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Acute and repeated intranasal oxytocin administration exerts anti-aggressive and pro-affiliative effects in male rats

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Summary Socio-emotional deficits and impulsive/aggressive outbursts are prevalent symptoms of many neuropsychiatric disorders, and intranasal administration of oxytocin (OXT) is emerging as a putative novel therapeutic approach to curb these problems. Recently, we demonstrated potent anti-aggressive and pro-social effects of intracerebroventricular (icv) OXT administration in male rats. The present study tested whether similar behavioral effects are induced when OXT is delivered intranasally. Heart-rate and blood-pressure responses were telemetrically monitored to investigate whether peripheral physiological effects were provoked after intranasal OXT administration. Intranasal OXT administration in resident animals reduced offensive aggression and increased social exploration toward an unfamiliar male intruder. Using a partner-preference test, intranasal OXT also strengthened the bonding between the male resident and its female partner. No changes in cardiovascular (re)activity were found, indicating an absence of direct peripheral physiological effects after intranasal OXT treatment. In conclusion, although the precise route and mechanisms of nose-to-brain transport/communication remain to be elucidated, our data demonstrated intranasal OXT to be an effective application method for suppressing intermale aggression and enhancing social affiliation.

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1. Introduction

Intranasal administration of oxytocin (OXT) has been shown to facilitate ''pro-social'' feelings and behaviors in healthy subjects (Domes et al., 2007; Kosfeld et al., 2005; Naber et al., 2010; Zak et al., 2007). Based on these findings, synthetic OXT analogs are emerging as novel therapeutic treatment approaches for mental disorders characterized by social dysfunction, such as autism (Guastella et al., 2010), social anxiety (Hall et al., 2012), and schizophrenia (Pedersen et al., 2011). Since minimal side-effects were reported across 38 randomized controlled trials (MacDonald et al., 2011), intranasal OXT administration found extensive use in clinical investigations for its easy and non-invasive delivery method and its putative rapid and direct access route to the brain.

The privileged access of the intranasal method is presumed to result from direct connections between the environment and the central nervous system afforded by the nasal mucosa (Guastella et al., 2013). To date, however, no clear evidence is available to support a direct transport pathway of OXT from the nasal cavity to the brain. Moreover, the mechanism and efficacy of penetration from the nose to either the cerebrospinal fluid (CSF) or the extracellular fluid is dependent on the distribution of the compound along the nasal epithelium.

A large expansion of trials testing nasal spray synthetic OXT effects on human social behaviors followed the initial study by Born et al. (2002), demonstrating a very small rise in human CSF vasopressin level, i.e. of a non-apeptide structurally closely related to OXT, within 10 min after its intranasal application. Only very recently, Striepens and colleagues provided clear evidence that a behaviorally effective dose of intranasal OXT (24 IU) elevated CSF (+60%) and blood (+250%) OXT concentrations in humans but that the kinetics in these compartments were considerably different (Striepens et al., 2013). Increased OXT concentration in human plasma (Gossen et al., 2012) and saliva (Weisman et al., 2012) has been reported more frequently and consistently after intranasal application, raising interpretative debates. Considering the great array of physiological activities affected by this peptide, a rise in plasma OXT level after intranasal application may provoke peripheral physiological changes thereby indirectly altering the behavioral performance with a similar, if not greater, impact than the effect induced by small rise in OXT CSF (Churchland and Winkielman, 2012). In primates, humans, and rats, for instance, peripheral administration of OXT is often associated with a decrease in blood pressure (Petersson et al., 1996), heart rate and body temperature (Hicks et al., 2014). Similarly, intracerebroventricular (icv) injected OXT decreased blood pressure, while inhibition of brain OXT synthesis by an anti-sense oligonucleotide increased blood pressure in rats (Maier et al., 1998). Moreover, deletion of the OXT gene in mice appeared to be associated with high blood pressure and heart rate (Bernatova et al., 2004).

Given that OXT is already prescribed off-label by health practitioners in the United States (Bales et al., 2013), animal studies should be pursued in a coordinated way with human studies addressing research questions concerning the spatial and temporal dynamics of the intranasal route, the dose-dependent effects on behavioral changes, as well as on the kinetics of both plasma and CSF OXT levels, while moreover verifying central availability of synthetic OXT after intranasal application.

Recent work by Neumann and colleagues showed, in rats and mice, increased OXT levels in the extracellular fluid of brain regions that are targeted by OXTergic projections (amygdala) and regions that are free of them (dorsal hippocampus), providing evidence that intranasally applied OXT is able to enhance CSF OXT (Neumann et al., 2013). Of relevance is also the recent study of Modi and colleagues reporting that aerosolized OXT resulted in significant increases in both lumbar CSF and plasma OXT levels over baseline for the full 120 min after administration (Modi et al., 2014).

Although, based on the lack of a barrier between the extracellular fluid and the CSF, changes in CSF OXT concentration are likely to be indicative of changes in OXT concentrations in brain and thus its bioavailability for behavioral effects, no study has provided a definite description of the route and/or mechanisms by which intranasally delivered OXT enhances brain OXT levels.

To date in preclinical research, behavioral effects of synthetic OXT have mainly been examined after either an icv infusion or direct local delivery into a brain region. Only few animal studies have tried to employ the intranasal route for inducing behavioral changes. In macaques, inhaled OXT enhanced pro-social choices (reward to another monkey) when there was no potential cost to self, but provoked an increase in selfish decisions when there was potential for direct self-reward. Moreover, the OXT-treated group showed a significantly increased CSF OXT concentration compared to the vehicle group (Chang et al., 2012). Parker and colleagues described a significantly reduced stress-induced hypothalamic—pituitary—adrenal axis activation only after chronic, but not acute, intranasal OXT administration in adult female squirrel monkeys (Parker et al., 2005). Bales and colleagues showed an impairment in partner-preference formation in male voles when treated long-term with low doses of the neuropeptide, while the acute administration facilitated partner preference (Bales et al., 2013).

As we recently showed clear anti-aggressive and pro-social explorative effects after acute and chronic icv infusion of synthetic OXT in male wild-type Groningen (WTG) rats (Calcagnoli et al., 2013), our current focus is to replicate these behavioral effects by applying the neuropeptide intranasally. Hence, the effects of acute and repeated intranasal administrations of OXT on the behavioral response of male resident rats are assessed during a standard resident-intruder (RI) test. According to the literature (Cho et al., 1999; Williams et al., 1994), we also hypothesized that intranasally administered OXT would promote pair-bonding formation during a partner-preference (PP) test. Moreover, heart-rate and blood-pressure (re)activities were monitored after acute intranasal application of OXT in order to control for potential peripherally provoked cardiovascular effects that may moderate the behavioral response to social challenges.

2. Methods

2.1. Animals

Four cohorts of adult male WTG rats (*Rattus norvegicus*) were used to perform the experiments as described below. All experimental and behavioral procedures were approved by the Animal Ethics Committee on Care and Use of Laboratory Animals (DEC 5824) of Groningen University and were conducted in agreement with Dutch laws (WoD 1996) and European regulations (Guideline 86/609/EEC).

2.2. Experiments

Each cohort of WTG rats was divided in 2 groups, and each group randomly assigned to either vehicle or OXT treatment condition. In experiments (1)—(3) the groups were matched according to the duration of offensive and social explorative behaviors displayed during the baseline RI test.

(1) thirteen animals received intranasal administration once a day, for 7 days of either vehicle ($N = 6$) or OXT ($N = 7$) and were tested using the RI test. This test was performed at baseline (day −4), and repeated at the start (day 1) and the end (day 7) of the treatment period, as well as 7 days after treatment cessation (day 14). In this way, we checked for acute (day 1 vs. day −4), repeated (day 7 vs. day 1 and vs. day −4) and long-lasting effects (day 14 vs. day −4);
(2) sixteen rats received intranasal administration once a day, for 7 days of either vehicle ($N = 8$) or OXT ($N = 8$) and their behavior was evaluated using the RI test. This test was performed at day −4, and then repeated at days 1, 7 and 14. Since the effects appeared to be washed out completely at day 14, the group-treatment combination was inverted in a cross-over design, i.e., animals that had received vehicle the first 7 days, were treated with OXT, and vice versa;
(3) sixteen rats received intranasal administration once a day, for 7 days of either vehicle ($N = 8$) or OXT ($N = 8$), and were tested using the PP test. The test was performed at days −4, 1, 7 and 14;
(4) thirteen animals were used for heart rate and blood pressure recordings before, during and after a single intranasal application of either vehicle ($N = 6$) or OXT ($N = 7$). After 5 days wash-out, the group-treatment combination was inverted in a cross-over design, i.e. the animals that received first vehicle, were then treated with OXT, and vice versa.

2.3. Social behavioral tests and behavioral evaluation

In experiment (1)—(3), after a 7-day habituation period, the baseline level of aggression was measured using the standard protocol of the RI test, as earlier described (Calcagnoli et al., 2013).

In experiment (3), one week after the baseline RI test, the baseline PP test was performed in the home cage of the resident. As social stimuli we used the companion female (partner) of the resident, that was removed from the home cage 1 h prior the test, and a novel oviduct-ligated but gonadally intact WTG female rat. Each female animal was encaged in a wire-meshed cage, allowing full visual interaction and olfactory communication. Both cages were simultaneously placed at the opposite walls of the observational cage. The test was performed and video recorded for 10 min. At the end of the test, both cages were removed. The companion female was placed back into the resident cage 1 h after the test.

The evaluation of the videos from both the RI and PP test was conducted using a custom-made data acquisition system (E-Line), which allows the manual scoring of the duration of different behaviors. The researcher was blind to the treatment conditions.

For the evaluation of the RI test, we used the followed behavioral categories: (1) offensive behaviors (lateral threat, clinch, attack-bite, keep down, chase, upright posture), (2) social explorative behaviors (moving toward, investigation and ano-genital sniffing of the intruder, crawl over, social grooming), (3) non-social behaviors (ambulation, rearing, exploration of the cage), (4) inactivity (sitting, lying), and (5) self-grooming (washing, scratching).

In the PP test, the behavioral assessment included: (1) investigation of the partner female, (2) investigation of the novel female, (3) exploration of the cage (ambulation, rearing, other), (4) self-grooming (washing, scratching), and (5) inactivity (sitting, lying). Investigation of the social stimulus was recorded only when the male resident was in the near proximity of the wired meshed cage, visibly engaged with the female animal (i.e., sniffing, nose-to-nose contact).

In both tests, the duration of each displayed behavior was expressed as the percentage of the total duration of the confrontation (10 min). The results of the PP test were graphically presented as ratio of the percentage of time spent investigating the partner above the novel female.

2.4. Telemetric measurement of blood pressure and heart rate

For the biotelemetric recordings of the cardiovascular signals, a blood pressure transmitter (PA-C40, Data Sciences International, St. Paul, MN, USA) was implanted surgically in the intraperitoneal cavity and the catheter of the transmitter was secured in the ventral aorta. Rats were anesthetized with a mixture of isoflurane and oxygen. After surgery, animals were allowed 10 days to fully recover. Receiver platforms were placed underneath each cage of the individually housed experimental animal and heart rate and blood pressure were recorded using Dataquest Labpro software. Data were sampled continuously from $t = -15$ min to $t = 60$ min. At $t = 0$ the animal was taken out of the cage for less than 2 min in order to intranasally administer vehicle or OXT. The average of the samples between $t = -15$ and $t = 0$ was used as baseline. Data from heart rate and blood pressure recordings were expressed as area under the curve (AUC) between $t = 0$ and $t = 60$.

2.5. Intranasal pharmacological treatment

We tested the effects of the following experimental treatments: vehicle solution (sterilized saline 0.9%, 20 μl) or synthetic OXT ($C_{43}H_{66}N_{12}O_{12}S_2$; MW 1007.19; Tocris, Germany; 1 μg/μl).

For the intranasal administration, we used the methodology described by Lukas and Neumann (2012). To minimize non-specific stress responses, the experimental animals had one week of habituation to the holding position, as well as training to the procedure.

The nasal application was carried out within the first 3—4 h of the dark phase, and 30 min prior to any behavioral test. The conscious rat was held by the experimenter in a supine position with a horizontal head position (Dhuria et al., 2010). The solution (2 × 10 μl) was bilaterally applied using a 100 μl pipette and equally distributed on the squamous epithelium of both the left and right rhinarium, avoiding direct contact of the tip of the pipette with the rhinarium, or direct application into one of the nostrils or in proximity of the philtrum. After administration, the rats were returned to their home cage.

2.6. Exclusion cases

In experiment 4, the blood-pressure response of one animal was excluded because of an unreliable signal. Therefore, in the analysis we included $N = 13$ animals for the heart-rate responses and $N = 12$ for the blood-pressure recordings.

2.7. Statistical analysis

Statistical analyses were carried out using SPSS for Windows; version 20. For all data, Shapiro—Wilk test was conducted to check for normality.

For experiments (1) and (3), treatment effects were tested by General Linear Model (GLM) repeated measures analysis of covariance (ANCOVA), while adjusting for baseline by entering the corresponding baseline values as a covariate for the sake of the design's efficiency (power) and validity (Liu et al., 2009; Senn, 2006). The design consisted of one within-subject variable (time) with the four measurement levels [baseline (day −4), acute (day 1), chronic (day 7), and wash-out (day 14)], and one between-subjects variable (treatment) with two levels (vehicle and OXT). If an overall significant time × treatment interaction was found, post hoc comparisons were carried out on the contrasts of the within-subject variable (day −4 vs. day 1; day −4 vs. day 7, day −4 vs. day 14, and day 1 vs. day 7). The repeated measure analysis was then repeated for each experimental group separately, to reveal which treatment condition was determining the statistical difference.

For the analysis of experiment (2), we used a GLM repeated measures ANOVA, consisting of two within-subject variables: treatment (vehicle and OXT) and time [days −4, 1, 7, and 14], and one between-subjects variable (sequence) with two levels [sequence 1 (vehicle treatment first, followed by OXT) and sequence 2 (OXT treatment first, followed by vehicle)]. If an overall significant time × treatment interaction was found, post hoc tests were carried out on the contrasts of the within-subject variable, as mentioned above.

To account for possible violations of the sphericity assumption for factors with more than two levels (such as the factor time), Huynh—Feldt adjusted *p*-values and the epsilon correction factor are reported together with the unadjusted degrees of freedom and *F*-values. To account for possible violations of the equality of variances, adjusted *p*-values are reported together with the unadjusted degrees of freedom and *t*-values.

Experiment (4) was analyzed by means of a repeated measure design with one within-subject variable (treatment) with two levels (vehicle and OXT), and one between subjects variable (sequence) with two levels [sequence 1 (vehicle treatment first, followed by OXT) and sequence 2 (OXT treatment first, followed by vehicle)].

For all comparisons, next to the *p*-values, partial eta squared (η^2) or the converted *z*-score (*r*) are presented as measures of effect size, with $\eta^2 < 0.06$ and $r < 0.3$ reflecting a small effect; $\eta^2 \geq 0.06$ and $r \geq 0.3$ a medium effect; and $\eta^2 \geq 0.14$ and $r \geq 0.5$ a large effect. Group differences at different time points were tested by means of Student's *t*-test. $p = 0.05$ was adopted as the criterion for statistical significance.

3. Results

3.1. Experiment 1: acute, repeated and long lasting behavioral effects of intranasal OXT treatment

Aim of this experiment was to evaluate the effects induced by acute and repeated intranasal OXT application on the socio-behavioral profile of male residents when encountering an unfamiliar intruder, and to explore possible long-lasting effects.

Baseline-adjusted significant time × treatment effects were found only for the categories of offensive behavior [$F_{3,30} = 3.85$, $p < 0.05$, $\eta^2 = 0.28$] and pro-social exploration [$F_{3,30} = 3.18$, $p < 0.05$, $\eta^2 = 0.24$] (Fig. 1 and Table 1, experiment 1).

In particular, in the offensive behavior time × treatment effects were found when comparing baseline (day −4) measure with day 1 [$F_{1,10} = 12.52$, $p < 0.01$, $\eta^2 = 0.56$], and day 7 [$F_{1,10} = 5.54$, $p < 0.05$, $\eta^2 = 0.37$]. No difference was found between day 1 and day 7, neither between day −4 and day 14 (Fig. 1A). Further analysis revealed that the above mentioned effects on offensive behavior resulted from both its increase in the vehicle group {[$F_{3,12} = 3.83$, $p < 0.05$, $\eta^2 = 0.49$]; day −4 vs. day 1 [$F_{1,4} = 7.38$, $p < 0.05$, $\eta^2 = 0.65$]; day −4 vs. day 7 [$F_{1,4} = 12.55$, $p < 0.05$, $\eta^2 = 0.76$]} and its decrease in the OXT-treated animals {[$F_{3,15} = 8.52$, $p < 0.01$, $\eta^2 = 0.63$]; day −4 vs. day 1 [$F_{1,5} = 14.84$, $p < 0.05$, $\eta^2 = 0.75$]; day −4 vs. day 7 [$F_{1,5} = 33.53$, $p < 0.01$, $\eta^2 = 0.87$]}. To note, when inserting the baseline measure as covariate into the analysis, vehicle- but not OXT-induced time effects showed a *p* value near to significance with a high effect size [$F_{3,12} = 3.08$, $p = 0.065$, $\eta^2 = 0.43$], indicating the dependency of OXT effects on the baseline measure.

Similarly, for the social explorative behavior, time × treatment effects were found when comparing

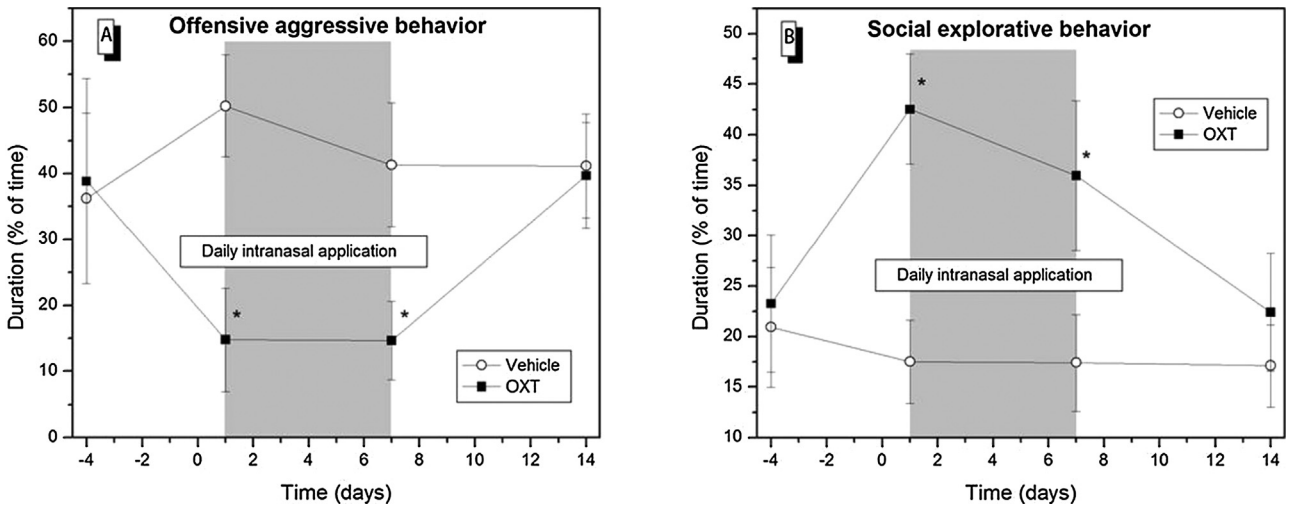

Figure 1 Changes in offensive aggression (A) and social exploration (B) before (day −4), during (days 1 and 7), and after daily intranasal treatment (day 14) of vehicle (20 μl) or oxytocin (OXT; 1 μg/μl, 2 × 10 μl). The gray area indicates the 7-day treatment period. Data are presented as mean ± SEM. Group differences at different time points are tested by means of *t*-test and *denotes significance ($p < 0.05$) between vehicle ($N = 6$) and OXT-treated ($N = 7$) groups.

baseline (day −4) measure with day 1 [$F_{1,10} = 13.03$, $p < 0.01$, $\eta^2 = 0.56$]. When comparing baseline with day 7, the time by treatment interaction failed significance but showed a high effect size [$F_{1,10} = 4.17$, $p = 0.07$, $\eta^2 = 0.29$], inviting to further investigate this effect on a single treatment level (see below). No difference was found between day 1 and day 7, neither between day −4 and day 14 (Fig. 1B). Independent of the baseline level of social exploration, OXT increased the duration of this behavior [$F_{3,15} = 4.13$, $p < 0.05$, $\eta^2 = 0.45$] after both single and repeated intranasal applications {day −4 vs. day 1 [$F_{1,5} = 14.07$, $p < 0.05$, $\eta^2 = 0.74$]; day −4 vs. day 7 [$F_{1,5} = 7.18$, $p < 0.05$, $\eta^2 = 0.59$]}. On the other hand, a baseline-dependent time effect was found in the vehicle condition [$F_{3,12} = 6.74$, $p < 0.001$, $\eta^2 = 0.63$] reflecting a significant decrease of social exploration compared to baseline (day −4) {day 7 [$F_{1,4} = 33.12$, $p < 0.01$, $\eta^2 = 0.89$], and day 14 [$F_{1,4} = 12.08$, $p < 0.05$, $\eta^2 = 0.75$]}.

Table 1 Summary of the durations (indicated as percentage of the total 10 min test) spent in the different behavioral categories evaluated during the intermale encounter (with the exclusion of the categories ''offensive aggressive behavior'' and ''social explorative behavior''), and the group means of the latency time to the first attack (ALT; indicated in seconds) ± the respective SEM.

		Day −4 Average ± SEM	Day 1 Average ± SEM	Day 7 Average ± SEM	Day 14 Average ± SEM
Experiment 1, $N = 13$					
Non-social exploration	Vehicle	40.50 ± 3.47	32.42 ± 5.29	44.87 ± 5.13	50.12 ± 6.61
	OXT	38.40 ± 6.70	34.61 ± 3.06	48.21 ± 5.06	44.19 ± 6.64
Inactivity	Vehicle	6.70 ± 1.32	5.82 ± 1.78	8.12 ± 1.25	7.18 ± 1.87
	OXT	6.53 ± 1.46	8.23 ± 1.44	6.97 ± 1.75	6.66 ± 1.32
Self-grooming	Vehicle	8.53 ± 2.66	5.38 ± 2.87	6.43 ± 3.03	5.68 ± 1.19
	OXT	3.36 ± 1.23	5.54 ± 1.60	6.39 ± 3.51	4.17 ± 1.34
ALT	Vehicle	492.50 ± 67.95	433.33 ± 49.01	428.33 ± 50.24	273.33 ± 46.61
	OXT	493.29 ± 49.97	458.00 ± 72.32	382.71 ± 64.19	211.43 ± 67.77
Experiment 2, $N = 16$					
Non-social exploration	Vehicle	28.24 ± 2.72	31.88 ± 2.75	32.44 ± 2.01	31.59 ± 1.89
	OXT	29.50 ± 2.92	39.58 ± 2.53	42.01 ± 2.64	30.76 ± 2.27
Inactivity	Vehicle	21.84 ± 2.15	20.21 ± 1.81	22.08 ± 1.56	21.13 ± 1.88
	OXT	19.77 ± 3.33	18.06 ± 1.62	18.52 ± 2.09	22.32 ± 2.43
Self-grooming	Vehicle	8.73 ± 1.85	7.24 ± 1.50	10.16 ± 1.61	9.77 ± 2.13
	OXT	6.39 ± 1.00	6.51 ± 1.45	8.90 ± 1.53	11.34 ± 1.61
[ALT	Vehicle	313.25 ± 53.11	306.38 ± 55.36	343.75 ± 56.70	307.81 ± 57.15
	OXT	321.06 ± 57.93	396.19 ± 63.72	396.69 ± 53.86	228.44 ± 42.79

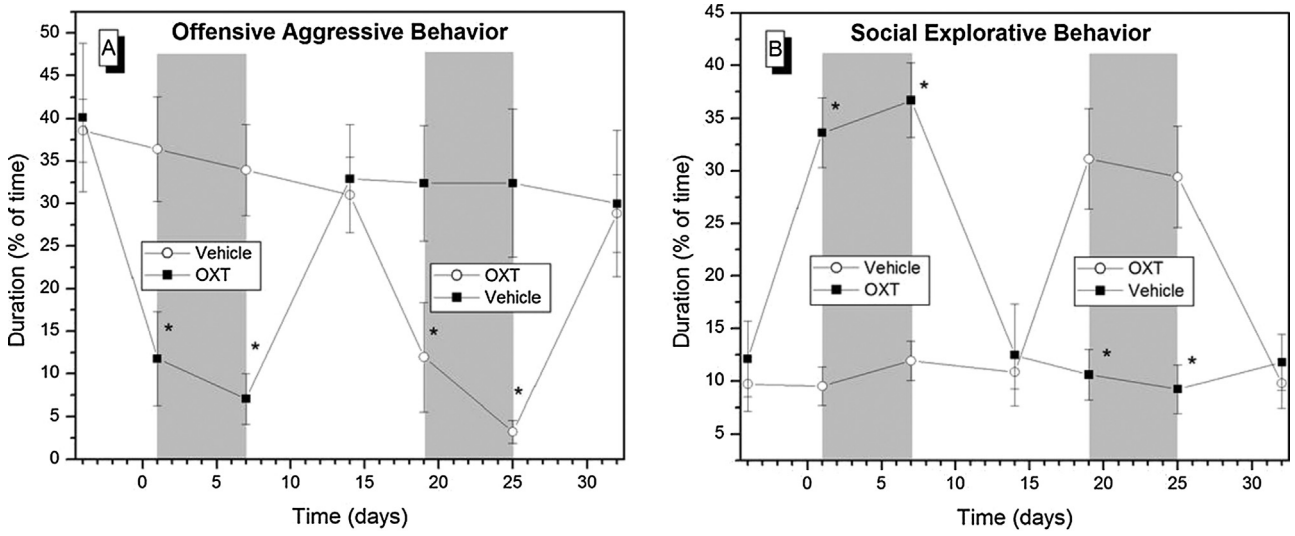

Figure 2 Changes in offensive aggression (A) and social exploration (B) before (days −4 and 14), during (days 1, 7, 19 and 25), and after (days 14 and 32) daily intranasal treatment of vehicle (20 μl) or oxytocin (OXT; 1 μg/μl, 2 × 10 μl). The gray areas indicate the 7-day treatment periods. Data are presented as mean ± SEM. Group differences at different time points are tested by means of *t*-test and *denotes significance ($p < 0.05$) between vehicle ($N = 8$) and OXT-treated ($N = 8$) groups.

3.2. Experiment 2: replication of the effects by a repeated measurement cross-over design

In this experiment, we aimed at confirming the findings of experiment (1), adopting a within-subject cross-over design. Inverting the initial group-treatment combination was possible since no long-lasting effects were found 7 days after the first treatment period [$F_{1,7} = 0.80$, $p > 0.05$, $\eta^2 = 0.10$].

No significance was found for the interaction treatment × time × sequence, excluding that treatment effects might have been due to the order of administration. Yet, significant overall treatment × time effects were found for both the category of offensive behavior [$F_{2,28} = 11.79$, $p < 0.001$, $\eta^2 = 0.46$] and pro-social exploration [$F_{2,28} = 13.00$, $p < 0.001$, $\eta^2 = 0.48$] (Fig. 2). As shown in Fig. 2A, only OXT treatment [$F_{2,30} = 4.57$, $p < 0.05$, $\eta^2 = 0.23$] remarkably reduced offensive behavior after both acute [$F_{1,14} = 14.12$, $p < 0.01$, $\eta^2 = 0.50$] and repeated [$F_{1,14} = 30.26$, $p < 0.001$, $\eta^2 = 0.68$] administration. Concomitantly, only OXT treatment [$F_{2,30} = 3.98$, $p < 0.05$, $\eta^2 = 0.21$] increased social exploration after both acute [$F_{1,14} = 25.04$, $p < 0.001$, $\eta^2 = 0.64$] and repeated [$F_{1,14} = 14.91$, $p < 0.01$, $\eta^2 = 0.52$] administration (Fig. 2B). No statistical difference was found between measurements at day 1 and day 7 in any of the two categories, neither were long-lasting effects found 7 days after the cessation of the second treatment period [$F_{1,7} = 0.22$, $p > 0.05$, $\eta^2 = 0.06$]. No overall time × treatment was found in any of the other behavioral categories (Table 1, experiment 2).

3.3. Experiment 3: acute, repeated and long lasting behavioral effects of intranasal OXT treatment on the partner-preference test

In this experiment we tested WTG rats in a PP test, in order to investigate the effects of acute and repeated intranasal OXT on pair-bonding behavior. We found a significant overall time × treatment interaction in the ratio score partner/novel [$F_{3,39} = 4.07$, $p < 0.05$, $\eta^2 = 0.24$] when comparing baseline (day −4) measure with day 1 [$F_{1,13} = 5.55$, $p < 0.05$, $\eta^2 = 0.30$]. When comparing baseline with day 7 the interaction just failed significance, but yet showing a high effect size [$F_{1,13} = 4.28$, $p = 0.06$, $\eta^2 = 0.25$] (Fig. 3). No difference was found between day 1 and day 7, neither between day −4 and day 14. Although the overall time effect failed significance when separately investigated in the OXT condition [$F_{3,18} = 2.90$, $p = 0.06$, $\eta^2 = 0.33$], the high effect size invited us to investigate single time

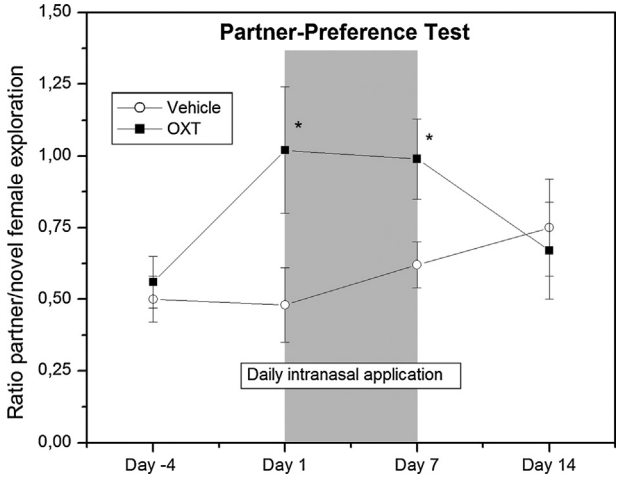

Figure 3 Changes in partner preference before (day −4), during (days 1 and 7), and after (day 14) daily treatment with either vehicle or oxytocin (OXT; 1 μg/μl, 2 × 10 μl). The gray area indicates the 7-day treatment period. Data are presented as mean ± SEM. Group differences at different time points are tested by means of *t*-test and *denotes significance ($p < 0.05$) between vehicle ($N = 8$) and OXT-treated ($N = 8$) groups.

contrasts. Here we found a greater preference to explore the partner rather than the novel female after both acute [$F_{1,6} = 10.13$, $p < 0.05$, $\eta^2 = 0.62$] and repeated [$F_{1,6} = 8.40$, $p < 0.05$, $\eta^2 = 0.58$] intranasal treatment. Even though the graph might suggest so, no effects were found for the vehicle group.

On the other hand, time × treatment effects were also found in the category self-grooming [$F_{3,39} = 4.56$, $p < 0.05$, $\eta^2 = 0.26$]. Both vehicle [$F_{3,18} = 5.12$, $p < 0.05$, $\eta^2 = 0.46$] and OXT [$F_{3,18} = 9.55$, $p = 0.001$, $\eta^2 = 0.61$] treatment increased the duration of self-grooming at day 14 as compared to baseline {vehicle [$F_{1,6} = 11.68$, $p < 0.05$, $\eta^2 = 0.66$] and OXT [$F_{1,6} = 41.26$, $p = 0.001$, $\eta^2 = 0.87$]}. No increase in self-grooming was found during the treatment period, excluding the possibility of confounding effects. No difference was found in the general locomotor activity.

3.4. Experiment 4: acute effects of intranasal OXT treatment on the cardiovascular baseline response

This experiment was designed to reveal potential intranasal OXT-induced peripheral effects that might bias centrally induced behavioral effects. We found no difference in either heart rate or blood pressure recordings between intranasal vehicle and OXT treatment (Fig. 4).

4. Discussion

This study provides the first evidence of robust anti-aggressive and pro-social explorative effects after intranasal application of synthetic OXT in adult male resident rats. A single intranasal administration of the nonapeptide selectively shifted the social behavior profile from a hostile toward a more positive/explorative interaction. The efficacy and selectivity of the effects persisted after repeated OXT administrations. Moreover, both acute and repeated intranasal OXT treatments strengthened the attention for the female companion in a PP test. No long-lasting effects were recorded 7 days after treatment cessation. As no alteration of heart rate and blood pressure was found after acute intranasal OXT application, we can exclude that effects on the cardiovascular system may have confounded the centrally OXT-induced acute behavioral changes.

The currently observed behavioral effects after intranasal OXT treatment resemble those found in our previous studies using acute and chronic icv administration (Calcagnoli et al., 2013) and are inline with other previous icv administration studies in animals (Williams et al., 1994; Young et al., 2011) and many clinical trials showing facilitated pro-social behavior, bonding formation and social engagement after intranasal OXT application (Bertsch et al., 2013; Liu et al., 2013).

Interestingly, we found that OXT decreased offensive behavior whereas an increase of offensive behavior was observed in the vehicle condition. Although this latter might be a batch-specific effect (it was not found in our second experiment), the phenomenon of increased aggressiveness with repeated exposure to a male intruder is well known and likely to be due to repeated winning experience (de Boer et al., 2003). Hence, the decrease resulting from intranasal OXT administration is even more remarkable. Moreover, as previously highlighted in our icv pharmacological manipulation (Calcagnoli et al., 2013), the efficacy of OXT in modulating offensive behavior appeared to be dependent on the baseline level of the behavior. Such dependency was not found for the OXT-induced effects in social explorative behavior. This prompts further research to investigate contextual and inter-individual factors that can moderate the effect of intranasal OXT, or even may confer a ''tend and defend'' response. In human trials, for instance, intranasal OXT was shown to decrease cooperation when participants interacted with strangers compared to familiar persons (Declerck et al., 2010). Similarly, OXT motivates non-cooperation in intergroup conflict to protect vulnerable in-group members (De Dreu et al., 2011), although a recent meta-analysis could not confirm that intranasal OXT significantly decreases out-group trust

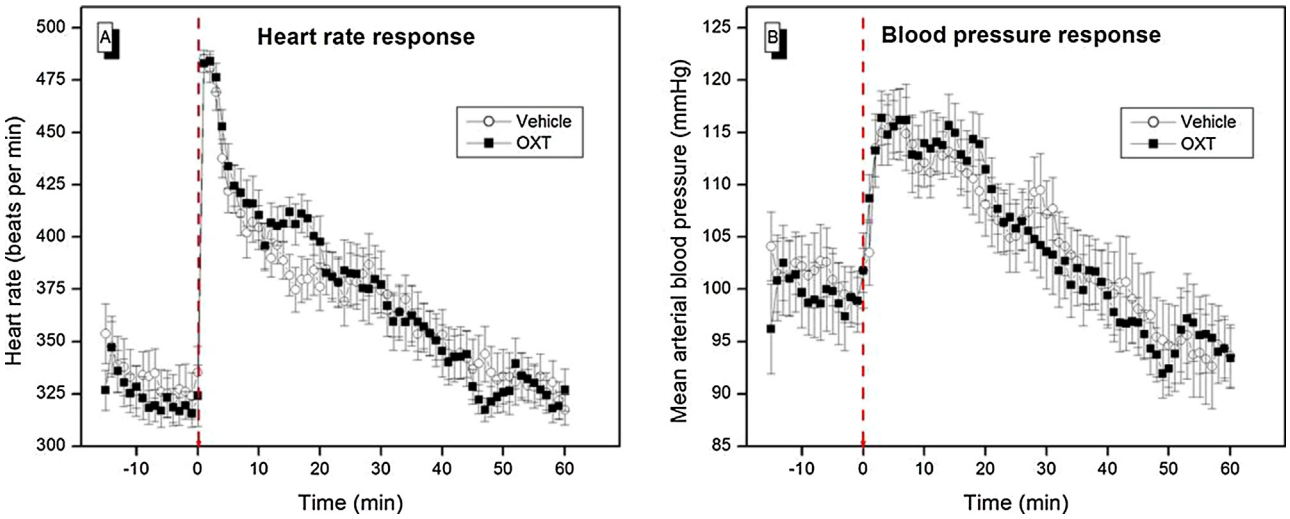

Figure 4 Heart rate (A) ($N = 13$) and blood pressure (B) ($N = 12$) responses of male rats that intranasally received either vehicle or oxytocin (OXT; 1 μg/μl, 2 × 10 μl) at $t = 0$ (red dashed line). (For interpretation of the references to color in this figure legend, the reader is referred to the web version of this article.)

(Van and Bakermans-Kranenburg, 2012). Moreover, in women, state anxiety has been found to moderate the intranasal OXT-induced reduction of hostility expressed in a competitive aggression game (Campbell and Hausmann, 2013).

In addition to the contextual factors and inter-subject variability, OXT-induced behavioral responses vary depending upon the application method, the therapeutic window and dose range. In rhesus macaques, aerosolized OXT, but neither intranasal nor intraveneous OXT administration, resulted in significant increases in lumbar CSF OXT levels (Modi et al., 2014). The discordance in effect on CSF OXT concentrations between the two routes of nasal OXT administration, aerosolized and intranasal, suggests the two methods of application may have different dynamics as to the nasal passage of rhesus monkeys. In male WTG rats, enduring anti-aggressive and pro-social explorative effects were found after a 7-day period of chronic and continuous enhancement of brain OXT levels via osmotic mini-pumps (Calcagnoli et al., 2014), but not after a 7-day period of repeated intranasal delivery of OXT. Absence of persistent behavioral changes may indicate that repeated OXT intranasal delivery is unable to provoke the putative lasting alterations in the endogenous OXTergic system (peptide expression, release patterns, receptor density, etc.) most likely occurring after continuous icv infusion of OXT.

Moreover, in male prairie voles, 3-weeks of low and medium doses of intranasal OXT during the developmental phase have been described to induce long-term impairment in partner-preference formation, despite the facilitation seen after acute intranasal treatment (Bales et al., 2013).

Although short-term administration may be safer or more effective than chronic administration, longitudinal studies should be carried out to assure safety, to exclude tolerance development, to determine the most effective therapeutic window and dose, and to verify the expected long-term effects. Moreover, studies exploring intranasal OXT effects should show corresponding central and plasma levels of OXT following intranasal dosing. In this way, we would know whether the rise in CSF OXT after intranasal application is appreciably greater than the one potentially triggered by the experimental challenges or contexts themselves. Collecting data of plasma OXT level after intranasal application is also of particular relevance when considering that pro-social effects (Ramos et al., 2013) and increased hypothalamic Fos expression (Carson et al., 2010) have been reported in male rats after intraperitoneal OXT injection.

The dose of OXT we applied intranasally is similar to what Neumann and colleagues have shown to induce increased brain OXT levels in adult rats (Neumann et al., 2013). Although those authors could not make a distinction between exogenous and endogenous OXT, the local rise in areas lacking OXTergic innervations (e.g., dorsal hippocampus and mediolateral part of caudate putamen) was interpreted as proof of transport of synthetic OXT from the nasal mucosa to the brain extracellular fluid. However, it cannot be excluded that the behavioral effect might also be due to endogenous OXT being released and transported from the hypothalamic area after binding of synthetic OXT on OXT-sensitive receptors located in the olfactory mucosal or brain regions (Yoshimura et al., 1993). Sensory input from the vomeronasal organ and main olfactory epithelium are received by and processed in the accessory olfactory bulb and main olfactory bulb, respectively (Sokolowski and Corbin, 2012). Already the olfactory bulb could be a site of action where OXT may alter the perception or processing of social behavior-relevant olfactory stimuli (Dluzen et al., 1998), and therefore the behavioral response in social context and recognition task, such as intermale confrontation and partner-preference test. However, in our experiments, the unaltered latency to attack seems to reject the hypothesis of OXT-induced olfactory deficits.

As alternative mechanism, OXT may modulate the activity of brain areas that receive projections from the accessory and main olfactory bulb, such as the olfactory/piriform cortex and amygdala, especially the medial region (Swanson and Petrovich, 1998). The amygdala is generally believed to be a crucial processing station where the level of salience is attributed to a given stimulus (LeDoux, 1993); in particular, the lateral and basal nuclei of the amygdala are strongly involved in processing sensory information and in the detection of biologically relevant stimuli in the environment, including olfactory cues (Sah et al., 2003). Microinjection of OXT into the central amygdaloid nuclei has been found to have potent anti-aggressive and pro-social exploratory effects in male WTG rats (Calcagnoli et al., unpublished), similarly to what we have here described after intranasal OXT application. From human literature, the pro-social ''tend and befriend''-like action ascribed to OXT appears to be associated with an OXT-dependent suppression of the amygdala activity in exposure to aversive and stressful conditions (Kirsch et al., 2005), and facilitated amygdala activity under positive/empathy-provoking conditions (Petrovic et al., 2008).

The amygdala is also known to have projections to the hypothalamus for further integration and coordination with the brain stem (LeDoux, 1993; Swanson and Petrovich, 1998). Therefore, the hypothesis that intranasal OXT application might induce endogenous release of the peptide via activation of the OXTergic neurons in the PVN and SON cannot be ruled out. To note, in a recent study where intranasal administration of large doses of OXT and vasopressin failed to show behavioral effects, the authors did not find any changes in neuronal activity that have otherwise been reported after icv infusion of low doses of these peptides (Ludwig et al., 2013).

Considering that a parallel but independent peripheral uptake is likely to occur after intranasal administration of OXT (Neumann et al., 2013; Striepens et al., 2013), more knowledge should also be gained about the contribution that peripheral OXT-evoked physiological changes may have to the centrally mediated behavioral output. Studies have shown, for instance, that blockage of peripheral vasopressin receptors not only prevented vasopressin-mediated physiological changes, but also abolished vasopressin-induced behavioral effects (Le Moal et al., 1981). In addition, inhaled vasopressin has been reported to increase sociality in rats, concomitantly with a reduction of temperature and heart rate (Ramos et al., 2014). Despite this evidence and the increased arterial pressure and bradycardia previously found after elevated peripheral OXT level (Ludwig et al., 2013), in our study we did not find any difference in cardiovascular (re)activity between vehicle and OXT-treated animals. Hence, the suggestion that our behavioral changes may have

been (partly) caused by peripheral cardiovascular changes induced by plasma OXT can be refuted.

Another method to ascertain that the intranasal OXT-induced behavioral effects are principally centrally mediated is combining intranasal OXT with a selective OXT receptor antagonist given centrally. However, different diffusion rates of the intranasal and icv route might limit the validity and conclusive value of this type of experiment. Alternately, imaging studies have been used to prove the nose-to-brain transport of relative big molecules in rats (Thorne et al., 2004). However, the influence that the radiolabelled ligand might have on the physical and chemical properties of the investigated molecule represents still the major concern for using this technique.

In conclusion, our findings provide evidence for the effectiveness of intranasal delivery of OXT in modulating social behavior in either an aggressive conflict with a conspecific or an affilitiative interaction with a familiar partner. The robustness and replicability of the behavioral effects encourage further investigation aimed at revealing the precise mechanistic underpinnings of the nose-to-brain transport/communication route.

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The University of Groningen had no further role in study design; in the collection, analysis and interpretation of data; in the writing of the report; and in the decision to submit the paper for publication.

Conflict of interests

The authors declare no conflict of interest.

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