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Intranasal oxytocin compensates for estrus cycle-specific reduction of conditioned safety memory in rats: Implications for psychiatric disorders

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ABSTRACT

Stress and anxiety disorder patients frequently fail to benefit from psychotherapies which often consist of inhibitory fear learning paradigms. One option to improve the therapy outcome is medication-enhanced psychotherapy. Research in humans and laboratory rodents has demonstrated that oxytocin (OT) reduces fear and facilitates fear extinction. However, the role of OT in conditioned safety learning, an understudied but highly suitable type of inhibitory fear learning, remains to be investigated. The present study aimed at investigating the effect of intranasal OT on conditioned safety. To test this, Sprague Dawley rats (♂n = 57; ♀n = 72) were safety conditioned. The effects of pre-training or pre-testing intranasal OT on conditioned safety and contextual fear, both measured by the acoustic startle response, and on corticosterone plasma levels were assessed. Furthermore, the involvement of the estrous cycle was analyzed. The present data show that intranasal OT administration before the acquisition or recall sessions enhanced conditioned safety memory in female rats while OT had no effects in male rats. Further analysis of the estrus cycle revealed that vehicle-treated female rats in the metestrus showed reduced safety memory which was compensated by OT-treatment. Moreover, all vehicle-treated rats, regardless of sex, expressed robust contextual fear following conditioning. Intranasal OT-treated rats showed a decrease in contextual fear, along with reduced plasma corticosterone levels. The present data demonstrate that intranasal OT has the capacity to compensate deficits in safety learning, along with a reduction in contextual fear and corticosterone levels. Therefore, add-on treatment with intranasal OT could optimize the therapy of anxiety disorders.

1. Introduction

Stress and anxiety disorders are one of the most prevalent neuropsychiatric disorders worldwide and primarily characterized by excessive levels of fear coupled with hyperarousal and an inability to inhibit fearful emotions (Jovanovic et al., 2010; Craske et al., 2017). With prevalence rates among females being about two-times higher than in males, sex seems to be an important risk factor. Apart from low circulating estradiol (E2) being a causative factor in the development of post-traumatic stress disorder (PTSD), several studies suggest a direct role of sex hormones in fear and fear inhibitory memories (Lebron-Milad and Milad, 2012; Zeidan et al., 2011; Graham and Milad, 2013).

International guidelines recommend psychotherapy as treatment of choice to reduce the symptoms of anxiety disorder patients (Foa et al., 2009). However, while only a limited amount of individuals benefits from psychotherapy, a substantial number of patients quit therapy, fail to respond to therapy or experience relapse (Craske et al., 2017; Roy-Byrne, 2015). One option to enhance psychotherapy outcome is the implementation of pharmacological compounds that support inhibitory fear learning (Sartori and Singewald, 2019). Recently, the neuropeptide oxytocin (OT) has moved into the focus of clinicians as a promising pharmacological agent for medication-enhanced psychotherapy (Meyer-Lindenberg et al., 2011; Eckstein et al., 2019). OT acts on various midbrain and frontal regions, thereby modulating a wide range of behaviors, including those associated with fear and anxiety (for reviews, see (Meyer-Lindenberg et al., 2011; Insel, 2010; Lee et al., 2009)). In rodents, systemic or intracerebral OT administration facilitates fear extinction learning and reduces the expression of fear in brain region-

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and temporal-dependent manners (Missig et al., 2010; Viviani et al., 2011; Toth et al., 2012; Campbell-Smith et al., 2015). In humans, the effects of OT have been investigated using an intranasal (IN) administration procedure, with the general finding that OT improves the recall of fear extinction and generates anxiolytic effects (Born et al., 2002; Eckstein et al., 2015; Koch et al., 2016; Koch et al., 2016b).

While past studies have discovered the beneficial effects of OT on fear and fear extinction, the effects on conditioned safety learning have not been investigated so far. Conditioned safety learning is a rather understudied form of inhibitory fear learning, in which a previously neutral stimulus develops the ability to predict the absence of an aversive stimulus, thereby acting as an inhibitor of anxiety and fear (Kong et al., 2014). Interestingly, impaired safety learning has repeatedly been observed in patients suffering from anxiety or stress-related disorders (e.g., PTSD), indicating a behavioral significance and potential function as biomarker of these disorders (Jovanovic et al., 2010; Lissek et al., 2009; Apergis-Schoute et al., 2017).

The aim of the present study was to investigate the effect of IN OT on the acquisition and recall of conditioned safety in male and females Sprague Dawley rats. Because anxiety disorder patients often show alterations in contextual fear, we further investigated IN OT effects on contextual fear learning and plasma corticosterone (CORT) levels. For female rats, the potential effects of the estrus cycle stage were evaluated. We hypothesized that IN OT would facilitate conditioned safety memory and decrease contextual fear along with a reduction in CORT levels in both sexes. We further expected that females in the met- and diestrus phase would show reduced recall of conditioned safety memory.

2. Material and methods

2.1. Animals and housing conditions

Experimental subjects were adult male and naturally cycling female Sprague Dawley rats (♂n = 57; ♀n = 72), aged 8–10 weeks (200–320 g). Rats were bred inhouse (original breeding stock: Taconic, Denmark) and housed in groups of 4–6 in transparent Makrolon type IV cages (1820 cm^2) with wood chip bedding and cage enrichment. The rats had free access to standard chow (Ssniff® R/M-H, V1534-0) and tap water, with a fixed 12:12 h light/dark photoperiod (lights on at 06:00 h) in a temperature- (22 ± 2 °C) and humidity-controlled room (50 ± 5%). In female rats, the phase of the estrous cycle was determined daily by collection of a vaginal smear (see Section 2.2).

All experimental procedures were approved by the local authorities (Landesverwaltungsamt Sachsen-Anhalt, 42502-2-1309 Uni MD) and conducted in agreement with international guidelines and regulations for animal experiments (2010/63/EU).

2.2. Vaginal smear

Vaginal secretion was collected from female rats every morning between 07:00 and 08:00 h for 8–14 consecutive days using a slightly modified protocol described elsewhere (McLean et al., 2012). In short: Vaginal lavage was performed by holding the rat in an upright position and placing a filtered pipette tip filled with 100 µl sterile saline (Fresenius Kabi, Bad Homburg, Germany) at the opening of the vaginal canal. 50 µl of the saline was gently released and withdrawn. This procedure was repeated 4–5 times using the same pipette tip. The vaginal sample was placed on a microscope slide and examined under a brightfield microscope (Leica MZ125, Leica Biosystems, Germany). The stage of estrous cycle was determined by the ratio of cells present at time of sample collection. While the proestrous and estrous phases (Pro/Est) are characterized by a high proportion of nucleated epithelial cells (proestrus) and cornified epithelial cells (estrous), metestrus and diestrus (Met/Die) are characterized by a large proportion of leukocytes.

2.3. Pharmacological intervention

Synthetic OT (MW 1007.19; Tocris Bioscience, Bristol, UK) was dissolved in sterile saline (Fresenius Kabi, Bad Homburg, Germany) in a concentration of 1 µg/µl. The dose and methodological procedure of the IN administration were selected on the basis of studies showing that 20 µl are completely absorbed within 2 min and has shown to induce increased brain OT levels in adult rats (Neumann et al., 2013; Lukas and Neumann, 2012; Calcagnoli et al., 2015).

The IN applications were carried out 25–35 min before the respective experimental session (Fig. 1A or 1B). To this end, the conscious rats were held in a supine position (Fig. 1C) and the solution was bilaterally applied (10µl/nostril for 20 µl total) and equally distributed on the squamous epithelium of the rhinarium. Direct contact of the pipette tip with the rhinarium and direct application into one of the nostrils or in proximity of the philtrum was avoided. The applications lasted about 2 min. Then, the rats were returned to their home cage. In order to minimize non-specific stress responses, the rats were habituated daily for seven days to the administration procedure prior to behavioral testing, with saline being IN applied on the last three days of habituation.

2.4. Behavioral testing

Behavioral experiments were performed during the first hours of the light phase with a safety conditioning protocol previously described in detail elsewhere (Kreutzmann et al., 2020a). In short, on the first and second test day rats underwent startle baseline measurements with 10 startle stimuli. On Day 3 the rats underwent a pre-conditioning test (Pre-Test) to determine mean startle magnitudes and to exclude potential unconditioned effects of the to-be-learned light stimulus: After 5 min acclimation and 10 startle stimuli for habituation, 20 startle stimuli were presented in a pseudo-randomized order, 10 without light (Startle Alone) and 10 upon presentation of the to-be-learned light CS (CS Startle). On the fourth and fifth day, rats underwent safety conditioning. In each safety conditioning session, rats received 15 electric stimuli (US) that were explicitly unpaired from the 5 s-light CS (ITI: 12–120 s), meaning, following conditioning, the light CS would predict the absence of an aversive stimulus. On the last day (Day 6), rats underwent a post-conditioning memory recall session (Post-Test) that was identical to the one pre-conditioning.

Noise bursts with a duration of 40 ms and an intensity of 96 dB SPL were used as startle stimuli. As aversive stimuli, scrambled electric stimuli (0.5 s, 0.6 mA) were administered via a floor grid.

2.5. Blood sampling

Blood samples were consistently drawn in the morning and 30 min after behavioral testing between 08:30 and 10:00 a.m. Animals were handled and habituated to the blood collecttion procedure one week prior to the first blood sample. For pre-training administration, blood samples were collected at five points in time: Baseline, Pre-Test (Day 3), Safety Conditioning (Day 4 and 5) and Post-Test (Day 6). For pre-testing administration, blood samples were collected at three points in time: Baseline, Pre-Test (Day 3) and Post-Test (Day 6) (Fig. 1).

For blood collection, the rats were gently restrained, a small tail vain incision was made and approximately 120 µl of blood was collected in EDTA-coated microtubes (Microvette® CB 300 K2E, Sarstedt AG & Co., Nümbrecht, Germany). Samples were immediately put on ice and centrifuged at 4 °C with 3000 rpm for 10 min (Eppendorf AG, Hamburg, Germany). Plasma (approximately 50–80 µl) was collected and stored at −80 °C until further processing.

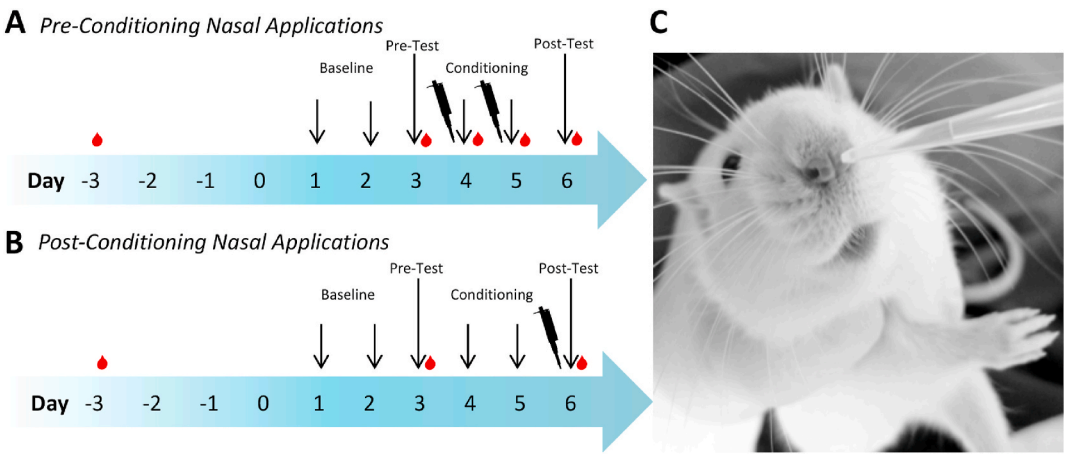

Fig. 1. Experimental Design of the Study and Intranasal Application Procedure. Upon one week of handling to the intranasal application procedure, the first blood sample (−3 Day) was drawn. On Day 1 and 2, rats were submitted to startle baseline sessions for habituation. 24 h later, rats underwent the pre-test (Day 3), followed by two safety conditioning sessions (Day 4 and 5). On the last day, the rats' memory for conditioned safety was tested in the recall session (Post-Test, Day 6). Intranasal administration of oxytocin either took place before each of the two conditioning sessions (pre-conditioning, A) or before the recall session (post-conditioning, B). Blood samples were drawn as indicated. (C) Holding position of the rat during the intranasal administration procedure.

2.6. Enzyme-linked immunosorbent assay (ELISA): corticosterone (CORT)

To determine plasma CORT-levels, an ELISA kit specific for CORT (Enzo Life Sciences GmbH, Lörrach, Germany, Catalog No. ADI-901-097) was applied. The assay was performed as per instructions provided by the manufacture. In short, plasma samples were diluted 1:100 in ELISA assay buffer, two 100 µl duplicates of each sample were added to the assay plate and incubated for two hours at room temperature. After several washing steps, the substrate (p-nitrophenyl phosphate, p-Npp) was added, and following one hour of incubation, the reaction was terminated and absorbance read on a microplate reader (ASYS HITECH GmbH, Eugendorf, Austria) at 405 nm.

2.7. Graphical and statistical analysis

To analyze conditioned safety memory in the Pre- or Post-Test, the mean startle magnitudes of the startle trials in the absence (Startle Alone) and in the presence of the light stimulus (CS-startle) was calculated for each animal. The percent difference scores were calculated to evaluate the safety learning effect independent of potential effects on the startle alone magnitude. For the analysis of contextual fear conditioning (referred to as context startle), the baseline startle measurements, i.e. the 10 startle stimuli before the measurement of startle alone and CS startle, from the Pre- and Post-test were used. To evaluate the shock-induced activity, the mean locomotor response to the electric stimuli for analyzed.

Due to high variation in the startle magnitudes within and between groups, startle magnitudes were either normalized to the Startle Alone magnitude or to the pre-conditioning baseline magnitude (for non-normalized data, see Supplementary Information, Supplementary Figs S2 and S3). Results are represented as means +SEM. For statistical analysis, Prism 8.0 (GraphPad Software Inc., La Jolla, CA, USA) was used. Normal distribution of the data was checked with the D'Agostino-Pearson omnibus normality test. Analyses of variance (ANOVA) with treatment as between-subject factor and startle trial type, blood sample session or estrus phase as within-subject factors were used. Post-hoc comparisons were made using Sidak's multiple comparisons test. The percent changes of startle magnitudes were analyzed with Student's t-test. Effects were deemed significant with $p \leq 0.05$.

3. Results

3.1. Intranasal oxytocin does not affect conditioned safety memory in male rats but reduces plasma corticosterone levels along with diminished contextual fear-potentiated startle

To investigate whether administration of IN OT affects the acquisition of conditioned safety memory, male rats received IN OT prior to each of the two safety conditioning sessions (Fig. 1A). The increase of baseline startle magnitudes (context startle) following conditioning with aversive stimuli can be used as an indicator of contextual fear conditioning (McNish et al., 2000). Analysis of pre- and post-conditioning context startle (i.e., the first 10 startle stimuli for habituation) revealed a significant main effect of session (Fig. 2A; session: $F_{(1, 26)} = 4.59$, $p = 0.04$), indicating contextual fear conditioning. There was neither a main effect of treatment (treatment: $F_{(1,26)} = 1.27$, $p = 0.27$) nor an interaction between treatment and session ($F_{(1,26)} = 1.27$, $p = 0.27$). Post-hoc comparisons showed significant contextual fear conditioning in vehicle-treated rats only (pairwise comparisons: vehicle: $t_{(26)} = 2.31$, $p = 0.05$; OT: $t_{(26)} = 0.72$, $p = 0.73$).

Pre-conditioning, the light stimulus did not affect startle magnitude in neither of the two treatment groups (Fig. 2B, left; trial type: $F_{(1,26)} = 1.13$, $p = 0.30$; treatment: $F_{(1,26)} = 1.46$, $p = 0.24$; interaction: $F_{(1,26)} = 1.46$, $p = 0.24$). In the recall session (Post-Conditioning), the safety CS significantly attenuated the startle magnitude (Fig. 2B, right; trial type: $F_{(1,26)} = 49.96$, $p < 0.0001$; pairwise comparisons: vehicle: $t_{(26)} = 4.59$; $p = 0.002$; OT: $t_{(26)} = 5.40$, $p < 0.0001$). There was no effect of treatment ($F_{(1,26)} = 0.33$, $p = 0.57$) and no interaction between treatment and trial type ($F_{(1,26)} = 0.33$, $p = 0.57$), indicating that both treatment groups learned conditioned safety.

Plasma CORT levels were affected by session and treatment (Fig. 2C; session: $F_{(3,78)} = 18.60$, $p < 0.0001$; treatment: $F_{(1,26)} = 6.35$, $p = 0.02$). OT-treated animals had significantly reduced plasma CORT levels in Conditioning Session 1 ($t_{(13)} = 6.29$, $p = 0.003$), Conditioning Session 2 ($t_{(13)} = 13.22$, $p < 0.0001$) and Post-Conditioning ($t_{(13)} = 5.52$, $p = 0.009$) when compared to the Pre-Conditioning. In vehicle-treated rats, plasma CORT levels were significantly reduced when comparing Pre-Conditioning levels to Conditioning Session 2 ($t_{(13)} = 4.74$, $p = 0.02$). Moreover, there was a significant difference between the first and second conditioning session ($t_{(13)} = 7.34$, $p = 0.0009$). Further comparisons to vehicle-treated rats showed that IN OT only significantly reduced CORT levels during the two conditioning sessions (Conditioning 1: $t_{(26)} = 3.53$, $p = 0.006$; Conditioning 2: $t_{(26)} = 3.11$, $p = 0.02$). Of note, IN OT

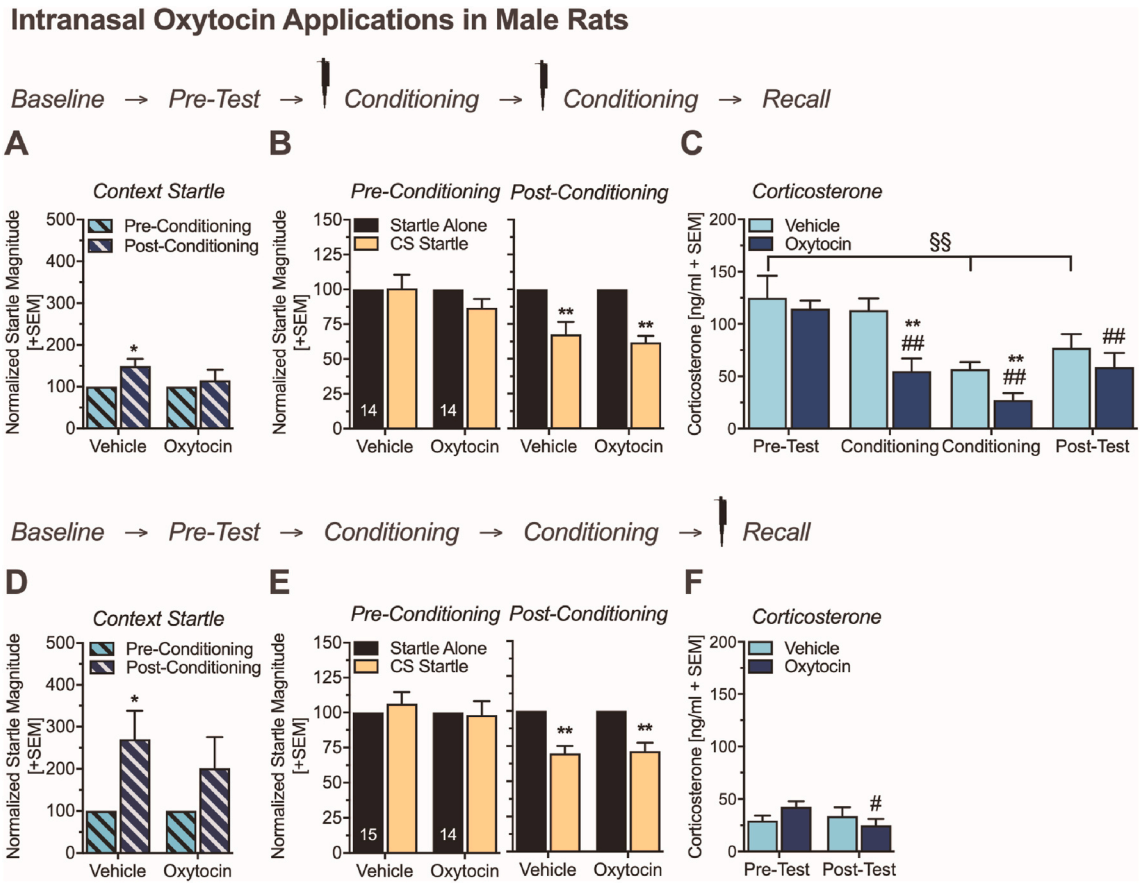

Fig. 2. Pre- or post-conditioning intranasal administration of oxytocin does not facilitate conditioned safety memory in male rats but reduces plasma corticosterone levels along with diminished contextual fear-potentiated startle.
In male rats, intranasally administered oxytocin (OT) before safety conditioning (A-C) or before the recall session (D-F) did not facilitate conditioned safety memory but reduced contextual fear and plasma corticosterone. Context startle was measured by assessing the baseline startle of the Pre-Test and Post-Test (A and D). Intranasal applications of VEH or OT pre- (A) or post-conditioning (D) revealed that while there was no difference between treatment groups in startle magnitude during the baseline before safety conditioning (Pre-Conditioning), while context startle in the recall session (Post-Conditioning) was increased in VEH-treated rats only, suggesting contextual fear conditioning (A and D) (*$p < 0.05$, comparison to Pre-Conditioning). Male rats that received intranasal OT pre-conditioning or post-conditioning did not display a significant increase in context startle (A and D). Pre-conditioning, the to-be-learned safety CS had no effect on the startle response, neither in VEH nor in OT-treated male rats (B, left panel; E, left panel). In the recall session (Post-Conditioning), both treatment groups significantly attenuated their startle magnitude upon presentation of the safety CS (B, right panel; E, right panel) (**$p < 0.01$, comparison to Startle Alone). There was no effect of treatment on the effect of the safety CS. Intranasal administration of OT pre- and post-conditioning significantly reduced plasma corticosterone (CORT) levels (C and F) (*$p < 0.05$, **$p < 0.01$, comparison to VEH-treated rats; #$p < 0.05$, ##$p < 0.01$, within-group comparison of OT-treated rats to Pre-Test CORT levels; §§$p < 0.01$, within-group comparison of VEH-treated rats to Pre-Test CORT levels). Data are represented as group averages +SEM. Numbers depicted in the bars represent the n of each group.

did not affect the reactivity to the aversive stimuli during safety conditioning (see Suppl. Fig. S1A).

To investigate whether IN OT affects the recall of conditioned safety memory, male rats received IN OT prior to the recall session (Fig. 1B). Analysis of context startle pre- and post-conditioning revealed a main effect of test session (Fig. 2D; session: $F_{(1, 27)} = 7.38$, $p = 0.01$; treatment: $F_{(1,27)} = 0.48$, $p = 0.50$; interaction: $F_{(1,27)} = 0.48$, $p = 0.50$). Vehicle- but not OT-treated male rats showed a significant increase in the context startle, indicating contextual fear conditioning (vehicle: $t_{(27)} = 3.85$, $p = 0.001$; OT: $t_{(27)} = 0.71$, $p = 0.73$).

Pre-conditioning, the light stimulus did not affect startle magnitudes in both groups (Fig. 2E, left; trial type: $F_{(1,27)} = 0.11$, $p = 0.75$; treatment: $F_{(1,27)} = 0.39$, $p = 0.54$; interaction: $F_{(1,27)} = 0.39$, $p = 0.54$). Post-conditioning, the safety CS significantly attenuated the startle magnitude regardless of treatment (Fig. 2E, right; $F_{(1,27)} = 54.83$, $p < 0.0001$). In both treatment groups, the startle response was significantly reduced by the light CS (vehicle: $t_{(27)} = 5.48$; $p < 0.0001$; OT: $t_{(27)} = 4.99$, $p < 0.0001$). Treatment had no main effects ($F_{(1,27)} = 0.05$, $p = 0.83$) and there was no treatment and trial type interaction ($F_{(1,27)} = 0.05$, $p = 0.83$).

Analysis of plasma CORT levels revealed no main effects but a significant interaction between test session and treatment (Fig. 2F; session: $F_{(1,27)} = 2.12$, $p = 0.16$; treatment: $F_{(1,27)} = 0.08$, $p = 0.78$; interaction: $F_{(1,27)} = 5.77$, $p = 0.02$). OT-treated rats had significantly decreased plasma CORT levels post-conditioning as compared to pre-conditioning ($t_{(27)} = 2.68$, $p = 0.03$).

3.2. Intranasal oxytocin facilitates the acquisition and recall of conditioned safety memory in female rats and reduces plasma corticosterone levels along with diminished contextual fear-potentiated startle

To further test the effect of IN OT before the acquisition of conditioned safety memory in female rats, animals received IN OT prior to each of the two safety conditioning sessions (Fig. 1A).

Analysis of context startle revealed a significant interaction between test session and treatment (Fig. 3A; $F_{(1,32)} = 5.39$, $p = 0.02$), as well as main effects (session: $F_{(1, 32)} = 4.72$, $p = 0.04$; treatment: $F_{(1,32)} = 5.39$, $p = 0.02$). Vehicle-treated females significantly increased their context startle post-conditioning, indicating contextual fear conditioning. No

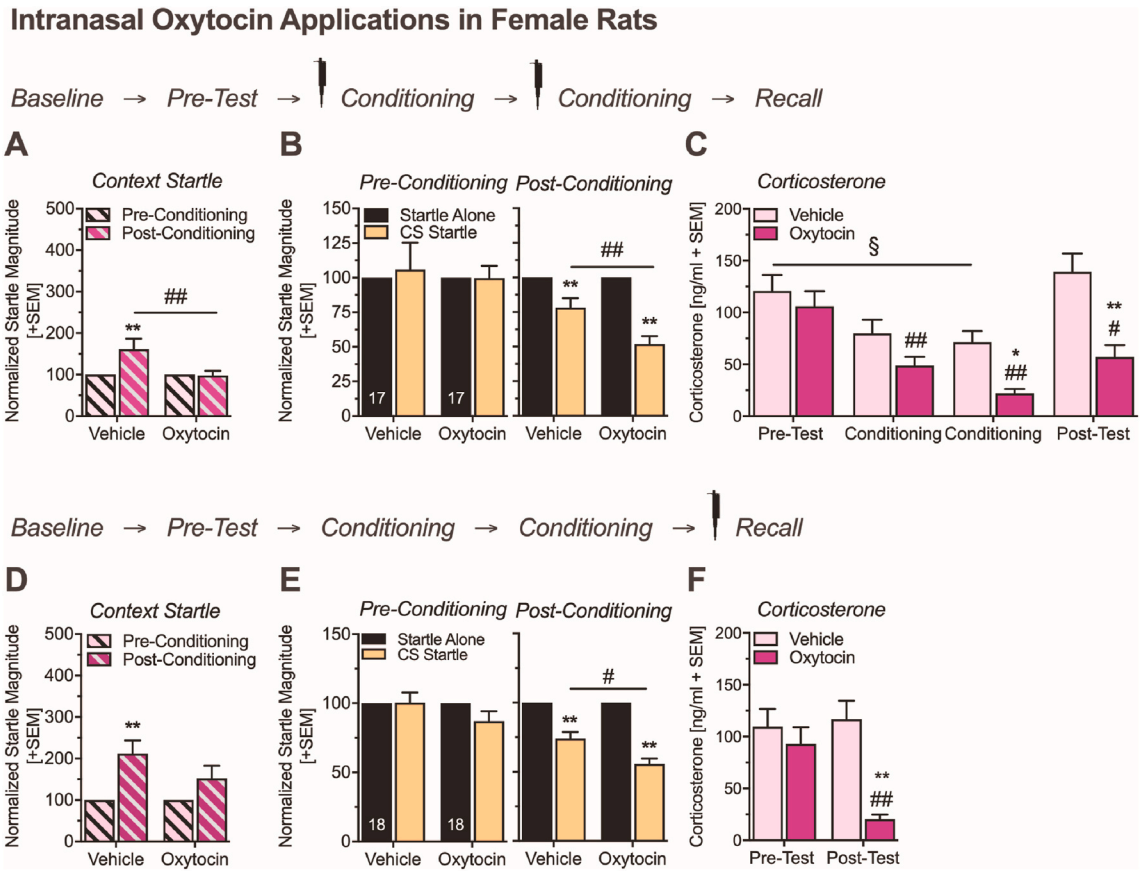

Fig. 3. Intranasal oxytocin facilitates the acquisition and recall of conditioned safety memory in female rats and reduces plasma corticosterone levels along with diminished contextual fear-potentiated startle.

In female rats, intranasally administered oxytocin (OT) before safety conditioning (A-C) or before the recall session (D-F) enhanced conditioned safety memory and reduced contextual fear and plasma corticosterone. Context startle was measured by assessing the baseline startle of the Pre-Test and Post-Test (A and D). Intranasal applications of vehicle (VEH) or OT pre- (A) and post-conditioning (D) revealed that while there was no difference between treatment groups in context startle before safety conditioning (Pre-Conditioning), context startle in the recall session (Post-Conditioning) was increased in VEH-treated rats only, suggesting contextual fear conditioning (A and D) (**$p < 0.01$, comparison to Pre-Conditioning). Female rats that received intranasal OT pre-conditioning or post-conditioning did not display such a significant increase in context startle (A and D) (##$p < 0.01$ comparison to VEH-treated rats). Pre-conditioning, the to-be-learned safety CS had no effect on the startle response, neither in vehicle (VEH) nor in OT-treated male rats (B, left panel; E, left panel). In the recall session (Post-Conditioning), both treatment groups significantly attenuated their startle magnitude upon presentation of the safety CS (B, right panel; E, right panel) (**$p < 0.01$, comparison to Startle Alone). Notably, OT-treated females showed an enhanced recall of safety memory as compared to VEH-treated females (#$p < 0.05$, ##$p < 0.01$, comparison to VEH treatment). Alongside, intranasal administration of OT pre- and post-conditioning significantly reduced plasma corticosterone (CORT) levels in female rats (C and F) (*$p < 0.05$, **$p < 0.01$, comparison to VEH-treated rats; #$p < 0.05$, ##$p < 0.01$, within-group comparison of OT-treated rats to Pre-Test CORT levels; §§$p < 0.01$, within-group comparison of VEH-treated rats to Pre-Test CORT levels). Data are represented as group averages +SEM. Numbers depicted in the bars represent the n of each group.

such increase could be observed in the OT-treated group (vehicle: $t_{(32)} = 3.18$, $p = 0.006$; OT: $t_{(32)} = 0.11$, $p = 0.99$). Further comparisons revealed that while context startle did not differ between the two treatment groups pre-conditioning ($t_{(64)} = 0.00$, $p > 0.999$), vehicle-treated female rats showed significantly increased context startle post-conditioning as compared to OT-treated female rats ($t_{(64)} = 3.28$, $p = 0.003$). These analyses indicate that IN OT blocks the acquisition of contextual fear.

Pre-conditioning, the light stimulus did not affect startle magnitude (Fig. 3B, left; trial type: $F_{(1,32)} = 0.06$, $p = 0.80$; treatment: $F_{(1,32)} = 0.08$, $p = 0.78$; interaction: $F_{(1,32)} = 0.08$, $p = 0.78$). Post-conditioning, startle magnitudes were significantly attenuated by the safety CS (Fig. 3B, right; trial type: $F_{(1,32)} = 60.79$, $p < 0.0001$). Notably, there was a main effect of the treatment ($F_{(1,32)} = 8.60$, $p = 0.006$) and a significant interaction between treatment and trial type ($F_{(1,32)} = 8.60$, $p = 0.006$). In both treatment groups, the startle response was significant reduced by the safety CS (Sidak's post-hoc comparisons; vehicle: $t_{(32)} = 3.44$; $p = 0.003$; OT: $t_{(32)} = 7.59$, $p < 0.0001$), however, a significantly larger startle inhibition by the safety CS could be observed in OT-treated animals.

CORT levels were affected by treatment and test session (Fig. 3C; session: $F_{(3,96)} = 12.54$, $p < 0.0001$; treatment: $F_{(1,32)} = 21.45$, $p < 0.0001$) and there was a significant interaction between session and treatment ($F_{(3,96)} = 2.80$, $p = 0.04$). There was no group difference between vehicle- and OT-treated females pre-conditioning ($t_{(32)} = 0.70$, $p = 0.93$) or during the first conditioning session ($t_{(32)} = 1.74$, $p = 0.21$). However, CORT levels of OT-treated animals were significantly decreased after the second conditioning session ($t_{(32)} = 4.32$, $p = 0.001$) and post-conditioning ($t_{(32)} = 3.92$, $p = 0.002$). Furthermore, OT-treated females had significantly lower plasma CORT levels after both conditioning sessions and post-conditioning when compared to pre-conditioning (Conditioning 1: $t_{(16)} = 4.52$, $p = 0.002$; Conditioning 2: $t_{(16)} = 5.17$, $p = 0.001$; Post-Test: $t_{(16)} = 2.92$, $p = 0.05$). Vehicle-treated females showed a significant decrease in CORT when comparing the conditioning session 1 ($t_{(16)} = 3.10$, $p = 0.04$) or conditioning session 2 ($t_{(16)} = 3.15$, $p = 0.04$) to post-conditioning. Notably, IN OT did not affect the reactivity to the aversive stimuli during safety conditioning in female rats (see Suppl. Fig. S1B).

To investigate whether IN OT affects the recall of conditioned safety memory, female rats received IN OT prior to the recall session (Fig. 1B).

Analysis of context startle pre- and post-conditioning revealed a main effect of test session (Fig. 3D; session: $F_{(1, 34)} = 14.08$, $p = 0.0007$; treatment: $F_{(1,34)} = 1.87$, $p = 0.18$; interaction: $F_{(1,34)} = 1.87$, $p = 0.18$). Post-hoc comparisons revealed significantly increased context startle post-conditioning in vehicle- but not in OT-treated rats (vehicle: $t_{(34)} = 3.62$, $p = 0.002$; OT: $t_{(34)} = 1.68$, $p = 0.19$).

IN OT administration before the recall session in female rats showed that the light stimulus did not affect startle magnitude pre-conditioning (Fig. 3E, left; trial type: $F_{(1,34)} = 1.50$, $p = 0.23$; treatment: $F_{(1,34)} = 1.71$, $p = 0.20$; interaction: $F_{(1,34)} = 1.71$, $p = 0.20$). Post-Conditioning, startle magnitudes were significantly attenuated by the safety CS (Fig. 3E, right; $F_{(1,34)} = 131.3$, $p < 0.0001$), as confirmed by post-hoc comparisons (Vehicle: $t_{(34)} = 5.98$; $p < 0.0001$; OT: $t_{(34)} = 10.23$, $p < 0.0001$). There was a main effect of treatment ($F_{(1,34)} = 9.10$, $p = 0.006$) and a significant interaction between treatment and trial type ($F_{(1,34)} = 9.10$, $p = 0.006$). Post-hoc comparisons further showed that treatment had an effect on the CS startle magnitude between groups ($t_{(68)} = 4.25$, $p = 0.0001$), indicating that OT significantly enhanced the recall of safety memory.

Analysis of plasma CORT levels revealed a main effect of session and treatment, as well as a significant interaction between session and treatment (Fig. 3F; session: $F_{(1,34)} = 9.83$, $p = 0.004$; treatment: $F_{(1,34)} = 9.70$, $p = 0.004$; interaction: $F_{(1,34)} = 14.55$, $p = 0.0005$). While there was no difference between treatment groups pre-conditioning ($t_{(68)} = 0.80$, $p = 0.67$), OT-treated rats showed reduced plasma CORT levels post-conditioning when compared to vehicle-treated rats ($t_{(68)} = 4.60$, $p < 0.0001$). Moreover, compared to pre-conditioning levels, OT-treated rats had significantly reduced CORT levels post-conditioning ($t_{(34)} = 4.91$, $p < 0.0001$). This effect was not observed in vehicle-treated rats ($t_{(34)} = 0.48$, $p = 0.87$).

3.3. Phase of the estrus cycle influences the recall of safety memory in vehicle-treated rats but has no effect on plasma corticosterone levels

We further investigated whether the phase of the estrus cycle of females at the time of the recall session influenced the effects of the safety CS. While the proestrous and estrous phases (Pro/Est) are usually accompanied with a high plasma concentration of estrogen, estrogen levels are low in the metestrus and diestrus (Met/Die) phase of the estrous cycle. We found a significant main effect of cycle phase and treatment, as well as a significant interaction (Fig. 4A, left; cycle phase: $F_{(1,66)} = 13.65$, $p = 0.0004$; treatment: $F_{(1,66)} = 8.93$, $p = 0.004$; interaction: $F_{(1,66)} = 4.04$, $p = 0.04$). Vehicle-treated females in the metestrus or diestrus (Met/Die) had significantly reduced safety memory than females in the proestrus or estrus phase (Pro/Est) ($t_{(66)} = 3.85$, $p = 0.0005$). This was not observed in OT-treated females ($t_{(66)} = 1.26$, $p = 0.38$). OT significantly enhanced the effect of the safety CS in female rats that were in their Met/Die phase ($t_{(66)} = 4.83$, $p < 0.0001$) but had no effect in females that were in their Pro/Est phase ($t_{(66)} = 0.57$, $p = 0.82$). This effect remained visible when splitting the groups based on the time point of IN OT treatment (Fig. 4A, right). Moreover, the phase of the estrous cycle did not significantly alter absolute levels of startle magnitudes, indicating that the observed effects were specific to the safety memory (see Supplementary Information, Fig. S4).

Because estrogen, as well as the OT system strongly interact with hypothalamic-pituitary-adrenal axis (HPA-axis) reactivity, we also investigated whether CORT levels obtained after the recall session interacted with the estrus phase or treatment. We found a main effect of treatment but not of cycle phase (Fig. 4B, left; cycle phase: $F_{(1,66)} = 2.1$, $p = 0.15$; treatment: $F_{(1,66)} = 22.79$, $p < 0.0001$; interaction: $F_{(1,66)} = 2.91$, $p = 0.09$; comparison vehicle vs. OT: Pro/Est: $t_{(66)} = 1.79$, $p =$

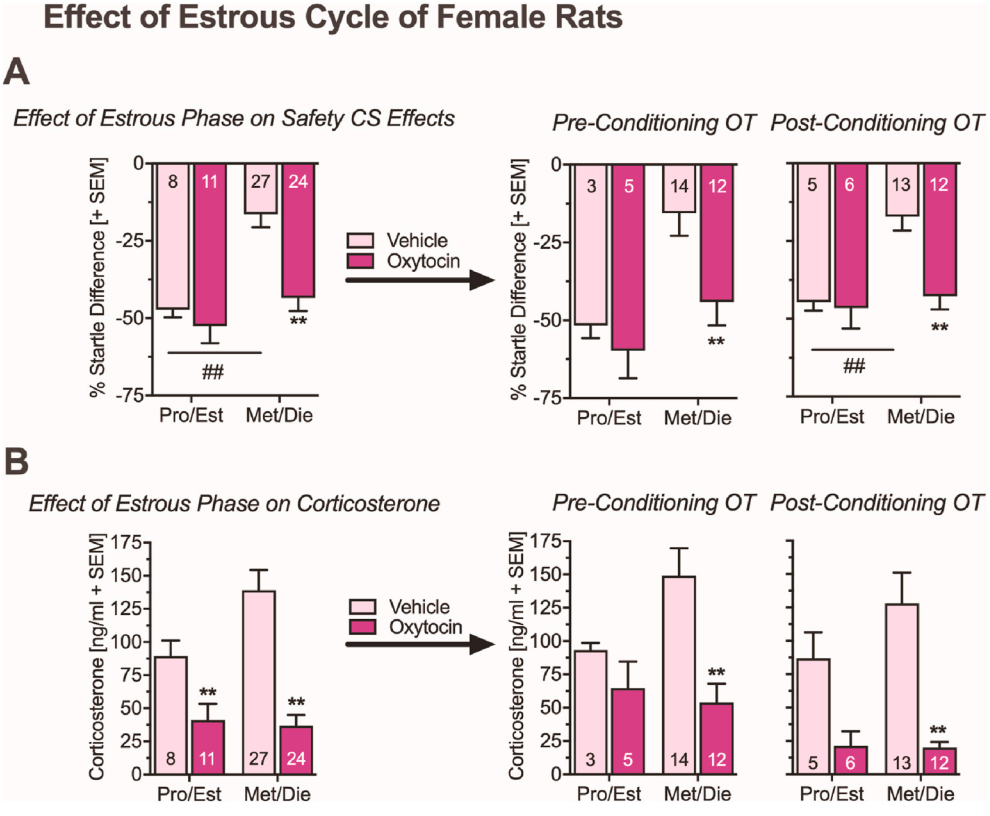

Fig. 4. Estrus cycle influences the recall of safety memory in vehicle-treated rats but has no effect on plasma corticosterone levels.
(A) The effect of the phase of the estrus cycle at the time point of the recall session was investigated in all females, regardless of when IN OT was administered (A, left panel). While VEH-treated females in the Pro/Est showed enhanced recall of safety memory, this effect was not apparent in VEH-treated females in the Met/Die. In OT-treated female rats, phase of the estrus cycle did not seem to matter as both groups showed enhanced safety memory (A, left panel) (**$p < 0.01$, between-group comparison; ##$p < 0.01$, within-group comparison). Splitting the groups based on the time point of IN OT treatment showed a similar pattern (A, right panel). (B) The influence of cycle phase or OT-treatment on plasma corticosterone (CORT) levels was further assessed in female rats (B, left panel). While there was a small trend in VEH-treated females showing less CORT when in the Pro/Est, this effect was not significant. OT-treated female rats, in turn, showed reduced plasma CORT, regardless of estrus cycle phase (B, left panel) (**$p < 0.01$, between-group comparison). Splitting the groups based on the time point of IN OT treatment showed a similar pattern in CORT release (B, right panel), with rats receiving IN OT pre-conditioning showed higher CORT levels in general, possibly due to undergoing a conditioning session prior to blood sampling. Data are represented as group averages +SEM. Numbers depicted in the bars represent the n of each group.

0.15; Met/Die: $t_{(66)} = 6.27$, $p < 0.0001$). Splitting the groups based on the time point of IN OT treatment showed a similar pattern (Fig. 4B, right; Pre-Conditioning OT: cycle phase: $F_{(1,30)} = 0.80$, $p = 0.38$; treatment: $F_{(1,30)} = 6.00$, $p = 0.02$; interaction: $F_{(1,30)} = 1.75$, $p = 0.19$, comparison vehicle vs. OT: Pro/Est: $t_{(30)} = 0.64$, $p = 0.78$; Met/Die: $t_{(30)} = 3.97$, $p = 0.0008$; Post-Conditioning OT: cycle phase: $F_{(1,32)} = 1.00$, $p = 0.33$; treatment: $F_{(1,32)} = 19.14$, $p = 0.0001$; interaction: $F_{(1,32)} = 1.14$, $p = 0.29$).

4. Discussion

The aim of the present study was to investigate whether IN administered OT affects the acquisition and recall of conditioned safety memory in rats. To test this, male and female rats received IN OT either prior to the conditioning sessions or the recall session. We found that IN OT enhanced the recall of conditioned safety in a sex-specific manner. In female rats, IN OT facilitated the recall of safety memory, along with a reduction in plasma CORT levels and diminished contextual fear. Further analyses revealed that vehicle-treated female rats in the metestrus/diestrus showed reduced (but still intact) safety memory which was compensated by OT-treatment. In male rats, IN OT did not affect the recall of safety memory but reduced levels of plasma CORT and tended to reduce contextual fear.

To investigate the effect of IN OT on conditioned safety, we applied a single-cued safety conditioning protocol in which the aversive US and the safety CS were explicitly unpaired. The single-cued safety conditioning protocol is a well-established protocol that allows an animal to predict the absence of an aversive event (Kong et al., 2014). As behavioral read-out, we utilized the acoustic startle response, which is a bivalent measure that can be attenuated by stimuli with positive valence and potentiated by stimuli with negative valence (Fendt and Koch, 2013). While the prospective safety cue had no effects on the startle magnitude pre-conditioning, it significantly attenuated the startle magnitude after single-cued safety conditioning in male and female rats. This, as well as data from other studies applying the same type of training indicate that a positive associative memory to the safety cue was formed (Kong et al., 2014; Kreutzmann et al., 2020a, 2020b, 2021; Rogan et al., 2005; Camp et al., 2012; Ostroff et al., 2010; Kreutzmann and Fendt, 2020). We further observed that vehicle-treated rats, regardless of sex, expressed potentiated context startle magnitudes following safety conditioning. This suggests that rats were fear conditioned to the context in which electric stimuli had previously been received (Kreutzmann and Fendt, 2020; Kreutzmann et al., 2021). Of note, the pre-conditioning IN application procedures seem to generally attenuate contextual fear conditioning in both treatment groups.

In female rats, the phase of the estrus cycle modulated the strength of safety conditioning. Vehicle-treated females in the Met/Die phase during the recall session showed deficits in safety memory recall when compared to females in the Pro/Est phase. This finding emphasizes the importance of the estrus phase during learning tasks and is in accordance with several studies showing that high levels of estrogen facilitate fear extinction while low estrogen levels or respective manipulations interfere with emotional learning (Lebron-Milad and Milad, 2012; Zeidan et al., 2011; Leuner et al., 2004; Chang et al., 2009; Toufexis et al., 2006, 2007). These facilitating effects of estrogen on fear inhibition are possibly mediated by the ventromedial prefrontal cortex (vmPFC), the hippocampus and the amygdala – brain regions with an abundant expression of estrogen receptors and important for fear inhibition (Blurton-Jones and Tuszynski, 2002; Ostlund et al., 2003; Tovote et al., 2015).

IN application of OT seems to be well tolerated and efficient in clinical studies (MacDonald et al., 2011). To optimize the translational meaning of our experiment, we administered OT intranasally in our rats. We found that IN OT enhanced the safety memory in a sex-specific manner: While OT had no effects in male rats, it facilitated the safety memory in female rats. Further analyses revealed that vehicle-treated females in the Met/Die phase had a reduced safety memory which was compensated by IN OT treatment (see Fig. 4A, Left). In the Pro/Est phase, IN OT had no significant effects, possibly due to a ceiling effect. More specifically, females in the Pro/Est phase may have already displayed a maximal safety memory. Hence, IN OT seems to only enhance reduced safety memory as observed in female rats in the Met/Die phase. Nevertheless, it is important to note that when splitting the analysis between the experiments (see Fig. 4A, Right), the number of female rats in the Pro/Est phase is very low ($n = 3–6$). Therefore, caution is needed when interpreting the results or drawing conclusions. In all rats, IN OT reduced plasma CORT levels and led to diminished contextual fear. In female rats, this effect was independent of cycle phase.

Our findings in female rats are in line with those of rodent studies that investigated the effect of systemic or central OT administration on a different form of inhibitory fear learning, namely fear extinction (Missig et al., 2010; Toth et al., 2012; Campbell-Smith et al., 2015). These effects are likely mediated by OT receptors in the lateral part of the central amygdala (CeA) (Viviani et al., 2011; Huber et al., 2005; van den Burg and Hegoburu, 2020). Enhanced OT signaling or administration of synthetic OT may activate OT-receptors in the lateral CeA. Through disinhibition of basal forebrain nuclei projecting to the neocortex, as well as through activation of GABAergic interneurons that in turn inhibit medial CeA neurons, fear responses are suppressed, primarily, by attenuated CeA-periaquaductal gray communication (Viviani et al., 2011; van den Burg and Hegoburu, 2020). Reduced fear levels probably affect the learning and retention of safety signals. Indeed, a previous study has shown that safety learning seems to be associated with contextual fear since the individual effect of a safety CS was correlated with the fear-inducing properties of the experimental context (Uzuneser and Fendt, 2020). Of note, conditioned safety, similar to our findings, was still observed with low levels of contextual fear and could even be measured in a neutral context, i.e. without contextual fear (Uzuneser and Fendt, 2020).

Interestingly, we only observed this IN OT effect in female rats, indicating sex-specific differences in the OT brain system. These have previously been described, with the tendency of OT levels and OT-receptor expression being higher in females than males (for comprehensive reviews, see (Dumais and Veenema, 2016; Love, 2018)). Therefore, one possible explanation for the sex-dependent effects on safety memory recall could be that the IN administered OT activated the OT brain system much more efficiently in female rats as compared to males, and was therefore able to inhibit CeA output more profoundly.

The OT system strongly interacts with sex steroids and HPA-axis reactivity (Love, 2018; Windle et al., 2004). For instance, sex steroid receptor modulation, particularly at the level of the estrogen receptor β (ERβ) within one of the main OT-producing brain regions, the paraventricular nucleus of hypothalamus, has been shown to promote OT mRNA expression, enhance OT neurotransmission, attenuate HPA responses, such as CORT release, and reduce anxiety-like behavior (Windle et al., 2004; Li et al., 2016; Acevedo-Rodriguez et al., 2015; Kudwa et al., 2014; Liu et al., 2012; Nomura et al., 2002). These observations are in line with our findings that IN OT reduced plasma CORT levels and diminished contextual fear in both sexes. Although male rats only showed a trend towards diminished contextual fear, OT administration in female rats prior to the recall session significantly reduced contextual fear as compared to vehicle-treated rats, again confirming that females seem to be more sensitive to OT-mediated effects and suggesting sexually dimorphic circuits within the brain (Li et al., 2016). Previous studies showed that subcutaneous OT administration attenuated the startle magnitudes following fear conditioning, indicating reduced anxiety (Missig et al., 2010; Ayers et al., 2011). Although only males were tested, these data are in line with our findings and suggest that OT has the potential to reduce anxiety levels. However, OT has also been shown to affect social behavior (social buffering) (Kikusui et al., 2006). Although we did not observe any difference in social behavior between vehicle- or OT-treated rats, we cannot fully rule out that

OT-induced social buffering is involved in the observed effect.

In order to optimize the translational perspective, the current study investigated the effect of IN OT on conditioned safety by utilizing the startle paradigm. Several studies demonstrated that fear-potentiated startle is enhanced and generalized in anxiety disorder patients, whereas fear inhibition is impaired (Jovanovic et al., 2010; Lissek et al., 2009; Apergis-Schoute et al., 2017; Grillon, 2002). Moreover, context effects on startle are more pronounced in these patients since contextual fear may mimic the general aversive expectations that characterize pathological anxiety better than cued fear (Grillon, 2002). In humans, the effects of OT on behavioral or neural correlates have been studied by IN administration which is based on the observation that intranasally administered peptides can bypass the blood-brain-barrier to enter the central nervous system (Born et al., 2002). In healthy humans, IN OT has been shown to (a) have anxiolytic properties, (b) enhance fear extinction recall, (c) elicit a faster recovery of neuroendocrine/autonomic stress responses, and (d) attenuate amygdala reactivity along with enhanced amygdala-vmPFC connectivity (Eckstein et al., 2015; Heinrichs et al., 2003; Sripada et al., 2013). To the best of our knowledge, there are currently no studies published testing the ability of OT to support psychotherapy in anxiety disorder patients. However, several studies investigating the effects of a single dose of IN OT in these patients provide promising results. In these studies, IN OT has been shown to have anxiolytic properties, accompanied by physiological changes (such as decreased heart rate, skin conductance, electromyography and brain amygdala reactivity) and decreased PTSD symptoms (Koch et al., 2016; Koch et al., 2016b; Yatzkar and Klein, 2010; Koch et al., 2019).

In the light of the promising results of the above-mentioned studies, it is essential to conduct more animal and human studies to provide answers regarding the basic principles of the nasal administration route, the action mechanism of OT, as well as to include more advanced clinical trials in stress and anxiety disorder patients. The present study shows that IN administration of OT enhances estrus cycle-specific deficits of conditioned safety in female rats. It would be of great interested to investigate whether deficient safety learning in rats after early life stress or a 'traumatic' experience can also be compensated by IN OT, and whether the same holds true to clinical trials investigating the effect of IN OT in medication-enhanced psychotherapy.

CRediT authorship contribution statement

Judith C. Kreutzmann: Conceptualization, Methodology, Formal analysis, Investigation, Writing – original draft, Writing – review & editing, Visualization. **Markus Fendt:** Resources, Writing – review & editing, Supervision, Project administration, Funding acquisition.

Declaration of competing interest

Both authors have no biomedical financial interests or potential conflicts of interest.

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Appendix A. Supplementary data

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