Chapter 3

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

Published as

Skvortsova A, Veldhuijzen DS, Pacheco-Lopez G, Meijer OC, Bakermans-Kranenburg M, van IJzendoorn M, Smeets MAM, Wilderjans TF, Dahan A, van den Bergh O, Chavannes NH, van der Wee NJA, Grewen KM, van Middendorp H, Evers AWM. Placebo effects in the neuroendocrine system: conditioning of the oxytocin responses. *Psychosom Med.* 2019 (in press)

Chapter 3
Conditioning of the oxytocin responses

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 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 investigated 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 is it 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.

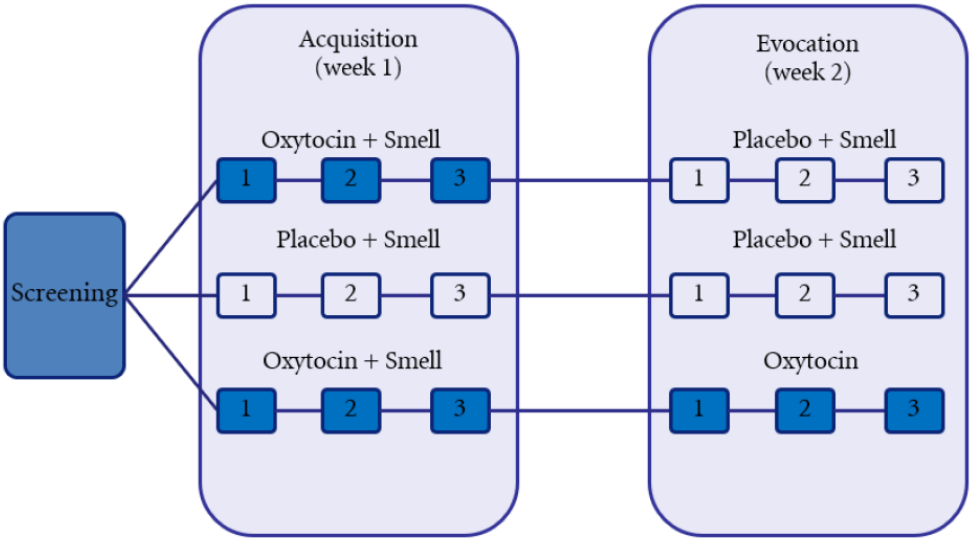

Figure 1. Study design

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.

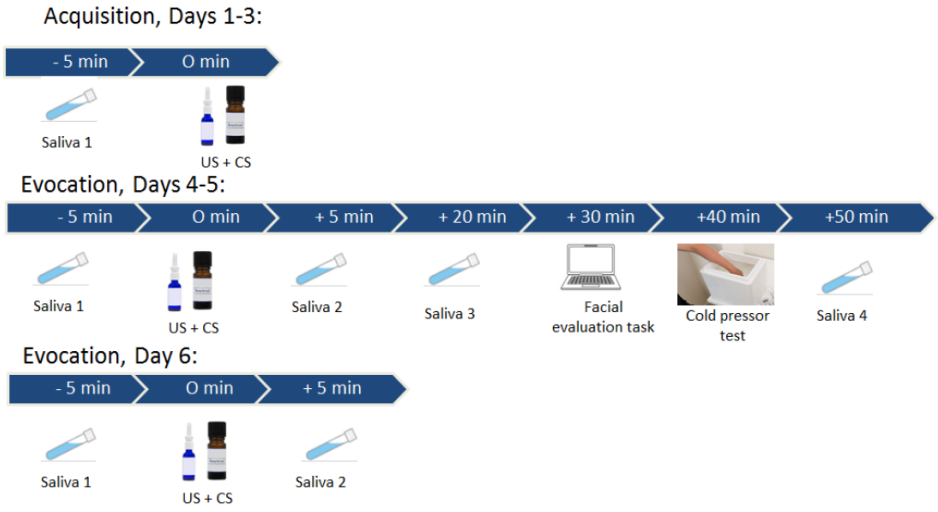

Figure 2. Timeline of the experimental days.

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) M (SD)	(n = 33) 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, ηp2 =0.114) after controlling for the baseline levels. No effect of time (F(2, 61)=0.642, p=0.53, ηp2 =0.01) or a time x group interaction (F(2, 61)=1.10, p=0.56, ηp2=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, ηp2 =0.028), No significant effect of time (F(2, 61)=0.86, p=.43, ηp2 = 0.014;) or a time x group interaction (F(1, 61)=1.53, p=.22, ηp2 =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, ηp2 =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)

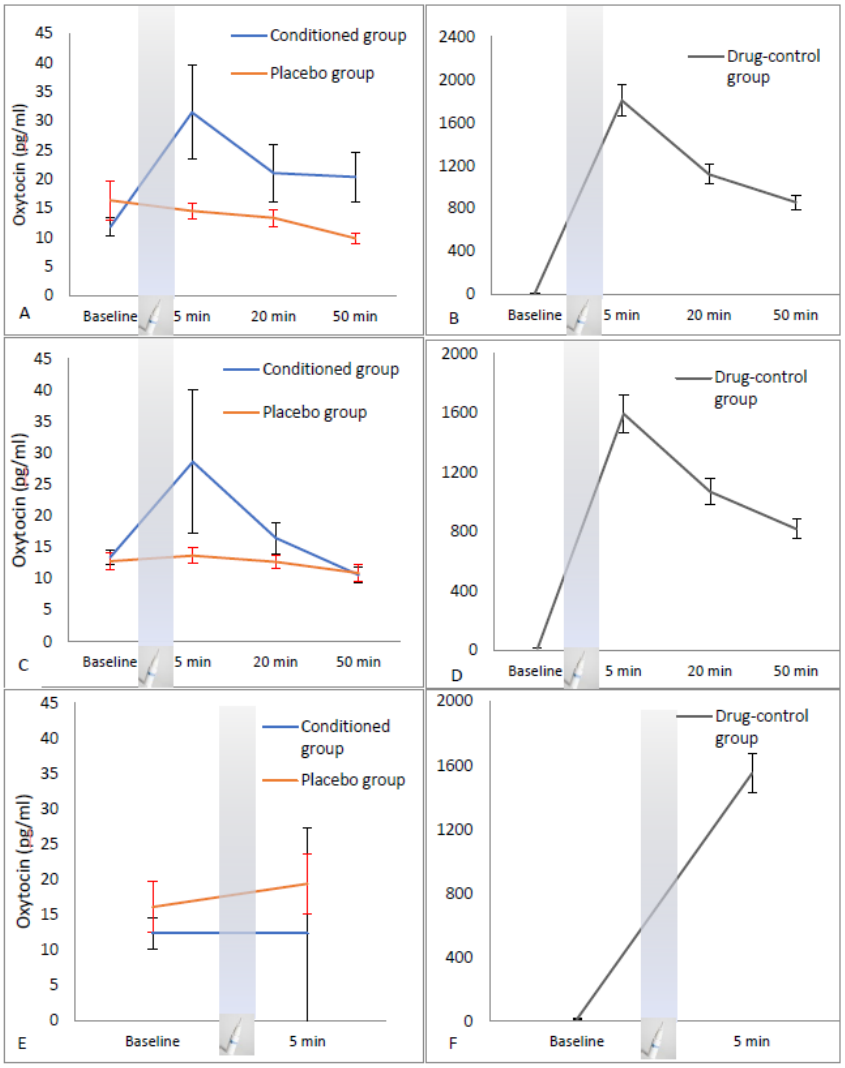

Figure 3. Salivary oxytocin levels (pg/ml) on evocation days 1 (A, B), 2 (C, D) and 3 (E, F) separately for each group (n= 99). Error bars indicate standard errors.

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	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	Evocation 1	4.0 (0.7)	4.0 (0.8)	4.1 (0.6)
score	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 intanasally 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) discuses 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.

References

1. Forsberg JT, Martinussen M, Flaten MA. The placebo analgesic effect in healthy individuals and patients: a meta-analysis. Psychosom Med. 2017;79(4):388-94.
2. Shaibani A, Frisaldi E, Benedetti F. Placebo response in pain, fatigue, and performance: possible implications for neuromuscular disorders. Muscle Nerve. 2017;56(3):358-67.
3. Pollo A, Carlino E, Benedetti F. Placebo mechanisms across different conditions: from the clinical setting to physical performance. Philos Trans R Soc Lond B Biol Sci. 2011;366(1572):1790-8.

4. Benedetti F, Amanzio M, Thoen W. Disruption of opioid-induced placebo responses by activation of cholecystokinin type-2 receptors. Psychopharmacology (Berl). 2011;213(4):791-7.
5. Benedetti F, Amanzio M, Rosato R, Blanchard C. Nonopioid placebo analgesia is mediated by CB1 cannabinoid receptors. Nat Med. 2011;17(10):1228.
6. Skvortsova A, Veldhuijzen DS, Kloosterman IE, Meijer OC, van Middendorp H, Pacheco-Lopez G, Evers AW. Conditioned hormonal responses: a systematic review in animals and humans. Front Neuroendocrinol. 2018.
7. Stockhorst U, de Fries D, Steingrueber HJ, Scherbaum WA. Unconditioned and conditioned effects of intranasally administered insulin vs placebo in healthy men: a randomised controlled trial. Diabetologia. 2011;54(6):1502-6.
8. Sabbioni ME, Bovbjerg DH, Mathew S, Sikes C, Lasley B, Stokes PE. Classically conditioned changes in plasma cortisol levels induced by dexamethasone in healthy men. FASEB J. 1997;11(14):1291-6.
9. Benedetti F, Pollo A, Lopiano L, Lanotte M, Vighetti S, Rainero I. Conscious expectation and unconscious conditioning in analgesic, motor, and hormonal placebo/nocebo responses. J Neurosci. 2003;23(10):4315-23.
10. Petrakova L, Boy K, Kügler M, Benson S, Engler H, Möller L, Schedlowski M. Plasma cortisol response cannot be classically conditioned in a taste-endocrine paradigm in humans. Psychopharmacology. 2017;234(21):3249-57.
11. Rief W, Bingel U, Schedlowski M, Enck P. Mechanisms involved in placebo and nocebo responses and implications for drug trials. Clinical pharmacology and therapeutics. 2011;90(5):722-6.
12. Bell AF, Erickson EN, Carter CS. Beyond labor: the role of natural and synthetic oxytocin in the transition to motherhood. J Midwifery Womens Health. 2014;59(1):35-42.
13. Gaudin S, Chaillou E, Wycke Ma, Cornilleau F, Moussu C, Calandreau L, Lainé A-L, Nowak R. All bonds are not alike: A psychoendocrine evaluation of infant attachment. Dev Psychobiol. 2018;60(1):90-103.
14. Bakermans-Kranenburg M, Van IJzendoorn M. Sniffing around oxytocin: review and meta-analyses of trials in healthy and clinical groups with implications for pharmacotherapy. Transl Psychiatry. 2013;3(5):e258.
15. Cong X, Ludington-Hoe SM, Hussain N, Cusson RM, Walsh S, Vazquez V, et al. Parental oxytocin responses during skin-to-skin contact in pre-term infants. Early Hum Dev. 2015;91(7):401-6.
16. Ditzen B, Schaer M, Gabriel B, Bodenmann G, Ehlert U, Heinrichs M. Intranasal oxytocin increases positive communication and reduces cortisol levels during couple conflict. Biol Psychiatry. 2009;65(9):728-31.
17. Green L, Fein D, Modahl C, Feinstein C, Waterhouse L, Morris M. Oxytocin and autistic disorder: alterations in peptide forms. Biol Psychiatry. 2001;50(8):609-13.
18. Aydın O, Lysaker PH, Balıkçı K, Ünal-Aydın P, Esen-Danacı A. Associations of oxytocin and vasopressin plasma levels with neurocognitive, social cognitive and meta cognitive function in schizophrenia. Psychiatry Res. 2018.
19. Jobst A, Albert A, Bauriedl-Schmidt C, Mauer MC, Renneberg B, Buchheim A, et al. Social exclusion leads to divergent changes of oxytocin levels in borderline patients and healthy subjects. Psychother Psychosom. 2014;83(4):252-4.
20. Flanagan JC, Sippel LM, Wahlquist A, Moran-Santa Maria MM, Back SE. Augmenting Prolonged Exposure therapy for PTSD with intranasal oxytocin: A randomized, placebo-controlled pilot trial. J Psychiatr Res. 2018;98:64-9.
21. Ooi YP, Weng S-J, Kossowsky J, Gerger H, Sung M. Oxytocin and autism spectrum disorders: a systematic review and meta-analysis of randomized controlled trials. Pharmacopsychiatry. 2017;50(01):5-13.
22. Bürkner P-C, Williams DR, Simmons TC, Woolley JD. Intranasal oxytocin may improve high-level social cognition in Schizophrenia, but not social cognition or neurocognition in general: a multilevel bayesian meta-analysis. Schizophr Bull. 2017;43(6):1291-303.
23. Servan A, Brunelin J, Poulet E. The effects of oxytocin on social cognition in borderline personality disorder. L'Encéphale. 2018;44(1):46-51.
24. Onaka T, Yagi K. Oxytocin release from the neurohypophysis after the taste stimuli previously paired with intravenous cholecystokinin in anaesthetized rats. J Neuroendocrinol. 1998;10(4):309-16.
25. Salonia A, Nappi RE, Pontillo M, Daverio R, Smeraldi A, Briganti A, et al. Menstrual cycle-related changes in plasma oxytocin are relevant to normal sexual function in healthy women. Horm Behav. 2005;47(2):164-9.
26. Tekampe J, van Middendorp H, Sweep FC, Roerink SH, Hermus AR, Evers AW. Human pharmacological conditioning of the immune and endocrine system: Challenges and opportunities. Int Rev Neurobiol. 138: Elsevier; 2018. p. 61-80.

27. Lundström JN, Gordon AR, Alden EC, Boesveldt S, Albrecht J. Methods for building an inexpensive computer-controlled olfactometer for temporally-precise experiments. International J Psychophysiol. 2010;78(2):179-89.
28. Grewen KM, Davenport RE, Light KC. An investigation of plasma and salivary oxytocin responses in breast and formula feeding mothers of infants. Psychophysiology. 2010;47(4):625-32.
29. McCullough ME, Churchland PS, Mendez AJ. Problems with measuring peripheral oxytocin: can the data on oxytocin and human behavior be trusted? Neurosci Biobehav Rev. 2013;37(8):1485-92.
30. Langner O, Dotsch R, Bijlstra G, Wigboldus DH, Hawk ST, Van Knippenberg A. Presentation and validation of the Radboud Faces Database. Cogn Emot. 2010;24(8):1377-88.
31. Sanderman R, Arrindell WA, Ranchor AV. Eysenck personality questionnaire (EPQ). Groningen: Noordelijk Centrum voor Gezondheidsvraagstukken. 1991.
32. Scheier MF, Carver CS, Bridges MW. Distinguishing optimism from neuroticism (and trait anxiety, self-mastery, and self-esteem): a reevaluation of the Life Orientation Test. J Pers Soc Psychol. 1994;67(6):1063.
33. Bjelland I, Dahl AA, Haug TT, Neckelmann D. The validity of the Hospital Anxiety and Depression Scale: an updated literature review. J Psychosom Res. 2002;52(2):69-77.
34. Bates D, Maechler M, Bolker B, Walker S. lme4: Linear mixed-effects models using Eigen and S4. R package version. 2014;1(7):1-23.
35. Ober K, Ober S, Benson M, Vogelsang A, Bylica D, Günther O, et al. Plasma Noradrenaline and State Anxiety Levels Predict Placebo Response in Learned Immunosuppression. Clin Pharmacol Ther. 2012;91(2):220-6.
36. Pavlov IP. Conditional reflexes: an investigation of the physiological activity of the Cereb Cortex. 1927.
37. Burbach J, Young LJ, Russell J. Oxytocin: synthesis, secretion, and reproductive functions. Knobil and Neill's physiology of reproduction. 2006;2:3055-128.
38. Van IJzendoorn MH, Bhandari R, Van der Veen R, Grewen KM, Bakermans-Kranenburg MJ. Elevated salivary levels of oxytocin persist more than 7 h after intranasal administration. Front Neurosci. 2012;6.
39. Weisman O, Zagoory-Sharon O, Feldman R. Intranasal oxytocin administration is reflected in human saliva. Psychoneuroendocrinology. 2012;37(9):1582-6.
40. Barraza JA, Zak PJ. Empathy toward strangers triggers oxytocin release and subsequent generosity. Ann N Y Acad Sci. 2009;1167(1):182-9.
41. Morhenn V, Beavin LE, Zak PJ. Massage increases oxytocin and reduces adrenocorticotropin hormone in humans. Altern Ther Health Med. 2012;18(6):11.
42. Dobashi Y, Watanabe H, Sato Y, Hirashima S, Yanagawa T, Matsubara H, et al. Differential expression and pathological significance of autocrine motility factor/glucose-6-phosphate isomerase expression in human lung carcinomas. J Pathol. 2006;210(4):431-40.
43. Theodoridou A, Rowe AC, Penton-Voak IS, Rogers PJ. Oxytocin and social perception: Oxytocin increases perceived facial trustworthiness and attractiveness. Horm Behav. 2009;56(1):128-32.
44. Mameli S, Pisanu G, Sardo S, Marchi A, Pili A, Carboni M, et al. Oxytocin nasal spray in fibromyalgic patients. Rheumatol Int. 2014;34(8):1047-52.
45. Skvortsova A, Veldhuijzen DS, Van Middendorp H, Van den Bergh O, Evers AW. Enhancing placebo effects in somatic symptoms through oxytocin. Psychosom Med. 2018;80(4):353-60.
46. Chan C, Harris JA. Extinction of Pavlovian conditioning: The influence of trial number and reinforcement history. Behav Processes. 2017;141:19-25.
47. Albring A, Wendt L, Benson S, Nissen S, Yavuz Z, Engler H, et al. Preserving learned immunosuppressive placebo response: perspectives for clinical application. Clin Pharmacol Ther. 2014;96(2):247-55.
48. Walum H, Waldman ID, Young LJ. Statistical and methodological considerations for the interpretation of intranasal oxytocin studies. Biol psychiatry 2016;79(3):251-7.
49. Nave G, Camerer C, McCullough M. Does oxytocin increase trust in humans? A critical review of research. Perspect Psychol Sci. 2015;10(6):772-89.
50. Zak PJ, Kurzban R, Matzner WT. The neurobiology of trust. Ann N Y Acad Sci. 2004;1032(1):224-7.
51. Conlisk J. Professor Zak's empirical studies on trust and oxytocin. J Econ Behav Organ. 2011;78(1-2):160-6.
52. Leng G, Ludwig M. Intranasal oxytocin: myths and delusions. Biol Psychiatry. 2016;79(3):243-50.
53. Heinrichs M, Baumgartner T, Kirschbaum C, Ehlert U. Social support and oxytocin interact to suppress cortisol and subjective responses to psychosocial stress. Biol Psychiatry. 2003;54(12):1389-98.
54. Kosfeld M, Heinrichs M, Zak PJ, Fischbacher U, Fehr E. Oxytocin increases trust in humans. Nature. 2005;435(7042):673.

56. Striepens N, Kendrick KM, Hanking V, Landgraf R, Wüllner U, Maier W, Hurlemann R. Elevated cerebrospinal fluid and blood concentrations of oxytocin following its intranasal administration in humans. Sci Rep. 2013;3:3440.
57. Anagnostou E, Soorya L, Chaplin W, Bartz J, Halpern D, Wasserman S, et al. Intranasal oxytocin versus placebo in the treatment of adults with autism spectrum disorders: a randomized controlled trial. Mol Autism. 2012;3(1):16.
58. Lawson EA. The effects of oxytocin on eating behaviour and metabolism in humans. Nat Rev Endocrinol. 2017;13(12):700.
59. Jankowski M, Bissonauth V, Gao L, Gangal M, Wang D, Danalache B, et al. Anti-inflammatory effect of oxytocin in rat myocardial infarction. Basic Res Cardiol. 2010;105(2):205-18.
60. Szeto A, Rossetti MA, Mendez AJ, Noller CM, Herderick EE, Gonzales JA, et al. Oxytocin administration attenuates atherosclerosis and inflammation in Watanabe Heritable Hyperlipidemic rabbits. Psychoneuroendocrinology. 2013;38(5):685-93.
61. Colli VC, Okamoto R, Spritzer PM, Dornelles RCM. Oxytocin promotes bone formation during the alveolar healing process in old acyclic female rats. Arch Oral Biol. 2012;57(9):1290-7.
62. Rief W, Glombiewski JA. The hidden effects of blinded, placebo-controlled randomized trials: An experimental investigation. Pain. 2012;153(12):2473-7.
63. Ader R, Mercurio MG, Walton J, James D, Davis M, Ojha V, et al. Conditioned pharmacotherapeutic effects: a preliminary study. Psychosom Med. 2010;72(2):192.
64. Perlis M, Grandner M, Zee J, Bremer E, Whinnery J, Barilla H, et al. Durability of treatment response to zolpidem with three different maintenance regimens: a preliminary study. Sleep Med. 2015;16(9):1160-8.
65. Kirchhof J, Petrakova L, Brinkhoff A, Benson S, Schmidt J, Unteroberdörster M, Wilde B,. Kaptchuk T, Witzke O, Schedlowski M. Learned immunosuppressive placebo responses in renal transplant patients. Proc Natl Acad Sci U S A. 2018:201720548.