5.38	-22	-80	-10
<i>WB</i> right OFG extending to lateral occipital cortex	232	5.15	28	-88	-10	376	6.25	22	-88	-10
<i>WB</i> right stg posterior division	123	4.83	46	-36	4					
<i>WB</i> right inferior temporal gyrus, temporooccipital part	24	4.29	46	-52	-8					
<i>ROI</i> right amygdala	16	3.39	28	6	-22					

Table 3. Effects of the sound valence across the groups (second level analysis)

<i>ROI</i> left insula	310	7.64	-50	-2	-2	94	6.26	-46	-8	0
<i>ROI</i> right insula	300	7.56	50	4	-4	69	6.15	50	-4	-4
<i>ROI</i> left IFG, pars triangularis	56	3.75	44	32	4					

Note: WF- results obtained with the whole brain analysis; ROI- results obtained with the region of interest analysis; STG- superior temporal gyrus; IFG = inferior frontal gyrus. Reported activations are corrected for multiple comparisons with the threshold-free cluster enhancement. Coordinates are reported using the Montreal Neurologic Institute space.

Table 4. Effects of the pain stimulation across the groups (second level analysis)

	Placebo group (n = 25)					Oxytocin group (n = 23)					Conditioned group (n = 25)				
	Cluster size	T max	X (mm)	Y (mm)	Z (mm)	Cluster size	T max	X (mm)	Y (mm)	Z (mm)	Cluster size	T max	X (mm)	Y (mm)	Z (mm)
pain>control															
<i>WB</i> right postcentral gyrus with extension to precentral gyrus	10776	6.82	48	-26	64	455	6.36	48	-20	58	21	4.81	60	-4	28
<i>WB</i> left temporal occipital fusiform cortex	1786	5.23	-28	-38	-24										
<i>WB</i> left temporal occipital fusiform cortex with extension to						27763	8.18	-26	-46	-10					

left occipital fusiform gyrus						
<i>W/B</i> right parahippocampal gyrus with extension to temporal fusiform cortex	623	6.77	26	-24	-24	32709 7.79 22 -22 -18
<i>W/B</i> bilateral occipital pole	248	5.19	0	-88	40	
<i>W/B</i> right lateral occipital cortex	217	4.89	44	-68	26	
<i>W/B</i> bilateral precuneous cortex	162	4.15	10	-60	14	

<i>WB</i> left thalamus	118	4.8	-14	-32	10				
<i>WB</i> left middle temporal gyrus	87	4.35	-68	-8	-10	25	4.01	-52	-12 -18
<i>WB</i> brain stem	81	4.68	-2	-26	-22				
<i>WB</i> angular gyrus	33	3.45	48	-54	30				
<i>WB</i> right cingulate gyrus	21	3.57	8	-50	6				
<i>WB</i> bilateral cuneal cortex	15	3.26	-2	-84	36				
<i>WB</i> frontal pole with extension to						1212	5.54	0	66 4

right paracingulate gyrus									
<i>W/B</i> left superior frontal gyrus	278	5.12	-24	22	42				
<i>ROI</i> left amygdala	18	5.03	-20	-16	-18				
<i>ROI</i> right amygdala	173	4.17	24	2	-26				
pain < control									
<i>W/B</i> right precentral gyrus	837	6.52	46	10	26				

<i>W/B</i> left precentral gyrus	23	5.55	-58	6	4				
<i>W/B</i> right frontal operculum cortex to insula and central opercular cortex	1484	11	48	18	0				
<i>W/B</i> right central opercular cortex						121	5.79	52	0 8
<i>W/B</i> right frontal pole	68	5.53	40	38	8				
<i>W/B</i> right insular cortex						422	6.95	34	12 10

Note: WF- results obtained with the whole brain analysis; ROI- results obtained with the region of interest analysis. Reported activations are corrected for multiple comparisons with the threshold-free cluster enhancement. Coordinates are reported using the Montreal Neurologic Institute space.

Discussion

This is the first study that investigated the effects of pharmacological conditioning with oxytocin on brain activity. We hypothesized that conditioned oxytocin responses would demonstrate patterns of brain activation similar to exogenous oxytocin administration and that both these groups would differ from the placebo group. Differences in the brain activation between the oxytocin administration and placebo groups were demonstrated in the right amygdala and in two clusters in superior temporal gyrus for the emotional faces task only, while brain activation of the conditioned oxytocin group was in between that of the oxytocin administration and the placebo group but did not significantly differ from either group.

The amygdala has been shown to play a role in negative emotion processing (35) and its activation has been found in response to threat (36). Previous research has demonstrated that presentation of faces with fear expression increases amygdala activation and 24 IU oxytocin has been repeatedly shown to dampen this effect (12, 13, 37). We replicated these previous findings and furthermore showed that there is a careful indication that conditioning with oxytocin might slightly affect this activity pattern as well but to a lesser extent than exogenous oxytocin, however, since no significant differences between the conditioned group and other two groups was found, this finding should be interpreted cautiously.

Moreover, the same fear > neutral contrast yielded a significant difference between the oxytocin administration and placebo group in two clusters of the superior temporal gyrus (STG). The STG plays an important role in the processing of emotional stimuli and social cognition (38) and particularly processing of fearful faces (12, 39). With this finding we thus replicated previous results showing increased STG activity in response to the presentation of the fearful faces. However, the direction of this oxytocin effect does not correspond to most previous studies. Several previous studies showed that oxytocin enhanced STG activity in response to emotional stimuli (34, 40). However, in our study we found that participants in the oxytocin group had lower activation in the STG on the contrast fear > neutral in comparison to the placebo group. In line with our findings, some other studies also found such effects of oxytocin on STG activation. For example, a decrease in STG activity after oxytocin administration was found in response to social rejection (41). The findings on the directionality of STG brain activity to oxytocin are thus mixed in

the current literature. In our study, we found an increase in STG in response to fearful faces in the placebo condition and this increase was dampened by oxytocin in the oxytocin condition, corresponding to our findings in the amygdala. Again, in the STG the activity of the conditioned oxytocin group was in between the oxytocin and placebo groups but did not significantly differ from both groups. This finding is indicative of a smaller response of the conditioned group in comparison to the effect of oxytocin administration however this should be interpreted with caution again as the conditioned group did not significantly differ from the other groups.

The sounds of a crying baby activated the auditory cortex in all groups and the amygdala and the inferior frontal gyrus, pars triangularis in the placebo group, as was expected (29). Even though significant activation by the cry > control contrast was found in the amygdala and inferior frontal gyrus pars triangularis in the placebo group and not in the oxytocin administration and conditioned oxytocin groups on the second level analyses, the between-group comparison in the third level analysis did not reach significance. A previous study (29) found that oxytocin reduced activation in the amygdala and increased activation in the insula and the inferior frontal gyrus pars triangularis on the contrast cry > control. We could not replicate these results. Speculatively, this could be due to design differences, as Riem and colleagues (29) included twins in their sample, and performed the task 45 minutes after the oxytocin administration while our task was done approximately 60 minutes after the spray (as it followed the faces task).

The pain task activated large clusters across the brain, including primary and secondary somatosensory cortex, thalamus, cingulate gyrus and amygdala, the areas that have been repeatedly shown to be activated by acute pain (42, 43). A few studies showed that oxytocin dampens amygdala activation in response to experimentally induced pain (18, 20). Indeed, the increased activation on the contrast pain > control in the left and right amygdala was found only in the placebo group and not in the conditioned and oxytocin administration groups in the second level analyses, however, again, the between-group comparison was not significant. At this moment we do not have enough evidence to make definite conclusions about the role of oxytocin in the brain responses to pain. Singer and colleagues (18) proposed that the effects they

found were driven by selfish participants: effects of oxytocin on the amygdala activation were found only in selfish, but not prosocial participants. Moreover, Zunhammer and colleagues (19) did not find effects of oxytocin on the brain activity in response to heat pain. Speculatively, oxytocin might influence emotional aspects of pain perception that have not been captured by our study.

The results of both the crying baby sounds and pain task showed that the second level analyses are partially in line with the previous literature as heightened amygdala activation in response to the crying sounds and pain stimulation was found in the placebo group but not in the oxytocin administration group. However, the effects were not strong enough to be seen in the between-group comparisons. One possible explanation for this lack of significance can be the timing of the experiment. We conducted the MRI scanning on the third evocation day to avoid interference with the conditioning procedure, because it has been previously shown that presenting a distinctive additional stimulus during the conditioning might inhibit the conditioned response (44) and the whole MRI environment can be perceived to be stressful and distracting. On the third evocation day, the conditioned response in saliva had already been extinguished even though it was found on the first evocation day (24). Since salivary oxytocin levels might be not be the only indicator of the conditioned response, we still hypothesized that we could observe the conditioned response in the brain. Speculatively, if the fMRI experiment was done on the first evocation day, a stronger response in the brain might have been found. Future studies are needed to confirm this hypothesis. Moreover, the fMRI scan started approximately 50 minutes after the oxytocin and placebo administration. This time frame was chosen because the neuronal effects of exogenous oxytocin administration have been demonstrated to be the strongest around this time (45). However, the conditioned response does not necessarily correspond to the timing of the effects of exogenous oxytocin administration. The only study on endocrine conditioning that investigated the conditioned response temporally was a study on conditioned insulin release (5) which found that the conditioned insulin release appeared around 40 minutes after the first placebo administration. However, insulin and oxytocin are distinct systems and the results from insulin conditioning cannot necessarily be directly generalized to the timing of oxytocin conditioning. As the highest conditioned oxytocin levels in saliva were found

immediately after the conditioned stimulus administration on the first evocation day (24), the strongest response in the brain might possibly also immediately follow the conditioned stimulus administration and may already have decreased 50 minutes later. The fact that the only difference between the groups was found on the first task and not on the subsequent tasks supports this speculation to some extent. For future studies, it is advised that the effects of oxytocin conditioning are studied on the first evocation day, and possibly immediately following the placebo administration with repeated measurements across time to find the peak of the conditioned response.

Changing hormonal levels with a behavioral manipulation can have important clinical implications especially for disorders related to dysfunction of the endocrine system. For example, it has been demonstrated that immunosuppressive treatment for renal transplant patients can be enhanced by using classically conditioned immunosuppression (26). Nevertheless, classically conditioned endocrine responses have not been investigated in clinical practice. The possibility to induce classically conditioned insulin release as demonstrated by Stockhorst and colleagues (5), might for example be applied for improving therapies for patients with diabetes type-2 who suffer from dysfunctional insulin release.

Classical conditioning of oxytocin responses could be tested in populations with mental disorders related to emotional deficits, such as autism, schizophrenia and borderline personality disorder as oxytocin has been shown to have promising effects for treatments of these disorders (46-48). Knowing what brain areas are involved in endocrine conditioning might be helpful for making the effects of the endocrine conditioning stronger and manipulating these effects more optimally.

To conclude, our study was the first study investigating the effects of classical conditioning with oxytocin on brain activity. We have found preliminary indications that the conditioned oxytocin response might affect the activity in the left STG and right amygdala in a direction similar to exogenous oxytocin, however future studies are needed to confirm this hypothesis as no significant group difference between conditioned and other two groups was found in the current study. Moreover, these effects remained small and were not generalizable across the tasks. Future research should explore different time frames of the conditioned oxytocin brain responses and extend this paradigm to the conditioning of other hormones as

comparisons to oxytocin conditioning. Unraveling the neural mechanisms of endocrine conditioning might help us to implement this potentially beneficial mechanism in clinical practice.

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Part II

Influencing Placebo Effects with Oxytocin

Chapter 5

Enhancing placebo effects in somatic symptoms through oxytocin

Published as

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Abstract

Objective: Placebo effects relieve various somatic symptoms but it is unclear how they can be enhanced in order to maximize positive treatment outcomes. Oxytocin administration may potentially enhance placebo effects but few studies have been performed with conflicting findings. The study aim was to investigate the influence of positive verbal suggestions and oxytocin on treatment expectations and placebo effects for pain and itch.

Methods: 108 Female participants were allocated to one of four groups: 1) oxytocin with positive verbal suggestions, 2) placebo with positive verbal suggestions, 3) oxytocin without suggestions, and 4) placebo without suggestions. The administration of 24 IU oxytocin or a placebo spray was preceded by positive verbal suggestions regarding the pain- and itch-relieving properties of the spray or no suggestions, depending on group allocation. Pain was assessed with a cold pressor test and itch was assessed with histamine iontophoresis.

Results: Positive verbal suggestions induced expectations of lower pain ($F= 4.77, p= .031$) and itch ($F= 5.38, p= .022$). Moreover, positive verbal suggestions elicited placebo analgesia ($F= 5.48, p= .021$), but did not decrease itch. No effect of oxytocin on the placebo effect or on expectations was found.

Conclusions: Positive suggestions induced placebo analgesia but oxytocin did not enhance the placebo effect. Study limitations are that we only included a female sample and a failure induce placebo effect for itch. Future studies should focus on how oxytocin might influence placebo effects, taken into account the role of gender, dose-dependent effects and various expectation manipulations.

Trial registration: The study was registered as a clinical trial on www.trialregister.nl (number 6376).

Key words: placebo effect, oxytocin, pain, itch

Abbreviations: CPT = cold pressor test; HI = histamine iontophoresis; NRS = numerical rating scale

Introduction

The placebo effect, which represents a positive outcome after receiving an inert treatment, has been repeatedly shown to relieve a variety of symptoms or conditions such as pain, depression, anxiety, addiction, and Parkinson's disease amongst others (1-4). An important mechanism that has been suggested to underlie placebo effects is that of positive outcome expectations that are linked to better treatment outcomes (2,5,6,7).

In experimental or clinical settings, positive outcome expectations can be induced by verbal suggestions regarding the positive effects of a treatment. Placebo effects induced by verbal suggestions of positive outcome expectations have been extensively investigated in the context of placebo analgesia (1,8).

Numerous studies demonstrated that mere positive suggestions about the analgesic properties of a placebo treatment may result in decreased pain (9-11). Placebo effects of positive verbal suggestions in the context of itch, a symptom closely related to pain (12), have been less studied. Initial studies have shown that verbal suggestions can increase itch, indicative of a nocebo effect (13), whereas verbal suggestions combined with conditioning, another important proposed mechanism underlying placebo effects, can reduce itch, indicative of placebo effects in itch (14). Inconclusive findings have been reported on the effects of verbal suggestions alone in inducing placebo effects for itch. Bartels and colleagues (13) and Van Laarhoven and colleagues (12) found no decrease in itch after giving participants positive verbal suggestions. However, Darragh and colleagues (15) used specific verbal suggestions accompanied with a video about the itch-relieving properties of a placebo cream and demonstrated a decrease in subjective ratings of itch induced by histamine iontophoresis.

Despite an increasing body of literature on placebo effects induced by positive verbal suggestions, it is currently not yet clear what exact neurobiological mechanism drives the possible effects of verbal suggestions. Moreover, it is currently unknown how placebo effects can be enhanced in order to obtain the best therapeutic results.

Enck and Klosterhalfen (16) proposed that oxytocin, a peptide hormone produced in the hypothalamus, might be a facilitator of the placebo effect. Oxytocin is a well-studied hormone that has many positive behavioral effects. Among others, it increases trust (17), empathy and social learning (16-18). In addition, oxytocin has anxiolytic effects (18-20) and it stimulates the secretion of nitric oxide that has been linked to the relaxation response (21). Oxytocin might therefore maximize positive treatment expectations induced by verbal suggestions or lead to better treatment outcomes by inducing a generally more positive affective state. Few studies have investigated the effect of oxytocin on placebo analgesia, with mixed results. Kessner and colleagues (22) induced placebo analgesia in male participants by giving positive suggestions regarding a placebo cream. Participants who received a 40 IU dose of oxytocin spray, demonstrated a higher placebo response to the cream than participants who received a placebo spray. Colloca and colleagues (23) induced placebo analgesia in both female and male participants by giving positive suggestions about pain-relieving effects of a sham electrode. Contrary to Kessner and colleagues (22), they found no effect of 24 IU of oxytocin on placebo analgesia. They however demonstrated that vasopressin, a peptide hormone closely related to oxytocin, enhanced placebo analgesia, but only in women and not in men. In addition to these findings, a few studies have demonstrated that oxytocin itself can influence pain symptoms (24-27), however, this evidence is inconclusive because other studies (22, 23, 28, 29, 30) found no pain-relieving effects of oxytocin. Considering these mixed results, the question remains whether oxytocin can potentially enhance placebo analgesia. Furthermore, no studies to date have examined the effect of oxytocin on placebo effects for other somatic symptoms, such as itch. In the present study we investigated whether positive verbal suggestions would induce placebo effects for somatic symptoms of pain and itch and whether oxytocin administration would increase these placebo effects in healthy women. Firstly, we hypothesized that participants who receive positive verbal suggestions would expect and experience less pain and itch in response to validated pain (cold pressor test, CPT) and itch (histamine iontophoresis, HI) tests than participants who received no suggestions. Secondly, we expected that oxytocin would boost the placebo effect induced by positive verbal suggestions and participants who receive oxytocin in combination with suggestions would expect and

experience less pain and itch than participants who receive only suggestions or only oxytocin. Similar effects for both pain and itch were expected. We also explored whether oxytocin in combination with verbal suggestions had an effect on the pain unpleasantness after the CPT, skin redness and wheal size, and skin temperature in response to HI. We further studied whether oxytocin reduced pain and itch sensitivity. Questionnaires were administered to control for potential group differences in psychological characteristics such as anxiety, mood, neuroticism, optimism and extraversion.

Methods

Study design

The study design was a randomized controlled trial. It was approved by the Medical Ethical Committee of the Leiden University Medical Center (number NL55922.058.15). The randomization was performed by the Clinical Pharmacy of the Leiden University Medical Center. After the screening, participants were randomly allocated to one of the following groups: 1) oxytocin with positive verbal suggestions, 2) placebo with positive verbal suggestions, 3) oxytocin without verbal suggestions, and 4) placebo without verbal suggestions. Both participants and experimenters were blind regarding the drug condition. One of the two experimenters was aware of the verbal suggestion condition due to the positive verbal suggestions instructions that were given by this experimenter, however, the second experimenter who performed the pain and itch tests was blind regarding allocation of the condition as to avoid influencing the results of the tests.

Participants

Female participants were eligible to participate when they were healthy and between 18 and 35 years old. The exclusion criteria consisted of: current use of medication, skin conditions such as eczema, current diagnosis of a mental disorder, Raynaud's syndrome, acute or chronic pain complaints, and (intended) pregnancy. Prior to the experiment, participants were screened for these exclusion criteria via an online questionnaire in Qualtrics (Provo, UT). Participants were asked to refrain from taking analgesic medication, drinking alcohol and doing intense physical exercise up to 24 hours before the experiment and drinking caffeine drinks up to 2 hours before the experiment. At the start of the experiment, participants

signed an informed consent form and after completion of the experiment they received a monetary compensation.

Procedure

The data was collected between April, 2016 and April, 2017. The experimental timeline is presented in Figure 1.

The experiment was conducted by a male and a female experimenter. A male experimenter explained the procedure and gave the verbal suggestions, while a female experimenter performed the CPT and HI. Fixed genders of the experimenters were chosen to minimize the possible influence of the gender of the lab personnel on the pain and itch test outcomes. On the test day, the participant was asked to fill in several baseline questionnaires on a computer. Subsequently, the baseline CPT took place. After the test, positive suggestions were given to participants in the two positive suggestions groups and participants received oxytocin or placebo nasal spray depending on group allocation. During a waiting period of 30 minutes allowing oxytocin to reach peak concentration (31), participants filled in some questionnaires and were offered magazines with neutral content to read. After the waiting period, the HI was performed; no baseline HI was performed in order to avoid habituation effects that appear during second HI administration (12). In order to minimize possible crossover effects of the HI on pain sensitivity, an additional waiting period of 10 minutes was completed. After that the post-intervention CPT took place. At the end of the session, participants were asked about their perceived group allocation, debriefed, and paid for their participation in the study.

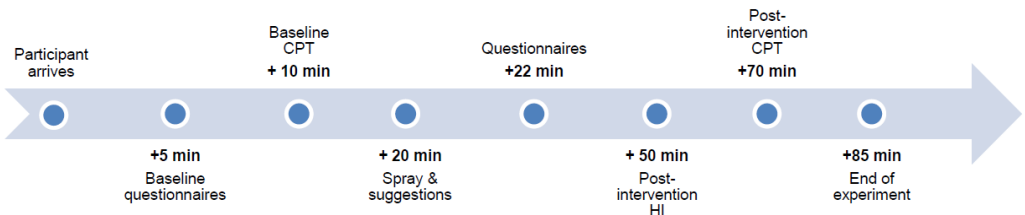


Figure 1. Experimental timeline.

Experimental interventions

Expectation induction. Positive expectations about the nasal spray were induced in the two groups with positive verbal suggestions. Participants received the following verbal suggestions: “Now you will receive a nasal spray. This spray contains oxytocin. It has been demonstrated in previous studies that oxytocin decreases pain and itch sensitivity. We expect that after receiving oxytocin you will also experience less pain during the cold pressor test and less itch during the histamine test”. Participants in the groups without suggestions were told the following: “Now you will receive a spray. This spray contains either oxytocin or placebo”.

Drug administration. Depending on the group allocation, participants received 24 IU of oxytocin (Syntocinon) or placebo nasal spray. Placebo spray looked and tasted identically to oxytocin and was prepared by the Clinical Pharmacy of the Leiden University Medical Center. The nasal spray was administered by the experimenter with two puffs (one puff per nostril) using a MAD Nasal mucosal atomization device (Teleflex, Inc., Research Triangle Park).

Materials and Questionnaires

Cold Pressor Test

Pain sensitivity was assessed by the CPT. The waterbath consisted of a 2.7 liter styrofoam tank with cold water, which was maintained at a fixed temperature of 4°C. Participants were asked to hold their dominant hand in the water for 1 minute (32). During this minute, every 15 seconds their pain levels were assessed on a numerical rating scale (NRS) with a question “How much pain do you have now?”. The participant gave an answer on a 0-10 scale (0 = no pain at all, 10 = worst pain ever experienced). In case participants were unable to hold their hand in the water for the entire minute, they could stop the test at any time. The pain intensity scores in response to the baseline and post-intervention CPT were calculated as the mean scores of the four pain rating measurement points during each CPT. Before the test participants were asked if they experienced any pain and itch and the test was performed only in case of the absence of both. After the test, participants were asked to indicate how unpleasant the test was on a 0-10 NRS scale (0 = not unpleasant at all, 10 = very unpleasant).

Histamine iontophoresis

Itch was induced by means of HI (12). Histamine dihydrochloride (0.5%) was dissolved in a gel of methylcellulose and propylene glycol in distilled water (manufactured by the Clinical Pharmacy, Leiden University Medical Center) and 2.5 ml was placed in a disposable iontophoresis electrode (IOGEL medium, Chattanooga, Hixson, USA). The electrode was placed on the non-dominant forearm, 2 cm distal to the lateral epicondyle of the humerus. The reference electrode was applied to the skin on the lateral side of the triceps brachial muscle. The histamine solution was delivered with a dose controller (Chattanooga ionto, Chattanooga Group, Hixson, USA) for 2.5 minutes at a current level of 0.4 mA. Itch was measured 6 times during 2.5 minutes (immediately, and after 30, 60, 90, 120 and 150 seconds from the beginning of the test) by asking the participants the following question: “How much itch do you have now?”. The participant gave an answer on an NRS scale ranging from 0-10 (0 = no itch at all, 10 = worst itch ever experienced). The itch intensity score was calculated as a mean score of the 6 itch rating measurement points during the HI. Before the test participants were asked if they experienced any pain and itch and the test was performed only in case of the absence of both. Upon completion of the test, the size of skin redness and wheal size were measured with a ruler, and skin temperature was measured using a hand-held infrared digital thermometer (held vertically approximately 1 cm above the area; accuracy ± 2.0 °C, resolution 0.1 °C, BaseTech, Conrad Electronic Benelux B.V.).

Questionnaires

Expectations about pain and itch were measured with questions “How much pain do you expect to experience during the cold pressor test?” and “How much itch do you expect to experience during the histamine test?”. Participants gave answers on an NRS from 0-10 (0 = no pain/itch at all is expected, 10 = worst pain/itch ever experienced is expected). Participants answered these questions twice: upon coming to the lab (baseline expectations) and after receiving the spray with or without the combined suggestions (post-intervention expectations).

State anxiety was measured with the short state version of the State Trait Anxiety Inventory, (STAI-Ss) (33). The questionnaire consists of 6 items and the participants were asked to give the answers on a 4-

point Likert scale. The score ranges from 6 to 24, with a higher score indicating higher state anxiety.

Cronbach's alpha in our sample (.80) was similar to previously reports (33).

Positive and negative affect were measured with a short version of the Positive and Negative Affect Schedule (PANAS) (34). The short PANAS consists of 5 items for measuring positive affect and 5 items for measuring negative affect on a 5-point Likert scale. The score for both scales ranges from 5 to 25, with higher scores indicating higher positive and higher negative affect, respectively. The Cronbach's alpha for positive affect in our sample was .85 and for negative affect .74 which was similar to previous reports (34).

The short version of the Eysenck Personality Questionnaire (EPQ-RSS; 35) was used for measuring neuroticism and extraversion. Each scale consists of 12 items and the participants were asked to use a dichotomous scale for giving the answers ("yes" or "no"). The total score for neuroticism and extraversion ranges from 0 to 12, with higher scores indicating higher neuroticism and higher extraversion, respectively. The Cronbach's alpha in this sample was lower than previous reports (35): .74 for neuroticism and .42 for extraversion which reflects low reliability.

The revised Life Orientation Test (LOT-R) (36) was used to measure dispositional optimism. The questionnaire consists of 3 positive, 3 negative, and 4 filler items and answers are given on a 5-point Likert scale. The total score ranges from 0 to 24, with higher score indicating higher optimism. In this sample, Cronbach's alpha was .66.

Statistical Analysis

The data analysis was performed using SPSS Statistics version 21 (IBM Corporation) with a two-tailed significance level of $\alpha < .05$. The power calculation was based on two studies: a study on the effect of oxytocin on placebo analgesia (22), which reported an effect size of $d=0.495$, and a study on the effects of positive verbal suggestions on self-reported pain (1), which found an effect size of $d=0.50$. G-power (37) analyses yielded a sample size of 23 persons per group to obtain a power of .80 at an alpha level of .05.

Taking into account 15% of potential drop outs due to amongst others technical problems, the total sample size was adjusted to a more conservative number of 108 participants.

The data was screened for univariate outliers using z-scores. No z-scores above 3.29 or below -3.29 were found indicating the absence of univariate outliers. Skewness and kurtosis and Shapiro-Wilk test were calculated and indicated normality of distribution of all variables. Homogeneity of variances was checked with Levene's tests that indicated homogeneity of variances in the groups. Baseline characteristics (baseline CPT pain score, baseline CPT pain unpleasantness, expected pain, expected itch, state anxiety, mood, neuroticism, extraversion, and optimism) of the participants were compared between the groups with a one-way between-subject analysis of variance (ANOVA). Baseline CPT and post-intervention CPT pain scores were compared with a paired-sample t-test.

The effects of oxytocin and positive verbal suggestions on the pain and itch expectations were analyzed by using two 2x2 factorial ANOVAs with spray (placebo, oxytocin) and positive verbal suggestions (with, without) as between-group factors and pain and itch post-intervention expectations, respectively, as dependent variables in separate analyses.

To test the effects of oxytocin and positive verbal suggestions on the post-intervention CPT pain intensity ratings, a factorial 2x2 ANCOVA was performed, with spray and suggestions as between-group factors and the baseline CPT pain intensity rating as a covariate. A similar analysis was conducted to examine the effects of oxytocin and positive verbal suggestions on the post-intervention CPT unpleasantness rating, including the baseline CPT unpleasantness rating as a covariate.

To assess the effects of oxytocin and positive verbal suggestions on itch induced by the HI, a factorial 2x2 ANOVA was performed with spray and suggestions as between-group factors. Three additional 2x2 ANOVAs were performed to evaluate the effects of the spray and suggestions on the size of skin redness, wheal size, and the skin temperature after the HI.

To check whether the groups differ in their perceived group allocation, chi-square test was performed.

Finally, to assess whether oral contraception use influenced the results of the analyses, the same analyses were performed with pill intake as a covariate. As the analyses did not change the results, these results are not reported further.

As an effect size measure, partial eta squared was calculated for analyses on the primary and secondary outcomes.

Results

Demographics and baseline characteristics

A total of 108 participants (age 22.1 ± 2.4 ; 57.7% taking oral contraceptives) were equally assigned to the four groups ($n = 27$ per group). Baseline characteristics of the groups are presented in Table 1. There were no significant differences between the four groups in any of the baseline characteristics. The baseline CPT ($M = 4.6$, $SD = 2.1$) was experienced significantly as less painful than the post-intervention CPT ($M = 5.0$, $SD = 2.2$; $t(107) = -4.5$, $p < .001$).

Table 1. Baseline characteristics

	Oxytocin with positive suggestions ($n = 27$)	Placebo with positive suggestions ($n = 27$)	Oxytocin without suggestions ($n = 27$)	Placebo without suggestions ($n = 27$)	$F(1;$	p
					104)	
Age	22.2 (2.5)	21.9 (2.4)	22.2 (2.4)	22.2 (2.2)	.10	.96
Baseline expected pain	4.7 (1.8)	4.6 (2.3)	4.8 (2.5)	5.7 (2.1)	1.35	.26
Baseline CPT pain intensity	4.7 (2.3)	4.4 (2.0)	4.6 (2.1)	4.5 (2.2)	.12	.95
Baseline CPT unpleasantness	5.9 (2.4)	6.1 (2.2)	6.2 (2.1)	5.6 (2.6)	0.33	.80
Baseline expected itch	4.9 (2.2)	5.8 (2.5)	5.6 (2.3)	5.8 (2.3)	.10	.40

Chapter 5
Enhancing placebo effects with oxytocin

State anxiety (STAI-S)	9.8 (2.4)	9.0 (2.1)	9.0 (1.7)	9.8 (2.3)	1.21	.31
Positive mood (PANAS)	27.1 (6.1)	27.2 (6.2)	29.7 (5.0)	27.7 (7.0)	1.07	.36
Negative mood (PANAS)	12.6 (2.3)	11.3 (1.9)	12.7 (3.8)	13.0 (2.4)	2.23	.089
Neuroticism (EPQ- RSS)	4.5 (2.9)	3.7 (2.6)	3.48 (2.5)	4.2 (2.7)	0.80	.50
Extraversion (EPQ-RSS)	8.0 (1.6)	8.0 (1.9)	8.3 (1.6)	8.0 (1.9)	0.21	.89
Optimism (LOT-R)	22.6 (3.0)	22.2 (3.6)	22.2 (3.0)	21.9 (3.2)	0.18	.91

Means and standard deviations are given for all variables. Abbreviations: STAI-S- State Train Anxiety Inventory, State-Version; PANAS- Positive and Negative Affect Schedule; EPQ-RSS- Eysenck Personality Questionnaire short version; LOT-R- The revised Life Orientation Test; CPT- cold pressor test.

The effect of verbal suggestions and oxytocin on expected pain

Expected pain scores are presented in Table 2 and Figure 2a. A significant main effect of the positive verbal suggestions on expected pain was found ($F(1, 104) = 4.77, p = .031, \eta_p^2 = .04$): participants in the positive verbal suggestions groups expected significantly less pain than those in the groups that did not receive suggestions. However, neither a significant main effect of the spray ($F(1, 104) = 0.03, p = .86, \eta_p^2 < .001$), nor a significant interaction between the spray and the positive verbal suggestions ($F(1, 104) =$

0.19, $p = .66$, $\eta_p^2 = .002$) on expected pain was found, indicating that oxytocin did not influence the expected pain of the participants.

The effect of verbal suggestions and oxytocin on expected itch

Expected itch scores are presented in Table 2 and Figure 2b. There was a significant main effect of the positive verbal suggestions on the expected itch ($F(1, 104) = 5.38$, $p = .022$, $\eta_p^2 = .05$), indicating that participants in the positive verbal suggestions groups expected less itch in comparison to the groups that did not receive suggestions. However, neither the spray ($F(1, 104) = .04$, $p = .84$, $\eta_p^2 < .001$), nor the interaction between the spray and the positive verbal suggestions ($F(1, 104) = .041$, $p = .84$, $\eta_p^2 < .001$) had a significant effect on the expected itch.

Table 2. Somatic symptom outcome parameters

	Oxytocin with positive suggestions (n=27)	Placebo with positive suggestions (n=27)	Oxytocin without suggestions (n=27)	Placebo without suggestions (n=27)
Pain				
Post-intervention expected pain	5.3 (2.5)	5.4 (2.0)	6.0 (1.9)	6.2 (2.3)
Post intervention CPT pain intensity	5.1 (2.2)	4.5 (1.7)	5.4 (1.9)	5.2 (2.4)
Post intervention CPT pain unpleasantness	6.1 (2.4)	5.9 (2.4)	6.8 (2.1)	6.5 (2.3)
Pre- and post intervention difference score	-0.4 (1.0)	-0.1 (1.2)	-0.7 (1.3)	-0.7 (0.9)
Itch				

Chapter 5
Enhancing placebo effects with oxytocin

Post-intervention	4.2 (2.3)	4.4 (2.3)	5.4 (2.6)	5.4 (2.2)
expected itch				
Post intervention HI	3.2 (1.7)	3.1 (1.5)	3.7 (1.7)	3.2 (1.6)
itch intensity				
Redness size (mm)	93.9 (15.1)	94.5 (18.4)	94.8 (16.8)	99.1 (17.2)
Wheal size (mm)	58.6 (4.8)	58.8 (5.4)	60.5 (6.7)	58.9 (4.6)
Skin temperature	31.3 (1.5)	31.1 (1.7)	30.9 (1.4)	31.0 (1.3)
(degrees Celcius)				

Means and standard deviations are given for all variables. Abbreviations: CPT- cold pressor test; HI- histamine iontophoresis; mm- millimeters

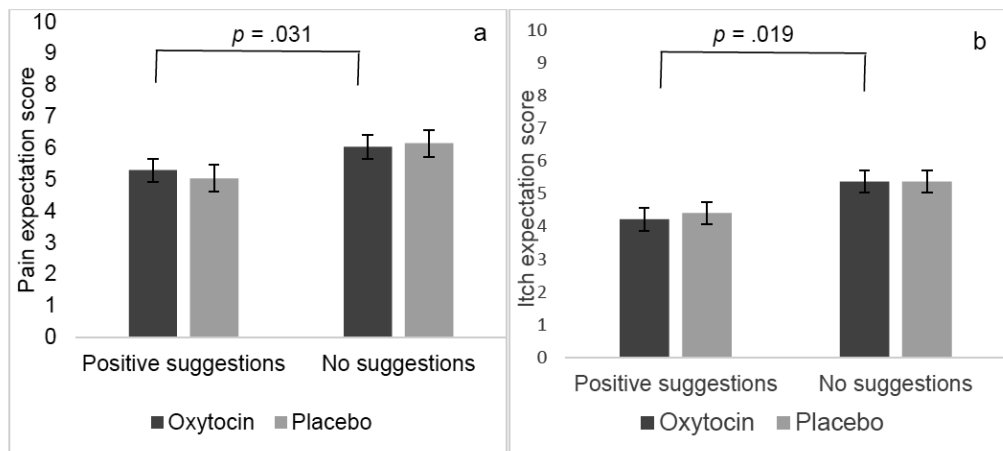


Figure 2. Post intervention pain (a) and itch (b) expectation scores for all groups. Error bars indicate standard errors.

The effect of verbal suggestions and oxytocin on pain outcomes

The pain intensity scores of CPT administration are presented in Figure 3a. There was a significant main effect of positive verbal suggestions on pain ($F(1, 103) = 5.48, p = .021, \eta_p^2 = .05$): participants who received positive verbal suggestions reported less pain in response to the post-intervention CPT in comparison with participants who did not, while controlling for baseline CPT levels. However, neither the main effect of the spray ($F(1, 103) = 0.78, p = .38, \eta_p^2 = .01$) nor the interaction between the spray and the positive verbal suggestions on pain scores ($F(1, 103) = 0.60, p = .44, \eta_p^2 = .01$) were significant. In addition, the results of the factorial ANCOVA indicated a significant main effect of suggestions on the unpleasantness ratings of the CPT ($F(1, 103) = 15.43, p = .002, \eta_p^2 = .09$): participants in the positive verbal suggestions group rated the post-intervention CPT as less unpleasant compared to the groups that received no suggestions, while controlling for baseline unpleasantness scores for the CPT. However, neither the main effect of the spray ($F(1, 103) < .001, p > .99, \eta_p^2 < .001$) nor the interaction between the spray and the positive verbal suggestions ($F(1, 103) = 1.55, p = .33, \eta_p^2 = .01$) were significant.

The effect of verbal suggestions and oxytocin on itch outcomes

The itch intensity scores of the four groups are presented in Figure 3b. No main effect of the positive verbal suggestions ($F(1, 104) = 0.93, p = .34, \eta_p^2 = .009$), the spray ($F(1, 104) = 1.11, p = .30, \eta_p^2 = .01$) or the interaction between the spray and the positive verbal suggestions ($F(1, 104) = 0.21, p = .65, \eta_p^2 < .001$) were found for itch. Three factorial 2x2 ANOVAs indicated also no significant main effects of the positive verbal suggestions and the spray on the size of skin redness, wheal size and skin temperature after the HI (see Table 2).

Perceived group allocation

The four groups did not differ in their perceived group allocation ($\chi^2(3, N = 108) = .49, p = .92$).

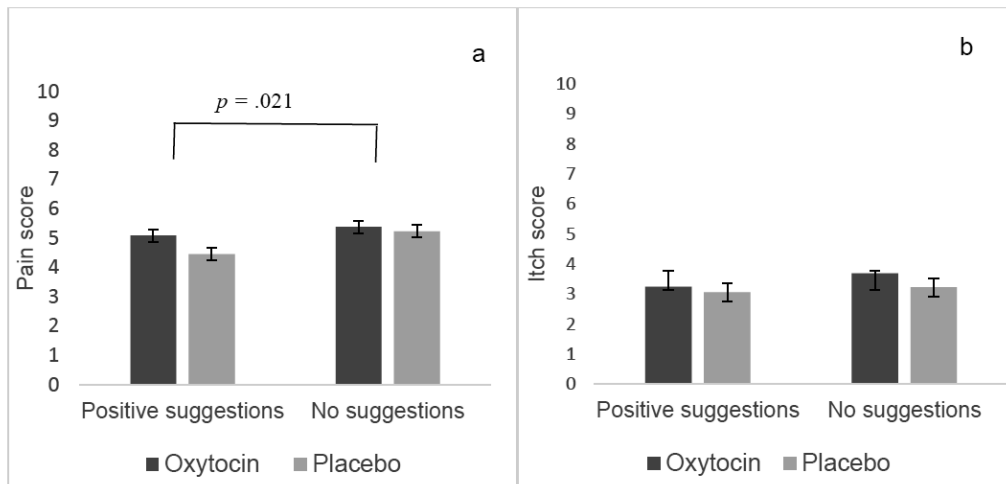


Figure 3. Subjective pain (a) and itch (b) intensities scores after the cold pressor test and histamine iontophoresis, respectively, for all groups. Error bars indicate standard errors.

Discussion

This is one of the first studies that investigated the influence of oxytocin and positive verbal suggestion on the placebo effects for both pain and itch. The study findings demonstrate that positive verbal suggestions were successful in inducing expectations of lower pain and itch, and subsequently were able to elicit small but significant placebo analgesia, but did not decrease itch sensitivity. However, oxytocin did not influence the placebo effects induced by positive verbal suggestions of pain or itch and also did not affect pain or itch sensitivity.

Firstly, we aimed to study whether positive verbal suggestions about nasal spray would change the expectations of participants about pain and itch and elicit placebo effect. The results demonstrated that the verbal suggestions successfully influenced expectations about pain and itch and elicited placebo analgesia. These findings are in line with studies that also showed that positive verbal suggestions can induce placebo analgesia (e.g., 9-11). We were however unable to demonstrate that these verbal suggestions can induce itch reduction, corresponding to previous research (13, 14, 32). The fact that participants

performed a baseline pain test but no baseline itch test, could explain the difference in the results between these two outcome measures. It might be that to exhibit a placebo effect, people need to experience the symptom before the suggestions of the improvement are given. This baseline exposure to the symptom might be a reference point that plays an essential role in the expectations of the participants regarding the effects of the treatment. In line with this hypothesis, Darragh and colleagues (15) found placebo effect for itch and wheal size only after participants experienced a baseline itch-inducing test.

Secondly, we studied whether the placebo effects induced by verbal suggestions can be enhanced by oxytocin. The results showed that oxytocin influenced neither the pain and itch expectations nor the placebo effect induced by positive verbal suggestions for pain or itch in women. It was previously hypothesized that oxytocin can influence positive treatment expectations and boost the placebo effect (16). Only two studies so far investigated oxytocin effects on the placebo effect with contradictory results. Our study is in line with the results of the study by Colloca and colleagues (23), who did not find the enhancing effect of oxytocin on placebo analgesia neither in men nor in women. Nevertheless, Kessner and colleagues (22), demonstrated that oxytocin can boost the placebo effect in men. There are several possible explanations of these divergent results. Kessner and colleagues (22) used a higher dosage of oxytocin (40 IU) than the study of Colloca and colleagues (23) or the present study where 24 IU was used. However, it is not clear whether a higher oxytocin dosage induces a stronger physiological or psychological effect. Van IJzendoorn and colleagues (38) demonstrated that there was no difference in the elevation of the endogenous oxytocin levels after the administration of 16 or 24 IU of oxytocin. Also, Cardoso and colleagues (18) showed that a 24 IU dose of intranasal oxytocin has a stronger effect on the cortisol response to vigorous exercise compared to a dose of 48 IU. A possible explanation for these findings is that by providing a larger amount of nasal spray, this will be swallowed and therefore cannot be absorbed (38). Alternatively, it has been proposed that higher doses of oxytocin bind to vasopressin receptors (39, 40), and that vasopressin instead of oxytocin enhances the placebo effect (23). Finally, gender differences in the effects of oxytocin might play an important role. It was shown that oxytocin has an opposite effect on brain activation patterns (41) and social interactions (42, 43) in men and women.

Possibly, oxytocin enhances placebo analgesia more in men than women (22) although different findings have been found as well (23). Various factors might possibly underlie gender differences such as for example, varying levels of oxytocin and other hormones in women during the cycle (44), gender difference in the baseline levels of gonadal hormones that were shown to modulate oxytocin receptor expression (45), differences in the brain structure of men and women (46). Future research should focus on gender differences in the effects of oxytocin on outcomes such as trust, anxiety, treatment expectations and the placebo response.

As an additional finding, there was also no evidence that oxytocin influences pain or itch ratings directly. The literature about the analgesic effects of oxytocin is contradictory while the effects of oxytocin on itch has not been studied so far. It remains unclear what underlies these contradictory findings. Some studies found that oxytocin can decrease experimentally induced pain (24-27), while others did not (22, 23, 28, 29, 30). A recent study by Tracy and colleagues (30) found pain-enhancing effects of oxytocin in women with chronic pain but no effects of oxytocin on pain sensitivity in healthy men and women. Most studies that found significant effects of oxytocin included male participants (24, 25, 27), however, there are also studies in men that found no analgesic results of oxytocin (22, 23, 30). The research about the effects of oxytocin on the pain sensitivity in women is scarce and it is not possible to state whether sex differences might play role in this relationship.

This study has several unique features. Our research design allows investigating the separate effects of verbal suggestions and oxytocin on pain and itch sensitivity and their interaction. Moreover, it was the first study to research the effect of oxytocin on pain and itch expectations. Besides these strong points, this study also has several limitations. First, as only women were included in this study, it is not possible to generalize the results to men. Second, we observed a placebo effect for pain but not for itch. Since no placebo effects for itch were found, this makes it impossible to generalize the results of the lack of oxytocin effects to itch. Moreover, even though the placebo effect for pain was found to be statistically significant, this effect was quite small and possibly would not be perceived by patients in clinical settings. Future studies should focus on ways to strengthen placebo effects by, for example, combining positive

verbal suggestions with conditioning. In addition, as the post-intervention expectations were measured after the baseline CPT, our pain and itch expectation measures might be not identical. It is recommended that future research either includes two separate sessions or uses other methods of itch induction that can be repeated twice. Additionally, in contrast to studies in which placebo effects were induced by giving positive verbal suggestions about a placebo cream (22) or a sham electrode (23), we gave positive verbal suggestions about the nasal spray itself. This procedure does not require an extra placebo treatment and might be clearer for the participants. However, future studies need to compare different placebo applications in order to find out which of the methods are more effective. Furthermore, the administration of a nasal spray with a particular taste and smell might have elicited a placebo response in both groups regardless of the verbal suggestions. Another limitation is that menstrual cycle phase was not measured in this study. Cycle phase can have an effect on the experience of pain (47). However, as we controlled for the baseline pain sensitivity that was measured at the same day, we expect these possible effects of the menstrual cycle to be negligible. Also, only one dosage of oxytocin was administered in this study. Future studies should preferably compare the influence of different dosages of oxytocin on the placebo effect. Finally, a general limitation of oxytocin research is the lack of knowledge of the underlying mechanisms of the intranasal oxytocin effects. There is an ongoing debate in the literature about how the intranasal oxytocin reaches the brain and via what pathways, central or peripheral, it influences behavior (48).

To conclude, we found that positive verbal suggestions can modify the expectations regarding pain and itch and induce small but significant placebo analgesia, however, oxytocin does not seem to influence expectations or the placebo effect for pain in healthy women. Future studies should investigate the effects of oxytocin on placebo effect at various methods of placebo effect induction and different doses for both genders. This will advance our knowledge of the role of oxytocin in placebo effect and the possible ways to maximize the placebo effect in clinical settings.

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Chapter 6

Effects of oxytocin on placebo and nocebo effects in
a pain conditioning paradigm: a randomized
controlled trial

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Abstract

Oxytocin is a peptide hormone that has been shown to increase trust, decrease anxiety and affect learning as has been observed in conditioning paradigms. Trust, anxiety and learning are important factors that influence placebo effects. In this study we investigated whether oxytocin can increase placebo analgesia, decrease nocebo hyperalgesia, and influence extinction processes of both. Eighty healthy male volunteers were assigned to a 40 IU of oxytocin nasal spray group, or to a placebo control group. Placebo analgesia and nocebo hyperalgesia were induced by a conditioning procedure in combination with verbal suggestions. The results demonstrate that the conditioning procedure successfully elicited significant placebo analgesia and nocebo hyperalgesia responses ($p < .001$). Furthermore, extinction was observed ($p < .001$), although placebo and nocebo responses did not return to baseline and remained significant. Oxytocin did not influence placebo analgesia or nocebo hyperalgesia and had no effect on extinction. This study provides support against the placebo-boosting effects of oxytocin and was the first one to demonstrate that it also did not influence nocebo effects or extinction processes. As managing placebo and nocebo effects has widespread clinical implications, further research should investigate other neurobiological or behavioral pathways to boost placebo and decrease nocebo effects.

Trial registration: The study protocol was preregistered on the website www.trialregister.nl under the number NTR6506.

Perspective: The present study demonstrated that placebo analgesia and nocebo hyperalgesia can be successfully induced by conditioning and verbal suggestions. We could not confirm the hypothesis that oxytocin affects either of these phenomena. Other pharmacological agents and behavioral manipulations for increasing placebo and decreasing nocebo effects should be investigated.

Keywords: oxytocin, placebo effect, nocebo effect, analgesia, hyperalgesia

Introduction

Accumulating findings demonstrate the pain reducing effects of placebo analgesia (1) and pain enhancing effects of nocebo hyperalgesia (2). Expectations and conditioning are two of the most important mechanisms proposed to underlie these phenomena (3, 4). They have been shown to induce robust reductions in pain in experimental and clinical settings (1), while expectations of negative treatment outcomes were associated with higher pain ratings (5). Classical conditioning, a learned association between an initially neutral stimulus (conditioned stimulus, CS) and a physiologically relevant unconditioned stimulus (US), has been also frequently applied to elicit both placebo and nocebo effects for pain (6, 7). Classical conditioning is furthermore characterized by the extinction of the conditioned response with time and when not reinforced (8). This extinction process is particularly found for placebo effects for pain (9, 10). In contrast, a lack of extinction of nocebo effects for pain was repeatedly demonstrated (7, 11). These negative learned associations are particularly relevant for clinical practice as these may be crucial processes underlying various chronic pain syndromes.

Despite the importance of managing placebo and nocebo effects for the treatment of pain, not much research has been done on the neurobiological pathways of these effects as a potential target to enhance placebo effects, and decrease nocebo effects. So far, some evidence exists regarding possibilities to block nocebo effects by proglumide, the cholecystokinin agonist (12), but almost no evidence for the enhancement of placebo effects is available. A recent study aimed to boost placebo analgesia using dopaminergic agonist was not successful (13). Colloca and colleagues, however, found that vasopressin enhances placebo effects in pain but only in women (14). Finding ways to influence placebo and nocebo effects is however crucial for application in clinical practice.

Oxytocin, a peptide hormone produced in the hypothalamus, was proposed as a possible mediator of the placebo effect (15) due to its trust-inducing and stress-relieving properties. A few studies investigated the influence of oxytocin on placebo effects in experimental settings. Kessner and colleagues (16) demonstrated boosting effects of oxytocin on verbally-induced placebo analgesia for experimentally induced pain. In contrast, Colloca and colleagues (14) and Skvortsova and colleagues (17) found no effects of oxytocin on verbally-induced placebo analgesia. These three previous studies used verbal suggestions alone to induce placebo effects. However, it well-known that the combination of verbal suggestions and conditioning induces the largest placebo and nocebo effects (18). No studies so far examined the effects of oxytocin on the placebo effect triggered by classical conditioning along with verbal suggestions and, furthermore, no studies looked at whether oxytocin is able to minimize nocebo effects. As oxytocin facilitates both learning performance during pain conditioning (19) and conditioned fear extinction (20), it is possible that it could affect the conditioning process and also the extinction of placebo and nocebo responses.

In this study, we investigated the effects of oxytocin administration on placebo and nocebo effects for pain induced by a combined conditioning and verbal suggestions approach. Moreover, we explored the extinction of placebo and nocebo effects and possible modulating effects of oxytocin on it.

Methods

Study design

A randomized placebo-controlled double-blind design was used for this experiment. Participants were randomly allocated to one of two groups: an oxytocin group, which received 40 IU of oxytocin nasal spray, and a placebo group, which received the same volume of a placebo nasal spray. The study protocol was approved by the Medical Ethical Committee of the Leiden University Medical Center (number NL60185.058.16) and the study was preregistered as a clinical trial on www.trialregister.nl (NTR6506). The randomization was performed by the Clinical Pharmacy of the Leiden University Medical Center using block randomization with the block size of 8.

Participants

Eighty healthy male volunteers between 18 and 36 years old participated in this study. Male participants were recruited since previous studies reported on the oxytocin boosting placebo analgesia in men (16) and not in women (17) and oxytocin fluctuations during the menstrual cycle can be an interfering factor in studying oxytocin effects in women (21). Exclusion criteria were heart and lung diseases, high or low blood pressure, chronic or acute pain complaints, heavy use of alcohol or other drugs, current diagnosis of psychiatric disorders, and current use of analgesic medications. Participants were asked to refrain from drinking alcohol and intense physical exercises up to 12 hours prior to the experiment and from drinking caffeinated drinks and smoking up to 2 hours before the experiment.

Procedure

Prior to being invited for the experiment, prospective participants were asked to give their informed consent and to complete an online questionnaire on Qualtrics (Provo, UT) to screen for exclusion criteria. All eligible candidates were invited to the laboratory for a 2 hour session.

The experimental timeline is presented in a Figure 1. Upon arrival to the laboratory, participants were given the information about the experiment and were asked to sign a written informed consent form. The experiment was conducted by two experimenters: one gave instructions and the other controlled the equipment. First, participants were asked to fill in several short questionnaires on the computer. Then the pain calibration took place. After determining individual temperatures that elicited low, moderate and high pain levels, participants were administered oxytocin or placebo nasal spray, depending on their group allocation, in a double-blind randomized manner. After the spray administration, participants were provided with verbal suggestions regarding the subsequent conditioning task and a 30-minute waiting time followed to allow oxytocin to reach its peak effects (22). During this break, participants were given

magazines with a neutral content to read. After the break, the conditioning task took place. After the end of the task, participants filled in a closing questionnaire, were debriefed and paid for their participation. The data was collected at the laboratory of the Faculty of Social Sciences of Leiden University between March and November 2017.

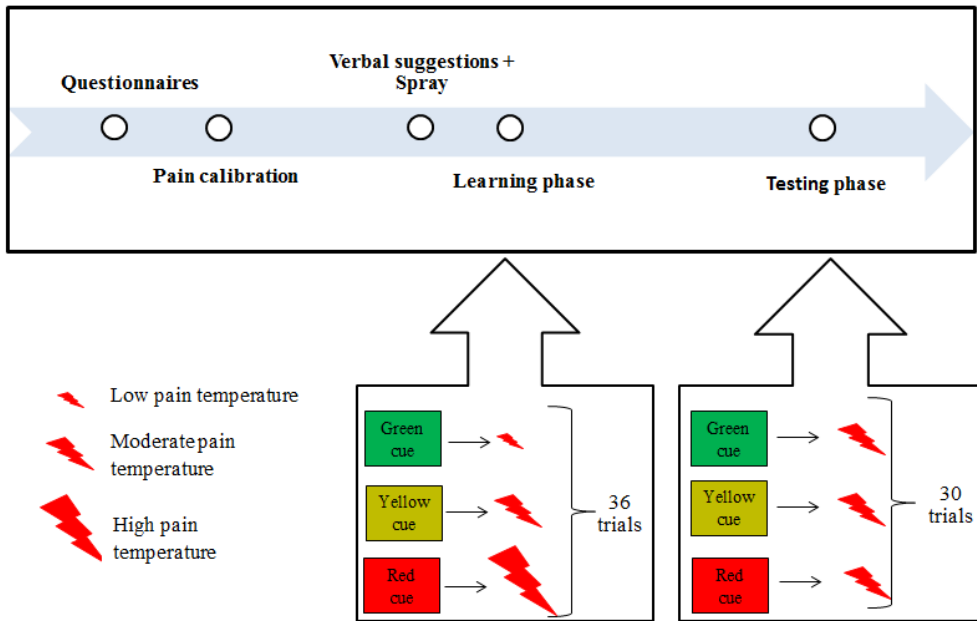


Figure 1. The experimental timeline.

Experimental manipulations

Nasal spray. Participants received 40 IU of oxytocin (Syntocinon) or placebo nasal spray depending on group allocation. The spray was administered by the experimenter with four puffs (two puffs per nostril) using a MAD Nasal Mucosal Atomization Device (Teleflex, Inc., Research Triangle Park). The placebo spray looked and tasted identically to oxytocin. The medication was prepared by Clinical Pharmacy of the Leiden University Medical Center that also was responsible for the randomization. The pharmacy assigned participants numbers and assigned participants to oxytocin or control groups. The researchers received the randomization list with the groups assignments only after the end of the study.

Verbal suggestions. After the nasal spray administration, participants received verbal instructions about the experiment. They were told that the aim of the experiment was to investigate how the oxytocin spray

would influence a TENS (transcutaneous electrical nerve stimulation) device. It was explained to them that during the next task, a TENS electrode would be placed on their arm and that this electrode was able to regulate their pain sensitivity levels as it acted on the pain processing pathways. They were told that when they would see a green cue on a computer screen, the TENS would decrease their pain sensitivity, when they would see a yellow cue this would indicate that the TENS would be inactive, and when they would see a red cue this would indicate that the TENS would increase their pain sensitivity. However, the TENS remained inactive during the experiment; the information was used as a cover story for inducing expectations about the influence of TENS on pain sensitivity and thereby possibly strengthening the conditioning procedure.

Pain calibration. Pain stimuli were delivered with a standardized heat pain application device (ATS-II, Medoc Advanced Medical Systems, Ramat Yishai, Israel). The stimuli were applied to the dorsal site of the not dominant arm. During the calibration procedure, three levels of heat stimulation were determined for each participant individually: 1) a temperature that elicited low pain (pain detection threshold, equal to around 1 on a 0-10 numeric rating scale (NRS); 2) a temperature that elicited moderate levels of pain (equal to 4 on the 0-10 NRS); 3) a temperature that elicited high but bearable levels of pain (equal to 7 on the 0-10 NRS). In order to determine these three levels, we applied one sequence of ascending temperatures to participants' arms with a peak temperature lasting for 4 seconds and a between-stimulus interval of 15 seconds. Participants were asked to rate each stimulus on an NRS ranging from 0 to 10 (0 = no pain at all, 10 = worst pain ever experienced). After the pain of 7 was reached, the calibration procedure was stopped.

Pain conditioning task. The pain conditioning task consisted of two phases: a learning phase and a testing phase. In the learning phase, a green cue on a computer screen was coupled to a low pain stimulus, a yellow cue to a moderate pain and a red cue to a high pain stimulus. The learning phase consisted of 36 stimuli (12 of each color/intensity).

In the test phase, only medium pain temperatures were presented coupled with green (placebo condition), yellow (control condition) and red (nocebo condition) lights. The test phase consisted of 30 stimuli (10 of each color). There was no break between the learning and the test phase. The peak temperature lasted 4 seconds with a ramp-up and ramp-down speed of 8 degrees per second and an inter-stimulus interval of 4 seconds. The stimuli were presented in a randomized fixed order: four random sequences of stimuli were created prior to the experiment and each participant was randomly allocated to one of the four sequences. To avoid habituation and sensitization to pain, the thermode was moved to another place on the forearm twice: after the 22nd stimulus of the learning phase and 8th stimulus of the test phase (every 22 stimuli). After each pain stimulus, participants were asked to rate the pain intensity on a 0 (no pain) - 10 (most intense pain imaginable) NRS verbally.

Instruments and materials

Visual stimuli. E-Prime (version 2.0) software installed on a desktop computer was used to present the visual cues (green, yellow, red) during the pain conditioning task. The resolution of the screen was 1280x1024 pixels and participants were sitting approximately 60 cm from the screen. The visual cues were shown two seconds before the start of each heat stimulus. During the cues, the whole screen turned green-, yellow-, or red-colored.

Questionnaires. A short version of the Positive and Negative Affect Schedule (PANAS) (23) was used to measure the mood of participants at baseline. The questionnaire consisted of 10 items: 5 for measuring positive affect and 5 for measuring negative affect. Scores were obtained on a 5-point Likert scale and could range from 5 to 25. Higher scores indicated higher positive and higher negative affect, respectively. The Cronbach's alpha in our sample was .85 for positive affect and .80 for negative affect, indicating a good internal consistency.

The State Trait Anxiety Inventory – state version (STAI-Ss) (24) was used for measuring baseline state anxiety of the participants. The questionnaire consisted of 6 statements, and participants had to indicate on a scale from 1 (not at all) to 4 (very much) how much this statement applied to them at that specific moment. Scores could range from 6 to 24 with higher scores indicating higher state anxiety. In the present study, the Cronbach's alpha was .81 indicating a good internal consistency.

The revised Life Orientation Test (LOT-R) (25) was used to measure optimism. The LOT-R consists of 3 positive, 3 negative, and 4 filler items and participants had to indicate whether they agree or disagree with each on a scale from 0 (strongly disagree) to 4 (strongly agree). Scores could range from 0 to 24 with higher scores indicating higher optimism. In the present study, the Cronbach's alpha was .75, indicating acceptable internal consistency.

The neuroticism and extraversion scales of the short version of the Eysenck Personality Questionnaire (EPQ-RSS) (26) was used for measuring neuroticism and extraversion. The questionnaire consisted of 24 items: 12 items for measuring neuroticism and 12 for measuring extraversion. The participants were asked to use a dichotomous scale for giving the answers ("yes" or "no"). Scores could range from 0 to 12 with higher scores indicating higher neuroticism and higher extraversion, respectively. The Cronbach's alpha in our sample was .77 for neuroticism, indicating acceptable internal consistency, and .84 for extraversion, indicating a good internal consistency.

Finally, a closing questionnaire was designed to ask participants about their experiences. They were asked to answer the following questions: "Do you think you received oxytocin or placebo?"; "What do you think was the aim of this experiment?"; "Have you heard anything about this experiment from other people? If yes, what?".

Data analyses

The power calculation was performed with software G*Power 3 (27). The input for the power calculation was derived from a study on the effect of oxytocin on placebo analgesia (16) with an effect size of $d=0.495$. A sample size calculation for a repeated measurements within-between analysis of variance with 2 groups indicated that 48 participants in total (24 in each group) would be needed to obtain a power of .95 at an alpha level of $\alpha=0.05$. Taking into account the conflicting results of the previous studies and an additional 10% of possible technical failures, we adjusted the total sample size to the conservative number of 80 participants; 40 in each group.

The data analysis was performed using SPSS Statistics version 21 (IBM Corporation) with a two-tailed significance level of $\alpha < .05$. The data was screened for univariate outliers using z-scores. Z-scores above 3.29 or below -3.29 were considered to indicate univariate outliers. One outlier was found for the negative affect score and one outlier for the state anxiety score, with z scores of 6.21 and 5.05 respectively. Non-parametric tests were applied for the analysis of the variables with outliers. Skewness, kurtosis and Shapiro-Wilk tests indicated normality of distribution of all variables. Levene's tests indicated homogeneity of variances in the groups. Sphericity was measured with the Mauchly's test of sphericity and in case of the violation of this assumption, Greenhouse-Geisser corrections were used. The heat stimuli directly following the repositioning of the thermode, elicited significantly higher pain ratings. For this reason, the two stimuli directly following the repositioning of the thermode, were excluded from the analysis. In total, 35 learning trials and 29 test trials were included in the analysis. Independent samples t-tests or non-parametric Mann-Whitney tests were used to compare the groups on the baseline and personality characteristics, as appropriate: age, heat pain detection threshold (temperature that was rated as 1 on the VAS pain scale), positive affect, anxiety, optimism, extraversion and neuroticism.

A 2x3 (group (oxytocin vs placebo) x cue color (green vs yellow vs red)) factorial analysis of variance (ANOVA) was used to compare the groups on the mean pain scores in response to green, yellow and red cues in the learning phase of the conditioning task. To examine whether the placebo and nocebo effects were significant and differed between the groups, a 2x3 (group (oxytocin vs placebo) x cue color (green vs yellow vs red)) factorial ANOVA was used to compare the groups on their mean pain ratings in response to the three color cues in the test phase of the conditioning task. Furthermore, to examine the extinction process in more detail, placebo and nocebo effects were calculated for each pair of green-yellow and yellow-red trials of the test phase. Specifically, placebo effects were calculated as the difference between pain ratings in response to the yellow versus the green cues whereas nocebo effects were calculated as the difference between the pain ratings in response to the red versus the yellow cues. In total, 9 placebo and 9 nocebo effects (corresponding to 9 red and yellow or green and yellow comparisons) were calculated. Repeated measures ANOVA was done with time (placebo effect order number) as a within-subject factor,

group as a between-subject factor and placebo effect as a dependent variable to evaluate the effects of the group and time on the placebo effect. Bonferroni corrected post-hoc tests were applied to investigate the difference between the time moments. The same analysis was run with nocebo effect as a dependent variable. To compare the strength of placebo and nocebo effects, the mean placebo effect was compared to the mean nocebo effect with a one-sample t-test. Finally, to check if the groups differed in their perceived group allocation, a chi-square test was performed.

To examine the effects of psychological and personality characteristics on the placebo effect, linear regression analyses were performed with positive affect, anxiety, optimism, extraversion and neuroticism as independent variables and the placebo effect as a dependent variable. The same analysis was repeated with nocebo effect as a dependent variable.

To examine whether the perceived group allocation has an effect on placebo and nocebo effects, independent samples t-tests were used to compare people who thought they received placebo and people who thought they received oxytocin spray on their placebo and nocebo effects.

Partial eta squared was calculated for analyses as the indication of the effect sizes.

Results

Baseline characteristics

Data of 76 participants were available for the analysis; data of 4 participants (1 from oxytocin group, 3 from placebo group) were excluded due to technical problems during the conditioning task. The baseline and personality characteristics are presented in Table 1. There were no differences between the groups on any of these variables, except for extraversion: participants randomized to the oxytocin group ($M = 10.69$, $SD = 2.68$) reported a higher extraversion in comparison to the control group ($M = 9.11$, $SD = 3.43$; $t(74) = 2.25$, $p = .027$).

	Oxytocin group (n = 39)	Control group (n = 37)	t	p
Age	23.30 (3.44)	23.11(2.94)	0.26	.80
State anxiety *	8.99 (2.46)	8.70 (2.23)	682	.68
Positive mood	31.90 (8.22)	30.81 (7.07)	0.62	.54
Negative mood*	12.56 (2.58)	13.28 (2.83)	611	.24
Neuroticism	2.59 (2.66)	3.62 (2.78)	-1.65	.10
Extraversion	10.69 (2.68)	9.11 (3.43)	2.25	.027
Optimism	22.13 (3.43)	22.27 (3.85)	-.017	.87

Pain detection threshold	43.39 (1.72)	43.87 (1.70)	-1.22	.23
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Table 1. Baseline and personality characteristics with standard deviations across the groups. *Mann-Whitney U test is presented instead of t-test, as non-parametric test was applied to these variables

Learning phase

The mean levels of pain during each trial of the learning phase are presented in Figure 2. The temperatures that were calibrated to elicit the pain of 1, 4 and 7 on the 11-point NRS, caused lower pain during the learning phase of the conditioning task (pain of 0.5, 2.85 and 5.88 respectively). The 2x3 (group x cue color) factorial ANOVA with a Greenhouse-Geisser correction demonstrated that there was a significant main effect of the cue color on the pain ratings ($F(1.65, 121.86) = 634.27, p < .001, \eta^2 = .90$), while the effect of the group ($F(1, 74) = 0.69, p = .69, \eta^2 = .01$) and the group-cue color interaction ($F(1.65, 121.86) = 0.55, p = .54, \eta^2 = .01$) were non-significant. Post-hoc tests using Bonferroni corrections indicated that participants rated the stimuli following the red cues ($M = 5.88, SD = .18$) as significantly more painful than stimuli following yellow ($M = 2.88, SD = 0.14$) and green cues ($M = 0.49, SD = 0.06$); also the yellow cues stimuli were rated as significantly more painful than the green cues stimuli (all $ps < .001$; Figure 2). Oxytocin did not influence the pain perception during the learning phase.

Test phase

The 2x3 (group x cue color) factorial ANOVA with a Greenhouse-Geisser correction demonstrated that there was a significant main effect of the cue color on the pain ratings ($F(1.41, 104.61) = 61.71, p < .001, \eta^2 = .46$), while the main effect of the group ($F(1, 74) = 2.31, p = .13, \eta^2 = .01$) and the group x cue color interaction ($F(1.41, 104.61) = 0.63, p = .48, \eta^2 = .01$) were non-significant. Post-hoc tests using Bonferroni corrections indicated that participants rated the stimuli following the red cues ($M = 3.37, SD = 0.19$) as significantly more painful than stimuli following yellow ($M = 3.07, SD = 0.17$) and green cues ($M = 2.62, SD = 0.15$), and green cues stimuli as significantly less painful than yellow and red cues stimuli (all $ps < .001$).

The difference scores between the yellow and green trials (placebo effect) and red and yellow trials (nocebo effect) of the test phase are presented in Table 2. The repeated measures ANOVA with a Greenhouse-Geisser correction for the placebo effect showed that the main effect of the group ($F(1, 73) = 0.06, p = .82, \eta^2 = .001$) and time x group interaction ($F(6.34, 456.80) = 0.80, p = .58, \eta^2 = .01$) were non-significant; however, there was a significant main effect of time on the placebo effect ($F(6.34, 458.6) = 5.07, p < .001, \eta^2 = .07$). Post-hoc tests using Bonferroni corrections indicated that the first placebo effect was significantly larger than the following placebo trials.

The repeated measures ANOVA with a Greenhouse-Geisser correction on the nocebo effect showed that the main effect of the group ($F(1, 73) = 0.67, p = .41, \eta^2 = .01$) and the time x group interaction ($F(6.14,$

448.19) = 0.61, $p = .77$, $\eta^2 = .01$) were non-significant; however, there was a significant main effect of time for the nocebo effect ($F(6.14, 448.9) = 3.67$, $p = .001$, $\eta^2 = .05$). Post-hoc tests using the Bonferroni corrections indicated that only one nocebo effect in the middle of the test phase was significantly smaller than all other nocebo effects. No other significant differences over time were found.



Figure 2. Pain ratings during the learning and test trials in response to green, yellow and red cues. Oxytocin group on the top panel, control group on the down panel, the first test trial is highlighted.

Table 2. Placebo and nocebo effects with standard deviations per test trial across the groups

Placebo effect		Nocebo effect	
Oxytocin group (n = 39)	Control group (n = 37)	Oxytocin group (n = 39)	Control group (n = 37)

Test trial 1	1.15 (0.20)	1.27 (0.21)	0.58 (0.21)	0.35 (0.21)
Test trial 2	0.75 (0.19)	0.44 (0.19)	0.35 (0.16)	0.60 (0.16)
Test trial 3	0.50 (0.23)	0.21 (0.23)	0.51 (0.26)	0.88 (0.26)
Test trial 4	0.57 (0.23)	0.53 (0.23)	-0.25 (0.212)	-0.07 (0.22)
Test trial 5	0.13 (0.18)	0.61 (0.18)	0.17 (0.15)	0.08 (0.15)
Test trial 6	0.34 (0.17)	0.46 (0.17)	0.29 (0.14)	0.23 (0.15)
Test trial 7	0.29 (0.18)	0.26 (0.18)	0.01 (0.1)	0.31 (0.16)
Test trial 8	0.25 (0.17)	0.31 (0.18)	0.23 (0.19)	0.39 (0.19)
Test trial 9	0.20 (0.18)	0.31 (0.19)	0.42 (0.20)	0.40 (0.20)

Secondary outcomes

It was demonstrated that the mean placebo effect across all test trials ($M = 0.47$, $SD = 0.50$) was larger than the mean nocebo effect ($M = 0.30$, $SD = 0.51$; $t(75) = 2.40$, $p = .019$). None of the personality characteristics significantly predicted placebo (all $p > .08$) or nocebo (all $p > .19$) effects.

Participants in the two groups did not differ in their perceived group allocation (chi square (1, $N = 76$) = 1.88, $p = .171$). The majority of participants thought that they received a placebo spray: 71.8% in the oxytocin group and 56.8% in the placebo group. There was no difference between people who thought they received oxytocin (placebo effect: $M = .56$, $SD = 0.71$; nocebo effect: $M = .48$, $SD = 0.74$) and who thought they received placebo (placebo effect: $M = .64$, $SD = 0.68$; nocebo effect: $M = .23$, $SD = 0.60$) in their placebo ($t(74) = -0.51$, $p = .61$) and nocebo ($t(74) = 1.64$, $p = .11$) effects.

Finally, to explore whether the baseline personality characteristic of extraversion that differed between the groups, influenced the results of the analyses, the same analyses were performed with extraversion as a covariate. As the analyses did not change the results, these results are not reported further.

Discussion

The results of this study demonstrate that conditioning can successfully induce placebo analgesia and nocebo hyperalgesia. Importantly, we showed for the first time that intranasal oxytocin administration did not influence analgesia and hyperalgesia induced by conditioning combined with verbal suggestions or the extinction of these effects.

Our findings regarding on the induction of placebo analgesia and nocebo hyperalgesia by conditioning with verbal suggestions are in line with previous research (1, 2). We used conditioning together with verbal suggestions as it was previously demonstrated that the combination of these two methods is more effective than applying them separately (18). The placebo effect of the first test trial was larger than 1 on the 11-point scale, what is consistent with previous literature (6, 28) and decreased during the test phase showing an extinction pattern. Interestingly, nocebo effects at the first test trial were smaller (0.5 on the 11-point scale), however, they seemed to be affected by extinction less than placebo effects: the greatest extinction of the placebo effect occurred after the first test trial, while the nocebo effect remained stable with only one trial in the middle of the sequence that was significantly lower than others, indicating that the nocebo effect in the present study did not extinct over time. These findings are in line with previous studies that found significant extinction of placebo (9, 10) but not of nocebo effects (7). This phenomenon can be explained by the fact that threatening information is more relevant for an organism's survival from an evolutionary point of view and that is why it should be stored in memory longer than positive information (29). Moreover, Colagiuri & Quinn (30) showed that anticipatory anxiety and elevated autonomic arousal contribute to the persistence of nocebo effects in comparison to placebo effects. Intranasal administration of 40 IU oxytocin did not influence pain-related placebo and nocebo effects and their extinction. These findings are in line with our earlier study in which we also did not find boosting effects of oxytocin on placebo effects for pain and itch induced by verbal suggestions in healthy females and with lower oxytocin doses (17). These findings further confirm previous findings from the study by Colloca and colleagues (14) where no effects of oxytocin on placebo analgesia in either men or women were found. The important addition of the current study is that we employed a conditioning procedure along with verbal suggestions to induce placebo effects and we furthermore explored the effects of oxytocin on nocebo effects. Previous research indicated that oxytocin facilitates learning during fear conditioning when given prior to the conditioning procedure (19) and enhances neural indicators of fear extinction when given prior to the extinction phase (20). We hypothesized that since these effects of fear conditioning were induced by painful stimuli, they could possibly also be applied to the response to a pain conditioning paradigm which essentially represents associative learning. Several explanations can be provided for the lack of confirming these expectations. Placebo analgesia and hyperalgesia are not identical to psychophysiological and neurological responses to fear conditioning used as outcome measures in previous research, such as amygdala activation, skin conductance and reaction times (19, 20). Possibly oxytocin influences neurological conditioned responses but not the conditioned pain perception. By contrast, social aspects seem to play an important role in oxytocin effects. It has been demonstrated that oxytocin enhances learning with social reinforces (emotional faces) in comparison to non-social reinforces (color cues) (31). As the conditioning procedure did not include any social stimuli, possibly

non-social cue based learning mechanisms remained unaffected by oxytocin. On the other hand, some studies (19, 20) found effects of oxytocin on fear conditioning regardless of any social aspect of the CS. Therefore, it remains unclear whether oxytocin influences only the perception of stimuli with a social component.

Another methodological difference of our study from previous research is the dose of oxytocin used. The only experiment that found boosting effects of oxytocin on the placebo effect (16) used 36 IU of oxytocin while two other studies with null findings (14, 17) used a more standard dose of 24 IU. The evidence regarding the correlation between the dose and effectivity of oxytocin is mixed. For example, lower (8 IU) doses of oxytocin have been found to affect amygdala activation in men in response to emotional faces more than higher (24 IU) doses (32), while another study demonstrated that 24 IU had the largest effect on amygdala activation in men in comparison to 12 IU and 48 IU (33). Also, a 24 IU dose seems to have a stronger effect on cortisol levels than a dose of 48 IU (34). On the other hand, 48 IU of oxytocin has been found to improve the performance on a face emotion recognition task, while 24 IU did not (35). Furthermore, a U-shaped relationship between oxytocin dose, social reward, and neural activity has been recently proposed (36), indicating the dose–response relationship is initiated at lower doses in females than males. Considering this mixed evidence, future research needs to determine the exact role of the dose of oxytocin for such phenomena as learning and extinction. Moreover, sex differences may also explain the mixed findings in oxytocin research. For example, oxytocin dampens amygdala activation in response to emotional stimuli in males but enhances activation in females (37). Evidence for opposite effects of oxytocin on social processing in men and women is also present (38, 39). Placebo enhancing effects of oxytocin were previously found in men (16), therefore we recruited a male sample in this experiment. However, we could not confirm these previous results and our results are more in line with our previous study performed in women (17) and with a study by Colloca (14) conducted in both sexes. Sex differences in oxytocin effects remain underexplored and future research should focus on the comparison of male and female samples.

Additionally, we did not find effects of oxytocin on pain sensitivity. A lot of research focused on possible analgesic effects of oxytocin, but the results remain contradictory. A number of studies found pain reducing effects of oxytocin (40–42), however, others could not replicate these results (17, 43–45). A few limitations of the current study should be mentioned. First of all, repeated administration of the heat stimuli leads to the habituation to pain: the temperatures that during the calibration caused low, medium and high pain levels, during the pain conditioning task elicited lower levels of pain. As participants experienced smaller ranges of pain sensations, it could have decreased the magnitude of placebo and nocebo responses. Despite this fact, this procedure was successful in eliciting placebo analgesia and nocebo hyperalgesia. Additionally, many participants had relatively high pain thresholds and it was

therefore not possible to reach high levels of pain because of the protection mechanisms of the ATSS termode that does not allow giving temperatures above certain thresholds. Finally, including only males into the study makes it impossible to generalize the results of the study to females.

To summarize, although placebo analgesia and nocebo hyperalgesia could be successfully induced by conditioning and verbal suggestions, there was no evidence that 40 IU oxytocin given intranasally affected placebo analgesia and nocebo hyperalgesia, nor the extinction of both. Our study adds to the accumulating evidence towards the absence of the ability of oxytocin to influence placebo effects. Researchers interested in possibilities to strengthen placebo effects and reduce nocebo effects might benefit from focusing on other neurobiological and behavioral mechanisms that influence learning mechanisms of placebo and nocebo effects.

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Chapter 7

Summary and General Discussion

The aim of the current dissertation was to explore the link between the endocrine system and placebo effects with a focus on the hormone oxytocin. Two perspectives were examined: the possibility to elicit placebo effects in the endocrine system (**Part I**) and influencing placebo and nocebo effects by hormones (**Part II**).

With regard to the first perspective where the focus was on eliciting placebo effects in the endocrine system, we have systematically reviewed studies on classical conditioning of endocrine responses in animals and humans in **Chapter 2**. Classical conditioning is one of the underlying mechanisms of the placebo effect and with this systematic review we aimed to investigate whether it was possible to elicit hormonal changes through this type of placebo manipulation. Overall, we demonstrated that a substantial body of evidence exists on the possibility to trigger endocrine changes with conditioning in animals and that the results of animal studies correspond to the results from human research. However, we have also found that human research is quite limited, as evidence of classically conditioned hormonal responses in humans exists so far only for insulin, cortisol, and growth hormone. No standard protocol for eliciting conditioned endocrine response was found: different studies used different numbers of sessions, different conditioning procedures and different conditioned stimuli. In general, our review demonstrated that there is a large potential for endocrine conditioning, however, no consensus yet exists in this field regarding a standard conditioning design and not all endocrine systems have been investigated, especially in humans. In **Chapters 3** and **4** we described the results of a large randomized controlled trial (RCT) in which we aimed to expand the knowledge from our systematic review and investigated the possibility to condition a hormone that has not been studied before in this context, oxytocin. In **Chapter 3** we described the results of the RCT demonstrating that we succeeded to classically condition endogenous oxytocin release and that this conditioned response became extinct within three evocation days. We showed conditioned effects in endogenous oxytocin levels; however, conditioning did not influence participants' responses to a social task on the evaluation of faces or a pain sensitivity task, which have both been shown to be sensitive to oxytocin effects previously. **Chapter 4** focused on the imaging part of this RCT. The data showed that there was some indication for conditioned oxytocin response in the brain, particularly in the amygdala and

the superior temporal gyrus in response to pictures of fearful faces, stimuli that are commonly used in oxytocin research. However, the conditioned response was relatively small in comparison to actual oxytocin effects and did not generalize to other tasks previously shown to be affected by oxytocin.

Part II of this dissertation was dedicated to the link between placebo and nocebo responses and oxytocin.

Chapter 5 presents results of an RCT in females in which we investigated whether it was possible to enhance placebo effects for pain and itch by using oxytocin. In this study we demonstrated that giving verbal suggestions regarding the pain- and itch-relieving properties of a nasal spray induced a significant placebo analgesia but did not affect itch sensitivity. Importantly, there was no boosting effect of intranasal oxytocin on the placebo effect found. To address the possible limitations of this study, we designed a follow-up study that is described in **Chapter 6**, in which we explored the effects of intranasal oxytocin on placebo and nocebo effects for pain. In contrast to the study described in Chapter 5, this trial included male participants, a higher dosage of oxytocin, and the placebo effects were induced by a combination of verbal suggestions and classical conditioning. The results of this trial are congruent with the results of the study described in Chapter 5: we demonstrated that the combination of verbal suggestions and classical conditioning induced significant placebo and nocebo effects for pain, but that oxytocin did not influence either effect or the extinction of both. With these two studies we conclude that there is no evidence to state that different dosages of oxytocin influence placebo and nocebo effects in male and female samples.

To sum up, the current dissertation described the link between placebo effects and the endocrine system, particularly oxytocin. We demonstrated that there is accumulating evidence in the literature that placebo effects can be elicited in the endocrine system. Moreover, we described results of a randomized controlled trial in which we successfully conditioned oxytocin release. Finally, we showed a lack of evidence that oxytocin can influence placebo and nocebo effects.

General discussion

The aim of this dissertation was to investigate the connection between placebo effects and the endocrine system with a focus on the hormone oxytocin. We looked at this link from two perspectives: eliciting placebo effects in the endocrine system, particularly oxytocin, and influencing placebo effects by oxytocin. First, we did a systematic review of the literature to summarize the existing evidence on classically conditioning the endocrine system. Then, we performed a randomized controlled trial and studied the possibility to classically condition oxytocin effects. We looked at the effects of classical conditioning on endogenous oxytocin release, performance of a social task, pain sensitivity, and brain activation. Furthermore, in two randomized controlled trials we studied whether oxytocin increased placebo and decreases nocebo responses in pain and itch. To summarize, the performed studies provide a novel perspective on the link between placebo effects and oxytocin, and emphasize the importance of studying the connection between the endocrine system and placebo effects. In this chapter we discuss the results of the dissertation, mention several limitations that should be addressed in future research, and discuss clinical implications and scientific relevance of this work.

Placebo effects in the endocrine system

Our review indicated that placebo effects in the endocrine system have been studied in the context of pharmacological conditioning. In pharmacological conditioning an association between a drug (unconditioned stimulus, US) and an initially neutral conditioned stimulus (CS) is established and further presentation of the conditioned stimulus triggers a physiological response similar to the drug response. Pharmacological conditioning of hormonal responses attracted a lot of interest in the 20th century since Pavlov described the principles of classical conditioning (1): especially a lot of animal research focused on the possibility to trigger hormone release or decrease by classical conditioning. In the 1980s, two reviews were written on this topic, summarizing the accumulating evidence on the endocrine conditioning at that time (2, 3). However, as we have demonstrated in Chapter 2, the interest in this topic significantly dropped in more recent years and only few recent studies looked at conditioning of hormones in humans. In this dissertation we come back to this clinically relevant topic as we believe that being able to modify

hormonal levels with placebo effects might have a lot of potential clinical implications, for example, for patients who require hormonal treatments.

Chapter 2 describes a systematic review of the literature on pharmacological conditioning in the endocrine system and demonstrates that there is a large body of animal research supporting the hypothesis that hormonal levels can be modified by conditioning. Moreover, a smaller number of human studies conducted in this area support the evidence coming from the animal research. Evidence exists for conditionability of corticosterone, insulin, sex hormones, oxytocin, adrenaline, and noradrenaline in animal research (4), but only limited hormones, namely cortisol (5-7), growth hormone (6), and insulin (8), have been investigated in human conditioning research. One of the main questions from the systematic review could not be conclusively answered based on the current evidence base: are all hormonal responses malleable by classical conditioning?

To shed more light on this question, we investigated classical conditioning of a hormone never researched in humans with this paradigm before, oxytocin. The results of this study are described in Chapter 3. We applied a standard conditioning design with acquisition and evocation sessions. During the acquisition session, oxytocin was coupled with a conditioned stimulus, a smell of rosewood oil. During the evocation sessions, oxytocin was replaced with placebo and presented with the smell, and several outcome variables were measured. We found a conditioned oxytocin release in saliva after participants received a placebo spray with the smell. This is the first study that investigated the possibility to condition oxytocin placebo release in humans. The only other study that explored this topic was done in rats (9) and also found conditioned oxytocin release in rats in response to placebo given with the CS. Therefore, we were successful to confirm conditionability of oxytocin in humans. Moreover, in Chapter 4, we describe the results of the fMRI scan done on the third evocation day, when the conditioned effect on oxytocin release had already gone extinct. We demonstrated that there is some indication for a conditioned response in the brain, particularly in the amygdala and superior temporal gyrus, although these effects were very small and not generalizable across tasks. This study was the first study about the effects of classical conditioning

with oxytocin on brain activity and even though the effects we found were quite small, these results indicate the potential of studying brain responses to hormonal conditioning.

Optimizing the strength of the conditioned response

It is important to mention that not all studies aimed at hormonal conditioning were successful. For instance, Petrakova and colleagues (10) investigated the possibility to elicit conditioned cortisol release by using intravenous injections of corticotrophin-releasing hormone as an unconditioned stimulus and a distinctive flavored drink as a conditioned stimulus. After two acquisition trials in which they coupled the CS and the US, no change in endogenous cortisol level was found on the evocation trials in response to CS with a placebo. Moreover, Stockhorst and colleagues (11) found conditioned glucose decrease but no conditioned insulin release in a study aimed at conditioning of insulin responses. Classical conditioning, possibly, depends to a large extent on the unconditioned stimulus chosen. The unconditioned response is not necessarily identical to the drug, but rather the changes that happen in the body due to the drug. The afferent signal sent to the brain by these changes constitute the unconditioned stimulus (12). Therefore, to successfully condition changes in certain hormone levels, it is important to understand the physiological action of the US used. In Chapters 2 and 3, we chose intranasal oxytocin as the US. It has been proposed that intranasal oxytocin administration triggers endogenous oxytocin release by feed-forward mechanisms, so that circulating oxytocin stimulates further oxytocin release (13-15). Possibly, because of the direct effects of the intranasal oxytocin on the brain and further endogenous oxytocin release, we were successful in establishing a connection between the US and CS.

Another important factor of classical conditioning is the strength and the duration of the conditioned response. It remains unknown what number of acquisition sessions (the sessions in which the associations between US and CS are established) is necessary to cause a clinically significant and long-lasting conditioned response. From a learning perspective, a larger number of acquisition sessions should lead to a stronger conditioned response (1). However, endocrine conditioning research on this topic remains quite scarce. In the review described in Chapter 2 we found some evidence for a link between the number of acquisition sessions and the strength of conditioned corticosterone responses in animals: conditioned

corticosterone decrease (16) and release (17) were shown to be stronger after a larger number of acquisition trials. No human studies so far looked at the link between the number of acquisition trials and the strength of the endocrine conditioned responses.

In the study described in Chapters 3 and 4 we show that 3 acquisition trials were enough to trigger conditioned oxytocin release. Moreover, we found a fast extinction of this response: on the second evocation day there was only a trend of the conditioned oxytocin release left and on the third evocation day no conditioned response was found anymore in saliva, with only a modest indication of the conditioned response in brain activity. To our knowledge, our study was the first one to examine extinction of the conditioned endocrine responses in humans. It remains unknown if a larger number of acquisition trials would lead to a stronger conditioned response and slow down the extinction process. Another way to measure a conditioned hormonal response and possibly increase it, would be including a challenge, that would stimulate hormone production, during the evocation session (18). Possibly, during the challenge the effects of classical conditioning would be more visible. An example of such a challenge is proposed in the pilot study of Tekampe (19) where Trier Social Stress test was added to the evocation phase of the cortisol conditioning experiment. In case of oxytocin conditioning, such a challenge would be a task that can stimulate oxytocin production, such as watching an emotional video (20) or receiving a massage (21). However, this remains a hypothesis before more studies investigate the conditioned hormonal responses during such challenges and find the suitable challenges for different hormonal systems.

The role of oxytocin in placebo and nocebo effects

Part II of this dissertation considers the link between placebo effects and the endocrine system from another perspective, namely the extent to which oxytocin can potentially influence placebo and nocebo effects. The hypothesis that oxytocin might be a mediator of the placebo effect was initially proposed by Enck and Klosterhalfen (22). They named several reasons why oxytocin might influence placebo effects: oxytocin has been shown to increase trust (23) and reduce stress (24). Moreover, oxytocin stimulates the secretion of nitric oxide that has been linked to the relaxation response (25), another possible mediator of

the placebo effect. So far, the findings on the possible role of oxytocin were contradictory. Kessner and colleagues (26) have found that exogenous oxytocin boosted placebo analgesia induced by verbal suggestions in men. However, Colloca and colleagues (27) have found no effects of exogenous oxytocin on the placebo effect in pain induced by verbal suggestions in men or in women. Since it is important to find ways to boost placebo effects and decrease nocebo effects, in Chapters 5 and 6 we investigated the possible impact of oxytocin on placebo and nocebo effects. In these two randomized controlled trials we employed two different methods of placebo effect induction: verbal suggestions and classical conditioning, tested female and male samples, investigated placebo effects in two somatic symptoms (pain and itch), and used two different dosages of oxytocin: 24 IU and 40 IU. The results of these two studies are consistent: placebo and nocebo effects for pain were successfully induced by the chosen procedures, but no effect of oxytocin was found.

These two studies have several unique aspects. The trial presented in Chapter 5 was the first study investigating the effects of oxytocin on placebo effects in itch. Most placebo research focuses on pain, and the previous studies of Kessner and colleagues (26) and Colloca and colleagues (27) have also looked at this symptom. Interestingly, our placebo manipulation, giving verbal suggestions about pain- and itch-relieving properties of a nasal spray, induced a significant placebo pain reduction but did not affect itch sensitivity. Research on the possibility to induce placebo effects for itch varies: Darragh and colleagues (28) have used verbal suggestions accompanied with a video about the itch-relieving properties of a placebo cream and demonstrated a decrease in subjective ratings of itch induced by histamine iontophoresis. On the other hand, several studies (29, 30) have failed to induce placebo effects for itch using positive verbal suggestions. Even though pain and itch are closely related symptoms (30), more evidence exists about the possibility to induce placebo effects with verbal suggestions for pain than for itch and our study adds to that.

Chapter 6 presents the results of the first study that investigated a possible influence of oxytocin not only on placebo but also on nocebo effects for pain. The underlying physiological mechanisms of nocebo effects are in general less investigated and the only study that focused on possible hormonal influences of

nocebo responding showed that nocebo effects might be enhanced through the hormone cholecystokinin (31). We did not have a directional hypothesis about whether oxytocin would enhance or decrease nocebo hyperalgesia. We expected that it might speed up the extinction of the nocebo effect for pain, since it has previously been shown that oxytocin facilitates the extinction of conditioned fear that was also induced by pain stimulation (32). However, our hypotheses were not confirmed: no effect of oxytocin on either the size of placebo and nocebo effects or their extinction was found.

Possibly, minor differences in the study designs between our studies and the study of Kessner and colleagues (26) could explain the differences in the results. For instance, in the study of Kessner and colleagues there was a male experimenter while we had two experimenters, one male and one female working together. It has previously been shown that the laboratory personnel gender can influence pain reports of participants (33) and possibly, it could also influence placebo effects; however, this has not been investigated before. Additionally, the precise methods of placebo effect induction differed between the studies: Kessner and colleagues used verbal suggestions about a placebo ointment, while we gave verbal suggestions about a nasal spray (Chapter 5) and used conditioning with suggestions about a (nonfunctional) transcutaneous electrical nerve stimulation device (Chapter 6). Nevertheless, to be able to use oxytocin in clinical practice, it is important that its effects are clinically significant and reliably generalizable across different samples and placebo effect induction procedures. The results of Part II of the dissertation, however, point at a lack of evidence that oxytocin might play a role in placebo and nocebo effects. The results of the two conducted studies were consistent: neither the gender of the sample nor the dose of oxytocin made a difference. Possibly, oxytocin does not influence placebo and nocebo effects at least in the described procedures, leaving unanswered whether other hormones could be instigated in future research as possible enhancers of the placebo effect.

Limitations

The current dissertation presents novel theoretical and empirical evidence about a link between the endocrine system and placebo effects. However, several limitations of this research have to be mentioned.

First of all, due to the lack of research in human studies on pharmacological conditioning of the endocrine system, we were unable to find an optimal conditioning protocol even after performing a systematic review of the available evidence. Therefore, we cannot say whether the design applied in our study on oxytocin conditioning was optimal to elicit the strongest conditioned oxytocin release. We have chosen three acquisition sessions, as this was the average number of acquisition sessions in the human endocrine conditioning studies described in our systematic review. Indeed, by applying this design we succeeded to classically condition oxytocin release. This response was, however, short-lasting and only a modest indication for a conditioned response in brain activity was found. Potentially, a longer acquisition phase would trigger a stronger and more long-lasting conditioned effect, but more research is needed to demonstrate that. Additionally, partial reinforcement, e.g. presenting the unconditioned stimulus at certain points of the evocation phase to reestablish the association between US and CS, could be applied to decrease extinction processes (34). So far this principle has not been studied in the context of hormonal conditioning. To be able to apply conditioning of hormones in clinical practice, it is necessary to be able to reliably induce clinically significant hormonal release and to find ways to avoid fast extinction of the conditioned hormonal responses.

Moreover, the brain mechanisms that trigger endocrine conditioning remain an underexplored topic. Our study from Chapter 4 was a first attempt to explore the brain responses accompanying conditioning of, in this case, oxytocin. The timing of the fMRI experiment is an important limitation of the current work, as the conditioned response in saliva had already gone extinct by the third evocation day when the scanning was done. However, including an fMRI scanning into a conditioning design might be an important confounding factor, as the whole MRI environment can be stressful for participants and might elicit hormonal changes, such as an increase in cortisol levels. Therefore, to minimize these possible confounding effects, we performed the scanning at the end of the whole experiment. Possibly, because of the timing, the effects of conditioning on brain activity were weak and not generalizable across the tasks. Another important limitation is the controversy about the efficacy of exogenous oxytocin administration. There is an ongoing debate in the literature about how intranasal oxytocin reaches the brain and via which

pathways, central or peripheral, it influences behavior. Leng (35) points out that according to mostly animal studies that measured oxytocin levels in cerebrospinal fluid after intranasal oxytocin administration, only a small fraction of the hormone reached cerebrospinal fluid and it is unclear how these small amounts can influence behavior. Moreover, the timing of the behavioral effects remains a point of discussion. Up to this moment only one study measured levels of oxytocin in cerebrospinal fluid in humans after intranasal oxytocin administration (36). This study found a significant increase in oxytocin levels only 75 minutes after the nasal spray administration, which is later than most behavioral and neural effects that are usually recorded at 45 minutes after administration (e.g., 37, 38, 39) and the conditioned oxytocin release in saliva that was already present at 5 minutes after the CS administration, as we demonstrated in Chapter 3. However, Striepens and colleagues (36) included only 3 or 4 participants per measurement and it remains unclear how generalizable the results collected on such a small sample are. Still, this inconsistency between the peak of cerebrospinal fluid levels and behavioral and neural results is disturbing, and therefore, more research on the pharmacokinetics of intranasal oxytocin is required.

Finally, the studies described in this dissertation failed to find any behavioral effects of oxytocin: exogenous oxytocin administration did not influence perceived facial trustworthiness and attractiveness in the experiment described in Chapter 3, pain sensitivity in Chapters 3 and 5, and no effects of oxytocin on the placebo and nocebo effects were found in Chapters 5 and 6. Regarding the facial trustworthiness and attractiveness task, these were not the primary outcomes of our studies and the experiments were not specifically powered to detect these effects. This could be a possible explanation of the null results. There is also a lot of contradiction in the literature regarding possible analgesic effects of oxytocin: some studies found that oxytocin decreases pain sensitivity (40), while other studies could not confirm these findings (41, 42). Our study contributed to the growing body of evidence about the absence of analgesic effects of oxytocin. Additionally, the effects of oxytocin on brain activity in response to the crying baby sounds and pain stimulation were found only sporadically on the second (group) level analysis and not when a comparison between the groups was made in Chapter 4. The fMRI study from Chapter 4 demonstrated

that oxytocin modified brain responses to the presentation of fearful faces, stimuli previously used in oxytocin research (43-45), and consistently with our hypotheses decreased the activation in the amygdala and the superior temporal gyrus. However, these effects were not found in the other two tasks previously shown to be affected by oxytocin on the between-group comparison. A possible limitation could be the timing of the tasks. Most of the previous research that used these tasks, administered them 45 minutes after oxytocin administration (46, 47). However, our scanning session was longer: the crying sounds task and pain task followed the structural scans and the task with emotional faces. The crying sounds task started around 65 minutes after the spray administration and the pain task- around 80 minutes after. Spengler and colleagues (37) recently showed that the strongest oxytocin effects on brain activation are found in the window between 45 and 70 minutes after oxytocin administration. Therefore, possibly due to the delay in time, the effects of the drug might have already been decreased in our experiment.

Clinical implications

In this dissertation, we addressed several issues that have possible implications, both for the theoretical development of the field and for future implementation of placebo effects in clinical practice.

In Part I of the dissertation, we summarized the knowledge on conditioning of endocrine responses and for the first time demonstrated that oxytocin can be classically conditioned in humans. Hormones underlie almost all physiological processes in the organism: from metabolism to reproduction. Hormonal disruptions are involved in a wide range of disorders: diabetes, thyroid disease, adrenal insufficiency, polycystic ovary syndrome and so on. For the treatment of most of these diseases, patients have to take either synthetic versions of hormones they lack (for example synthetic thyroxine in hypothyroidism) or a medication that regulates the imbalance caused by hormonal malfunctioning (for example, metformin that decreases glucose levels in diabetes type-2). Finding ways to stimulate endogenous production of hormones, therefore, might be very useful for optimizing the treatment of endocrine disorders. For example, patients with diabetes type-2 suffer from malfunctioning of the insulin system and slowly develop inability to secrete insulin. In our systematic review, we demonstrated that there is convincing evidence that insulin effects (including insulin release and glucose reduction) can be classically

conditioned. Boosting the organism's production of insulin by a conditioning manipulation, might have great potential benefits for these patients. Another hormone that has been shown to be malleable by classical conditioning, is cortisol. Classically conditioned cortisol responses might be used to boost psychotherapy of phobic patients as cortisone (a prodrug of cortisol) can have fear-reducing effects in this group of patients (48). Stimulating endogenous oxytocin production with classical conditioning, demonstrated in Chapter 3, has also many possible clinical implications. Oxytocin has been extensively investigated as a treatment for a range of mental disorders, such as autism (49), schizophrenia (50), and post-traumatic stress disorder (51). Potentially, classical conditioning of oxytocin responses could be added to the treatment of these patients to improve their social functioning.

Even though conditioned endocrine responses have not been investigated in the context of treatment, there are a few successful examples in which pharmacological conditioning has been shown to be beneficial in clinical practice. Pharmacological conditioning has been applied in placebo-controlled dose reduction studies, i.e., a treatment schedule in which a part of the pharmacological treatment is replaced by a placebo while maintaining the efficacy of the treatment (52). It is hypothesized that patients develop a conditioned reaction in response to their medicine intake, and if a part of the medicine gets replaced with placebo, the treatment efficiency would remain as a result of the conditioned response. Ader and colleagues (53) employed a placebo-controlled dose reduction treatment schedule for the treatment of psoriasis. They demonstrated that the group that received a placebo-controlled dose reduction treatment had a greater skin lesion reduction than the group whose treatment dose was simply reduced, although both groups received the same treatment dose overall, and that this dose-reduction treatment was as efficient as the full-treatment group. Similar results have been demonstrated by Sandler and colleagues (54) in the treatment of attention deficit and hyperactivity disorder. They found that children in a placebo-controlled dose reduction group, where 50% of active medication was replaced with placebo, remained stable during the treatment and did not differ in the severity of symptoms from the group that received the full dose of medication.

Classical conditioning can be used not only for reducing the dosages of medication but also for enhancing the effects of standard treatments. Kirchhof and colleagues (55) have employed a conditioning paradigm to the immunosuppression treatment of renal transplant patients. Immunosuppressive medication was paired with a gustatory stimulus during the acquisition phase and during the evocation phase patients were re-exposed to the gustatory conditioned stimulus. The authors have demonstrated that the group that was exposed to the conditioning procedure in addition to their standard treatment had a reduced T-cell proliferative capacity in comparison to the standard treatment group. These results demonstrate that adding pharmacological conditioning to a standard treatment can improve the efficiency of this treatment. As we have demonstrated in this dissertation, pharmacological conditioning has a potential to elicit conditioned hormonal responses in healthy participants. If this holds true for patients, these methods might be very beneficial for the treatment of hormonal disorders. Placebo-controlled dose reduction and adding conditioning to standard treatment are two methods that can be applied to patients who require hormonal treatments.

Directions for future research

The present dissertation raised several new questions about the link between the endocrine system and placebo effects. First of all, as we have demonstrated in the systematic review, the field of classical conditioning of the endocrine system has a long history and has drawn a lot of attention in animal physiological research. At the same time, human studies on this topic remain limited with a strong focus on the conditionability of cortisol and insulin effects. In Chapter 3, we demonstrated that another hormone, oxytocin, can be also classically conditioned. An important theoretical implication of our finding is that we pointed out that possibly more hormones can be successfully modified by this conditioning paradigm. However, more research into conditioning of other hormones besides cortisol and insulin responses is needed to be able to generalize the animal research to humans.

Another question that was raised from the results of Part I of the dissertation concerns the optimal conditioning design. In the systematic review from Chapter 2, we were unable to describe an optimal conditioning protocol that would guarantee successful and long-lasting endocrine conditioning. The main

reason was the heterogeneity of the designs used in previous research and a lack of studies that would compare effects of several conditioning procedures on the conditioned hormonal responses. There is no agreement on the number of acquisition sessions necessary to induce conditioned endocrine responses and the number of evocation sessions necessary to investigate the extinction of them. It might be, however, impossible to develop one standard protocol, as conditioning depends on various factors: the conditioned stimulus used, the hormonal system investigated, the outcomes measured, etcetera. Nevertheless, it would be very relevant for future research to focus on developing clear conditioning designs per endocrine system.

Another interesting finding of the RCT described in Chapters 3 and 4 is the possible presence of responders and non-responders in the conditioned oxytocin responses. Overall, a significant conditioned oxytocin release has been found on the first evocation day. However, large standard deviations of oxytocin levels in the conditioned group indicated that not all participants demonstrated a conditioned oxytocin release. This is not the first study on pharmacological conditioning that demonstrated the presence of non-responders. Petrakova and colleagues (10) found overall no significant conditioned cortisol increase in the study aimed at conditioning of cortisol. However, they showed that some of the participants did respond to the conditioning manipulation. Also Ober and colleagues (56) showed a presence of non-responders in a study on classical conditioning of immunosuppression. We were unable to find variables that would explain the differences between responders and non-responders. Neither baseline oxytocin levels nor psychological characteristics of participants such as personality and affect were related to the conditioned response. Several other factors, however, could have played a role. For instance, genetic factors might influence responsiveness to oxytocin conditioning. There is evidence that variations in the oxytocin receptor gene influence the effects of oxytocin administration on brain activity (57). Possibly these genetic variations make some people more sensitive to oxytocin administration and this sensitivity potentially could also influence conditioning with oxytocin. Moreover, baseline hormonal levels might also influence hormonal conditioning. Even though we did not find an association between baseline oxytocin levels and conditioned response, it does not exclude other hormonal factors that could have influenced our results.

For instance, there is a complex interplay between the stress hormone cortisol and oxytocin: these hormones have been shown to have a positive correlation (58) and be released at the same time in response to stress (59), with oxytocin down-regulating cortisol-induced stress responses. Therefore, cortisol might have an influence on the secretion of oxytocin and the variability in baseline cortisol levels could explain responders and non-responders to conditioning with oxytocin. We believe that in order to be able to apply hormonal conditioning to clinical practice, it is crucial to understand why not all people are susceptible to pharmacological conditioning. Therefore, it is important that future research focuses on factors that could help distinguish responders from non-responders.

Another relevant theoretical question was raised in Part II of the dissertation. Despite the lack of evidence that oxytocin influences the placebo and nocebo effect, it is at this moment impossible to rule out any role of oxytocin in placebo responding. In our experiments we used endogenous oxytocin administration to test our hypothesis. Future research could further look into the possibility that endogenous oxytocin levels might underlie placebo effects. As oxytocin is known for its prosocial functions, future research may consider more social aspects in placebo induction, such as warmth of the contact between patient and doctor, trust and empathy (60-62). Possibly, oxytocin might increase placebo effects when a longer and more emphatic interaction between the participant and experimenter/doctor is involved. Additionally, oxytocin might play a role in observational learning of placebo and nocebo effects. It has been demonstrated that oxytocin decreases brain activity in the pain circuitry when seeing pain in others (63); therefore, it might have potential to decrease nocebo effects learned by observation.

It is also important that future studies look for other endocrine moderators of placebo and nocebo effects. For instance, vasopressin seems to be a promising candidate, as Colloca and colleagues (27) have found that it enhances placebo analgesia in women only. Moreover, sex differences in the placebo response have been recently described in placebo responding: men seem to respond stronger to placebo treatment and women to nocebo treatment (64, 65). This can indicate a possible important role of sex hormones in placebo effects.

Finally, there are several possible implications of the knowledge from this dissertation for clinical practice. Since no studies so far applied classical conditioning to patients with endocrine disorders despite the promising results coming from the studies on immune conditioning, it would be relevant for future research to investigate whether classical conditioning can influence hormone levels not only in healthy participants, but also in patients with endocrine disorders, for example in patients with diabetes who suffer from malfunctioning of the insulin system. In case of promising results of such studies, it could be tested whether placebo-controlled dose reduction and conditioning boosting of the treatment can be applied to improve the treatment of endocrine disorders and reduce side effects of the standard treatment schedules.

Conclusions

In the present dissertation we studied the possible connection between placebo effects and oxytocin from two perspectives: conditioning of oxytocin and enhancing placebo effects by oxytocin. We have demonstrated that endocrine parameters can be manipulated by classical conditioning in animals and humans and that it is possible to classically condition endogenous oxytocin release. We furthermore found no support that oxytocin increases placebo and decreases nocebo responses. Understanding how we can influence the endocrine system with placebo effects and, alternatively, how we can affect placebo effects with hormones is important to enable starting to use placebo effects in clinical practice. Applying placebo effects in medical treatments, for example, for endocrine conditions, has a great potential to improve the efficiencies of standard treatment protocols, decrease costs of medications, and minimize side effects.

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Nederlandse Samenvatting

Placebo effecten zijn positieve effecten van behandelingen die niet door de actieve ingrediënten van een behandeling optreden maar door mechanismen zoals positieve verwachtingen over een behandeling.

Placebo effecten beïnvloeden niet alleen symptomen van verschillende ziekten maar ook fysiologische processen zoals de afscheiding van hormonen.

Het doel van dit proefschrift is om het verband tussen het endocriene systeem en placebo effecten te onderzoeken, met daarbij een speciale focus op het hormoon oxytocine. Oxytocine is een hormoon dat veel positieve effecten heeft: het vermindert stress en angst en verhoogt vertrouwen. Het verband tussen oxytocine en placebo effecten is vanuit twee perspectieven onderzocht: het oproepen van placebo effecten in het endocriene systeem (**Deel I**) en de invloed van hormonen op placebo en nocebo effecten (**Deel II**).

Deel I van dit proefschrift focust op het oproepen van placebo effecten in het endocriene systeem door middel van het leerprincipe klassieke conditionering, een belangrijk onderliggend mechanisme van placebo effecten. In **Hoofdstuk 2** wordt een systematische literatuur review beschreven waarin we hebben onderzocht of het mogelijk is om hormonale veranderingen op te roepen met behulp van klassieke conditionering. Bij klassieke conditionering van hormonale veranderingen wordt een twee-fase conditioneringsparadigma gebruikt. In de eerste fase wordt een associatie aangeleerd: een hormonale medicatie wordt gekoppeld aan een geconditioneerde stimulus (bijvoorbeeld een geur). In de tweede fase wordt de geconditioneerde stimulus gegeven in combinatie met een placebo en worden de lichamelijke reacties op deze geconditioneerde stimulus gemeten. In dit review werd overtuigend bewijs gevonden dat endocriene veranderingen kunnen worden geconditioneerd in dieren. Deze resultaten van dierstudies zijn tevens in overeenstemming met die van studies bij mensen. Echter, het onderzoek bij mensen is beperkt tot het conditioneren van insuline, cortisol en groeihormonen. Over het algemeen toont dit review aan dat conditionering van het endocriene systeem veelbelovend is. Er is echter geen consensus over een standaard design, het onderzoek bij mensen is beperkt en niet alle endocriene systemen zijn onderzocht. Het doel van de beschreven studies in **Hoofdstukken 3 en 4** was om de kennis zoals beschreven in de systematische review uit te breiden en te onderzoeken of het mogelijk is om oxytocineresponsen te conditioneren. In **Hoofdstuk 3** beschrijven we de resultaten van een gerandomiseerde en gecontroleerde

studie (RCT) in gezonde vrouwen en laten we zien dat oxytocineresponsen opgeroepen kunnen worden met behulp van klassieke conditionering. Daarnaast doofden deze responsen na conditionering in het laboratorium binnen enkele dagen uit. Er was geen effect van conditioneren op taken die in eerder onderzoek door oxytocine beïnvloed werden, zoals een taak waarin gezichten moesten worden geëvalueerd of op een taak voor pijngevoeligheid. In **Hoofdstuk 4** wordt het neuroimaging deel van deze conditioneringsstudie beschreven. Er werden aanwijzingen gevonden voor geconditioneerde responsen in hersenactiviteit, met name in de amygdala en de superior temporal gyrus in reactie op angstige gezichten. Deze geconditioneerde effecten waren echter klein in vergelijking met de effecten van extern toegediende oxytocine en werden bovendien niet op andere taken gevonden.

Deel II van dit proefschrift beschrijft het verband tussen placebo en nocebo effecten en oxytocine. Er werd onderzocht of oxytocine placebo en nocebo effecten beïnvloedt. **Hoofdstuk 5** beschrijft de bevindingen van een RCT, waarin we in gezonde vrouwen hebben onderzocht of het mogelijk is om placebo effecten in pijn en jeuk te versterken met behulp van oxytocine. In deze studie bleken verbale suggesties over de vermindering van pijn en jeuk middels een neusspray een significante placebo analgesie op te roepen, maar hadden deze suggesties geen effect op de jeukgevoeligheid. Bovendien werd er geen placebo- versterkend effect van oxytocine gevonden. Om de beperkingen van deze studie nader te onderzoeken, hebben we een follow-up studie ontworpen, die in **Hoofdstuk 6** beschreven staat. In deze follow-up studie hebben we de effecten van oxytocine op placebo en nocebo effecten onderzocht bij pijn, dit keer in gezonde mannen en met een hogere dosering van oxytocine dan in de studie in Hoofdstuk 5. Daarnaast zijn in deze studie placebo en nocebo effecten geïnduceerd door verbale suggesties te combineren met een conditioneringsprocedure. De bevindingen van deze studie zijn vergelijkbaar met die zoals beschreven in Hoofdstuk 5: Een combinatie van klassieke conditionering en verbale suggesties riep placebo en nocebo effecten op, terwijl oxytocine geen aanvullend effect had op placebo en nocebo effecten, of op de uitdoving hiervan. Met deze twee studies zoals beschreven in Hoofdstukken 5 en 6 kunnen we concluderen dat er geen bewijs is dat verschillende doseringen van oxytocine een invloed hebben op placebo of nocebo effecten bij vrouwen en mannen.

Samenvattend werd in dit proefschrift een verband tussen placebo effecten en het endocriene systeem onderzocht met een speciale focus op oxytocine. Vanuit de literatuur werd er bewijs gevonden dat placebo effecten veranderingen in het endocriene systeem kunnen oproepen. Daarnaast werd aangetoond dat oxytocineresponsen oproepen kunnen worden door middel van klassieke conditionering. Ten slotte was er geen bewijs dat oxytocine een invloed heeft op placebo en nocebo effecten. Vervolgonderzoek naar het verband tussen placebo effecten en het endocriene systeem kan bijdragen aan mogelijke toepassingen waarin placebo effecten in de klinische praktijk kunnen worden gebruikt.

Curriculum Vitae

Aleksandrina Skvortsova was born on July 11, 1987 in Kokchetav, former USSR, and grew up in Estonia. She attended Narva High School #6 in Estonia between 2003 and 2006. Aleksandrina did her Bachelor's degree in Clinical Psychology in Saint-Petersburg State University between 2006 and 2011. Afterwards she worked for a year as a clinical psychologist in Narva Hospital, in Estonia. Pursuing her interest in science, she moved to the Netherlands and did a Research Master in Behavioural Sciences at the Radboud University, Nijmegen between 2012 and 2014. During her master degree, she performed two independent research projects and did a research internship in the Center of Anxiety and Related Disorders of Boston University, the USA. Additionally, she was involved in a junior research project of The European Federation of Psychology Students' Associations.

After receiving her Master degree, Aleksandrina started her PhD-project under the supervision of professor Andrea Evers, dr. Judy Veldhuijzen and dr. Henriët van Middendorp at Leiden University. Aleksandrina worked on a project sponsored by the European Research Consolidator Grant of Andrea Evers "Empowering expectations for health and disease: Training the immune and endocrine system" and investigated the link between placebo effects and the endocrine system. In addition to the research activities, she supervised multiple bachelor and master students and was a member of the organizing committee of the 2nd Official Society for Interdisciplinary Placebo Studies Conference which took place in July 2019 in Leiden, the Netherlands.

Currently, Aleksandrina works as a postdoctoral researcher and investigates the possibility to classically condition insulin responses and the role of sex differences in diabetes patients.

Articles in peer reviewed journals:

Skvortsova A, Veldhuijzen DS, van Middendorp H, Colloca L, Evers AWM. Effects of oxytocin on placebo and nocebo effects in a pain conditioning paradigm: a randomized controlled trial. *J of Pain*. 2019 (in press).

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Articles submitted for publication:

Skvortsova, A., Veldhuijzen, D.S., de Rover, M., Pacheco-Lopez, G., Bakermans-Kranenburg, M., van IJzendoorn, M., Chavannes, N.H., van Middendorp, H., Evers, A.W.M. (2019). Effects of oxytocin administration and conditioned oxytocin on the perception of emotional faces, crying baby sounds and heat pain: an fMRI study. Manuscript submitted for publication.

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