



Salivary oxytocin concentrations in response to running, sexual self-stimulation, breastfeeding and the TSST: The Regensburg Oxytocin Challenge (ROC) study



Trynke R. de Jong^{a,*}, Rohit Menon^a, Anna Bludau^a, Thomas Grund^a, Verena Biermeier^a, Stefanie M. Klampfl^a, Benjamin Jurek^a, Oliver J. Bosch^a, Juliane Hellhammer^b, Inga D. Neumann^a

^a Department of Behavioural and Molecular Neurobiology, University of Regensburg, Regensburg, Germany

^b Diagnostic Assessment and Clinical Research Organization (DAACRO) GmbH & Co, KG, Science Park Trier, Trier, Germany

ARTICLE INFO

Article history:

Received 21 July 2015

Accepted 26 August 2015

Keywords:

Oxytocin
Saliva
challenge
Radioimmunoassay
Sexual self-stimulation
Running
TSST

ABSTRACT

Intranasal oxytocin (OXT) application is emerging as a potential treatment for socio-emotional disorders associated with abnormalities in OXT system (re-) activity. The crucial identification of patients with such abnormalities could be streamlined by the assessment of basal and stimulus-induced OXT concentrations in saliva, using a simple, stress-free sampling procedure (i.e. an OXT challenge test). We therefore established the Regensburg Oxytocin Challenge (ROC) test to further validate salivary OXT concentrations as a practical, reliable and sensitive biomarker.

OXT concentrations were quantified by radioimmunoassay in samples collected at home by healthy adult male and female volunteers before and after running (“Run”) or sexual self-stimulation (“Sex”). In lactating women, salivary OXT concentrations were quantified before, during and after breastfeeding. Salivary OXT along with salivary cortisol and heart rate were monitored in healthy adult participants undergoing the Trier Social Stress Test (TSST).

The home-based “Run” and “Sex” challenges as well as the laboratory-based TSST caused quantifiable, rapid, and consistent increases in salivary OXT (approximately 2.5-fold after 10–15 min), which were similar for men and women. Breastfeeding did not result in measurably increased salivary OXT levels, probably because the short pulses of OXT release characteristic for lactation were missed.

Taken together, ROC tests reliably assess the responsiveness of the OXT system (i.e., the increase in salivary OXT concentrations as compared to basal levels) to challenges such as “Run” and “Sex” at home or psychosocial stress (TSST) in the laboratory. Further studies with larger sample numbers are essentially needed in order to reveal individual differences in ROC test outcomes depending on, for example, genetic or environmental factors.

© 2015 Elsevier Ltd. All rights reserved.

1. Introduction

The nonapeptide oxytocin (OXT) has received considerable interest in recent years as a modulator of social behaviors on the one hand, and anxiety and stress-coping on the other (Lee et al., 2009; Neumann and Landgraf, 2012). Chronic or transient imbalances in OXT system activity driven by genetic and epigenetic variables, or by stressful environmental factors, have been associated with socio-emotional dysfunctions in both animals and humans (for reviews see Meyer-Lindenberg

et al., 2011; Neumann and Landgraf, 2012). Polymorphisms in the OXT gene or its receptor and/or alterations in plasma or cerebrospinal fluid (CSF) OXT levels have been described, for example, in patients suffering from conduct disorder (Dadds et al., 2014), autism spectrum disorders (Ruggeri et al., 2014), borderline disorder (Bertsch et al., 2013), Prader-Willi Syndrome (Tauber et al., 2014), and (social) anxiety disorders (Neumann and Slattery, 2015; Ziegler et al., 2015).

In line with the profound pro-social, anxiolytic and anti-stress effects of OXT described in animals, intranasal administration of synthetic OXT is extensively investigated to assess its effects in healthy adults as a function of OXT receptor gene polymorphisms (Feng et al., 2015), or to reveal potential benefits for patients suffering from socio-emotional disorders (Zik and Roberts, 2015). Due

* Corresponding author at: Department of Behavioural and Molecular Neurobiology, University of Regensburg, 93053 Regensburg, Germany. Fax: +49 941 943 3052. E-mail address: trynke.de-jong@ur.de (T.R.d. Jong).

to the multifactorial etiology, there is a pressing need to identify patients with OXT system deficits, for example using endocrine biomarkers reflecting the activity of the OXT system. However, the assessment of OXT responses to defined stimuli has been identified as a major research gap (Neumann and Slattery, 2015).

To date, OXT concentrations have been typically analyzed in blood plasma (Crockford et al., 2014; Landgraf et al., 1982; McCullough et al., 2013) and, less commonly, in urine (Seltzer et al., 2010) or CSF (Carson et al., 2015; Jokinen et al., 2012; Kagerbauer et al., 2013). These methods each have their own advantages and limitations. Obtaining CSF, for example, requires an invasive lumbar puncture procedure and trained medical care, but reflects the central OXT system best (Carson et al., 2015; Kagerbauer et al., 2013; Neumann and Landgraf, 2012). Measuring OXT in urine is not invasive, but it is not well validated and provides information with a low and unspecific temporal resolution (McCullough et al., 2013). Blood sampling requires medical care, but is modestly invasive and provides good temporal resolution. Therefore, plasma OXT is the most commonly used global biomarker of OXT system activity (Crockford et al., 2014). Though often warranted, it needs to be kept in mind that the temporal dynamics of peripheral OXT release may substantially differ from those of central release in a stimulus-dependent way (Landgraf and Neumann, 2004; Neumann and Landgraf, 2012). Further, measuring plasma OXT requires a time-consuming extraction procedure prior to radioimmuno-assay (RIA) or enzyme-linked immunosorbent assay (ELISA) to avoid the detection of non-OXT fragments resulting in artificial, supra-physiological values (McCullough et al., 2013).

Aside from blood, urine, and CSF, OXT can also be measured in saliva, which provides various advantages. Saliva samples can be collected repeatedly in a short time frame, allowing a detailed monitoring of peripheral OXT secretion both under basal and challenged conditions (Carter et al., 2007). In addition, saliva collection does not require medical care, a laboratory setting or interaction with a researcher, eliminating considerable sources of (psychosocial) stress that are likely to interfere with OXT system activity, especially in patients with socio-emotional disorders and children. Although initially greeted with skepticism (Horvat-Gordon et al., 2005), reliable basal levels of OXT have now been repeatedly measured in saliva (Blagrove et al., 2012; Grewen et al., 2010; Holt-Lunstad et al., 2011; Javor et al., 2014; White-Traut et al., 2009). Much less is known, however, about the sensitivity of saliva samples to detect dynamic changes in OXT levels in response to relevant physiological and psychological challenges. Such knowledge is needed for two important reasons: first, to avoid certain stimuli when assessing basal OXT concentrations, and second, to select appropriate challenges to monitor OXT release patterns in order to identify patients with dysfunctional OXT system reactivity.

The aim of the present Regensburg Oxytocin Challenge (ROC) study was, therefore, to measure OXT levels in saliva samples in response to four stimuli known to trigger OXT release into blood: physical exercise (“Run”) (Hew-Butler et al., 2008; Landgraf et al., 1982), sexual self-stimulation (“Sex”) (Blaicher et al., 1999; Carmichael et al., 1987; Murphy et al., 1987), and psychosocial stress during the Trier Social Stress Test (TSST) (Pierrehumbert et al., 2010) in healthy men and women, and breastfeeding in lactating women (McNeilly et al., 1983; Nissen et al., 1996; Ueda et al., 1994; Ueda et al., 1994).

2. Methods

2.1. Participants

For the three home-based ROC tests (“Run”, “Sex” and “Breastfeeding”), healthy adult volunteers were recruited from the

environment of the researchers. For “Run” and “Sex”, participants (males: $n = 10$, median years of age: 28, range: 26–65; females: $n = 7$, median years of age: 29, range: 23–52) performed both tasks with at least 24 h in between. For “Breastfeeding”, lactating mothers ($n = 4$) of healthy, fully breastfed singleton infants (≤ 4 months old) and well established nursing routines volunteered to collect two sets of three saliva samples at home, with at least 24 h between the sets. For the TSST, 15 healthy adult males (median years of age: 24, range: 19–34) and 15 healthy adult females (median years of age: 23, range: 18–27) were recruited using the in-house subject database of DAACRO, as well as advertisements, posters and flyers distributed in the Trier region.

Participants received detailed verbal and written information on the study procedures and gave their consent to the experiments. For the TSST, ethical approval was obtained via the International Medical and Dental Ethics Commission (Freiburg, DE), and the study was performed in accordance with the ethical principles originating in the Declaration of Helsinki and consistent with the Good Clinical Practice (GCP) guidelines of the International Conference on Harmonization (ICH) and applicable regulatory requirements on bioethics.

2.2. Procedures for home-based ROC tests (“Run”, “Sex” and “Breastfeeding”)

The three ROC tests consisted of the self-controlled collection of saliva samples under basal conditions 30 min prior to the start of the stimulus, and 10 min as well as 40 min (or 60 min, for breastfeeding) after onset of the stimulus (see Fig. 1). Each volunteer received pre-coded Salivettes® (Sarstedt, Nuembrecht, DE) as well as written instructions for the behavioral and sampling procedures. “+10” samples were collected 10 min after onset of moderate running (“Run”), after 10 to 15 min of masturbation including orgasm (“Sex”), or 10 min after onset of breastfeeding. The “Run” and “Sex” stimulations were terminated at the “+10” sample collection, whereas “Breastfeeding” continued for approximately 20 min after sampling until the infant was satisfied.

Volunteers were instructed to complete the tasks between 18:00 h and 0:00 h, and to refrain from consuming food and drinks (other than water), chewing gum/candy, brushing teeth, or considerable activity/stress (aside from the stimulus) from at least 1 h before the basal sample until completion of the task. For the “Sex” stimulus, volunteers were also instructed to perform the masturbation alone. For “Breastfeeding”, volunteers were instructed to minimize interaction with their infant for 1 h prior to collection of the basal sample (but not between the “+10” and “+60” samples).

For each saliva sample, volunteers were instructed to gently chew on the cylindrical swab of the Salivette for approximately one minute, to securely place the swab back into the Salivette and to store it at -20°C .

2.3. Procedures for the TSST

TSST procedures were derived from Kirschbaum et al. (1993). TSST participants visited DAACRO’s study site in Trier twice. During the first visit, participants received an introduction and instructions; during the second visit the TSST took place between 13:00 h and 15:30 h. The TSST consisted of 4 components: introduction (2 min), preparation (3 min), speech (5 min) and arithmetic task (5 min) using the following procedure: the participant was lead into the test room, introduced to the setting by the study manager, and was asked to prepare a speech as part of a job interview, which had to be subsequently given in the presence of two members of a committee board trained in behavioral observation. The participant was informed that video and voice recording are taken during the interview for evaluation of the content and style of presenta-

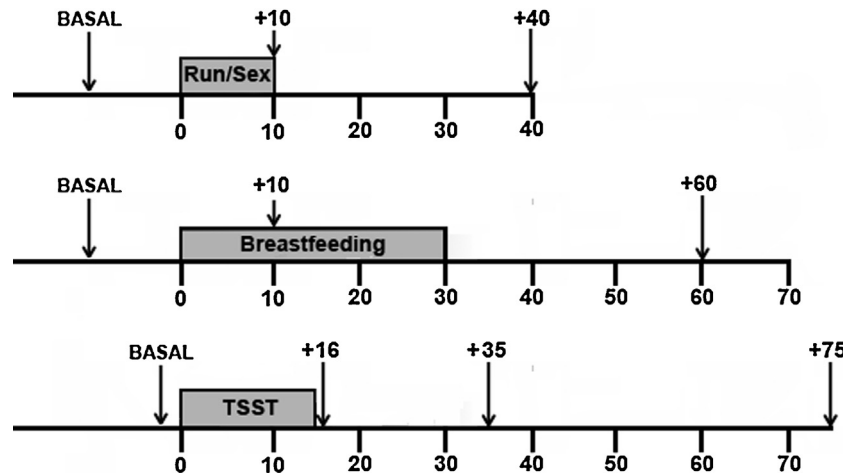


Fig. 1. Timing of collection of saliva samples during the different challenges to monitor changes in OXT concentrations. Timing of basal samples was somewhat variable for the “Run”, “Sex” and “Breastfeeding” challenges (i.e. 10–30 min prior to the start of the stimulus), but not for the “TSST” challenge (always 2 min prior to the start of the TSST). The total duration of the “Breastfeeding” stimulus was controlled by the infant, resulting in a variable timing of the third sample (30 min after the end of breastfeeding, i.e. approximately 50–70 min after the start of the stimulus).

tion. Immediately after the speech, the participant was asked to subtract 17 from 2023 stepwise as quickly and correctly as possible. After completion of the TSST (15 min total, a duration unknown to the participants), the study manager guided the participants to another room where they rested until the final saliva sample was obtained.

Seven saliva samples were taken from each subject (see above), collected 2 min prior to the TSST (“basal”) as well as 16, 25, 35, 45, 60 and 75 min after the start of the TSST (“+16”, “+25” etc.). CORT was measured in all samples, whereas OXT was measured in the basal sample and the “+16”, “+35” and “+70” samples, see Fig. 1. After collection, samples were frozen at -20°C until analysis.

2.4. Quantification of salivary OXT

Salivary OXT concentrations were quantified by Radioimmunoassay (RIA) (RIAGNosis, Munich, Germany). In brief, for each sample 300 μl of saliva was evaporated (SpeedVac, Thermoscientific Inc, Waltham, MA, USA), and 50 μL of assay buffer was added followed by 50 μL antibody (raised in rabbits against OXT). After a 60-min preincubation interval, 10 μL ^{125}I -labeled tracer (PerkinElmer, Waltham, MA, USA) was added and samples were allowed to incubate for 3 days at 4°C . Unbound radioactivity was precipitated by activated charcoal (Sigma–Aldrich, St Louis, MO, USA). Under these conditions, an average of 50% of total counts are bound with <5% non-specific binding. The detection limit is in the 0.5 pg/sample range, depending on the age of the tracer, with typical displacements of 20–25% at 2 pg, 60–70% at 8 pg and 90% at 32 pg of standard neuropeptide. Cross-reactivity with arginine vasopressin (AVP), ring moieties and terminal tripeptides of both OXT and AVP and a wide variety of peptides comprising 3 (α -melanocyte-stimulating hormone) up to 41 (corticotropin-releasing factor) amino acids are <0.7% throughout. The intra- and inter-assay variabilities were <10%. Saliva samples were analyzed in different batches; however, all samples from an individual challenge were always assayed in the same batch. Serial dilutions of saliva samples containing high levels of endogenous OXT run strictly parallel to the standard curve indicating immuno-identity.

2.5. Salivary CORT levels (TSST)

Free salivary CORT levels were determined at Trier University (Laboratory of the Psychobiology Department, Trier, Germany) employing a time-resolved immunoas-

say with fluorometric detection (DELFI; Wallac, Turku, Finland), described in detail in a previous report (Dressendörfer et al., 1992). The intra-assay coefficient of variation was 4–6.7% and the corresponding inter-assay coefficient of variation was 7.1–9%.

2.6. Heart rate (TSST)

HR was monitored continuously over a period of 55 min, starting 20 min prior to and ending 20 min after termination of the TSST. Assessment was carried out using a polar watch device (S610i, S710i and S810i, Polar Electro GmbH, Büttelborn, Germany) that recorded every 5 s. Data were transferred with a Polar–electro interface to the Polar Precision Performance SW Program (Polar Version 4.03.050, Polar Electro Oy 2007) on a personal computer. Mean values of HR were calculated over 7 different time windows: 10 min sitting before and after the TSST, 10 min standing before and after the TSST, 5 min of introduction and speech preparation, 5 min interview, and 5 min mental arithmetic.

2.7. Statistics

All data were analyzed by SPSS 21.0 (IBM Corp., Armonk, NY, USA) with $p \leq 0.05$ considered statistically significant. All data are shown as means \pm S.E.M.

Changes in salivary OXT, CORT, and HR were analyzed using ANOVA for repeated measures, using time as within-subjects factor and gender (if applicable) as a between-subjects factor. Greenhouse-Geisser corrections were used when the assumption of sphericity was violated according to Mauchly’s test. In case of a main effect of time, post-hoc Bonferroni-corrected pair-wise comparisons between the basal value and all other values were performed. In case of an interaction effect of time and gender, these pair-wise comparisons were made separately in male and female participants.

For the TSST, two additional analyses were performed: (i) in female participants, ANOVA for repeated measures was used to determine the effects of time as within-subjects factor and contraceptive use (either daily oral pills containing 20–30 μg ethinylestradiol, $n = 11$, or no hormone-based contraceptives, $n = 4$) as between-subjects factor; (ii) Pearson’s correlation coefficients were determined between basal and peak values of salivary OXT, salivary CORT and HR.

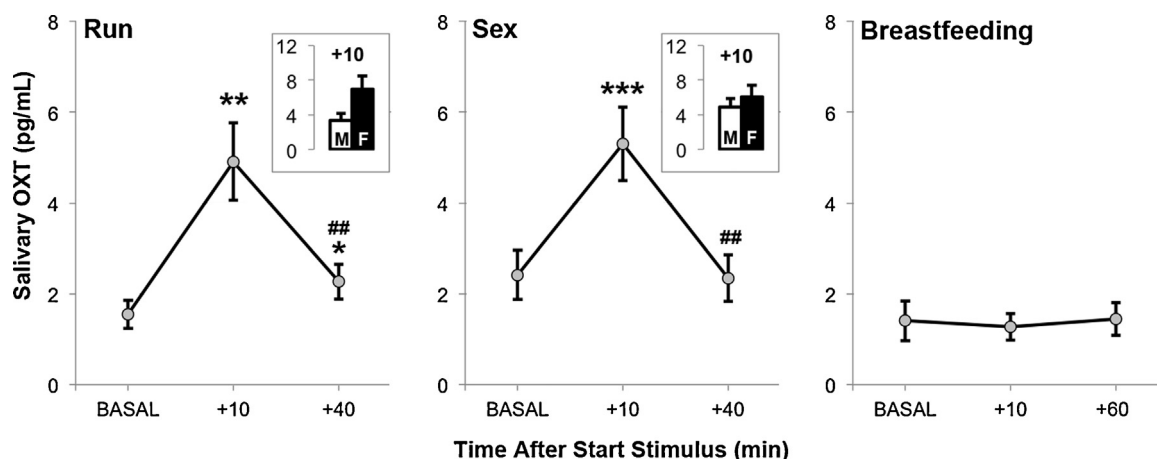


Fig. 2. OXT concentrations in saliva (pg/mL) in response to three ROC tests: running (“Run”), sexual self-stimulation (“Sex”), and breastfeeding. Saliva samples were collected at three time points (see Fig. 1): during resting state before the start of the stimulus (“basal”), 10 min after the start of the stimulus (“+10”) and 30 min after the end of the stimulus (“+40” for Run and Sex, “+60” for Breastfeeding). Inserts represent salivary OXT measured at “+10” separated for male (M) and female (F) participants. For main and interaction effects of time and gender: see text.

Significant differences with corresponding basal value: * $p < 0.05$; ** $p < 0.01$; *** $p < 0.001$.

Significant difference with corresponding stimulus value: ## $p < 0.01$.

3. Results

3.1. Home-based ROC test “Run”

Repeated measures ANOVA showed that salivary OXT concentrations changed significantly over time (factor time: $F[1.16] = 16.74$, $P < 0.001$), with a main effect of gender ($F[1] = 5.38$, $P = 0.039$), but no interaction effect of time \times gender ($F[1.16] = 2.27$, $P = 0.151$; Fig. 2). In more detail, while salivary OXT levels were modestly higher in females than in males at all three time points, both genders showed an increase in salivary OXT after 10-min of running compared to both the basal sample ($P = 0.001$) and the sample taken 40 min after the start of running ($P = 0.002$). Salivary OXT was still increased 40 min after the start of running ($P = 0.013$ versus basal).

3.2. Home-based ROC test “Sex”

Repeated measures ANOVA revealed that 10-min of sexual self-stimulation caused an increase in salivary OXT concentration (significant main effect of time: ($F[1.41] = 17.50$, $P < 0.001$), but neither a main effect of gender ($F[1] = 0.625$, $P = 0.442$) nor an interaction effect of time \times gender ($F[1.41] = 1.81$, $P = 0.195$; Fig. 2). Post-hoc pair-wise comparisons revealed a significant increase in salivary OXT concentrations 10 min after the start of sexual self-stimulation ($P \leq 0.001$ versus basal and 40 min after the start of the stimulus). At 40 min after the start of the stimulus, salivary OXT levels were no longer different from basal ($P = 0.438$).

3.3. Home-based ROC test “Breastfeeding”

Repeated measures ANOVA showed that salivary OXT concentrations changed neither in response to 10 min of nursing nor 60 min after the infant started suckling ($F[2] = 0.16$, $P = 0.851$; Fig. 2).

3.4. Exposure to the TSST

Statistics are summarized in Tables 1 (general effects) and 2 (effects of contraceptive use in females). In brief, repeated measures ANOVA indicated that exposure to the TSST caused significant changes over time in salivary OXT concentrations, salivary CORT and HR (Fig. 3). A main effect of gender and significant interaction

effect of time \times gender was only found for salivary CORT. Within females, (marginally) significant main effects of contraceptive use were found for salivary OXT levels and salivary CORT levels. In addition, significant interactions of time \times contraceptive use were found for salivary OXT levels and salivary CORT levels.

Post-hoc pair-wise comparisons showed that in both men and women, salivary OXT concentrations peaked at 16 min after the start of the TSST and had returned to basal levels 35 min and 75 min after the start of the TSST. In female participants, taking contraceptive pills significantly reduced basal, but not stress-induced salivary OXT levels.

Salivary CORT levels were higher in males than in females, and peaked at 35 min after the start of the TSST. In male participants, salivary CORT concentrations were significantly increased compared to basal levels at all time points. In female participants, the rise in salivary CORT reached significance only at 45 min and 60 min after the start of the TSST. This gender-effect was mainly caused by women taking oral contraceptives, who displayed significantly blunted salivary CORT levels at 16, 25 and 35 min after the start of the TSST compared with women that did not use hormone-based contraceptives.

HR rapidly increased during the TSST, peaked at the “speech” and “compute” stages, and returned to basal within 20 min after the TSST in both sexes. Oral contraceptive use did not affect changes in HR of women.

Analysis of correlation coefficients between relevant parameters in the TSST (basal and peak levels of OXT, CORT and HR) yielded no significant correlations when both genders were included. When the analysis was performed in men only (see Table 1), we found a significant negative correlation between peak OXT levels and basal HR. In females, peak salivary CORT levels (measured 35 min after the start of the TSST) correlated positively with both basal levels of OXT (measured prior to the TSST) and peak levels of OXT (measured 16 min after the start of the TSST, see Fig. 3D).

4. Discussion

The present ROC study provides evidence that salivary OXT responds to a variety of stimuli that are known to activate the peripheral and/or central release of the neuropeptide. This is an important finding confirming that saliva sampling can be used as

Table 1
Summary of statistics (TSST) corresponding to Fig. 3.

Repeated measures ANOVA						
	OXT		CORT		HR	
Time	$F = 25.43$ $df = 2.08$ $P < 0.001$		$F = 30.95$ $df = 2.03$ $P < 0.001$		$F = 100.9$ $df = 2.26$ $P < 0.001$	
Gender	$F = 2.22$ $df = 1$ $P = 0.148$		$F = 6.78$ $df = 1$ $P = 0.015$		$F = 0.13$ $df = 1$ $P = 0.724$	
Time \times gender	$F = 1.96$ $df = 2.08$ $P = 0.149$		$F = 8.76$ $df = 2.03$ $P < 0.001$		$F = 0.80$ $df = 2.26$ $P = 0.466$	
Post-hoc Bonferroni-corrected pair-wise comparisons with basal values						
Time	OXT	CORT [M]	CORT [F]	Time	HR	
+16	$P < 0.001$	$P = 0.011$	$P = 0.334$	Stand	$P < 0.001$	
+25	–	$P = 0.002$	$P = 0.109$	Prepare	$P < 0.001$	
+35	$P = 1.000$	$P = 0.001$	$P = 0.096$	Speech	$P < 0.001$	
+45	–	$P = 0.001$	$P = 0.045$	Compute	$P < 0.001$	
+60	–	$P = 0.001$	$P = 0.028$	Stand	$P < 0.001$	
+75	$P = 1.000$	$P = 0.001$	$P = 0.203$	Sit	$P = 0.075$	
Pearson correlation coefficients (r)						
HR	Basal	M	OXT Basal	Peak	CORT Basal	Peak
		F	–0.44	–0.59	0.14	0.05
	Peak	M	–0.05	–0.23	–0.25	–0.13
		F	–0.35	–0.46	0.44	0.31
CORT	Basal	M	0.18	0.16	–0.32	–0.06
		F	–0.11	–0.15	–	–
	Peak	M	0.18	–0.02	–	–
		F	–0.19	0.01	–	–
			0.57	0.62	–	–

Table 2
Summary of statistics for female participants in the TSST that used either oral contraceptives containing ethinylestradiol (E+, $n = 11$) or no hormone-based contraceptives (E–, $n = 4$).

Repeated measures ANOVA						
	OXT		CORT		HR	
Time	$F = 10.79$ $df = 3.00$ $P < 0.001$		$F = 17.41$ $df = 1.72$ $P < 0.001$		$F = 36.11$ $df = 2.36$ $P < 0.001$	
Contraceptives	$F = 4.51$ $df = 1$ $P = 0.054$		$F = 5.25$ $df = 1$ $P = 0.041$		$F = 0.38$ $df = 1$ $P = 0.548$	
Time \times contraceptives	$F = 3.40$ $df = 3.00$ $P = 0.027$		$F = 6.97$ $df = 1.72$ $P = 0.006$		$F = 0.36$ $df = 2.36$ $P = 0.734$	
Values (means \pm S.E.M.) and post-hoc pair-wise comparisons						
Time	OXT [pg/mL]		CORT [nmol/L]			
	E+	E–	E+ vs. E–	E+	E–	E+ vs. E–
basal	0.8 ± 0.2	2.5 ± 0.4	$P = 0.001$	3.6 ± 0.5	4.7 ± 1.0	$P = 0.328$
+16	2.0 ± 0.4	3.5 ± 0.9	$P = 0.091$	4.7 ± 0.7	10.6 ± 3.0	$P = 0.014$
+25	–	–	–	6.4 ± 0.9	13.4 ± 4.4	$P = 0.030$
+35	1.2 ± 0.3	1.2 ± 0.1	$P = 0.925$	6.9 ± 1.0	16.3 ± 5.1	$P = 0.015$
+45	–	–	–	6.2 ± 1.0	11.0 ± 2.9	$P = 0.060$
+60	–	–	–	6.0 ± 0.9	8.8 ± 2.4	$P = 0.189$
+75	1.0 ± 0.2	1.1 ± 0.3	$P = 0.807$	5.3 ± 0.7	6.1 ± 1.8	$P = 0.586$

an alternative to blood sampling (Carter et al., 2007), providing three crucial advantages: (1) saliva sampling is non-invasive, (2) the sampling can be performed at home and without interference of a researcher, thereby strongly reducing stress, and (3) saliva samples do not need to be extracted prior to RIA assessment to eliminate high molecular weight proteins or other unknown substances, which otherwise interfere with the RIA or ELISA resulting in artificial, supraphysiological levels of OXT (McCullough et al., 2013).

Here, we demonstrate that three physiological challenges (running, sexual self-stimulation and psychosocial stress) triggered a robust (approximately 2.5-fold) increase in salivary OXT that was rapid (peak at 10–15 min after onset of the stimulus and return to baseline 30 min thereafter) and reliable (53 out of 61 stimulus samples showed a relative increase of 20% or more compared with basal OXT levels). The increases in salivary OXT were similar to those reported in plasma for physical exercise (Hew-Butler et al., 2008; Landgraf et al., 1982), sexual self-stimulation (Blancher et al.,

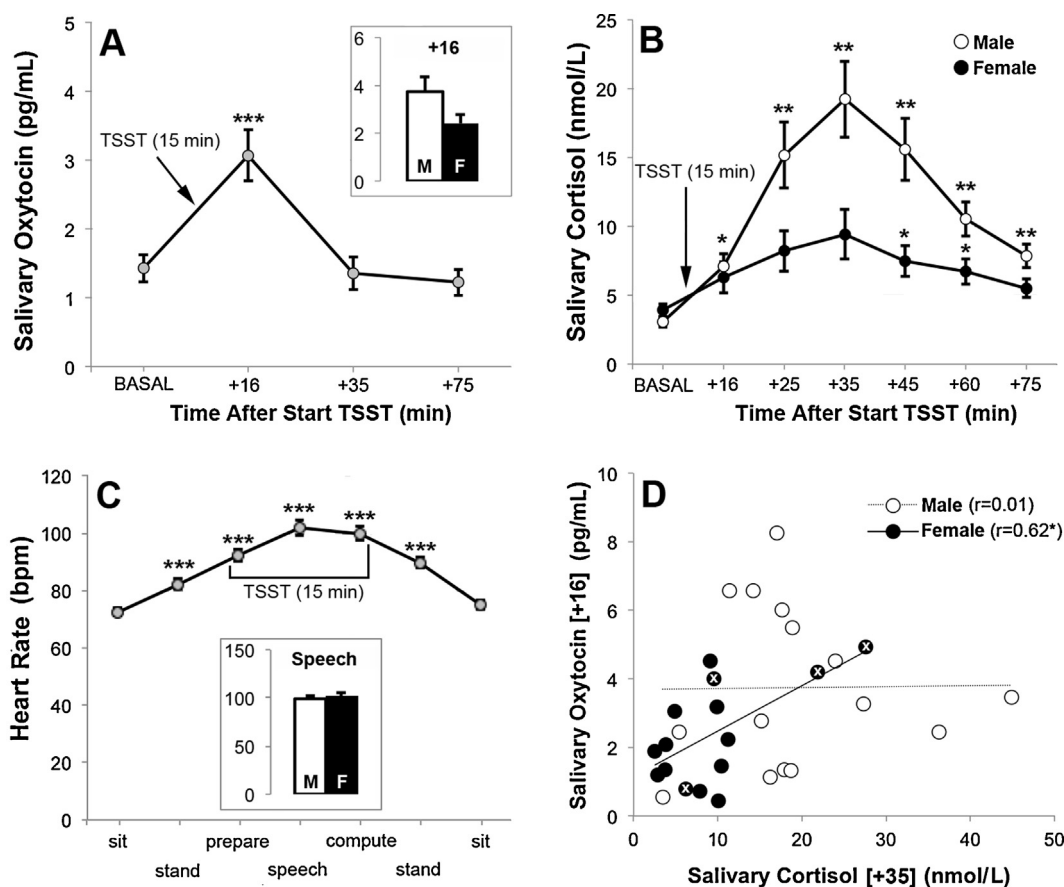


Fig. 3. Changes in (A) salivary OXT levels measured before (basal) and after the TSST; (B) salivary CORT levels measured before (“basal”) and after the TSST; and (C) heart rate before, during and after the TSST. Inserts depict peak salivary OXT levels (measured 16 min after the start of the TSST) and peak HR (measured during “speech”) for male (M) and female (F) participants separately. Peak levels (D) of salivary OXT (measured at 16 min after the start of the TSST) and salivary CORT (measured at 35 min after the start of the TSST) were positively correlated in female, but not male participants. Female participants not taking hormone-based contraceptives ($n=4$) are indicated by a white x-mark.

For main and interaction effects of time and gender: see Table 1.

Significant difference with corresponding basal value: * $P<0.05$; ** $P<0.01$; *** $P<0.001$.

1999; Carmichael et al., 1987; Murphy et al., 1987), and psychosocial stress (Kirschbaum et al., 1993; Pierrehumbert et al., 2010) and most likely reflect the release of OXT from the neurohypophysis into the blood stream. OXT then reached the salivary glands via blood circulation and entered the saliva through (as of yet undefined) active transport mechanisms (Gröschl, 2008; Proctor and Carpenter, 2014).

In contrast, breastfeeding did not measurably alter salivary OXT concentration despite its well-described activation of the OXT system in both humans (McNeilly et al., 1983; Ueda et al., 1994) and rats (Eriksson et al., 1996; Higuchi et al., 1983; Neumann et al., 1993). The most likely explanation for this discrepancy is the pulsatile nature of lactation-induced OXT release (2–3 pulses/20 min suckling period (McNeilly, 1983; Ueda, 1994). Combined with the short half-life of OXT in blood of approximately 3 min, as shown in women (Rydén and Sjöholm, 1969), these brief peaks are difficult to detect in plasma, and probably even harder to detect in saliva (note that the half-life of OXT in saliva is still unknown). Importantly, our data is in line with a recent study reporting that 10-min of breastfeeding was not sufficient to significantly increase either plasma or salivary OXT levels (Grewen et al., 2010). Another study using concentrated saliva samples showed an increase in OXT prior to breastfeeding followed by a decrease at initiation of the feeding (White-Traut et al., 2009), but this pattern was not seen in the present study (Fig. 2).

In this context it is of note that the basal salivary OXT concentrations as measured prior to the challenges “Run”, “Sex”, “Breastfeeding” and TSST were highly similar. Further, OXT levels did not depend on gender; either when measured separately for each challenge (see Section 3) or when basal levels for the three challenges were combined. This result is consistent with previous studies finding similar basal salivary or plasma OXT concentrations in healthy adult men and women (Graugaard-Jensen et al., 2014; Holt-Lunstad et al., 2011). Gender also did not play a significant role in salivary OXT responses to any of the ROC tests. Although previous studies indicated a somewhat higher increase in plasma OXT following sexual self-stimulation in women (Carmichael et al., 1987) and higher global OXT levels in women compared with men participating in a TSST (Pierrehumbert et al., 2010), these differences were modest. Interestingly, in our study daily use of oral ethinylestradiol-containing contraceptives resulted in lower basal salivary OXT levels in female TSST participants. This finding is in contrast to an oral contraceptive-induced increase in basal plasma OXT levels reported before (Stock et al., 1989). Since the sample of naturally cycling women in the present study was small ($n=4$), additional studies providing detailed information about the phase of the menstrual cycle at the time of sampling are needed to confirm and interpret this finding.

The increased HR and salivary CORT levels in response to the TSST confirmed that the test was indeed stressful. HR increased in all participants independent of gender or contraceptive use,

as shown before (Kirschbaum et al., 1999). Psychosocial stress-induced CORT levels were significantly higher in men compared with women, however, this is most likely an effect of contraceptive use of female participants rather than a gender-dependent difference in stress sensitivity: the use of daily oral ethinylestradiol-containing pills is known to enhance the level of corticosteroid binding globulin, thereby decreasing circulating free cortisol (Kirschbaum et al., 1999).

Salivary OXT peaked earlier than salivary CORT and, in fact, already returned to pre-stress levels when salivary CORT levels reached their peak. Combined with the fact that the lipid-soluble steroid hormone CORT can pass from the capillaries into the saliva more rapidly than the hydrophilic neuropeptide OXT (Gröschl, 2008), it can be concluded that TSST-induced OXT release precedes CORT release. Although salivary OXT (basal and peak levels) and peak salivary CORT levels correlated positively in women, this probably does not reflect a direct causal relationship as OXT has repeatedly been found to inhibit the release of ACTH and, subsequently, cortisol (Legros, 2001). Most likely, the use/non-use of oral contraceptives divided the women into a high CORT/high OXT and low CORT/low OXT group leading to a positive correlation. No strong or consistent correlations were found among HR, salivary CORT and salivary OXT when genders were combined or in men alone. Together, these results indicate that the TSST-induced OXT release occurred rather independently from HR and CORT release.

It is important to mention that the present study has considerable limitations. First, it needs to be kept in mind that OXT in saliva reflects primarily peripheral OXT release, which may differ substantially from central OXT release in terms of temporal dynamics (Landgraf and Neumann, 2004; Neumann and Landgraf, 2012). However, studies in rats have demonstrated that peripheral OXT release may occur in parallel to central OXT release within distinct brain regions in response to physical activity such as wheel running and swimming (Bakos et al., 2007; Wigger and Neumann, 2002; Wotjak et al., 1998), various sexual cues (Nyuyki et al., 2011; Waldherr and Neumann, 2007), and social defeat by an aggressive conspecific as a model for psychosocial stress (Bosch et al., 2004; Engelmann et al., 1999) (for review see Landgraf and Neumann, 2004). Thus, salivary OXT responses to the challenges used in the present study seem a proper representation of the global reactivity of the OXT system.

The second limitation is that for the “Run” and “Sex” challenges a relatively small sample size was studied, which did not allow to control for various parameters known to influence OXT release, such as the phase of the menstrual cycle, oral contraceptive use (Pierrehumbert et al., 2010) and the general fitness of participants (Hew-Butler et al., 2008). In addition, no restrictions based on age, cultural background, or relationship status were enforced. Lastly, we allowed unsupervised sample collection at home, likely resulting in some variability in the timing of saliva collection and intensity of the challenges. It is important to note that despite the many sources of variability, which will also be encountered in many patient cohorts, the OXT release in response to running and sexual self-stimulation was as robust as in the more strictly controlled TSST challenge. Future studies using larger sample sizes and stricter inclusion and exclusion criteria are needed to confirm and extend these findings of saliva OXT as a potential biomarker reflecting OXT system activity and reactivity.

In summary, with the use of ROC tests we advanced the validation of salivary OXT as a marker of global OXT system functioning by monitoring its responsiveness to physiological challenges known to raise plasma OXT and to trigger region-dependent intracerebral release of OXT. Indeed, running, sexual self-stimulation and psychosocial stress, but not breastfeeding, caused quantifiable, rapid and reliable increases in salivary OXT concentrations. These results support the use of ROC tests to assess the responsiveness of the OXT

system to relevant stimuli in individual patients, in order to identify high versus low OXT responders prior to the use of intranasal OXT as treatment option.

Conflict of interest

None.

Contributors

TRdJ and IDN were responsible for the conception and design of the study. TRdJ analysed data and drafted the manuscript. JH performed the TSST. All authors contributed to data acquisition. RM, TG, SMK, OJB and IDN assisted with literature research. SMK, BJ, OB, JH and IN critically revised drafts of the manuscript. SMK, BJ, OJB and IDN helped with the interpretation of results. IDN provided financial support.

All authors have approved the final article.

Sources of funding

No external funding source was associated with this study.

Acknowledgements

The authors would like to thank Prof. Dr. Rainer Landgraf for expert performance of the RIA and critical reading of the manuscript, Martin Kerwer, MSc for statistical assistance, and the anonymous volunteers for their valuable contribution to the experiments.

References

- Bakos, J., Hlavacova, N., Makatsori, A., Tybitanclova, K., Zorad, S., Hinghofer-Szalkay, H., Johansson, B.B., Jezova, D., 2007. Oxytocin levels in the posterior pituitary and in the heart are modified by voluntary wheel running. *Regul. Pept.* 139, 96–101.
- Bertsch, K., Schmidinger, I., Neumann, I.D., Herpertz, S.C., 2013. Reduced plasma oxytocin levels in female patients with borderline personality disorder. *Horm. Behav.* 63, 424–429.
- Blagrove, M., Fouquet, N.C., Baird, A.L., Pace-Schott, E.F., Davies, A.C., Neuschaffer, J.L., Henley-Einion, J.A., Weidemann, C.T., Thome, J., McNamara, P., et al., 2012. Association of salivary-assessed oxytocin and cortisol levels with time of night and sleep stage. *J. Neural Transm.* 119, 1223–1232, Vienna Austria 1996.
- Blaicher, W., Gruber, D., Bieglmayer, C., Blaicher, A.M., Knogler, W., Huber, J.C., 1999. The role of oxytocin in relation to female sexual arousal. *Gynecol. Obstet. Invest.* 47, 125–126.
- Bosch, O.J., Krömer, S.A., Brunton, P.J., Neumann, I.D., 2004. Release of oxytocin in the hypothalamic paraventricular nucleus, but not central amygdala or lateral septum in lactating residents and virgin intruders during maternal defence. *Neuroscience* 124, 439–448.
- Carmichael, M.S., Humbert, R., Dixon, J., Palmisano, G., Greenleaf, W., Davidson, J.M., 1987. Plasma oxytocin increases in the human sexual response. *J. Clin. Endocrinol. Metab.* 64, 27–31.
- Carson, D.S., Berquist, S.W., Trujillo, T.H., Garner, J.P., Hannah, S.L., Hyde, S.A., Sumiyoshi, R.D., Jackson, L.P., Moss, J.K., Strehlow, M.C., et al., 2015. Cerebrospinal fluid and plasma oxytocin concentrations are positively correlated and negatively predict anxiety in children. *Mol. Psychiatry* 20, 1085–1090.
- Carter, C.S., Pournajafi-Nazarloo, H., Kramer, K.M., Ziegler, T.E., White-Traut, R., Bello, D., Schwartz, D., 2007. Oxytocin: behavioral associations and potential as a salivary biomarker. *Ann. N. Y. Acad. Sci.* 1098, 312–322.
- Crockford, C., Deschner, T., Ziegler, T.E., Wittig, R.M., 2014. Endogenous peripheral oxytocin measures can give insight into the dynamics of social relationships: a review. *Front. Behav. Neurosci.* 8, 68.
- Dadds, M.R., Moul, C., Cauchi, A., Dobson-Stone, C., Hawes, D.J., Brennan, J., Urwin, R., Ebstein, R.E., 2014. Polymorphisms in the oxytocin receptor gene are associated with the development of psychopathy. *Dev. Psychopathol.* 26, 21–31.
- Dressendorfer, R.A., Kirschbaum, C., Rohde, W., Stahl, F., Strasburger, C.J., 1992. Synthesis of a cortisol-biotin conjugate and evaluation as a tracer in an immunoassay for salivary cortisol measurement. *J. Steroid Biochem. Mol. Biol.* 43, 683–692.
- Engelmann, M., Ebner, K., Landgraf, R., Holsboer, F., Wotjak, C.T., 1999. Emotional stress triggers intrahypothalamic but not peripheral release of oxytocin in male rats. *J. Neuroendocrinol.* 11, 867–872.

- Eriksson, M., Ceccatelli, S., Uvnäs-Moberg, K., Iadarola, M., Hökfelt, T., 1996. Expression of Fos-related antigens, oxytocin, dynorphin and galanin in the paraventricular and supraoptic nuclei of lactating rats. *Neuroendocrinology* 63, 356–367.
- Graugaard-Jensen, C., Hvistendahl, G.M., Frøkiær, J., Bie, P., Djurhuus, J.C., 2014. Urinary concentration does not exclusively rely on plasma vasopressin. A study between genders gender and diurnal urine regulation. *Acta Physiol.* 212, 97–105.
- Grewen, K.M., Davenport, R.E., Light, K.C., 2010. An investigation of plasma and salivary oxytocin responses in breast- and formula-feeding mothers of infants. *Psychophysiology* 47, 625–632.
- Gröschl, M., 2008. Current status of salivary hormone analysis. *Clin. Chem.* 54, 1759–1769.
- Feng, C., Lori, A., Waldman, I.D., Binder, E.B., Haroon, E., Rilling, J.K., 2015. A common oxytocin receptor gene (OXTR) polymorphism modulates intranasal oxytocin effects on the neural response to social cooperation in humans. *Genes Brain Behav.*, in press.
- Hew-Butler, T., Noakes, T.D., Soldin, S.J., Verbalis, J.G., 2008. Acute changes in endocrine and fluid balance markers during high-intensity, steady-state, and prolonged endurance running: unexpected increases in oxytocin and brain natriuretic peptide during exercise. *Eur. J. Endocrinol. Eur. Fed. Endocr. Soc.* 159, 729–737.
- Higuchi, T., Honda, K., Fukuoka, T., Negoro, H., Hosono, Y., Nishida, E., 1983. Pulsatile secretion of prolactin and oxytocin during nursing in the lactating rat. *Endocrinol. Jpn.* 30, 353–359.
- Holt-Lunstad, J., Birmingham, W., Light, K.C., 2011. The influence of depressive symptomatology and perceived stress on plasma and salivary oxytocin before, during and after a support enhancement intervention. *Psychoneuroendocrinology* 36, 1249–1256.
- Horvat-Gordon, M., Granger, D.A., Schwartz, E.B., Nelson, V.J., Kivlighan, K.T., 2005. Oxytocin is not a valid biomarker when measured in saliva by immunoassay. *Physiol. Behav.* 16, 445–448.
- Javor, A., Riedl, R., Kindermann, H., Brandstätter, W., Ransmayr, G., Gabriel, M., 2014. Correlation of plasma and salivary oxytocin in healthy young men—experimental evidence. *Neuro Endocrinol. Lett.* 35, 470–473.
- Jokinen, J., Chatzittofis, A., Hellström, C., Nordström, P., Uvnäs-Moberg, K., Asberg, 2012. Low CSF oxytocin reflects high intent in suicide attempters. *Psychoneuroendocrinology* 37, 482–490.
- Kagerbauer, S.M., Martin, J., Schuster, T., Blobner, M., Kochs, E.F., Landgraf, R., 2013. Plasma oxytocin and vasopressin do not predict neuropeptide concentrations in human cerebrospinal fluid. *J. Neuroendocrinol.* 25, 668–673.
- Kirschbaum, C., Pirke, K.M., Hellhammer, D.H., 1993. The trier social stress test—a tool for investigating psychobiological stress responses in a laboratory setting. *Neuropsychobiology* 28, 76–81.
- Kirschbaum, C., Kudielka, B.M., Gaab, J., Schommer, N.C., Hellhammer, D.H., 1999. Impact of gender, menstrual cycle phase, and oral contraceptives on the activity of the hypothalamus-pituitary-adrenal axis. *Psychosom. Med.* 61, 154–162.
- Landgraf, R., Neumann, I.D., 2004. Vasopressin and oxytocin release within the brain: a dynamic concept of multiple and variable modes of neuropeptide communication. *Front. Neuroendocrinol.* 25, 150–176.
- Landgraf, R., Häcker, R., Buhl, H., 1982. Plasma vasopressin and oxytocin in response to exercise and during a day-night cycle in man. *Endokrinologie* 79, 281–291.
- Lee, H.-J., Macbeth, A.H., Pagani, J.H., Young, W.S., 2009. Oxytocin: the great facilitator of life. *Prog. Neurobiol.* 88, 127–151.
- Legros, J.J., 2001. Inhibitory effect of oxytocin on corticotrope function in humans: are vasopressin and oxytocin ying-yang neurohormones? *Psychoneuroendocrinology* 26, 649–655.
- McCullough, M.E., Churchland, P.S., Mendez, A.J., 2013. Problems with measuring peripheral oxytocin: can the data on oxytocin and human behavior be trusted? *Neurosci. Biobehav. Rev.* 37, 1485–1492.
- McNeilly, A.S., Robinson, I.C., Houston, M.J., Howie, P.W., 1983. Release of oxytocin and prolactin in response to suckling. *Br. Med. J. Clin. Res. Ed.* 286, 257–259.
- Meyer-Lindenberg, A., Domes, G., Kirsch, P., Heinrichs, M., 2011. Oxytocin and vasopressin in the human brain: social neuropeptides for translational medicine. *Nat. Rev. Neurosci.* 12, 524–538.
- Murphy, M.R., Seckl, J.R., Burton, S., Checkley, S.A., Lightman, S.L., 1987. Changes in oxytocin and vasopressin secretion during sexual activity in men. *J. Clin. Endocrinol. Metab.* 65, 738–741.
- Neumann, I.D., Landgraf, R., 2012. Balance of brain oxytocin and vasopressin: implications for anxiety, depression, and social behaviors. *Trends Neurosci.* 35, 649–659.
- Neumann, I.D., Slattery, D.A., 2015. Oxytocin in general anxiety and social fear: a translational approach. *Biol. Psychiatry*, in press.
- Neumann, I., Ludwig, M., Engelmann, M., Pittman, Q.J., Landgraf, R., 1993. Simultaneous microdialysis in blood and brain: oxytocin and vasopressin release in response to central and peripheral osmotic stimulation and suckling in the rat. *Neuroendocrinology* 58, 637–645.
- Nyuyki, K.D., Waldherr, M., Baeuml, S., Neumann, I.D., 2011. Yes, I am ready now: differential effects of paced versus unpaced mating on anxiety and central oxytocin release in female rats. *PLoS One* 6 (8), e23599.
- Pierrehumbert, B., Torrisi, R., Laufer, D., Halfon, O., Ansermet, F., Beck Popovic, M., 2010. Oxytocin response to an experimental psychosocial challenge in adults exposed to traumatic experiences during childhood or adolescence. *Neuroscience* 166, 168–177.
- Proctor, G.B., Carpenter, G.H., 2014. Salivary secretion: mechanism and neural regulation. *Monogr. Oral Sci.* 24, 14–29.
- Ruggeri, B., Sarkans, U., Schumann, G., Persico, A.M., 2014. Biomarkers in autism spectrum disorder: the old and the new. *Psychopharmacology (Berl)* 231, 1201–1216.
- Rydén, G., Sjöholm, I., 1969. Half-life of oxytocin in blood of pregnant and non-pregnant women. *Acta Endocrinol. (Copenh.)* 61, 425–431.
- Seltzer, L.J., Ziegler, T.E., Pollak, S.D., 2010. Social vocalizations can release oxytocin in humans. *Proc. Biol. Sci.* 277, 2661–2666.
- Stock, S., Silber, M., Uvnäs-Moberg, K., 1989. Elevated plasma levels of oxytocin in women taking low-dose oral contraceptives: identification of the plasma oxytocin with high performance liquid chromatography. *Acta Obstet. Gynecol. Scand.* 68, 75–78.
- Tauber, M., Diene, G., Mimoun, E., Çabal-Berthoumieu, S., Mantoulan, C., Molinas, C., Muscatelli, F., Salles, J.P., 2014. Prader-Willi Syndrome as a model of human hyperphagia. *Front. Horm. Res.* 12, 93–106.
- Ueda, T., Yokoyama, Y., Irahara, M., Aono, T., 1994. Influence of psychological stress on suckling-induced pulsatile oxytocin release. *Obstet. Gynecol.* 84, 259–262.
- Waldherr, M., Neumann, I.D., 2007. Centrally released oxytocin mediates mating-induced anxiolysis in male rats. *Proc. Natl. Acad. Sci. U. S. A.* 104, 16681–16684.
- White-Traut, R., Watanabe, K., Pournajafi-Nazarloo, H., Schwertz, D., Bell, A., Carter, C.S., 2009. Detection of salivary oxytocin levels in lactating women. *Dev. Psychobiol.* 51, 367–373.
- Wigger, A., Neumann, I.D., 2002. Endogenous opioid regulation of stress-induced oxytocin release within the hypothalamic paraventricular nucleus is reversed in late pregnancy: a microdialysis study. *Neuroscience* 112, 121–129.
- Wotjak, C.T., Ganster, J., Kohl, G., Holsboer, F., Landgraf, R., Engelmann, M., 1998. Dissociated central and peripheral release of vasopressin, but not oxytocin, in response to repeated swim stress: new insights into the secretory capacities of peptidergic neurons. *Neuroscience* 85, 1209–1222.
- Ziegler, C., Dannlowski, U., Bräuer, D., Stevens, S., Laeger, I., Wittmann, H., Kugel, H., Döbel, C., Hurlmann, R., Reif, A., et al., 2015. Oxytocin receptor gene methylation: converging multilevel evidence for a role in social anxiety. *Neuropsychopharmacology* 40, 1528–1538.
- Zik, J.B., Roberts, D.L., 2015. The many faces of oxytocin: implications for psychiatry. *Psychiatry Res.* 226, 31–37.