Variation in DNA methylation of the oxytocin gene is associated with prosocial, reward-related, decision-making

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Abstract

Human and animal studies suggest a critical role for central oxytocin (OXT) in social behavior. While experimentally manipulating central OXT levels via intranasal administration is a well-suited noninvasive option in human research, existing findings are mixed, suggesting that the effects of exogenous OXT on social behavior are highly variable and dependent on other (e.g., biological) factors. Endogenous OXT synthesis is regulated by a wide range of molecular mechanisms including DNA methylation (DNAm). However, the link between DNAm of the OXT gene (*OXT*) and human social behavior has received little attention. We studied the relationship between *OXT* DNAm, intranasal OXT administration, and facets of human (pro-)social behavior and found preliminary evidence that *OXT* DNAm at three CpG sites (i.e., GRCh37/hg19=chr20: 3,052,319; chr20: 3,052,334; chr20:3,052,345) is negatively associated with trust game investments. Associations with (pro-)social traits or interactions between *OXT* DNAm and OXT administration on trusting behavior and generosity were not significant. Our results suggest that variation in *OXT* DNAm is associated with trusting behavior but no (main or moderating) effect of exogenous OXT administration was observed. Importantly, because functional indices of *OXT* gene expression or endogenous OXT levels were not incorporated into the present analyses, the findings cannot speak to whether DNAm variation translates into measurable differences in oxytocinergic system activity in the brain. Further, our findings highlight the need for future research examining whether, and how, *OXT* DNAm is related to reward-related processes and their neurobiological underpinnings, including potential interactions between oxytocinergic and other neuromodulatory systems implicated in reward processing (e.g., dopaminergic or serotonergic systems). Future studies should investigate the relationship between *OXT* DNAm and trust game investments, determine functional

consequences on the molecular level, and directly assess whether, and how, *OXT* DNAm is related to neurobiological processes involved in prosocial, reward-related, behavior.

Keywords: Oxytocin, DNA methylation, trust, reward, epigenetics

1. Introduction

Oxytocin (OXT) is a neuropeptide and hormone that is widely expressed throughout the body. In the brain, it functions as a neurotransmitter and neuromodulator, where it is assumed to mediate behavior and cognition. Outside of the brain, it acts as a hormone, playing a prominent role in uterine contractions during childbirth, and milk let-down during lactation (Gimpl & Fahrenholz, 2001; Jurek & Neumann, 2018). Central (i.e., brain-based) OXT has been strongly implicated in social behaviors in both animals and humans. Rodent studies, for example, demonstrated OXT's ability to shape behaviors such as social recognition (Gur et al., 2014), social avoidance (Lukas et al., 2011), maternal behavior (Pedersen et al., 1982), and partner preferences (Williams et al., 1994). Similarly, in humans, OXT has been linked to parent-infant (Kohlhoff et al., 2017) and romantic (Schneiderman et al., 2012) bonding. Moreover, OXT is assumed to play a role in more complex human social behaviors such as empathy, altruism and cooperation (for an overview see Marsh et al., 2021). Those positive social behaviors towards others can be coined under the term 'prosocial' behaviors, which are crucial in establishing and maintaining relationships among humans (Marsh et al., 2021).

OXT is synthesized in the paraventricular, supraoptic, and intermediate accessory nuclei of the hypothalamus and released from the posterior pituitary into the bloodstream (Gimpl & Fahrenholz, 2001; Jurek & Neumann, 2018; Meyer-Lindenberg et al., 2011). The synthesis of

OXT critically relies on a precursor protein that is encoded by the OXT gene *(OXT*). *OXT* expression is regulated by a wide range of molecular mechanisms, including epigenetic modifications such as DNA methylation (DNAm). DNAm is an epigenetic mechanism, in which a methyl group is transferred onto a cytosine to form 5-methylcytosine. DNAm mostly occurs on cytosines at cytosine-phosphate-guanine (CpG) sites and changes the organization and accessibility of chromatin. Higher DNAm at promotor regions, therefore, is generally associated with transcriptional repression, although exceptions to this rule exist (e.g., Moore et al., 2013).

Despite the widely demonstrated role of OXT in various aspects of human social behavior (e.g., Kohlhoff et al., 2017; Marsh et al., 2021; Schneidermann et al., 2012), the degree to which *OXT* DNAm is associated with these behaviors has received little attention. One of the few published studies reported that higher DNAm levels in the *OXT* promoter region in healthy participants were associated with higher levels of anxious (insecure) attachment styles, lower accuracy in recognizing emotional facial expressions, and reduced brain activity during a social-cognitive task in regions involved in social-cognitive functioning such as the right superior temporal sulcus activity (Haas et al., 2016). In an electroencephalogram (EEG) study in healthy women (Lesemann et al., 2020), DNAm in the promoter region of the *OXT* was linked to the N170 event-related potential, which peaks in response to human faces and is considered to reflect the fundamental processing of facial stimuli. It was found that DNAm was associated with an increased N170 response in response to viewing images depicting faces. This effect was independent of the amount of trustworthiness study participants felt towards the shown faces, and other image-related factors such as cuteness or group membership, possibly indicating that OXT is involved in salience and attention. Overall, findings to date suggest that *OXT* DNAm may contribute to interindividual differences in social-cognitive and socio-emotional functioning

by influencing the regulation of the OXT system, thereby affecting both relatively stable social tendencies (e.g., attachment-related traits) and context-dependent social behavior.

In addition to the naturally occurring regulation of OXT synthesis through gene expression, the amount of OXT available can be experimentally altered using intranasal OXT administration (e.g., Meyer-Lindenberg et al., 2011); a non-invasive approach that is well-suited for human research. Evidence suggests that exogenously administered OXT can indeed reach the central compartment (Lee at al., 2020) via diffusion-mediated transport, where it can then bind to brain-based OXT receptors (van der Aart et al., 2018). Studies suggest that intranasally administered OXT may modulate facets of social behaviors, mostly towards increased (pro-) social behavior (for an overview see Marsh et al., 2021). However, the reported mixed findings suggest that the effects of exogeneous OXT are highly variable and depend on both individual (psychological or biological) and contextual factors (e.g., Declerck et al., 2020; Marsh et al., 2021) as well as dose (e.g., Quintana et al., 2017). Further, while some of these effects may arise from central penetration, a recent overview by Yao and Kendrick (2025) suggests that many effects could occur via stimulation of peripheral OXT receptors that may influence brain function through vagal pathways. Here, oromucosal administration of OXT may produce comparable effects to intranasal administration despite not directly entering the brain.

Yao and Kendrick (2025) further propose a hierarchical “SSS” model of OXT functions, describing three interconnected domains, namely survival, security and sociability. Within the survival domain, OXT may enhance attention to salient social and environmental cues, and increase vigilance and stimulus detection. In the security domain, OXT is suggested to facilitate interpersonal bonding, emotional understanding, and caregiving behaviors. Lastly, in the sociability domain, OXT may promote social group cohesion, moral norm compliance, and

social learning by influencing emotions such as guilt and/or by modulating reinforcement of prosocial behaviors. These effects are thought to involve interactions with other neurotransmitters such as dopamine (DA), serotonin, or opioids, as well as GABA and glutamate systems. This integrative perspective may help to explain the inconsistent OXT effects in human research, as outcomes are shaped by a number of factors including individual traits, social context, or dosing. Understanding these mechanisms better is crucial for interpreting experimental OXT studies.

To explore how different parameters of the OXT system are associated with human (pro-)social behavior, we analyzed data collected in a recent study (Kroll et al., 2023) and examine relationships between *OXT* DNAm, intranasal OXT administration, and facets of human (pro-)social behavior collected via self-reports and task-based measurements. Although dispositional trust and SVO are higher-order, trait-like constructs influenced by multiple biological and environmental factors, variation in *OXT* DNAm may reflect longer-term regulatory differences in the OXT system that could, over time, bias stable social tendencies. Accordingly, we first expected that greater *OXT* DNAm (assumed to affect chromatin structure leading to repressed *OXT* expression and thus reduced OXT synthesis) is negatively associated with self-report measures of dispositional trust (*Hypothesis 1a*) and dispositional social value orientation (SVO; *Hypothesis 1b*). Second, we hypothesized that there is a negative association between *OXT* DNAm and two task-based measures of human (pro-)social behavior: investments in a trust game (*Hypothesis 2a*) and money allocated to an anonymous beneficiary in a so-called dictator game, as a proxy measure of generosity (*Hypothesis 2b*). Lastly, we expected that the effect of intranasal OXT administration on investments in a trust game (*Hypothesis 2c*) and money allocated to the beneficiary in a dictator game (*Hypothesis 2d*) increases with increasing DNAm,

reflecting a greater effect of intranasal OXT administration in participants with putatively lower (baseline) OXT levels. The study was preregistered (https://osf.io/vgah3/overview).

2. Methods

This study was pre-registered on the *Open Science Framework* (OSF; https://osf.io/vgah3). All hypotheses in this manuscript and the statistical models applied correspond to the pre-registered analysis plan.

2.1 Participants

Participants were recruited within a larger project examining prosocial effects of OXT. A detailed description of participants, statistical power simulations, laboratory procedures and tasks can be found in Kroll et al., 2023. Briefly, the sample consisted of 234 male participants (aged 18 to 32). Participants were reimbursed with a flat fee of €20 for study participation, plus their decision-based earnings from the trust game (range €0 - €72), and from the dictator game (range €0 - €10).

Initial exclusion criteria were a lifetime diagnosis of any DSM-V psychiatric (including substance dependence and abuse) or neurological disorder, current treatment for mental health-related issues, current use of psychotropic medication for mental-health related problems or a latex allergy which was self-reported by participants in an online screening. The consumption of water or other liquids was limited to a maximum of 1 liter within the two hours before the laboratory session to minimize side effects associated with OXT-induced urinary retention. All participants reported to have adhered to this water consumption instruction. In a set of questions at the end of the experiment, a total of 13 participants reported to have consumed alcohol or non-prescription drugs or smoked excessively (>20 cigarettes) in the 12 hours before the laboratory

session. Their data was excluded from analyses. Further, for all trust game-related analyses, data from ten participants was excluded because these participants interacted with mock participants during the trust game due to an unexpectedly high number of no-shows in three sessions. Mock participants were used to maintain the same conditions for all participants (i.e., always participating in a group of 12-24 in the trust game; see Kroll et al., 2023 for details). Regarding the dictator game-related analyses, data of all ten participants within a single session were not included due to technical issues preventing saving the data. No CpG DNAm data were available for five participants. Lastly, one participant showed implausible CpG values, including very low DNAm and minimal variation. Collectively, these exclusions resulted in a subsample of n=215 for all analyses concerning dispositional traits only (i.e., H1a&b). For trust game-related analyses (i.e., Hypothesis 2a&c), a subsample of n=206 was used (one participant that was excluded due to drug consumption also engaged with a mock participant), for dictator game-related analyses (i.e., Hypotheses 2b&d), a (different) subsample of n=205 participants was used. Approval of the study was obtained from the *Maastricht University/Maastricht Academic Medical Ethics Committee* (protocol NL74615.068.20 / METC 20-057) and written informed consent was obtained from all participants.

2.2 Study design

The experiment consisted of an initial online session (session 1), followed by a laboratory session (session 2). During session 1, participants completed several digital questionnaires within a pre-defined time interval using their preferred device with an internet connection. During session 2, a randomized double-blind placebo -controlled (PLC) between-subjects design was used, where 12 to 24 participants (depending on show-up) were randomized equally to either the OXT or the PLC treatment (between-subject randomization; random number generated in *Excel*).

During this laboratory session, these participants additionally provided pre-treatment DNA samples and made post-treatment decisions in the trust and dictator game.

2.3 OXT manipulation

To temporarily manipulate OXT levels, participants self-administered a 24 I/U of synthetically manufactured OXT (*Syntocinon*) via a nose spray in six puffs (three per nostril), or an equivalent number of puffs of a hypotonic saline (PLC) spray with similar ingredients but without OXT. Spray administration was performed strictly in line with the recommendations and guidelines provided in Guastella et al., 2013 which includes a standardized demonstration, test puff, and visual inspection by the experimenter. Both the trust game and the dictator game were performed within the expected 30-90 minutes post-administration peak window (Gossen et al., 2012) in the same order for all participants. Participants were not informed about the potential behavioral/prosocial effects of OXT before participating in the experiment. However, in the participant information it was stated that the spray could contain OXT.

2.4 *OXT* DNAm

DNA was collected pre-administration using *Oragene Discover OGR-500 kits* (*DNA Genotek*) and extracted using *prepIT•L2P reagent* (*DNA Genotek*). To quantify DNAm at the promoter region of the *OXT*, DNA was isolated and bisulfite-converted, followed by amplification of the target region and pyrosequencing. In all steps, samples were randomized using stratification by session and treatment to ensure balanced distribution over the plates to avoid potential batch effects.

2.4.1 DNA isolation

DNA was isolated using phenol-chloroform extraction. First, 500 µl of each sample were put in a heating block at 50°C for 1 hour. Then samples were incubated with 2 µl RNaseA (2 mg/ml, Promega Corporation) for 30 min at 21°C, after which 500 µl phenol/chloroform/isoamylalcohol 25:24:1 (pH 7.5) was added. Samples were vigorously vortexed and centrifuged for 5 min (13,000xg) at room temperature. The aqueous phase was transferred to a clean tube. One volume of the aqueous phase (about 400 µl) chloroform/isoamylalcohol was added and samples were vortexed vigorously. After centrifuging for another 5 min (15,000xg) at room temperature, the aqueous phase was again transferred to a clean tube. Samples were then incubated with 3.5 µl sodium acetate (3M) and 900 µl 96% ethanol for 10 min at -20°C. After washing with 500 µl 70% ethanol and centrifuging for 3 min (15,000xg), the supernatant was removed. Samples were centrifuged another time for 10 sec and supernatant was removed. Pellets were dried using a Savant DNA120 SpeedVac Concentrator (Thermo Electron corporation). Finally, the pellet was dissolved in 150 µl sterile water (Baxter Healthcare SA).

DNA concentrations were measured using 1 ul of DNA and the Qubit 1x dsDNA High Sensitivity and Broad Range Assay kit according to manufacturer's instructions, and the Qubit Flex Fluorometer (Thermo Fisher Scientific). Calibration standards were included. DNA was stored at 4°C until further processing.

2.4.2 Sodium bisulfite treatment

Sodium bisulfite treatment was performed using the EZ-96 DNA Methylation-Gold kit (D5008, Zymo Research). For each sample, 500 ng of DNA was treated following manufacturer's instruction, except for eluting the bisulfite-converted DNA in 50 µL of elution buffer to obtain a final concentration of 10 ng/µL bisulfite-converted DNA.

2.4.3 Primer design

The forward and reverse primers for polymerase chain reaction (PCR), and the sequencing primer for pyrosequencing were designed using the PyroMark Assay Design software (version 2.0.1.15, Qiagen), based on the Ensembl Genome Browser GRCh37 assembly. Primers were designed to target the promoter region of the *OXT* gene as region of interest. The PCR primers (5'-3') covered the region GRCh37/hg19=chr20:3,052,228-3,052,393 (166 bp product size), and the sequencing primer (5'-3') targeted region GRCh37/hg19=chr20:3,052,271-3,052,355, which included the following eight CpG sites (Koulousakis et al., 2024): CpG site 1, chr20:3,052,247; CpG site 2, chr20:3,052,290; CpG site 3, chr20:3,052,296; CpG site 4, chr20:3,052,307; CpG site 5, chr20:3,052,319; CpG site 6, chr20:3,052,334; CpG site 7, chr20:3,052,345; and CpG site 8, chr20:3,052,355.

2.4.4 Polymerase chain reaction

The region of interest was amplified using PCR. In brief, bisulfite-converted DNA was denaturated at 95°C for 5 min, followed by 45 cycles at 95°C for 30 sec, 54°C for 30 sec, and 72°C for 30 sec, with a final elongation step at 72°C for 1 min. For each sample, a reaction mix of 25 µl volume was produced, consisting of 2.5 µl PCR buffer (10x) with 20 mM $MgCl_2$, .5 µl (10 mM) dNTP mix (Roche Diagnostics GmbH , 1 µl of the forward primer (5 µM stock, metabion international AG), 1 µl of the reverse primer (5 µM stock, metabion international AG), 1 µl of bisulfite-converted DNA, and .2 µl (5 U/µl) FastStart Taq DNA Polymerase (Roche Diagnostics GmbH). To verify PCR product yield, PCR products were size-fractionated on a 2% agarose gel (in Tris-Acetate-EDTA (TAE) buffer). Samples were concentrated in 10 µl sterile

water (Baxter Healthcare SA) using a Savant DNA120 SpeedVac Concentrator (Thermo Electron Corporation).

2.4.5 Pyrosequencing

DNAm at cytosines (5-methylcytosine, 5-mC) was quantified using the PyroMark Q48 Autoprep system with the Pyro Q48 Autroprep 2.4.2 software (Qiagen), and the PyroMark Q48 Advanced Reagents according to the manufacturer's instructions. The sequencing primer (100 uM stock, metabion international AG) was diluted to 4 μM. Bisulfite-converted DNA standards from human origin were included (0% and 100% methylated, EpiTect PCR Control DNA Set, Qiagen), confirming assay sensitivity. Data of CpG sites not meeting the quality control by the Pyro Q-CpG 1..9 software were excluded from further analyses. The potential functional relevance of differential methylation at analyzed CpG sites was further investigated using JASPAR CORE 2024 (Rauluseviciute et al., 2024), predicting transcription factor binding sites, in the UCSC Genome Browser (GRCh37/hg19, February 2009).

2.5 Experimental tasks

2.5.1 Trust game

During session 2, all participants made two decisions in the trust game (Berg et al., 1995), first in the role of an investor (i.e., the trustor), and then in the role of a trustee. In each role, they were randomly paired with a different other participant in the opposite role. Participants made their first decision as investor without being aware that they would make a second decision in the role of a trustee. This design guarantees that behavior as investor cannot be affected by anticipated own behavior and beliefs about other investors' behavior when acting as trustee. Investors were endowed with 12 Experimental Money Units (EMU) and asked to invest either 0, 4, 8, or 12 EMU into the joint project with their trustee. The investment was

tripled and added to their trustee's endowment of 12 EMU. After having received the tripled investment from their investor, trustees could back-transfer any integer number of their new EMU holdings to their investor. After having made their decisions all investors were informed that they no act as a trustee towards another participant in the role of investor. Lastly, participants were informed about the back-transfers they received, and their total payoff. As investing any positive amount of money puts the investor at a risk to be exploited by the trustee, the investment amount served as proxy of interpersonal trust (Berg et al., 1995). See Kroll et al. 2023 for details and a visualization.

2.5.2 Dictator game

In the dictator game, all participants make their choice in the role of dictator. They received standardized instructions, in which they were also truthfully informed that their matched partner (i.e., the beneficiary) will be an anonymous, real, participant outside of the laboratory. Subsequently, they had to allocate an endowment of €10 between themselves and the other participant (integer values). The amount allocated to the other served as proxy of generosity.

2.6 Procedure

See *Table 1* for a visualization of the experimental procedure.

Screening: online	In-/exclusion questions	
Session 1: online	Baseline traits	
Session 2: lab	Water compliance	*Minute 0*
	Mood questionnaire	*1*
	Swab saliva sample + DNA saliva sample	*6*
	OXT vs. PLC nose spray	*24*

State and trait questionnaires	*35*
Minimal social condition	*42*
Mood questionnaire	*53*
Swab saliva sample	*58*
Trust game	*68*
Mood questionnaire	*90*
Swab saliva sample	*95*
Dictator game	*105*
Final ratings	*111*
Reimbursement form and session end	*Minute 117*

Table 1. Experimental procedure. OXT, oxytocin; PLC, placebo.

Measures of dispositional trust (i.e., IGTS scores; Yamagashi et al., 2015), dispositional SVO (Van Lange, 2000), and levels of chronic nasal obstruction (Lipan & Most, 2013) were collected using self-report questionnaires during the online session (session 1).

2.7 Statistical analyses

Mean methylation values for each CpG site at the promoter region of the *OXT* were used for statistical analyses. IGTS scores were calculated by taking the mean of the nine items (Yamagashi et al., 2015). We used SVO data to classify participants as prosocial, individualistic, or competitive using the criteria of at least six (out of nine) choices that align with these categories, in line with Van Lange, 200. Individualistic and competitive participants were summarized as proself. Participants that did not fit one of the three categories were labelled as "unclassified" (n=43) and not used in further analyses. NOSE scores were measured using five items on a 5-point Likert scale (range 0-4), summed, and multiplied by 5 to allow for a range of scores between 0 and 100. These scores were used to categorize mild (range 5-25), moderate (range 30-50), severe (range 55-75), or extreme (range 80-100) nasal obstruction (Lipan & Most, 2013), which was used as a covariate in interaction analyses related to OXT (vs. PLC) spray

administration. The trust game data consisted of the participants' investment choices, made from the four possible options (levels; 0, 4, 8 or 12 EMUs). In the dictator game, allocated money to the beneficiary was recorded as the participants' choice, with eleven possible levels (0, 1, 2, 3, 4, 5, 6, 7, 8, 9 or 10 €). Descriptive statistics includes the min, max, mean and *SD* of IGTS and of DNA methylation levels for all CpG sites and the frequency of SVO types and nasal obstruction levels.

Statistical analyses were performed using R version 4.4.0 (R Core team, 2024) and statistical tests were considered significant at $p<.05$. Because this study is one of the first studies that assesses the hypothesized associations, we viewed our approach as primarily exploratory in nature, and therefore, did not correct for multiple comparisons. We deemed minimizing the risk for *Type II* errors as more important, since missing out on a real effect might lead other researchers to not further investigate those associations.

2.7.1 Statistical models

Hypothesis 1a: To investigate if there is a negative association between *OXT* DNAm and dispositional trust (IGTS scores), we conducted a *Pearson* correlation analysis.

Hypothesis 1b: To investigate if there is a negative association between *OXT* DNAm and SVO, with participants classified as prosocial showing lower mean *OXT* DNAm levels than participants classified as proself, we conducted a Point-Biserial Correlation.

Hypothesis 2a: To investigate if there is a negative association between *OXT* DNAm and investments in the trust game we conduct a *Spearman* rank correlation due to the ordinal outcome variable (investment level) that has only four levels (0, 4, 8, 12) and the perceived difference between those investment values may not be equal across participants.

Hypothesis 2b: To investigate if there is a negative association between *OXT* DNAm and allocated money to the beneficiary, we conducted a *Pearson* correlation analysis.

Hypothesis 2c: To investigate if the effect of OXT (compared to PLC) nose spray increases with increasing *OXT* DNAm, we conducted an ordinal logistic regression model (OLR) with investment levels as dependent variable, treatment condition (OXT vs. PLC) as independent variable, and *OXT* DNAm as interaction factor. Levels of nasal obstruction are included as covariate that could potentially affect intranasal treatment administration. We first ran *brant* and *nnet* tests to check if the proportional odds assumption holds. Both tests for all individual CpG site test indicated no evidence to reject the proportional odds assumption (i.e., $p>.05$). We thus ran OLR models as pre-registered.

Hypothesis 2d: To investigate if the effect of OXT (compared to PLC) nose spray increases with increasing *OXT* DNAm, we conducted a linear regression model with allocated money to the beneficiary as dependent variable, treatment condition (OXT vs. PLC) as independent variable, and *OXT* DNAm as interaction factor. Levels of nasal obstruction are included as covariate that could potentially affect intranasal treatment administration.

3. Results

3.1 Descriptive statistics

Table 2 reports the min, max, mean, and SD of *OXT* DNAm levels for all CpG sites. Overall, methylation ranged from 7.60 to 67.43. For histograms and density plots of all *OXT* DNAm per CpG site see *Supplementary materials*.

	n	Min.	Max.	Mean	*SD*

CpG site 1	215	7.60	66.70	33.17	9.63
CpG site 2	206	23.05	67.43	39.59	7.35
CpG site 3	204	19.82	63.02	38.20	7.78
CpG site 4	198	10.59	37.43	21.47	5.01
CpG site 5	196	18.69	53.88	33.59	6.70
CpG site 6	196	8.47	35.91	16.97	4.50
CpG site 7	195	11.32	31.83	19.68	3.93
CpG site 8	177	15.57	45.16	26.81	5.46

Table 2. Description of *OXT* DNAm values per CpG site based on the dataset of *n*=215 participants (excluding those who consumed drugs or alcohol, or who smoked excessively in the 12h before the laboratory session). See 2.4.3 for the chromosome locations of the CpG sites.

The mean IGTS score was 4.25 (*SD*=.87, min=1.33, max=6.44; see *Figure 1a* for a histogram and density plot). Out of the 215 participants, 97 (45.12%) participants were classified as proself, 75 (34.88%) were classified as prosocial, and 43 (20%) remained unclassified. In the trust game subsample, 96 participants (46.60%) received OXT and 110 participants (53.40%) received PLC. In the dictator game subsample, 96 participants (46.83%) received OXT and 109 participants (53.17%) received PLC. *Figure 1b* and *1c* show trust game investments and money allocated to the beneficiary in the dictator game in both treatment groups.

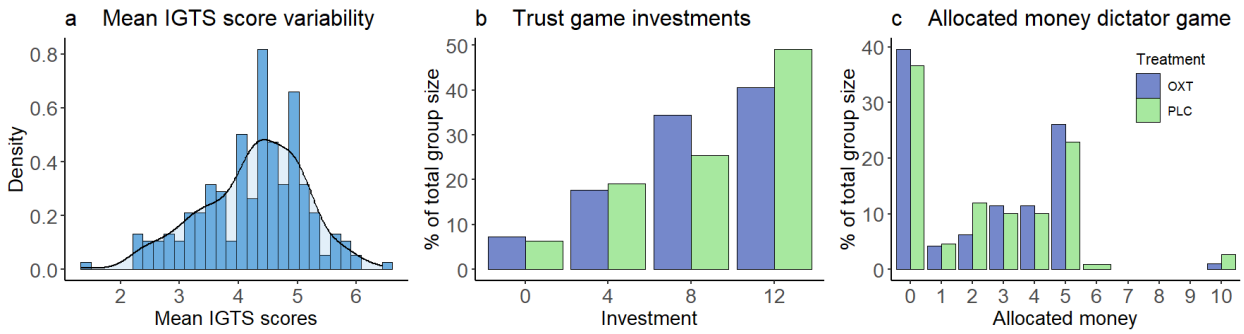

Figure 1. Distributions of a. mean IGTS scores, b. relative (% of total group size) trust game investments in the OXT and plc group and c. relative allocated money in the dictator game in the OXT and PLC group.

3.2 Hypotheses testing

Coefficients for all hypothesis tests per CpG site as well as links to study data and code can be found in the *Supplementary materials*.

3.2.1 Hypothesis 1a&b

Correlation analyses revealed no significant negative association between *OXT* DNAm on any CpG site and dispositional trust (IGTS scores) or between *OXT* DNAm on any CpG site and a participant's SVO classification.

3.2.2 Hypothesis 2a

Spearman's rank correlation tests revealed a significant negative association between *OXT* DNAm and investments in the trust game on three neighboring CpG sites (CpG site 5, ρ=-.16, p=.03; CpG site 6, ρ=-.15, p=.04; CpG site 7, ρ=-.15, p=.04). Boxplots for those significant negative correlations are shown in *Figure 2.* Trend-level significance was found for negative associations between trust game investments and *OXT* DNAm on two other CpG sites (CpG site

1, ρ=-.13, p=.07; CpG site 4, ρ=-.13, p=.07). The overall correlation between mean CpG values per participant and investments in the trust game was ρ=-.12, p=.09.

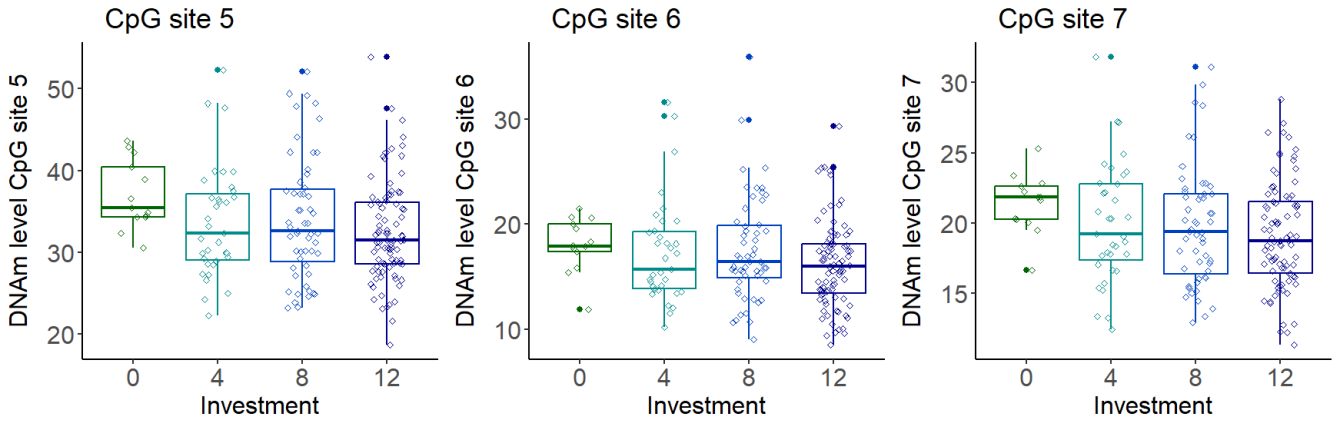

Figure 2. Significant negative associations between *OXT* DNAm on neighboring CpG sites 5, 6, and 7 and investments in the trust game.

3.2.3 Hypothesis 2b

Pearson correlation analyses revealed no significant negative association between *OXT* DNAm on any CpG site and allocated money to the beneficiary in the dictator game.

3.2.4 Hypothesis 2c

Independent samples *t*-tests indicated that *OXT* DNAm levels on any CpG site did not differ between participants randomized to OXT vs PLC. The OLR models revealed no significant interaction effect between treatment and *OXT* DNAm on any CpG site on trust game investments.

3.2.5 Hypothesis 2d

Independent samples *t*-tests indicated that *OXT* DNAm levels on any CpG site did not differ between participants randomized to OXT vs PLC. Linear regression models revealed no significant interaction effect between treatment and *OXT* DNAm on any CpG site on money allocated to the beneficiary in the dictator game.

3.2.6 Prediction of transcription factor binding

The JASPAR CORE database predicted several binding sites for transcription factors overlapping with the CpG sites that were significantly associated with investment in the trust game (*Hypothesis 2a*). ZNF460 was predicted to bind to the sequence that includes CpG site 5 (score 446, $p=10^{-4}$). ZfX (score 404, $p=10^{-4}$), ZNF135 (score 587, $p=10^{-5}$), and ZNF770 (score 443, $p=10^{-4}$) were predicted transcription factors for the sequence containing CpG site 6, and ZNF454 (score 408, $p=10^{-4}$) for CpG site 7.

3.2.7 Exploratory analyses

3.2.7.1 OXT DNAm differences in high- vs. low-trusting participants

To explore if *OXT* DNAm differed between participants with high vs. low dispositional trust, participants were divided into high- and low-trust groups using a median split of IGTS scores. Participants at or above the median (IGTS score of 39) were assigned to the high-trust group ($n=120$), participants below the median were assigned to the low-trust group ($n=95$). Next, we ran independent samples *t*-tests for each CpG site to examine group differences in *OXT* DNAm between high- vs. low trust groups. These analyses revealed no significant differences in OXT DNAm at any CpG site between high- and low-trust participants (see *Supplementary materials* for all coefficients).

3.2.7.2 Placebo effect

We ran additional analyses to explore the possibility of a placebo effect, i.e., if beliefs about (not) receiving OXT affected participant's trust game investments. Fifty percent of participants in the OXT group (n=48) and 65% in the PLC group (n=72) correctly guessed which treatment they had received, when asked after the experiment had concluded. A chi-square test was performed to examine if the ratio of correct guesses differed between the treatment groups which revealed that accuracy in guessing the correct treatment differed between the groups (χ^2=4.28, df=1, p=.04; n=96 for the OXT group and n=110 for the PLC group), indicating partial unblinding.

We then investigated if participants in the PLC group who did (not) believe they received OXT differed in their trust game investments. The *nnet* test revealed that the proportional odds assumption did not hold (p=.03) and we thus computed three separate binary logistic models (I: 0=0 and 4/8/12=1; II: 0/4=0 and 8/12=1; III: 0/4/8=0 and 12=1). None of the models revealed significant differences in trust game investments between treatment belief groups (I: OR=1.34, 95% confidence interval (CI)=[.27, 9.71], p=.73; II: OR=2.35, 95% CI=[.90, 6.94], p=.10; III: OR=.77, 95% CI=[.34, 1.68], p=.51). Next, we ran the corresponding analysis in the OXT group, investigating if participants in that OXT group who did (not) believe they received OXT differed in their trust game investments. Both *brant* (p=.43) and *nnet* (p=.38) tests indicate that the proportional odds assumption held. We therefore ran an OLR model which, like in the PLC analysis, revealed no significant group differences in trust game investments between treatment belief groups (OR=.99, 95% CI=[.48, 2.09], p=.99), indicating that the investment was not affected by treatment belief.

In line with the trust game investments, we also examined the possibility of a placebo effect on money allocated to the beneficiary in the dictator game. In the OXT group, 49% (n=47) correctly guessed the treatment they received, while in the PLC group, it was 67% (n=73). Similar to trust game data, a chi-square test was run to examine whether the ratio of correct guesses differed between the treatment groups, which indicated partial unblinding as the accuracy of guessing the correct treatment differed between treatment groups (χ^2=5.94, df=1, p=.01; n=96 for the OXT group and n=109 for the PLC group).

However, a follow-up linear regression revealed that, within neither the PLC (t(107)=-.27, β=-,13, SE=.49, p=.79) nor OXT group (t(94)=1.33, β=.61, SE=.46, p=.19), participants' treatment beliefs predicted money allocations in the dictator game.

4. Discussion

We aimed to assess the link between *OXT* DNAm, intranasal OXT administration, and human (pro-)social behavior in healthy male participants. In line with our hypotheses, we observed a modest, yet significant, association between higher *OXT* DNAm and reduced trust game investments on three neighboring CpG sites (i.e., 5, 6, and 7). In contrast, we found no significant associations with trait-like dispositional trust, proxy measures of generous behavior, or interactions between *OXT* DNAm and OXT administration on trusting behavior and generosity, and no evidence for differences in *OXT* DNAm between high- vs. low-trusting participants. In addition, although exploratory analyses revealed partial unblinding, participants' beliefs about treatment did not influence behavior in the trust nor the dictator game. This suggests that expectancy or placebo effects did not contribute significantly to participants' behavior. Below, we discuss the relevance of these findings.

The observed associations between *OXT* DNAm and reduced trust game investments may signify that reduced *OXT* synthesis capacity, and consequently lower baseline OXT, may negatively affect prosocial capacities, specifically trusting behavior. Importantly, however, this interpretation remains hypothetical. To date, no studies have examined whether variation at the investigated CpG sites in humans is associated with *OXT* gene expression, peripheral OXT levels, or indices of oxytocinergic tone. Therefore, we cannot determine within the scope of this report whether higher methylation reflects altered transcriptional activity and/or peptide availability. *OXT* DNAm should thus be interpreted here as an epigenetic marker associated with behavioral variation, rather than a validated proxy of endogenous levels.

This interpretation aligns with prior evidence showing that increased DNAm in the promoter region of the *OXT* was linked to more insecure attachment styles, poorer facial emotion recognition, and reduced activation in social-cognitive brain regions during social processing tasks (Haas et al., 2016). To our knowledge, the current study is the first to examine DNAm of the OXT gene itself, rather than the OXT receptor gene, in relation to pro-social behavior. While prior work Anzani et al. (2022) has shown that higher OXT receptor gene DNAm, implying lower receptor expression, was linked to heightened sensitivity to untrustworthiness, our results suggest a complementary mechanism related to OXT synthesis. Nevertheless, without transcriptional or hormonal indices, the specific functional level at which *OXT* DNAm may operate cannot be specified. Together, these findings support a broader model in which epigenetic regulation along the OXT pathway, from synthesis to receptor signaling, shapes individual differences in pro-social capacities. At the same time, we highlight that the present study does not directly test functional consequences within the OXT system.

In an attempt to identify candidate mechanisms that could explain the observed *OXT* DNAm–trust associations, we investigated which transcription factors within the JASPAR CORE database were predicted to bind at CpG sites 5-7. This approach was motivated by the notion that all identified CpG sites are located within the promotor region of the OXT gene and, therefore, may have regulatory relevance and functional consequences for gene expression. Importantly, results from this analysis consistently identified associations with transcription factors belonging to the zinc finger protein (ZNF) family, the largest group of DNA-binding proteins in humans (Li et al., 2022). ZNFs are involved in diverse biological processes such as cell growth and proliferation and many have been linked to diseases (e.g., Li et al., 2022). The association of these ZNFs with CpG sites in the promoter region of the *OXT* could suggest a role for ZNF-mediated transcriptional activity in neural processes relevant to social behavior, such as trust. Supporting this notion, a previous study reported that local overexpression of a gene coding for a putative ZNF in neurons in the prefrontal cortex promoted behavioral resilience to social stress in mice (Lorsch et al., 2019). This highlights a potential role for ZNFs in regulating OXT-mediated pro-social capacities that could be explored in future work.

Contrary to the significant relationship between *OXT* DNAm and trust behavior, we did not find an association with generous behavior in the dictator game. One potential reason for the absence of significant associations is that DNAm of these CpG sites does not affect all prosocial capacities equally (i.e., methylation may preferentially affect trusting, but not generous, behavior). Another reason could be of technical nature: while participants knowingly engaged with their fellow laboratory participants in the trust game, they were instructed that the dictator game would be played with a real, but anonymous, participant outside of the laboratory. This could not only have reduced social salience but may have also created a situation where

participants in the dictator game would not be concerned that their decisions could ever become public. Overall, the distribution of money allocated in the dictator game indicates that most participants (97.56%) allocated ≤ 5€, with 78 participants (38%) allocating no money at all (see *Figure 1c*).

Moreover, we did not find a link between trait-like trust propensity or SVO and *OXT* DNAm. Although these findings could signify that *OXT* DNAm contributes little to shaping complex pro-social dispositions, it is worth noting that, overall, there was low variability in trait scores due to our choice of recruiting participants without a lifetime diagnosis of DSM-V psychiatric or neurological disorder. Furthermore, it is possible that *OXT* DNAm is associated with social dispositional traits that were not assessed in this study, such as attachment style. For instance, in previous studies, higher methylation was associated with callous-unemotional traits (Maud et al., 2018) and with higher quantitative autism traits (Moerkerke et al., 2021). Of note, these two reports focused on DNAm of the oxytocin receptor gene, not on the OXT gene investigated in the current study.

Collectively, our finding that *OXT* DNAm is associated with trust game investments, but not with other prosocial behaviors and traits examined in this study, could suggest a specific role in one domain of prosocial behavior, namely, trusting behavior. Importantly, however, another explanation for the overall pattern of observed results could be that the association between *OXT* DNAm and trust game investments reflects individual differences in reward-related, domain-general, functioning rather than trust per se. While trust game investments are commonly interpreted as a proxy of trusting behavior, such investments may also reflect the motivation to increase one's own payoff, as higher investments are associated with a higher probability of reward increase (Mislin et al., 2015). This represents a crucial difference between the trust and

dictator game: while investing more in the trust game is associated with a higher probability of reward increase, allocating money in the dictator game leads to lower own rewards.

In addition to the domain-general reward-related processes discussed above, other general factors such as arousal or overall motivational state may also play a role in shaping trust game behavior. For instance, heightened arousal or approach-related motivation could increase general task engagement or sensitivity to potential gains (Liu et al., 2015; Radke et al., 2016; Sakaki et al., 2024), thereby leading to higher investments in the trust game without necessarily reflecting trust-related processes.

In line with this broader perspective, emerging behavioral and neurobiological evidence suggests that OXT is involved in domain-general constructs such as reward sensitivity and motivated behavior in both humans and animals (e.g., Ide et al., 2018; Mickey et al., 2016; Nawijn et al., 2016; Plessow et al., 2018; Roberts et al., 2019). Behaviorally, Ide et al. (2018) found that OXT administration decreased feedback learning (i.e., the ability to adjust behavior based on reinforcement) in humans in response to both positive and negative reward, while Roberts et al. (2019) reported that chronic administration of subcutaneous OXT in rats improved reward learning but not effortful motivation. On a neural level, OXT has the ability to modulate activity in canonical reward-responsive brain areas including midbrain, basal ganglia, and prefrontal cortex regions during tasks that assess (non-social) motivated behavior. In a functional Magnetic Resonance Imaging (fMRI) study, Mickey and colleagues (2016) reported that midbrain and medial prefrontal activity during motivated (non-social) behavior can be modulated by intranasal OXT administration. Further, in a sample of overweight and obese men, OXT administration reduced the Blood Oxygenation Level Dependent (BOLD) signal in reward-related food motivation brain regions such as the orbitofrontal cortex, insula, globus pallidus,

hippocampus, and amygdala (Plessow et al., 2018). OXT administration also increased neural responses in the striatum, dorsal anterior cingulate cortex and insula, all key regions in the reward pathway, during reward and loss anticipation in a traumatized sample, indicating a role for OXT in motivation for goal-directed approach behavior (Nawijn et al., 2016). Lastly, in a reinforcement learning paradigm, reduced learning under OXT was linked with a muted communication between key nodes within the reward circuit, i.e., amygdala, lateral (limbic) habenula and orbitofrontal cortex (Ide et al., 2018).

Together, these behavioral and neural findings suggest that the effects of OXT on reward sensitivity and motivated behavior are tied to neural circuits that are mediated by dopaminergic signaling (Baskerville & Douglas, 2010). Importantly, the present study did not assess OXT expression, dopaminergic activity, or neural reward processing directly. It should be noted that most evidence linking OXT and dopaminergic signaling is derived from animal work, and that one human study that investigated the effects of intranasal OXT on DA reported no change in striatal DA receptor availability following OXT administration (Striepens et al., 2014). Nevertheless, in terms of interindividual variation in endogenous OXT as indexed by DNAm, the above findings align with extensive evidence for a close anatomical and functional relationship between OXT and DA. The mechanisms through which OXT and DA interact and influence each other's release profiles include mutual innervation, co-activation and/or co-release, and receptor-receptor interactions (e.g., Amato et al., 2023; Baskerville et al., 2009; Baskerville & Douglas, 2010; Gimpel & Fahrenholz, 2001; Mason et al., 1983; Petersson & Uvnäs-Moberg, 2024). For example, OXT release has been shown to increase DA levels in regions such as the amygdala, ventral tegmental area, and medial preoptic area (Baskerville & Douglas, 2010; Gimpel & Fahrenholz, 2001; Petersson & Uvnäs-Moberg, 2024), and dopaminergic signaling

can in turn modulate oxytocinergic neurons via DA receptor-mediated mechanisms (Baskerville et al., 2009; Mason, 1982; Petersson & Uvnäs-Moberg, 2024).

Based on the above and from a mechanistic perspective, one possibility is that interindividual differences in *OXT* DNAm reflect longer-term regulatory variation in the OXT system that may be associated with differences in reward processing and motivated behavior. However, this interpretation remains speculative and cannot be directly evaluated with our data. Another potential mechanism is histone dopaminylation, a newly identified epigenetic mark that is connected to the regulation of gene transcription and through which DA can bind to proteins inside cells with functional and activational consequences (Lepack et al., 2020), and that could theoretically take place in *OXT*..

Beyond dopaminergic mechanisms, the serotonin system has also been implicated in OXT-mediated modulation of reward and social behavior. Levefre and colleagues (2017) for instance reported that OXT can influence serotonergic signaling in brain regions relevant for reward, social approach, and affective processing. In line with this, Dölen et al. (2013) suggest that coordinated OXT and serotonin activity in the nucleus accumbens is necessary for social reinforcement, with blockade of serotonergic receptors abolishing OXT-dependent social reward. Further, at a cellular level, OXT has been shown to excite serotonergic neurons and modulate their synaptic inputs, providing a mechanism for OXT to influence serotonergic output to forebrain targets (Oudbraim et al., 2023). Together, these findings suggest that serotonergic circuits provide an additional or complementary pathway through which oxytocinergic systems may impact reward-related and social decision-making. Again, it is important to note that we did not assess serotonergic activity, and this interpretation cannot be directly evaluated with data collected in this study. Regarding both, potential dopaminergic and serotonergic mechanisms,

future studies integrating epigenetic, neurochemical, and neuroimaging measures will be necessary to clarify whether, and how, *OXT* DNAm relates to reward-related neurobiological processes and social decision-making.Lastly, we examined whether individual differences in *OXT* DNAm modulates the effect of OXT administration on pro-social capacities. Recent large-sample studies (Declerck et al., 2020; Kroll et al., 2023) have reported the absence of a main effect of intranasally administered OXT on trust game behavior. The present study adds to this by demonstrating that OXT administration effects on trust (and generosity) also do not depend on variation in a meaningful biological mechanism that could mediate such effects. However, the hypothesized moderating role of *OXT* DNAm was conceptually based on the assumption that higher *OXT* DNAm may reflect lower endogenous OXT levels. Because functional indices of OXT system activity were not incorporated into the present analyses, this mechanistic assumption cannot be empirically evaluated which remains a priority for future work. Consequently, the absence of an *OXT* DNAm x OXT administration interaction does not conclusively rule out the possibility that biologically verified differences in endogenous OXT function moderate behavioral responses to OXT administration. Rather, our findings indicate that interindividual variation in peripheral *OXT* DNAm, as assessed in this study, was not associated with different behavioral responsiveness to a 24 IU dose of intranasal OXT.

Our study has some limitations that affect the interpretation of results, which could be addressed in future research. Importantly, while we observed an association between *OXT* DNAm and trust behavior consistent with our hypothesis, this association would not survive correction for multiple comparisons. This limitation, which frequently affects candidate gene and epigenetic association studies, indicates that the finding should be considered preliminary and primarily hypothesis-generating. However, associations with trust behavior were observed across

adjacent CpG sites, a pattern that suggests robustness even if the individual association is not particularly strong. Future studies with larger samples or pooled analyses and replication designs will be necessary to confirm these associations.

A further limitation is that we did not directly assess downstream functional consequences of *OXT* DNAm. Specifically, we did not test whether variation in *OXT* DNAm was associated with OXT gene expression or with peripheral or central OXT levels under basal or stimulated conditions. Therefore, we cannot empirically determine within the present study whether higher methylation at the investigated CpG sites is linked to altered *OXT* transcription, endogenous OXT availability, or oxytocinergic responsibility. As a result, the functional implications cannot be directly inferred.

At the same time, previous work has however suggested that *OXT* DNAm may be functionally relevant for social behavior and OXT-related processes (e.g., Haas et al., 2016). These authors reported that increased *OXT* DNAm was associated with attachment insecurity, reduced facial emotion recognition, and altered activation in social-cognitive brain regions, indicating that peripheral methylation variation can be related to meaningful behavioral and neural phenotypes. The present findings should thus be interpreted as indirect markers of potential regulatory mechanisms, and future studies combining epigenetic, transcriptional, and hormonal measures should be performed to further clarify the functional significance of *OXT* DNAm.

Another consideration concerns the efficacy and reliability of intranasal OXT delivery. While the use of a nasal spray has greater ease-of-use for participants and is widely applied (e.g., Declerck et al., 2020; Kroll et al., 2023), and research suggests that this OXT administration method reaches central areas (Lee et al., 2020; van der Aart et al., 2018), there is no direct

evidence for central penetration and physiological engagement in humans, and thus administration reliability remains uncertain. Consequently, the absence of significant findings related to intranasal OXT administration in this study cannot be taken as definitive evidence for a lack of OXT involvement. Our findings also call for a critical evaluation of OXT administration methods, including the potential advantages of more advanced delivery techniques such as a Breath Powered device, which may enhance central bioavailability and lead to more consistent pharmacodynamics (e.g., Djupesland & Skretting, 2012; Winterton et al., 2021).

In this study, we administered a dose of 24 IU in line with the design of a larger replication project in which the present study is embedded (Kroll et al., 2023), and this dose is also the most commonly employed one in human intranasal OXT research (Quintana et al., 2021). Nevertheless, prior work has reported dose-dependent effects of intranasal OXT on social-cognitive behavior (e.g., Quintana et al, 2017), and accumulating evidence suggests that OXT administration may not follow a linear dose-response relationship (Guoynes et al., 2018). It is therefore possible that the null effects observed in the present study reflect suboptimal dosing and timing, and that alternative dosing strategies could yield different outcomes. Future studies directly comparing multiple OXT doses, as in Quintana et al. (2017) will be important to further elucidate dose-response characteristics.

Another limitation is that we recruited a sample of healthy young adults, which limited confounding factors but may have resulted in relatively low trait variability. Since only male participants were tested, the generalizability of the present findings to females is limited. This design prevents the assessment of potentially sexually dimorphic effects of OXT which may arise from interactions with sex-specific hormonal and neurotransmitter systems (see Quintana et al., 2024 for an overview). In females, OXT effects may be further modulated by hormonal

variations across the menstrual cycle and by hormonal contraceptive use. For example, peripheral OXT levels have been shown to vary across the menstrual cycle, with increasing levels during the follicular phase, mirroring increasing estrogen levels, and a post-ovulatory decline to the mid-luteal phase (Engel et al., 2019), and to be elevated in hormonal contraceptive users, particularly those using formulations with ethinylestradiol (Garforth et al., 2020). Although peripheral OXT levels do not directly index central OXT activity, these findings underscore the complexity of hormonal interactions that may shape OXT effects and highlight the need for future studies including female participants while accounting for menstrual cycle phases and contraceptive status. Further, since OXT signaling activity is suggested to change with age (Audunsdottir & Quintana, 2022), our findings in a sample of younger adults may not extend to older adults.

A final point concerns the OXT manipulation. While both the trust game and the dictator game were performed during the expected post-administration peak window of 30-90 minutes as described by Gossen et al. (2012), these authors also stated that the course of individual OXT (plasma) levels over time exhibited variability, with some participant levels returning to baseline earlier than others. It is thus possible that variability in OXT levels, especially during the second task (i.e., the dictator game) may have impacted the OXT effect. Lastly, it should be noted that the observed *OXT* DNAm-trust associations were robust across the three neighboring sites, but their magnitude was overall small, with a correlation of ρ between -.15 and -.16, underscoring its subtle nature in the order of trait-like variation.

5. Conclusion

In conclusion, this is the first study investigating the link between *OXT* DNAm, intranasal OXT administration, and facets of human (pro-)social behavior. We found evidence

for an association between *OXT* DNAm on CpG sites 5, 6, and 7 (GRCh37/hg19=chr20: 3,052,319; chr20:3,052,334; and chr20:3,052,345, respectively) and trust game investments. However, these findings should be considered preliminary. Importantly, functional indices of OXT system activity were not incorporated into the present analyses, therefore the study cannot unambigously establish whether variation in *OXT* DNAm translates into measurable differences in *OXT* gene expression, peripheral levels, or central oxytocinergic signaling. Consequently, the results do not allow conclusions regarding OXT synthesis capacity or endogenous OXT availability and call for further mechanistic investigations into the nature of this association and the determination of functional consequences on a molecular level. Based on the overall pattern of results obtained, one possible interpretation is that *OXT* DNAm may be associated with individual differences in oxytocinergic regulation of reward-related behavior, potentially involving interactions with dopaminergic and/or serotonergic systems, although this remains to be tested empirically. Further, other general factors such as arousal or overall motivational state may also contribute to the observed associations.

CRediT authorship contribution statement

CK: Conceptualization, Methodology, Software, Formal analysis, Investigation, Data Curation, Writing – Original Draft, Writing – Review & Editing, Visualization; NL: Conceptualization, Methodology, Formal analysis, Writing – Original Draft, Writing – Review & Editing; CV: Methodology, Writing – Review & Editing; GK: Methodology, Resources; DvdH: Methodology, Resources, Writing – Review & Editing; AR: Conceptualization, Methodology, Writing – Review & Editing, Supervision, Funding Acquisition; KS: Conceptualization, Methodology,

Writing – Review & Editing; DH: Conceptualization, Methodology, Writing – Review & Editing, Supervision, Funding Acquisition.

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