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Placebo Effects in the Neuroendocrine System: Conditioning of the Oxytocin Responses

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ABSTRACT

Objective: There is evidence that placebo effects may influence hormone secretion. However, few studies have examined placebo effects in the endocrine system, including oxytocin placebo effects. We studied whether it is possible to trigger oxytocin placebo effects using a classical conditioning paradigm.

Methods: Ninety-nine women were assigned to a conditioned, control, 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]) for three acquisition days, whereas the control group received placebo spray. Subsequently, the conditioned and control groups received placebo spray with the CS and the drug control group received oxytocin spray for three evocation days. Salivary oxytocin was measured several times during each day. Pain sensitivity and facial evaluation tests previously used in oxytocin research were also administered.

Results: On evocation day 1, in the conditioned group, oxytocin significantly increased from baseline to 5 minutes after CS (B[slope] = 19.55, SE = 5.88, $p < .001$) and remained increased from 5 to 20 ($B = -10.42$, SE = 5.81, $p = .071$) and 50 minutes ($B = -0.70$, SE = 3.37, $p = .84$). On evocation day 2, a trend for increase in oxytocin was found at 5 minutes ($B = 15.22$, SE = 8.14, $p = .062$). No placebo effect was found on evocation day 3 ($B = 3.57$, SE = 3.26, $p = .28$). 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, such as 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).

Key words: 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), and depression (3). There are also studies that indicated that placebo affects not only subjective symptoms but also 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

ANCOVA = analysis of covariance, **ANOVA** = analysis of variance, **BMI** = body mass index, **CPT** = cold pressor task, **CS** = conditioned stimulus, **MRI** = magnetic resonance imaging, **US** = unconditioned stimulus

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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.

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, although 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 has 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). Oxytocin has especially attracted a lot of attention for its prosocial effects. However, despite that it has been proposed to play an important role in emotion recognition (14), emotional contact (15), and stress responsiveness (16), these results have been heavily criticized for low statistical power and possible biases (17). No consensus has been reached as of yet on the role of oxytocin in human emotional or human social behavior. Nevertheless, disruption of oxytocin responses has been found in several mental disorders such as autism (18), schizophrenia (19), borderline disorder, (20) and posttraumatic stress disorder (21), and currently, oxytocin is investigated as a treatment for these conditions (22–24). In case this potential is proven to be beneficial for patients, being able to influence oxytocin release with placebo manipulation would open additional perspectives for the treatment of conditions related to emotional deficits. However, it is important to note again that the exact relationship between oxytocin and prosocial behavior is unclear at this moment and needs careful and critical consideration.

So far, only one study demonstrated that oxytocin levels can be manipulated by classical conditioning in rats (25). In this randomized controlled trial, we investigated whether is it possible to trigger placebo oxytocin release by using 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 nonreinforced evocation trials.

METHODS

Participants

Ninety-nine healthy women participated in the study. Only women who were taking oral contraceptives were included because they have stable levels of oxytocin during the cycle (26). All women were tested while in their pill weeks and not in their stop weeks. Exclusion criteria were as follows: 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 2 weeks of testing, drinking alcohol and doing physical exercise 24 hours before each session, and drinking caffeinated drinks and eating a meal 2 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 part of their monetary reward (adding up to 200 euros in total for study completion).

Sample size was calculated on the basis of a pilot study performed in our laboratory aimed at conditioning of cortisol with a similar study design (27). 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: a) conditioned group, b) control group, and c) 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 control group.

In line with previous conditioning studies (7,10), a randomized placebo-controlled conditioning paradigm consisting of two phases (acquisition and evocation phases) was applied (Figure 1). Both acquisition and evocation phases lasted for three consecutive days with a 4-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 (24 IU 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 (28) 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 control 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 laboratory during the evocation phase to eliminate possible conditioning effects triggered by the CS and the environment of the CS administration. This was done to avoid a conditioned response in the drug control group.

The study was approved by Medical Ethical Committee of Leiden University Medical Center (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 nine participants per block.

Procedure

Screening

Upon arrival at the laboratory, 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

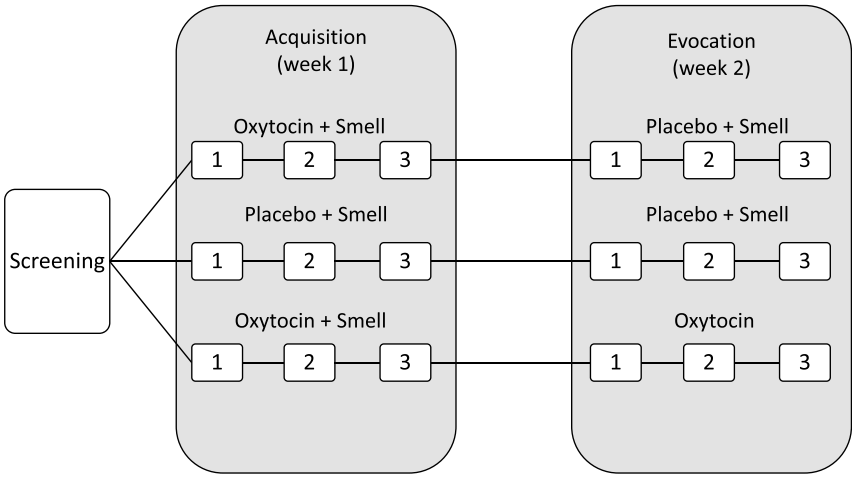


FIGURE 1. Study design.

was taken to establish baseline oxytocin levels. Afterward, 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 magnetic resonance imaging (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 PM and 6 PM. Upon arrival to the laboratory, a baseline saliva sample was taken and the participants were asked questions about their health and food and alcohol consumption. Afterward, 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 (Figure 2).

Evocation Phase

All evocation sessions started at the same time as acquisition sessions between 2 PM and 6 PM. Identical to the acquisition days, upon arrival at the laboratory, a baseline saliva sample was taken. Then depending on group allocation, participants were administered the CS and a placebo spray (in the conditioned and control groups) or oxytocin without the CS in a different setting (in the drug control group to avoid triggering the conditioned response and measure the response to the drug only). 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 were 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 Center. Details of the functional MRI part of this experiment will be reported separately. After the end of the last evocation day, participants were fully debriefed (Figure 2).

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 US was 24 IU of oxytocin (Syntocinon Spray) or a placebo nasal spray. The placebo spray looked and tasted identically to oxytocin and was

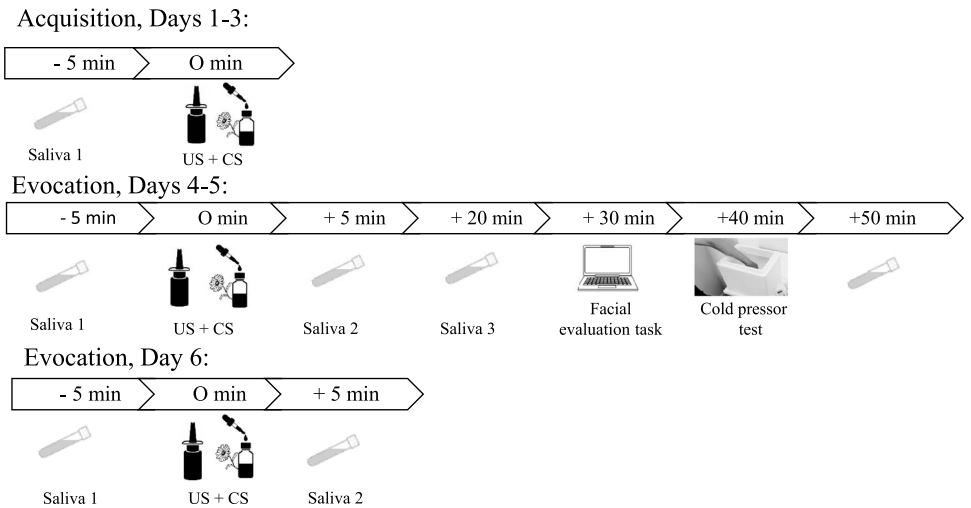


FIGURE 2. Timeline of the experimental days. US = unconditioned stimulus; CS = conditioned stimulus.

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, North Carolina).

Conditioned Stimulus

The CS was the administration of a smell of rosewood oil for 1 minute immediately before and 1 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 L/min through a jar with five 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, to prevent the smell from spreading into the room.

Materials and Measurements

Oxytocin levels were measured in saliva. 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 enzyme-linked immunosorbent assay kits with extraction (Enzo Life Sciences, Farmingdale, New York) 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 (29). This method is consistent with currently recommended best practices (30). Lower level of detection for oxytocin was 0.5 pg/ml after extraction; extraction efficiency was 99%; and intra-assay and interassay coefficients of variation were 10.2% and 11.8%, respectively.

The *CPT* was used to assess pain sensitivity. The waterbath consisted of a 2.7-L 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 to 10 scale with decimals (0, no pain at all; 10, worst pain ever experienced). The pain intensity scores in response to the baseline and postintervention CPT were calculated as the mean scores of the four pain rating measurement points during each CPT.

A *heat pain task* was used to determine individual temperatures that would cause a painful but bearable sensation (equal of 6 points on the 11-point numeric scale with 0 indicating no pain at all and 10 indicating worst pain ever experienced). Pain stimuli were administered using a standardized heat pain application device (ATS thermode; Medoc Advanced Medical Systems, Ramat Yishai, Israel). The thermode was attached to the dorsal site of the left arm of the participants. Participants received a sequence of ascending temperatures with a peak temperature lasting for 5 seconds and an interstimulus interval of 15 seconds and were asked to evaluate how painful each stimulus was. Stimuli that were given a pain score of 6 were used during the functional MRI part of the experiment that will be reported separately.

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 seven-point Likert scale (1, not attractive/trustworthy; 7, extremely attractive/trustworthy). In total, 32 pictures from the Radboud Faces Database (31) 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 (32). 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 (33). 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 (34). The Hospital Anxiety and Depression Scale is divided into two subscales: the depression subscale and the anxiety subscale, both containing eight items. The score per scale ranges from 0 to 8, with higher scores indicating higher depression and anxiety.

Statistical Analysis

To compare the groups on baseline characteristics such as baseline oxytocin levels, age, body mass index (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 participant. We used the lmer function of the lme4 package (35) 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 participants (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 (36), we determined conditioning responders and nonresponders 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 (37). This was separately done for evocation sessions 1, 2, and 3. Responders and nonresponders 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) using *t* tests. In addition, we used Pearson correlations to see whether there was an association between these variables and the endogenous oxytocin change in the conditioned group on evocation session 4.

To investigate the effects of the manipulations on the pain sensitivity in response to the CPT, a repeated-measures 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 CPT 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 ($F(2,96) = 0.76$, $p = .47$), BMI ($F(2,96) = 0.85$, $p = .43$), baseline pain sensitivity in response to the CPT ($F(2,95) = 0.30$, $p = .74$), and questionnaires measuring

TABLE 1. Baseline Characteristics Across the Groups With SDs

	Conditioned Group (n = 33), M (SD)	Control Group (n = 33), M (SD)	Drug Control Group (n = 33), M (SD)
Age, y	21.2 (2.8)	21.7 (1.9)	21.9 (2.5)
Body mass index, kg/m^2	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)

M = mean.

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).

Endogenous Oxytocin Release

Because of 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 carryover 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 ($B = 19.55$, SE = 5.88, $t(187) = 3.33$, $p < .001$), followed by a trend to decrease from 5 to 20 minutes ($B = -10.42$, SE = 5.81, $t(187) = -1.79$, $p = .071$) and nonsignificant changes 20 to 50 minutes ($B = -0.70$, SE = 3.37, $t(187) = -0.21$, $p = .84$) after the placebo spray with CS administration. In the control group, the changes from the baseline to 5 minutes after the placebo spray with CS administration ($B = -1.82$, SE = 5.79, $t(187) = -0.31$, $p = .75$), 5 to 20 minutes ($B = -1.21$, SE = 5.75, $t(187) = -0.21$, $p = .83$), and 20 to 50 minutes ($B = -3.45$, SE = 3.36, $t(187) = -1.03$, $p = .31$) 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, whereas no such increase was found in the control group. The oxytocin levels in the drug control group greatly increased from baseline to 5 minutes after oxytocin administration ($B = 1686$, SE = 236.80, $t(272) = 7.12$, $p < .001$) and then significantly decreased from 5 to 20 minutes ($B = -736.15$, SE = 161.47, $t(272) = -4.6$, $p < .001$) and from 20 to 50 minutes ($B = -212.41$, SE = 66.78, $t(272) = -3.18$, $p < .001$) after the spray administration.

On evocation day 2, a trend toward a significant increase of oxytocin levels from baseline to 5 minutes after the CS administration was found ($B = 15.22$, SE = 8.14, $t(187) = 1.87$, $p = .062$) followed by no change of oxytocin levels from 5 to 20 minutes ($B = -12.05$, SE = 7.80, $t(187) = -1.54$, $p = .12$) and a significant drop of oxytocin levels from 20 to 50 minutes ($B = -5.98$, SE = 1.87, $t(187) = -3.21$, $p < .001$) in the conditioned group. In

TABLE 2. Mean Salivary Oxytocin Levels (pg/ml) Across the Groups and Measurements With SDs

Session	Measurement	Conditioned Group (n = 33)	Control 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 min	31.5 (46)	14.6 (7.5)	1805.7 (2275.3)
	20 min	21.1(27.6)	13.4 (8.3)	1118.8 (1835.3)
	50 min	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 min	28.6 (64.4)	13.7 (6.5)	1595.7 (2139.6)
	20 min	16.5 (14.3)	12.6 (5.6)	1069.3 (1622.6)
	50 min	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 min	12.4 (7.3)	19.4 (42.6)	1762.9 (2519)

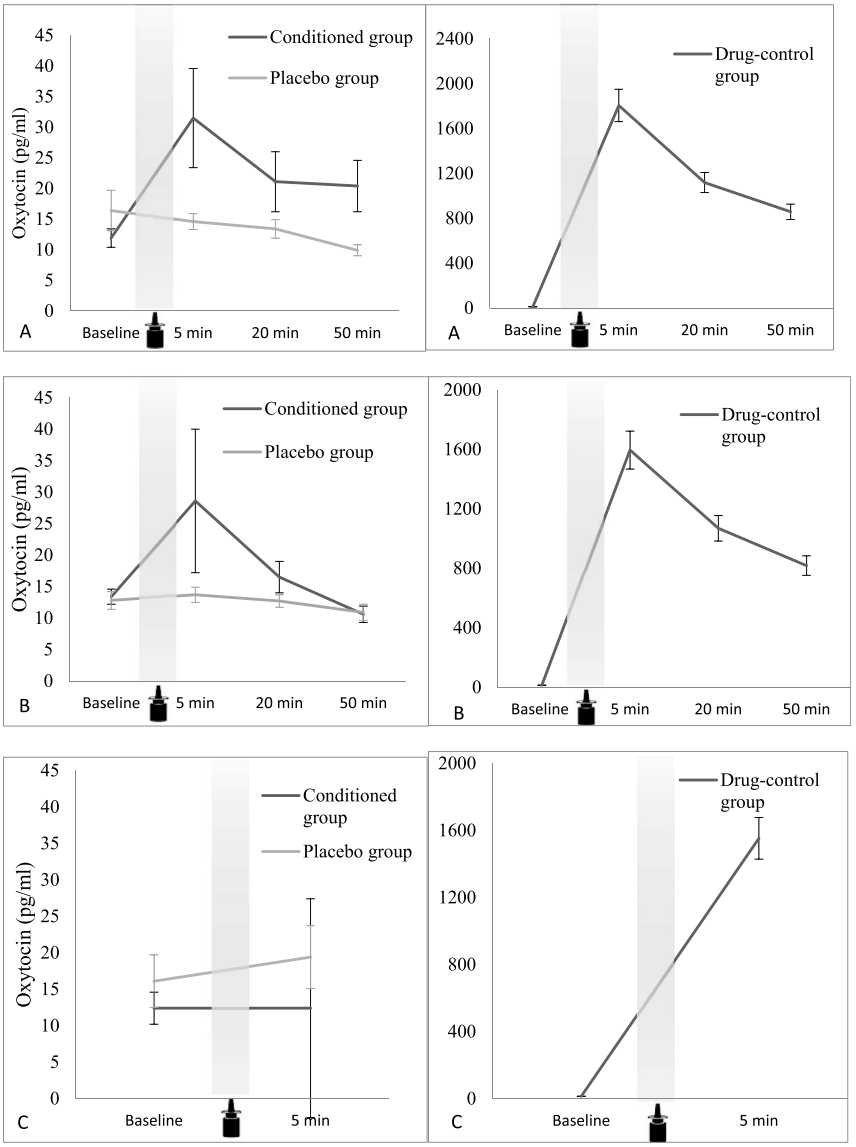


FIGURE 3. Salivary oxytocin levels (in picograms per milliliter) on evocation days 1 (A), 2 (B), and 3 (C) separately for each group (n = 99). Error bars indicate SEs. Color image is available only in the online version (www.psychosomaticmedicine.org).

the control group, the changes from the baseline to 5 minutes (B = 0.47, SE = 8.11, $t(187)$ = 0.06, p = .95), 5 to 20 minutes (B = −0.61, SE = 7.78, $t(187)$ = −0.08, p = .94), and 20 to 50 minutes (B = −1.76, SE = 1.84, $t(187)$ = 1.84, p = .34) were all not significant. This pattern indicated a trend for increased conditioned oxytocin levels in the conditioned group that remained present from 5 to 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, SE = 225.20, $t(272)$ = 6.57, p < .001), followed by a significant decrease from 5 to 20 minutes (B = −536.16, SE = 142.43, $t(272)$ = −3.76, p < .001) and from 20 to 50 minutes (B = −242.38, SE = 72.11, $t(272)$ = −3.36, p < .001).

Finally, on the evocation day 3, which 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, SE = 3.26, $t(59)$ = 1.10, p = .28) nor in the control group (B = 3.02, SE = 3.14, $t(59)$ = 0.96, p = .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, SE = 261.37, $t(87)$ = 5.94, p < .001).

The sensitivity analyses using a 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 in comparison with the control group ($F(1,61)$ = 7.84, p = .007, η_p^2 = 0.114) after controlling for the baseline levels. No effect of time ($F(2,61)$ = 0.642, p = .53, η_p^2 = 0.01) or a time by group interaction ($F(2,61)$ = 1.10, p = .56, η_p^2 = 0.01) on oxytocin levels during evocation session 1 was found. Salivary levels of the conditioned and control group did not significantly differ on evocation day 2 ($F(1,61)$ =1.76, p = .19, η_p^2 = 0.028). No significant effect of time ($F(2,61)$ = 0.86, p = .43, η_p^2 = 0.014) or a time by group interaction ($F(1,61)$ = 1.53, p = .22, η_p^2 = 0.024) on the salivary levels on evocation day 2 was found. Finally, the two groups

did not differ in the salivary oxytocin levels on evocation day 3 $F(1,61) = 1.27$, $p = .27$, $\eta_p^2 = 0.021$) after controlling for the baseline levels.

Participants in the conditioned group could be divided into responders and nonresponders. 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 nonresponders on baseline characteristics and questionnaires: age, BMI, baseline oxytocin levels, baseline pain sensitivity in response to the CPT, extraversion, neuroticism, optimism, pessimism, depression, and trait anxiety (all p values > .10). In addition, no significant correlations between the change in the endogenous oxytocin levels in the conditioned group on evocation session 4 and these baseline variables were found (all p values > .1).

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 (Table 3).

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 with 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).

DISCUSSION

The present study demonstrated for the first time that placebo effects can trigger endogenous oxytocin release. After 3 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 effect on the first evocation day as hypothesized. A trend toward 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 (37). No previous data on extinction of hormonal placebo effects in humans are available because 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 (38–40). 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 (39,40): the oxytocin levels increased 100 times (10,000%) from baseline after the oxytocin administration. The conditioned response was much smaller: salivary oxytocin levels increased two times (200%) compared with 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 (41), and a 17% increase was found in response to massage (42). These results show that classical conditioning can be an effective way to induce oxytocin release on demand; however, future research should investigate whether these conditioned responses are large enough to cause any clinically significant effects and whether effects of similar magnitude can be induced with fewer acquisition trials. Moreover, the large standard errors in the conditioned group indicate the presence of responders and nonresponders to the conditioning manipulation among the participants. These results add to the accumulative evidence on responders and nonresponders to pharmacological conditioning (10,36). 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 (36). Furthermore, baseline cortisol levels

TABLE 3. Mean Task Scores Across the Groups and Evocation Days With SDs

Task	Session	Conditioned Group	Control 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	Evocation 1	2.8 (0.5)	3.0 (0.6)	3.0 (0.6)
score	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)

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 conditioned endogenous oxytocin release and exogenous oxytocin on pain sensitivity and perceived facial attractiveness and trustworthiness. These two tasks were included into the experiment as additional measures of the behavioral and physiological effects of oxytocin conditioning. Previous studies reported that these parameters were influenced by exogenous oxytocin administration (43,44). Therefore, we expected that conditioned oxytocin responses could increase perceived facial trustworthiness and attractiveness and decrease pain sensitivity, mimicking th effects of exogenous oxytocin administration. We were unable to demonstrate that exogenous oxytocin administration or conditioned oxytocin release affects perceived facial attractiveness and trustworthiness or pain sensitivity in the current dose and method of administration. A previous study on the perceived facial attractiveness and trustworthiness included larger groups (44), and because 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. Although trust-enhancing effects of oxytocin have been demonstrated in a meta-analysis (45), Lane and colleagues (46) report several failures to replicate these results. Therefore, our results contribute to the mixed evidence regarding the trust-enhancing effects of oxytocin. 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 (47,48).

Several limitations of the current study have to be addressed. First of all, the results cannot be generalized to men and women who do not take hormonal contraceptives. Because 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 (28). Future research should expand our findings and examine whether endogenous oxytocin levels are conditionable in men and women who are not using contraception. Second, the conditioned responses we found were very short-lasting and seemed to start extinguishing already after the first evocation day. 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 (49). 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 intanasally administered oxytocin. Realizing this limitation, we did not directly compare the experimental group with the drug control group. One more limitation is that we did not measure the expectations of the participants regarding the treatment they received. Expectations are one of the important underlying mechanisms of placebo effect (3,11). Only one study so far measured the effect of expectations on conditioned hormonal responses and found no effect (9). Possibly, because the hormonal changes happen without conscious awareness, the role of expectations in endocrine conditioning is less important than in placebo effects of such subjective symptoms as pain. However, we cannot exclude the possibility that expectations of participants had an influence on our results. 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 (17,46,50) 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. In addition, an often-cited study on the trust-enhancing effects of oxytocin (51) has been criticized for not-standard interpretation of the statistics (52). Leng and Ludwig (53) discuss 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. In addition, the timing of behavioral effects (e.g., Refs. (54,55)) 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 (56). 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 a potential to improve social cognitions in autism (57), borderline personality disorder (23), and schizophrenia (22). Also, treatment with oxytocin was shown to increase insulin sensitivity and decrease weight in obese adults (58), reduce inflammation (59,60), and increase healing processes (61) in animals. However, other studies were unable to find beneficial behavioral effects of oxytocin (47,50), which is more in line with our findings. In sum, the findings on the clinical relevance of oxytocin are not conclusive because no consensus has been reached at this moment.

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 afterward, a part of their regular medication is replaced with placebo, following for example a partial reinforcement schedule (64). Because of 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 adverse 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, psoriasis, and chronic insomnia (62–64). 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. Using 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 adverse effects of standard treatments.

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