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Publication Date

2022-04-01

DOI

10.1016/j.yhbeh.2022.105126

Peer reviewed

HHS Public Access

Author manuscript

Horm Behav. Author manuscript; available in PMC 2022 July 03.

Published in final edited form as:

Horm Behav. 2022 April ; 140: 105126. doi:10.1016/j.yhbeh.2022.105126.

Long Term Effects of Chronic Intranasal Oxytocin on Adult Pair Bonding Behavior and Brain Glucose Uptake in Titi Monkeys (*Plecturocebus cupreus*)

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Abstract

Intranasal oxytocin (IN OXT) administration has been proposed as a pharmacological treatment for a range of biomedical conditions including neurodevelopmental disorders. However, studies evaluating the potential long-lasting effects of chronic IN OXT during development are still scarce. Here we conducted a follow-up study of a cohort of adult titi monkeys that received intranasal oxytocin 0.8 IU/kg (n=15) or saline (n=14) daily for six months during their juvenile period (12 to 18 months of age), with the goal of evaluating the potential long-lasting behavioral and neural effects one year post-treatment. Subjects were paired with an opposite-sex mate at 30 months of age (one year post-treatment). We examined pair affiliative behavior in the home

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cage during the first four months and tested for behavioral components of pair bonding at one week and four months post-pairing. We assessed long-term changes in brain glucose uptake using ^{18}FDG positron emission tomography (PET) scans. Our results showed that OXT-treated animals were more affiliative across a number of measures, including tail twining, compared to SAL treated subjects (tail twining is considered the "highest" type of affiliation in titi monkeys). Neuroimaging showed no treatment differences in glucose uptake between SAL and OXT-treated animals; however, females showed higher glucose uptake in whole brain at 23 months, and in both the whole brain and the social salience network at 33 months of age compared to males. Our results suggest that chronic IN OXT administration during development can have long-term effects on adult social behavior.

Keywords

Intranasal oxytocin; autism; Prader-Willi syndrome; PET scan; behavior

Introduction

Neurodevelopmental disorders such as autism and Prader-Willi Syndrome (PWS) can cause significant compulsivity, social, and behavioral challenges. Currently there are no medications to treat these behavioral impairments in childhood, and intranasal oxytocin (IN OXT) remains a promising treatment option due to its potential to ameliorate symptoms of PWS. A majority of previous IN OXT studies have focused on the immediate effects following a single dose (Andari et al., 2010; Auyeung et al., 2015; Guastella et al., 2010; Hollander et al., 2006) or repeated administration (Anagnostou et al., 2012; Feifel et al., 2012; Parker et al. 2017; Tachibana et al., 2013; Watanabe et al., 2015; Yatawara et al., 2016; Yamasue et al., 2018; Dadds et al., 2014; Guastella et al., 2015; Munesue and Higashida, 2016); however, animal research has shown that IN OXT administration during development may lead to long-term changes that are different from those seen during acute administration (Bales et al., 2013; Rault et al., 2013).

The current study examined the long-term behavioral and neural effects of chronic IN OXT treatment during adolescence in a non-human primate model. This research is a continuation of a previously published study on the behavioral and neural effects of chronic IN OXT in juvenile animals during treatment and one month after treatment (see Arias-del Razo et al., 2020). Our animal model, the titi monkey (*Plecturocebus cupreus*) is a socially monogamous primate which is a good animal model for human social behavior due to similarities in their close relationships (Bales et al., 2017). Titi monkey pair mates display a strong and enduring emotional bond, characterized by a strong preference for their familiar partner (Carp et al., 2016; Rothwell et al., 2020), mate-guarding behavior (Fisher-Phelps al., 2016; Witczak et al., 2018), territorial defense against intrusion (Mason, 1966), distress upon separation (Mendoza and Mason, 1986) and the ability of the partner to buffer against stress (Mendoza et al., 2000).

Animal research suggests that pharmacological manipulation of the OXT system during development may lead to long-term effects (Bales & Perkeybile, 2012). In socially

monogamous prairie voles, a single OXT exposure at certain dosages (1 mg/kg, 4 mg/kg and 8 mg/kg) but not others (2 mg/kg) during the neonatal period resulted in adult females no longer displaying a partner preference (Bales et al., 2007a), while a single OXT exposure in adulthood facilitated the formation of a pair-bond in females (Cho et al., 1999, Williams et al., 1994). Rault et al. (2013) showed that repeated exposure to IN OXT in neonatal pigs resulted in long-term disrupted social behavior, more aggressive behavior, and dysregulation of the HPA axis. Bales and colleagues (2013) examined the long-term effects of chronic IN OXT at different dosages in juvenile prairie voles and found that daily treatment with the medium (0.8 IU/kg) and the lowest dose used (0.08 IU/kg) resulted in longer term deficits in the ability of male subjects to form a partner preference, shown by a reduction in time spent with a familiar partner.

These findings highlight the importance of taking factors such as age, dose, and sex into consideration for the administration of IN OXT over an extended period of time. In addition, the existing literature on IN OXT also calls attention to the sensitivity of the neurobiological mechanisms of the oxytocin receptor (OXTR) system during development (Ellis et al., 2021; Horta et al., 2020), and the close relationship between OXT and arginine-vasopressin (Song & Albers, 2018).

Recently, human studies have started to focus on the long-term effects of continuous administration of IN OXT. Bernaerts et al. (2020) evaluated the long-term effects of a four-week IN OXT treatment (24 IU once daily) in adult men with ASD and found no specific improvements in core social symptoms; however, they did find beneficial effects in repetitive behaviors and feelings of avoidance at one month and even one-year post-treatment. These long-term behavioral effects were associated with neural changes in intrinsic functional connectivity of the amygdala to core regions of the “social brain”, particularly the orbitofrontal cortex and superior temporal sulcus (Alaerts et al., 2020). Preliminary data and a subsequent phase three industry trial of intranasal carbetocin (synthetic oxytocin with a longer half-life) for PWS report better clinical outcomes with decreased anxiety and distress behaviors in children ranging between 7–18 years (Tauber and Diene, 2021).

Imaging studies using ^{18}FDG PET scans co-registered with structural magnetic resonance imaging (MRI) in male titi monkeys have provided important information about the developmental timeline of pair-bond formation (Bales et al., 2007b, Maninger et al., 2017). FDG is a fluorinated form of glucose that is taken up in active areas of the brain; it correlates with brain oxygenation and is reflective of synaptic activity (Jueptner & Weiller, 1995). It is neurochemically non-specific (as are several other common measures of brain activity, including fMRI; unfortunately, validated ligands which would cross the blood-brain barrier and directly bind to OXT receptors still do not exist (Smith et al., 2016). Changes in brain activity exist 48 hours after pairing in two areas: the right nucleus accumbens and the ventral pallidum (Bales et al., 2007b). One week after pairing, males showed a significant increase in whole brain glucose uptake compared with the control condition (housed alone); this increase was maintained and even exaggerated at four months post-pairing. Glucose uptake in “motivational” areas [nucleus accumbens (NAcc), ventral pallidum (VP), caudate (Caud), and putamen (Put)], and emotion and social memory related areas [medial amygdala (MeA),

lateral septum (LS), medial preoptic area (MPOA), posterior cingulate cortex (PCC)] also showed higher increases in glucose uptake at one week and four months post-pairing (Maninger et al., 2017).

Ultimately, very few studies have examined the long-term effects of chronic intranasal OXT given during development; we are aware of only one other study in non-human primates (Parr et al., 2016), aside from our own. That study was carried out in rhesus monkeys, which do not form pair bonds, and followed the animals only to six months of age. In this study we focused on the possible long-term effects of developmentally administered, chronic IN OXT on pair bonds (Bales et al., 2021) in adult male-female pairs of titi monkeys, representing a much longer-term follow-up than has been seen in any non-human or human primate study. We examined pair affiliative behavior in the home cage during the first four months. We experimentally interrogated changes in species-typical behavior towards partners and strangers during pair-bond formation (one week post-pairing) and maintenance (4 months post-pairing). Finally, we examined changes in cerebral glucose metabolism at 23 months of age (pre-pairing) and at 33 months of age (3 months post-pairing).

We hypothesized that long-term effects of chronic IN OXT administration in adulthood would be more evident in OXT-treated males than females. Sex differences in the neurobiology of pair bonding are common (DeVries et al., 1996; Cho et al., 1999). Males have also shown greater sensitivity to developmental manipulations of OXT in previous studies, and females seem more resilient to developmental manipulations, typically responding only at higher doses of OXT (Bales et al. 2013; Arias-del Razo et al. 2020; Bales et al 2007a). We hypothesized that IN OXT in adult males would facilitate pair-bond formation, enhance sociability with strangers, and reduce the display of territorial and mate guarding behaviors towards strangers. Finally, using cerebral glucose uptake as a non-specific marker of regional brain activity, we hypothesized that the behavioral effects observed in OXT-treated males would be accompanied by an increase in glucose uptake in whole brain, and in regions of interest (ROIs) involved in motivation and social behaviors (as detailed in previous paragraph).

Methods

Subjects and housing

All titi monkeys (*Plecturocebus cupreus*, previously *Callicebus cupreus*) used in this study were born in captivity at the California National Primate Center (CNPRC) and housed in indoor home cages measuring 1.2 m x 1.2 m x 2.1 m. Rooms were maintained at 21° C on a 12:12 light-dark cycle (lights on at 6:00 AM and off at 6:00 PM). Animals were fed twice daily at 8:00 AM and 1:30 PM. The diet included monkey chow, carrots, bananas, apples, rice cereal, and water available *ad libitum*. Additional enrichment was provided twice a day and included two of the following items: spinach, apples, mealworms, puffed cereal, oats, green lentils and sunflower seeds. For further detail of husbandry practices see Tardif et al. (2006). The University of California Davis Institutional Animal Care and Use Committee approved all housing conditions and experimental procedures described in this paper.

Subjects included twenty-nine titi monkeys, OXT=8 females, 7 males; SAL=7 females, 7 males. Dosing, data collection, scoring, and analysis were done without knowledge of treatment. Throughout the study, several different color codes were used to represent oxytocin and saline to create additional avoidance of experimenter bias. From birth to twenty-nine months of age (Mean ± SE = 895 ± 12 days) subjects were housed with both parents and siblings (if present). At twenty-nine months of age, subjects were removed from their family group, moved into a new home cage, and paired with an unfamiliar partner of the opposite sex.

Pharmacological Treatment

Subjects received either 50 μl SAL (vehicle control), or oxytocin acetate salt (OXT) dissolved in 50 μl of saline (Santa Cruz Biotechnology). Doses were administered via intranasal administration, at a concentration of 0.8 IU/kg body weight, every day for six months starting at 12 months (360 ± 2 days) and lasting through 18 months (540 ± 2 days) of age. This age interval encompasses the late juvenile period as well as the pubertal period (Valeggia et al., 1999). Prior to initial treatment, subjects were habituated and trained using positive reinforcement techniques to all steps required to receive an intranasal dose.

Treatments were administered every morning between 9:00 AM and 10:00 AM, taking approximately 5 minutes per subject for both SAL and OXT groups. The treatment was administered using a pipette to drip 25 μl of OXT (Santa Cruz Biotechnology, Dallas, Texas) or SAL in each nostril (50 μl in total). Treatments were prepared every month to ensure each subject received the appropriate dose based on its weight. Aliquots of the treatment solutions were stored in microcentrifuge tubes at −80° Celsius until use.

The age and dosage were based on clinical trials (ClinicalTrials.gov identifiers: NCT01908205 and NCT01308749) that were in progress at the time of the study. These trials were testing the effects of IN OXT on social deficits in adolescents with ASD. The first trial administered 0.4 IU/kg twice daily, using a maximum of 24 IUs per dose; while the second trial administered IN OXT twice a day using maximum adjusted dose of 32 IU. Our dose (0.8 IU/Kg) would be equivalent to a 40 IU dose given to a 50 kg human subject, and is in the multidose ranges given in PWS and autism studies. This dose (0.8 IU/Kg) was also used in previous oxytocin studies involving prairie voles and mice as the medium dose (Bales et al., 2014, 2013). For further details of dosing, as well as behavioral tests performed when subjects were juveniles, see Arias-Del Razo et al. (2020).

Formation of pairs

At approximately thirty months of age (899 ± 12 days) each subject was paired with an unfamiliar individual of the opposite sex with previously proven fertility (subjects were reproductively naïve). Subjects were removed from their natal group the same day they were paired. An additional part of this study (results not reported here) was to examine whether the chronic intranasal oxytocin administration affected the reproductive system and fertility of the treated animals. In order to examine fertility, we had to pair subjects with animals that were known to have successfully reproduced – meaning that in many cases, we had to break up a current pair. Mates were housed alone for approximately two weeks prior to pairing as

titi monkeys will only re-pair and form new bonds if separated from their previous mate for at least one week (Bales, unpublished data). If we were to just take an animal from a current pair and put them with a new pair-mate directly, it is highly probable that they would be aggressive towards the new animal. A separation of at least one week is therefore absolutely indispensable for animal welfare. In some cases, after a subject completed testing around 36 months of age, they were separated from their original pair mate and paired with a different monkey, and their original pair mate ($n = 5$) was used as the pair mate for another subject in the study who had not yet started testing. In all, three females (1 SAL, 2 OXT) and two males (1 SAL, 1 OXT) had mates which had been used previously with another subject.

During the two weeks of single housing, the animal can still hear, see, and smell other titi monkeys. In addition to the standard dietary enrichment, all animals receive foraging and other physical enrichment. Animal behavioral health is monitored closely by the Behavioral Management department at the CNPRC, and relatively short spans of single housing, like these, have never been noted to result in development of stereotypies.

New pairs were monitored closely the first two days to ensure compatibility. If displays of low aggression (chase, grab) and/or distress were observed between the pairs, the initial monitoring period was extended as necessary. In three cases (two SAL-treated females and one OXT-treated male), pairs were separated on the first day of pairing and moved into two adjacent cages connected by a grated window (grate pairing) to allow visual and olfactory access with limited physical contact for one week before attempting a second full pairing. In all cases, the aggression was elicited by the males in the pair. This second pairing attempt was successful in all three cases.

Pair affiliation

Pair affiliation in the home cage was assessed using a scan sampling method 5-6 times a day, approximately 5 days a week (see Baxter et al., 2020; Rothwell et al., 2020), across the first four months of pairing. At each assessment, pairs were marked as either *Tail Twining* (a species-typical affiliative behavior), in *Contact*, in *Proximity* (within one arm's length), or *None*. For each pair and month, the percent of observations that the pair was observed in each of the affiliation states (*Proximity*, *Contact*, and *Tail Twining*) was determined by summing the total number of observations of each state and dividing by the total number of observations conducted that month for that pair. The total number of observations for each pair and month ranged from 46-119 (median = 97) and were obtained between 9-25 days (median = 19.5).

Behavioral testing

After being paired, subjects and mates underwent different behavioral tests to measure components of pair-bonding (Figure 1). After the first week, pairs were tested in two partner preference tests (Sequential Partner Preference Test and Simultaneous Partner Preference Test) and one Mirror Test. Tests occurred in that order and were scheduled one test per day. This set of tests was repeated at four months post-pairing.

We used two different stimulus animals per subject to represent potential mates in both Partner Preference Tests. Stimulus animals were selected by choosing an unfamiliar animal

that was age-matched to the subject's mate and was not directly related to the subject or pair mate. All stimulus animals came from one pool; however, each subject encountered a different, novel stimulus animal for each testing situation.

Sequential Partner Preference Test—This test allowed us to assess the subject's behavior towards an unfamiliar stimulus animal in the home cage rather than in a novel apparatus. This is notable because titi monkeys are very sensitive to alterations in their surroundings (Hennessy et al., 1995). We used three wire mesh test cages, measuring 30 cm high × 30 cm wide × 60 cm long, which allowed visual, olfactory, auditory, and limited physical contact (e.g., fingers through the mesh). Prior to the test, the partner and the stimulus animal were moved from their home cages and placed in two test cages, with an empty test cage used as a non-social control stimulus. Stimulus conditions were presented consecutively to the subject inside its home-cage for 5 minutes each. While the subject had access to each condition, visual contact from/to the other conditions and from/to stimulus animals was prevented. For scoring purposes, each presentation was divided into 20 behavioral scans at 15 second intervals, signaled by an audible beep. During this test we recorded the subject's location within the home cage, proximity, contact, latency to contact the stimuli at each beep, and whether contact occurred between beeps (Table 1). Each of the three stimuli was presented three times, in a random order (Jarcho et al., 2011).

Simultaneous Partner Preference Test

Test Apparatus.: The test apparatus consisted of three adjacent cages with two grated windows (30 x 30 cm) connecting each of the side cages to the center cage. The grated windows were made of a mesh wirework and allowed visual, olfactory, and limited physical contact between the test animal in the center and the side cages which held the stimulus animals (Carp et al, 2016; Rothwell et al., 2020). Each cage had two 1.2-meter perches. The perches of the center cage were marked into three equal sections of 40 cm each and classified as either a preference zone (the one-third of the perch closest to either left or right grate) or a neutral zone (the middle third). All the other areas of the center cage were considered no preference, or neutral zones. The two stimulus animals had visual access to one another when both animals were located near their respective grated windows. Likewise, when stimulus animals were near their grated windows, they could observe the test animal interacting with the other stimulus animal. While no other animals were within the visual field of the testing animals, there was auditory and olfactory contact with non-testing animals housed in the same room (Carp et al., 2016).

Procedure.: All tests took place from 10:00 AM to 1:15 PM. All animals were provided with food and water for the entire duration of the test. Prior to testing, a visual block was put in front of the three-cage apparatus to prevent visual access to other animals housed in the same room. The stimulus animals were then released into the side cages; the location of the stimulus animals was counterbalanced with each treatment that had been previously received as a juvenile (OXT and SAL).

Each test consisted of five consecutive 30-minute observations with 5-minute breaks in between, approximately 3 hours in total (during the observer breaks, the test continued

undisturbed; these just represented a break for the observer). The beginning of the first observation was signaled by the release of the subject into the center cage of the apparatus. We live-scored physical location of the subject including presence in the preference zones and contact with the grated window of either stimulus animal (Table 2). Behavioral data was collected using Behavior Tracker 1.5 (www.behaviortracker.com). Multiple trained observers were validated for live focal animal sampling with greater than 90% observer reliability prior to the start of the study.

Mirror test—When exposed to a mirror, titi monkeys react to their reflection in ways that indicate a lack of self-recognition. Even after repeated exposures, titi monkeys behave similarly to how they would respond to a live intruder (Fisher-Phelps et al., 2016; Mendoza & Mason, 1986). The use of a mirror has been proven to be a safe and effective method to examine mate-guarding and territorial behavior in response to simulated intrusion in titi monkeys, especially in males (Witczak et al., 2018; Rothwell et al., 2019; Mercier et al., 2020. When a subject is tested with their partner, their reflection in the mirror may be perceived as a same-sex stranger and/or an opposite-sex stranger, both of which may pose a threat to the pair bond (Fisher-Phelps et al., 2016).

We used a 33 x 22 cm mirror placed on top of a movable cart (82.6 cm in height). The mirror, concealed by a towel, was wheeled in front of the home cage so that animals sitting near the front of the cage would see their full reflection. The beginning of the test was signaled by removal of the towel and exposing the mirror to the animals for 5 minutes. We used two conditions: a mirror-condition (showing the reflective side of the mirror) and a control-condition (showing the non-reflective, back side of the mirror), which were performed on consecutive days. In total, pairs had two exposures to the mirror and two exposures to the back side of the mirror. The order of front side vs. back side presentation of the mirror was counterbalanced across subject, treatment groups and 1 week and 4 months test periods.

Subjects and pair mates were recorded during testing and behaviors were later scored by two validated observers (achieved >95% inter-rater reliability) using Behavior Tracker 1.5 (www.behaviortracker.com) and the ethogram on Table 3.

Imaging Study: To assess long-term neural changes due to early chronic IN OXT treatment, subjects underwent a PET scan at 23 months old (pre-pairing) and at approximately 33 months of age (3 months post-pairing) to identify differences in brain glucose uptake between both treatment groups (IN OXT/SAL). We focused on areas that have been shown to be involved in primate selective social behavior (paternal and pair mate attachment) and/or express OTR and AVPR1a. The following ROIs were measured for FDG uptake: anterior cingulate cortex (ACC), lateral septum (LS), caudate nucleus (C), nucleus accumbens (NACC), putamen (PUT); amygdala (AMY), hippocampus (HIPP), paraventricular nucleus of the hypothalamus (PVN), and supraoptic nucleus of the hypothalamus (SON).

For both scans, subjects, their family (23 months old scan/pre-pairing) or their pair-mate (33 months old scan/3 months post-pairing), were relocated to a metabolism room at CNPRC

48 hours prior to their PET scan in order to habituate the animals to the room and reduce the possible effect of novel housing on brain metabolism (Bales et al., 2007b). Animals were fasted 10 hours prior to each PET scan, with water available throughout the pre-scan period. On the day of the PET scan, the subject was removed from the cage, and manually restrained by trained personnel to receive a bolus injection of [^{18}F]-fluorodeoxyglucose (^{18}FDG, PETNET Solutions, Sacramento, CA, USA) administered in a volume of <2 ml) into the saphenous vein. The subject was returned to their cage with their attachment figure (their father for the 23 month scan, their pair mate for the 33 month scan) for 30 min of conscious uptake.

After the FDG uptake period, subjects were anesthetized with ketamine (25mg/kg IM). Anesthesia was maintained throughout the scan with isoflurane (1-2%), while the subject was positioned on the scanner bed. PET imaging was performed on a microPET P4 scanner (Siemens Preclinical Solutions, Knoxville, TN). Image acquisition was targeted to begin at approximately 65 minutes post-FDG administration, and static PET scans were acquired for 60 minutes. After the scan, animals were maintained in the metabolism room for 24 hours, at which time radiation was decayed to background levels and animals were returned to their home cages in the colony room. The resulting PET images were co-registered with a structural MRI performed at 23 months. The co-registration process enables the physiologic data obtained on the PET scan to be localized using the anatomical information from the MRI. MRI scans were conducted using a GE Signa LX 9.1 scanner (General Electric Corporation, Milwaukee, WI) with a 1.5 T field strength and a 3” surface coil. Both PET and MRI scans were performed as previously described (Bales et al., 2007; Maninger et al., 2017a, 2017; Arias-del Razo et al., 2020).

PET and MRI Co-registration and quantification of FDG Uptake.—Boundaries for the ROI structures, as well as for the whole brain, were drawn on each subject’s MRI image using Siemen’s Inveon Research Workplace software (IRW, Siemens Healthcare, USA) by methods previously described in Arias-del Razo et al. (2020). Static PET images were reconstructed with a 3DRP reconstruction protocol. MRI images were co-registered with PET scan images using the automatic rigid registration algorithm in IRW and checked visually for acceptable registration accuracy. Mean FDG activity was determined by applying the ROI boundaries that were defined on the MRI images to the PET images in IRW.

Data analysis

For the pair affiliation scan sampling, we performed linear mixed models to assess for treatment effects on pair affiliation using the *lmer* function from the *lme4* R package (Bates, Mächler, Bolker, & Walker, 2014). For each outcome measure (*Proximity*, *Contact*, *Tail Twining*, and *Contact or Tail Twining*), a series of stepwise regression models were tested. In the first model, we assessed the effects of treatment group. In the second model, month and the interaction between month and treatment group were added to the model. In the third model, sex was added with all two-way and three-way interactions. In all models, the outcome and predictor variables were z-scored. Each model included subject ID nested within pair mate ID as a random effect. This was done because there were repeated

(monthly) measurements across subjects, and because some of the subjects ($n = 10$) in this study were paired with the same pair mate ($n = 5$) as other subjects in the study. Each model AIC was compared to the AIC of a null model (a model with only the intercept plus random effects included as predictors) as well as to the model in the previous step. Models were considered significant if the decrease in AIC (relative to the previous model) was greater than 2 units. To interpret models with significant changes in AIC, p values for the main effects and interaction terms were calculated using 25 degrees of freedom were used (29 subjects - 3 predictors - 1).

To analyze the data from the Sequential Partner Preference Test we used a multiple analysis of variance (MANOVA) in R to determine differences between treatment and sex in the dependent variables (contact, proximity and latency to contact) for the three conditions (empty, stranger and partner) at each time point. We did multiple comparisons using the Bonferroni test for all significant interactions.

For the simultaneous Partner Preference Test, we calculated proportions for the time subjects spent in the partner and stranger preference zones and touching the corresponding grated windows using the total duration of the test (i.e., 150 min). For each treatment group (OXT, SAL), partner preference was determined by the subject spending significantly more time in the partner preference zone than in the stranger preference zone. We used a GLMM with a binomial distribution in R using the function glmer from the lm4 package. Preference (partner, stranger) and sex (females, males) were independent variables (fixed), and subject was a random factor. All p-values below 0.05 were considered significant. We provided marginal R^2 (R^2m) as a measure of variance explained by fixed effects of the model. Effect size for the significant pairwise comparisons was calculated using standardized beta coefficient (β).

To analyze behavioral data from the Mirror Study, we used the *nlme*: Linear and Nonlinear Mixed Effects Models package (Pinheiro, Bates, DebRoy, Sarkar and R core team 2020) in R. IN treatment (OXT, SAL), test condition and sex (females, males) were independent variables (fixed), and subject was a random factor. We used a square root or logarithmic transformations for variables that were not normally distributed. All p-values below 0.05 were considered significant. We provided R^2m as a measure of variance explained by fixed effects of the model. We performed multiple comparisons using the Bonferroni test for all significant interactions. Effect size for the significant pairwise comparisons was calculated using standardized beta coefficients.

For the imaging study, we multiplied the activity data by the cylinder calibration factor calculated for each subject. Then we averaged the activity data of the left and right hemisphere for the whole brain and all the ROIs. Finally, to correct for FDG uptake by injected dose we divided the activity data by the injected dose and multiplied by 100%. This normalized the FDG activity data for the amount injected into the subject. Data are presented in proportion of injected dose per gram of tissue. We tested the data for normality and equal variances using Shapiro Wilk test and Bartlett test of homogeneity of variances. The data did not satisfy normality and equal variances assumptions, so we used a logarithmic transformation to normalize data distribution.

For whole brain, we used a two-way ANOVA in R project to assess differences in glucose uptake by IN treatment (OXT, SAL), sex, or an interaction of treatment and sex at 23 months (pre-pairing) and at 33 months old (3 months post-pairing). We also calculated the difference in glucose uptake between the 33 months old (3 months post-pairing) PET scan and the 23 months old (pre-pairing) PET scan and performed a two-way ANOVA to assess if glucose uptake in whole brain increased or decreased from pre-pairing to post-pairing status based on treatment, sex and treatment and sex interaction. Effect size was calculated using ETA squared (η^2).

To analyze glucose uptake in ROIs we performed a Principal Component Analysis (PCA) with an orthogonal rotation for each time point (23 and 33 months) and for the difference between time points. Finally, we analyzed each principal social component with ANOVA in R project and assess if glucose was influenced by treatment, sex, or an interaction of treatment and sex. Effect size was calculated using ETA squared. We corrected for multiple comparisons using the Bonferroni test for all significant interactions. Effect size for the significant pairwise comparisons was calculated using standardized beta coefficients.

Results

Behavioral Testing

Pair affiliation—For all affiliation states, the model with affiliation predicted by treatment group (Model 1) significantly decreased model AIC relative to the null model (see Table S1). The analyses showed that, compared to subjects that received saline, subjects that received intranasal OXT exhibited more tail twining (β=0.30, p=0.043), and less home cage proximity (β=−0.42, p=0.002) and contact (β =−0.39, p=0.002; Figure 2 and Table 4) with their pair mate across the first four months of pairing. Please note that tail twining is scored mutually exclusively with contact and proximity. These results thus indicate the most time spent in the “highest” type of affiliation in that the category of tail twining also includes both contact and proximity.

For proximity and contact (but not for tail twining), the addition of month and the interaction between month and treatment group (Model 2) further improved model fit (see Table S1). Further exploration of these models showed that, across the first four months of pairing, there were increases in proximity (β=0.22, p=0.002) and contact (β=0.20, p=0.006; see Table 4). The interaction between month and treatment group was not significant in any model (p>0.09; see Table 4). The full factorial model that included sex (Model 3) did not significantly improve model fit for any affiliation variable (see Table S1).

Sequential Partner Preference Test

Summary.: At one week post-pairing, OXT-treated animals touched the stimulus cage during the empty condition more than SAL-treated animals. At four months post-pairing, OXT-treated males touched the partner and empty condition more than SAL-treated males.

Contact (frequency).: At one week post-pairing, OXT-treated animals touched the stimulus cage during the empty condition more than SAL-treated animals ($F_{1,83}$=8.55, p=0.004) (Figure 3). We found no significant differences for the stranger or partner conditions.

At four months post-pairing, we found a main effect of treatment ($F_{1,71}$=4.68, p=0.03) and a treatment by sex interaction ($F_{1,71}$=5.36, p=0.02) for the partner condition (Figure 4). OXT-treated animals touched the partner condition more than SAL-treated animals: this result was driven by OXT-treated males touching the partner condition significantly more than SAL-treated males. We also found a main effect for treatment for the empty cage condition ($F_{1,71}$=5.56, p=0.02): OXT-treated animals (6.12±0.79) touched the empty condition more than the SAL-treated animals (4.09±0.86) ($F_{1,71}$=5.56, p=0.02).

We found no significant treatment differences at one week post-pairing or four months post-pairing for proximity and latency to contact.

Simultaneous Partner Preference Test

Summary.: At one week post-pairing, IN OXT treated males and IN SAL females and males showed a significant preference for their partner versus the stranger, while IN OXT females did not show significant preference for their partner or the stranger.. At one week post-pairing neither IN OXT or SAL treated animals showed significant differences in the time spent touching the grated window of the partner or stranger.

At four months post-pairing, IN OXT and IN SAL subjects all showed a significant preference for their partner versus the stranger. At four months post-pairing, both IN OXT and SAL females and males touched their partner's grated window more than the stranger's grated window. However, the effect size for grate window touching for IN OXT subjects was greater than for IN SAL subjects, particularly for IN OXT males.

Preference by time spent in preference zones.—We considered subjects to have established a preference for their partner or the stranger when we found a significant difference between the time spent in these preference zones (Table 5). At one week post-pairing, we found a significant sex by preference interaction for OXT treated animals (z=−25.90, p<0.0001; proportion of the variance explained by the model was R^2m=0.40.) IN OXT treated males showed a significant preference for their partner versus the stranger (β=0.56, p<.0001), however, IN OXT females did not show a significant preference for their partner versus the stranger (z=10.31, p=0.318) (Figure 5A). At one week post-pairing, we found a significant sex by preference interaction for SAL treated animals (z=21.66, p<0.0001; proportion of the variance explained by the model was R^2m=0.56). IN SAL treated males and females showed a significant preference for their partner versus the stranger (males: β=0.318, p<0.0001; , females: β=0.683, p<0.0001) (Figure 5C)

At four months post-pairing, we found a significant sex by preference interaction for OXT treated animals (z=−56.73, p<0.0001; the proportion of the variance explained by the model was R^2m=0.88). IN OXT males and females showed a significant preference for their partner compared to the stranger (males: β=1.67, p<0.0001;females: β=.69, p<0.0001) (Figure 5B). At four months post-pairing, we found a significant sex by preference interaction for SAL treated animals (z=3.39, p<0.0001;the proportion of the variance explained by the model was R^2m=0.83). IN SAL males and females also showed a significant preference for their partner versus the stranger (males: β=0.51, p=<0.0001;females: β=0.93, p<0.0001) (Figure 5D).

Preference by time spent touching grated windows.—At one week post-pairing, neither IN OXT or IN SAL treated subjects showed a preference for touching the partner or the stranger's grated window (Table 6; Figure 6 A & C). However, we found a main effect for sex for OXT treated animals ($z=3.15$, $p=0.0004$; the proportion of the variance explained by the model was $R^2m=0.63$) and for SAL treated animals ($z=34.99$, $p<0.0001$; the proportion of the variance explained by the model was $R^2m=.23$). Males touched the grated window more than females in both treatment groups (IN OXT: $\beta=0.39$, $p<0.0001$; IN SAL: $\beta=0.30$, $p<0.0001$).

At four months post-pairing, we found a significant sex by preference interaction for OXT treated animals ($z=-6.72$, $p<0.0001$) and for SAL treated animals ($z=-8.31$, $p<0.0001$; proportion of the variance explained for the OXT model was $R^2m=0.52$ and $R^2m=0.29$ for the SAL model). Both IN OXT and SAL males and females touched their partner's grated window more than the stranger's grated window (IN OXT males: $\beta=2.03$, $p<0.0001$; IN OXT females: $\beta=1.794$, $p=<0.0001$; IN SAL males: $\beta=0.46$, $p<0.0001$; IN SAL females:$\beta=0.18$, $p<.0001$) (Figure 6 B & D). However, the effect size for grate window touching for IN OXT subjects (males: $\beta=2.03$ and females: $\beta=1.794$) was greater than for IN SAL (males: $\beta=0.46$ and females: $\beta=0.18$), particularly IN OXT males. IN OXT males touched the partner's grated window significantly more than IN SAL males ($\beta=3.97$, $p<.0001$) and OXT females ($\beta=0.50$, $p<0.0001$).

Mirror Test

Summary.: At one week post-pairing, intranasal treatment did not influence behavior during the test. At four months post-pairing, OXT-treated males approached the mirror and locomoted more when exposed to the mirror condition than when exposed to the back of the mirror. OXT-treated males also lip-smacked significantly more than SAL-treated animals and OXT-treated females when exposed to the mirror condition.

Both at one week and four months post-pairing, subjects, particularly males, displayed more affiliative and arousal behaviors in response to the reflective side of the mirror versus the control (see Supplementary Table S2).

Interactions with the mirror

Mirror Approach Latency.: Treatment did not affect mirror approach latency at one week or 4 months post-pairing.

Mirror approaches.: Treatment did not affect latency to approach the mirror at one week post-pairing. At four months, the interaction between treatment, sex and condition was significant ($F_{1,25}=4.56$, $p=0.04$; proportion of the variance explained for the model was $R^2m=0.29$). OXT males approached the mirror more times when exposed to the reflective side than when exposed to the control condition ($\beta=-1.21$, $p=0.008$) (Figure 7A).

Mirror proximity.: We found no significant main effects or interactions in the time subjects spent in proximity to the mirror at one week or four months post-pairing.

Interactions with the Partner

Proximity to partner.: Treatment did not affect time spent in proximity to partner at one week or four months post-pairing.

Partner contact.: At one week post-pairing, treatment did not affect time spent in contact with partner. At four months post-pairing, we found a significant treatment by sex interaction ($F_{1,25}$=5.40, p=0.03; proportion of the variance explained for the model was R^2m=0.41). OXT males spent more time in contact with their partner compared to OXT females (β =1.48, p=0.0006) and SAL females (β=−1.093, p= 0.01) , and showed a tendency to differ from SAL males (β=2.96, p=0.08; Figure 7B).

Partner withdraw.: At one week post-pairing, treatment did not have an effect on partner withdraw. At four months post-pairing, we found a non-significant trend for treatment ($F_{1,25}$=3.86, p=0.06), with OXT subjects (2.4±0.36) tending to end contact and/or proximity fewer times than SAL subjects (3.26±0.50).

Affiliative and agonistic behaviors

Lip-smack.: At one week post-pairing, treatment did not have an effect on lip-smacking. At four months, we found a significant interaction for sex by treatment by condition ($F_{1,25}$=5.75, p=0.02; proportion of the variance explained for the model was R^2m=0.65). OXT treated males lip-smacked more than SAL males (β=6.286, p=0.007), SAL females (β=−6.43, p=.005) and OXT females (β=−7.607, p=0.0006) when exposed to the mirror condition. All groups lip-smacked significantly more during the mirror condition compared to the control condition (p≤0.05), except OXT females (β=−4.25, p=0.09) (Figure 7C).

Back-arch and Tail-lash.: We found no significant effects or interactions by treatment at one week or four months post-pairing.

Movement.: Treatment did not have an effect on the time subjects spent moving during the test at one week or four months. We found significant treatment by sex by condition interaction for movement frequency at four months post-pairing ($F_{1,25}$=5.39, p=0.03; proportion of the variance explained for the model was R^2m=0.15). OXT-treated males moved more when exposed to the mirror condition compared to the control condition (β=−12.57, p=0.04) (Figure 7D).

Imaging Study

An exploratory analysis using a Pearson Correlation showed a high degree of correlation between the ROIs (r > 0.95) for all data sets. The results for the PCA showed the first factor explained variance at a level of 0.97 for 23 months (pre-pairing), 0.99 for 33 months (3 months post-pairing) and .97 for the difference between 33 and 23 months, allowing us to group them in one principal social component (social salience network) (see Supplementary Table 3 for Eigenvalues, proportions, and cumulative proportions obtained by principal component analysis).

Glucose uptake in the whole brain did not vary by treatment, however, we found a borderline significant main effect for sex at 23 months old (pre-pairing) ($F_{1,25}$=4.26, p=0.049; η^2=0.14) with females having greater glucose uptake than males (β=5.92, p=0.048) (Figure 8. A). This main effect for sex was stronger at 33 months old (3 months post-pairing) ($F_{1,25}$=6.12, p=0.02; η^2=1.96); females had greater glucose update than males (β=6.40, p=0.02) (Figure 8. B). The differences in glucose uptake in whole brain between the 33 months old (3 months post-pairing) PET scan and the 23 months old (pre-pairing) PET scan was not significant for treatment or sex.

When examining the glucose uptake in the social salience network, we found no differences by treatment at 23 or 33 months of age. However, we found a non-significant trend for sex at 23 months old (pre-pairing) ($F_{1,25}$=3.245, p=0.07, η^2=0.11), with females tending to have greater glucose uptake than males (t=−1.861, p=0.07, β=−0.681) (Figure 9. A). At 33 months old (3 months post-pairing) we found a significant main effect for sex ($F_{1,25,}$=5.78, p=0.023, η^2=0.18). Females had significantly greater glucose uptake in the social salience network than males (β=− 0.855 p=0.02) (Figure 9. B).

Finally, we calculated the differences in glucose uptake between the 33 months old (3 months post-pairing) PET scan and the 23 months old (pre-pairing) PET scan, to assess if glucose uptake in whole brain and ROIs in the social salience network increased or decreased from pre-pairing to post-pairing status. We found no significant effects or interaction by treatment and/or sex either in whole brain and in the social salience network.

Discussion

To our knowledge, this study, along with our previous study (Arias-del Razo et al., 2020), were the first to investigate the potential long-term effects of chronic IN OXT administration during development in a non-human primate species. Here, we conducted a follow-up study with a cohort of adult titi monkeys that received chronic IN OXT or SAL during their juvenile period and looked for long-term effects in pair-bonding behavior, changes in glucose brain uptake in the whole brain, across the social salience network, and changes between pre-pairing and post-pairing status.

Our previous study showed that during chronic IN OXT treatment, OXT-treated subjects had increased brain activity across the social salience network after one month of daily treatment compared with SAL-treated subjects. OXT-treated animals also displayed increased social behaviors, such as more time grooming family members. During preference tests, OXT-treated females displayed an enhanced preference for their parents, while OXT-treated males displayed an increased interest in unfamiliar pairs, without reducing time spent with their parents. These effects were moderate, and we could not attribute them to a decrease in anxiety, major changes in the timing of the pubertal transition or levels of steroid hormones (Arias-del Razo et al., 2020).

In the current study, we sought to determine if developmental exposure to IN OXT could have long-term effects in the formation and maintenance of an adult pair bond. The exact timeline for pair bond formation in titi monkeys is not known (Bales et al., 2017; Rothwell

et al., 2020), although partner preference has been observed in titi monkey partners that had been paired for approximately six months (Rothwell et al., 2020). A previous study on the neurobiology of pair bonding in titi monkeys showed that after being paired for one week, males showed a global increase in cerebral glucose metabolism, driven by areas involved in motivation, emotion, and social memory. This effect was maintained and enhanced at 4 months post-pairing (Maninger et al., 2017). Based on these findings, we assessed pair affiliation in the home cage 5 times a week across the first four months of pairing and chose one week and four months post-pairing as our key time points to assess behavioral components of the pair bond.

Pair affiliation across the first four months post-pairing

In the homecage, OXT-treated subjects exhibited more tail twining, but less proximity and contact with their pairmate across the first four months of pairing. There were no differences in total affiliation. Together, these findings suggest that IN OXT modulated the way that subjects affiliated with their pair mate, rather than the total amount of time spent affiliating. In other words, relative to SAL-treated subjects, OXT-treated subjects spent more time tail twining with their pair mate at the expense of contact and proximity with their pair mate. Tail twining is a species-typical behavior that is considered a more “intimate” form of affiliation than proximity or contact; for example, a previous study from our lab that used the same daily scan-sample paradigm showed that well-established pairs exhibited more tail twining than newly-formed pairs (Rothwell et al., 2020). In the context of this study, our findings suggest that IN OXT facilitated pair bonding by increasing the proportion of time that monkeys spent in more intimate forms of affiliation. This effect was constant across all four months investigated, suggesting that this effect of IN OXT operated from the moment that monkeys were paired together.

Behavioral changes following one week of pairing

During the Sequential Partner Preference Test at one week post-pairing, neither SAL nor OXT-treated animals had established a preference for their partner. OXT-treated animals interacted more with empty stimulus cage compared to SAL-treated animals. We found no differences in the subjects’ behavior towards the partner and stranger. Neither SAL nor OXT-treated animals displayed defensive or territorial behaviors towards the stranger.

During the Simultaneous Partner Preference Tests at one week post-pairing, IN OXT treated males showed significant preference for their partner versus the stranger, while IN OXT females did not show significant preference for either their partner or a stranger. Both IN SAL females and males, showed a significant preference for their partners over the stranger. During the Mirror Study at one week post-pairing, we found no differences in behavior between treatments. As expected, subjects behaved differently when exposed to the reflective side versus the control, including more affiliative and arousal behaviors in response to the reflective side. Only three males (two OXT and one SAL-treated) displayed restraining behavior towards their partner.

Behavioral changes following four months of pairing

At four months post-pairing, both OXT and SAL-treated animals showed a preference for their partner versus the opposite-sex stranger by spending more time in the partner preference zone during the Simultaneous Partner Preference Test, suggesting that the pair bond had been established in both groups. As a secondary measure, we considered the time subjects spent touching the grated window that separates the center test cage from the stimulus cages. Both OXT and SAL-treated animals showed a preference for touching their partner's grated window; however OXT-treated animals touched their partner's grated window more compared to SAL males and OXT females. Rothwell and colleagues (2020) suggested that the time touching the grated window of the partner could potentially indicate a greater motivation for physical contact with the partner in newly formed titi monkey pairs (~6 months paired).

During the Sequential Partner Preference Test at four months post-pairing, treatment influenced only contact, with OXT-treated males touching the partner and the empty condition more times than SAL-treated males. Four tests (two SAL-treated females, one SAL-treated male, and one OXT-treated male), were canceled for safety reasons due to high inter-sexual aggression displays between subjects and strangers. This observation was consistent with the findings reported by Rothwell and colleagues (2020), who found that aggressive displays during partner preference tests were more frequent in newly-formed bonds (six months) compared to well-established bonds (over one year), especially for females.

During the Mirror Study at four months post-pairing, subjects' behavior towards the reflective side versus the control side was significantly different, with subjects approaching the mirror quicker and displaying more affiliative, agonistic and arousal behaviors to their reflection. IN OXT treatment influenced male behavior, with OXT-treated males making a stronger distinction between the reflective and back side (control) of the mirror. OXT-treated males approached the mirror, moved, and lip-smacked more frequently when exposed to the reflective condition than when exposed to the back of the mirror. OXT-treated males also lip-smacked more than SAL-treated males and SAL-females during the mirror condition. Four SAL-treated males displayed restraint behavior when exposed to mirror condition while no OXT-males displayed this behavior.

The enhanced interest and tolerance towards both same-sex and opposite-sex strangers displayed by OXT-treated animals is not species typical of male titi monkeys, although it is consistent with our findings for these same OXT-treated males as juveniles (Arias-del Razo et al., 2020). Mate-guarding and territorial aggression (intra- and inter-sexual) are behavioral components of social monogamy in titi monkeys both in the field and in the laboratory, and they are important for the maintenance of monogamous bonds by potentially excluding extra-pair adults (Mendoza & Mason, 1986; Fisher-Phelps et al. 2016; Dolotovskaya et al. 2020). Previous studies have shown that when titi monkey pairs are exposed to the reflective side of the mirror, males display agonistic and arousal behaviors towards the mirror stimulus, such as back-arch, tail-lash and time in proximity to the mirror. Males also increased interactions with their pair mate, such as restraints, lip-smacking and partner approaches, when exposed to the reflective side of the mirror (Fisher-Phelps et al., 2016).

Witczak et al. (2018) showed that more aggressive males spent less time in contact with their mates and lip-smacked less when exposed to the reflective side of the mirror. OXT-treated males' interest and tolerance towards other unfamiliar animals could potentially compromise the maintenance of the pair-bond under circumstances where animals would have access to interact with strangers regularly. OXT-treated males' behavior towards strangers is consistent with results from other studies that found reduced aggression and increased social exploration towards unfamiliar animals in male rats after repeated administration of IN OXT (Calcagnoli et al., 2015).

Neural findings

Contrary to our hypothesis, behavioral effects observed in OXT-treated males were not accompanied by an increase in glucose uptake in whole brain or across the social salience network. It is possible that long term effects observed in OXT-treated males were too subtle or were more specific to interactions with unfamiliar animals. These functional changes would not have been detected by the PET scan, since subjects spent the FDG uptake period housed with their attachment figure in what was meant to be a non-stressed baseline scan.

We found sex differences in cerebral glucose uptake, with females having greater glucose uptake in the whole brain at 23 months old (pre-pairing) and in the whole brain and social salience network at 33 months old (post-pairing) than males. This sex difference was recently described in humans, with women displaying higher glucose uptake across the lifespan than men (Goyal et al., 2019). As glucose uptake declines with age (Martin et al., 1991), higher glucose uptake can be viewed as a metabolically "younger" brain, although the functional significance of this sex difference is not yet known.

It remains to be determined whether the sex differences in glucose uptake are associated with other components of parent and pair-mate attachment in titi monkeys. Studies in humans have shown that maternal and romantic love both involve areas that are specific to each, as well as an overlapping set of areas (Bartels and Zeki, 2000, 2004). Among these overlapping areas are the putamen, caudate nucleus, and the anterior cingulate cortex, which were included in our study.

Our results showed that chronic IN OXT administration during the juvenile period can induce long-term alterations in adult behavior at least one year after treatment. The observed long-term behavioral effects were mild and were more pronounced in OXT-treated males and at four months post-pairing, once animals had an established a pair bond. At 4 months post-pairing, OXT-treated males were more responsive to a social stimulus versus a non-social stimulus, and displayed more interest for their partner compared to SAL-treated males. This suggests that the effects of IN OXT were specific towards the figure of attachment and that IN OXT treatment might have enhanced the sensitivity of socially-relevant cues, modulating approach and avoidance motivational tendencies. The direction of these long-term effects in social behavior is consistent with the behavioral changes observed during chronic IN OXT treatment in the juvenile period (Arias-del Razo et al., 2020), however, the long-term effects appeared to be weaker than the ones observed during treatment.

Consistent with our results, Bernaerts and colleagues (2020) reported long-term effects of IN OXT at one month and one year post-treatment in adult males with ASD; after four weeks of IN OXT treatment (24 IU once a day), subjects in the OXT group self-reported reductions in repetitive behaviors and reduced avoidance towards others compared to the placebo group. The authors suggest that the observed effects that outlasted the period of actual IN OXT administration provide support to the notion that repeated administrations over an extended period of time might induce long-term adaptations in social brain circuits in an experience-dependent manner. Thus, the period of IN OXT administration may contribute to the observed long-term adaptations in the individual's motivational tendencies (i.e., increased feelings of social approachability). In a similar direction, the allostatic theory of oxytocin proposed by Quintana and Guastella (2020), suggests that manipulation of the oxytocin system via intranasal administration could modulate the core features of learning, interoception, and prediction to improve social outcomes.

The direction of the longer-term effects of IN OXT appears promising for the use of IN OXT as long-term treatment for psychiatric disorders involving social and behavioral impairments during adolescence; however, these results should be interpreted with caution, as effects of IN OXT administration have been shown to depend on context and individual differences, as well as to affect males and females differently (Carter et al., 2020). Our results encourage investigating the potential long-term effects of chronic IN OXT in humans during childhood and adolescence and to gain a deeper understanding of the neural mechanisms underlying the behavioral effects.

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

Acknowledgements

We gratefully acknowledge the following for contributions to this project: Madison Dufek, Elisa Arnaud, Gregoire Boulinguez-Ambroise, Floriane Poulain, Nicolas Almeida E Silva, Rebecca Cotterman, Charles Smith, Doug Rowland, Rich Larson, Veterinary Staff, Jaleh Janatpour and Kevin Theis, and Bales lab volunteers. This research was supported by NICHD grant HD071998, NIH grant OD011107 to the CNPRC, UC Mexus, and the Good Nature Institute.

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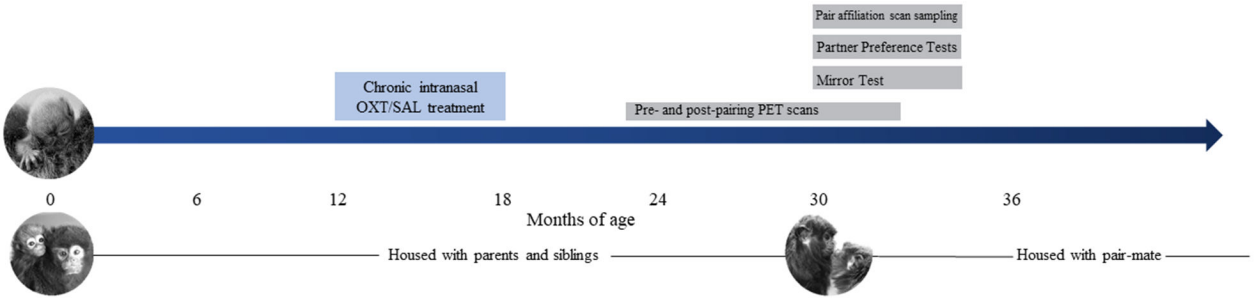

Figure 1. Study Timeline.

Subjects were housed with parents (and siblings if present) from birth until 30 months old (approximately). Chronic intranasal (OXT or SAL) treatment was administered daily from 12 to 18 months of age. Pre-pairing PET scan was at 23 months of age, followed by an MRI scheduled one to three weeks after scan. Post-pairing PET scan was at approximately 33 months of age (3 months post-pairing). A set of post-pairing behavioral tests (Sequential Partner Preference Test, Simultaneous Partner Preference Test and Mirror Test) were scheduled one week and 4 months after pairing. Pair affiliation in the home cage was assessed using a scan sampling method for the first four months post-pairing.

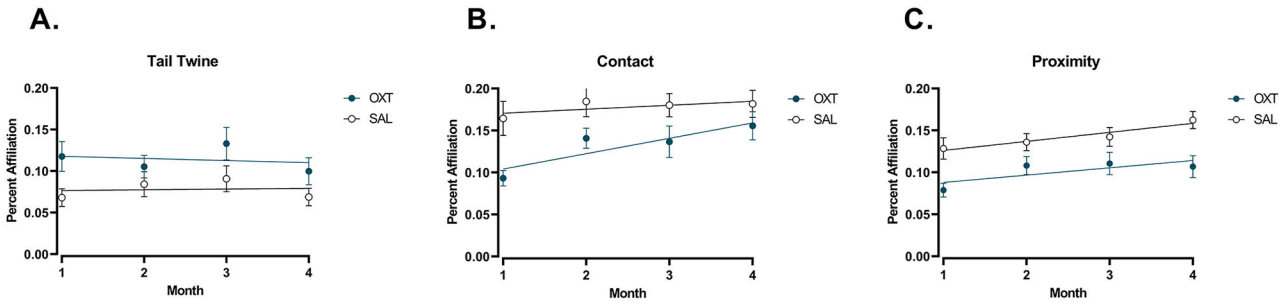

Figure 2. Pair affiliation.

Change in home cage pair mate affiliation across the first four months of pairing by treatment group. Subjects that received intranasal OT exhibited more Tail Twining (β=0.30, p=0.043), but less home cage Proximity (β=−0.42, = 0.002) and Contact (β=−0.39, p=0.002) with their pair mate across the first four months of pairing.

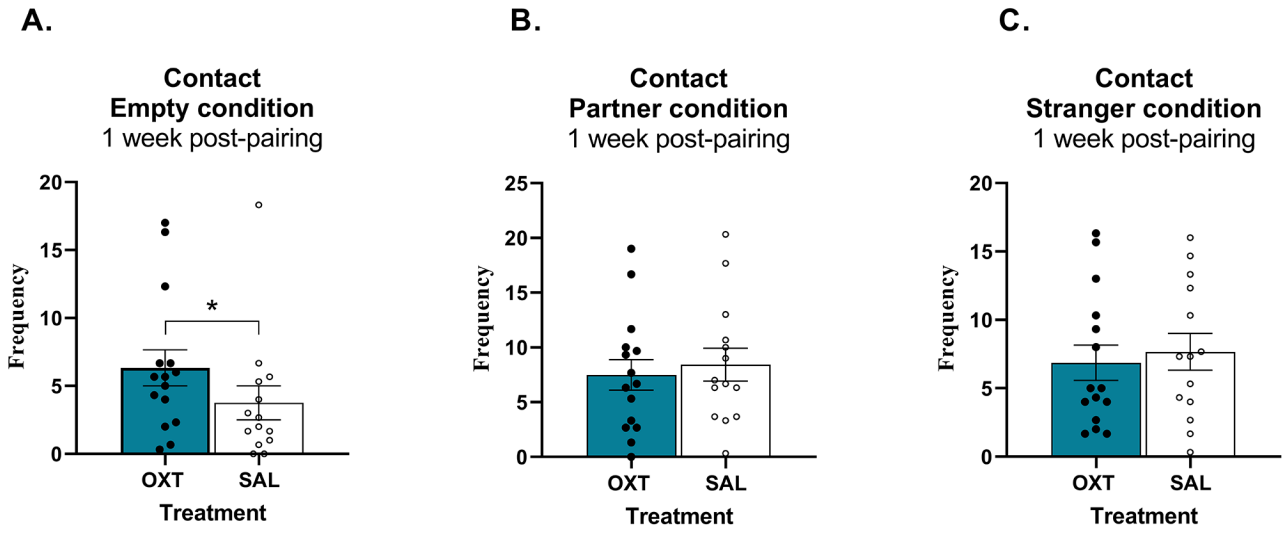

Figure 3. Sequential Partner Preference Test at one week post-pairing.

A. OXT-treated animals touched the empty condition more than SAL-treated animals ($F_{1,83}$=8.55, p=0.004). B. and C. No significant differences between treatment groups were found for the partner or stranger conditions. Height of the bars indicate means; error bars indicate standard errors.

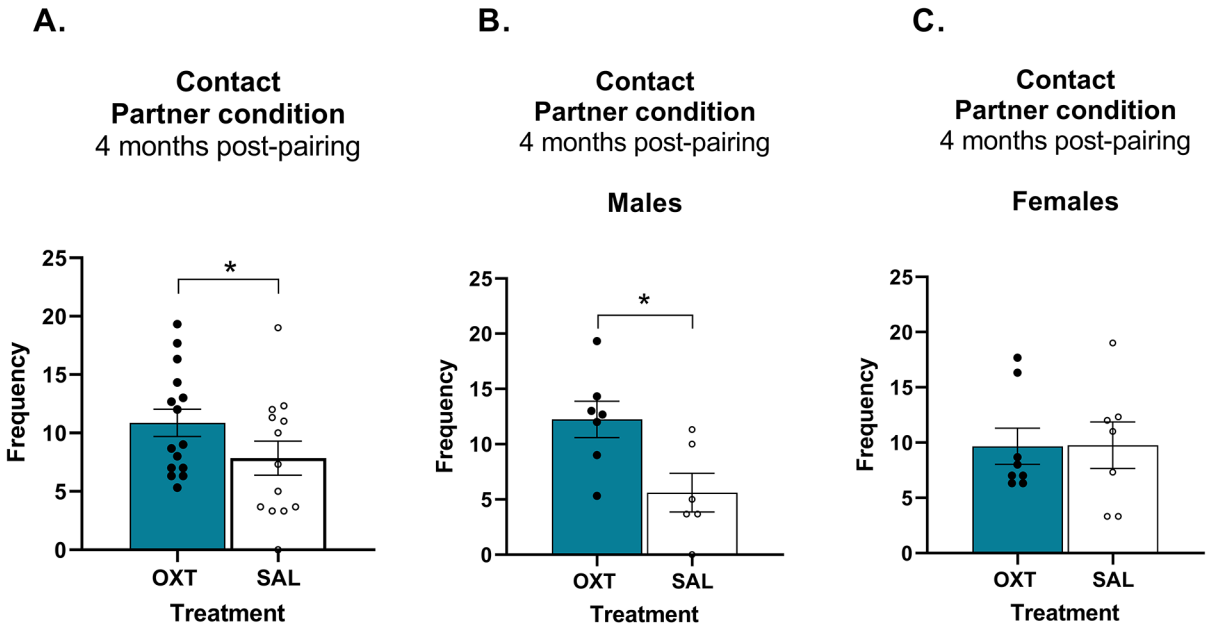

Figure 4. Sequential Partner Preference Test at 4 months post-pairing.

A. OXT-treated animals touched the stimulus cage during partner condition more times than SAL-treated animals ($F_{1,71}$=4.68, p=0.03). B. OXT-treated males touched the stimulus cage during the partner condition more times than SAL-treated males ($F_{1,71}$=5.36, p=0.02). C. No significant differences were found in the number of times OXT-treated females and SAL-treated females touched the stimulus cage during the partner condition. Height of the bars indicate means; error bars indicate standard errors.

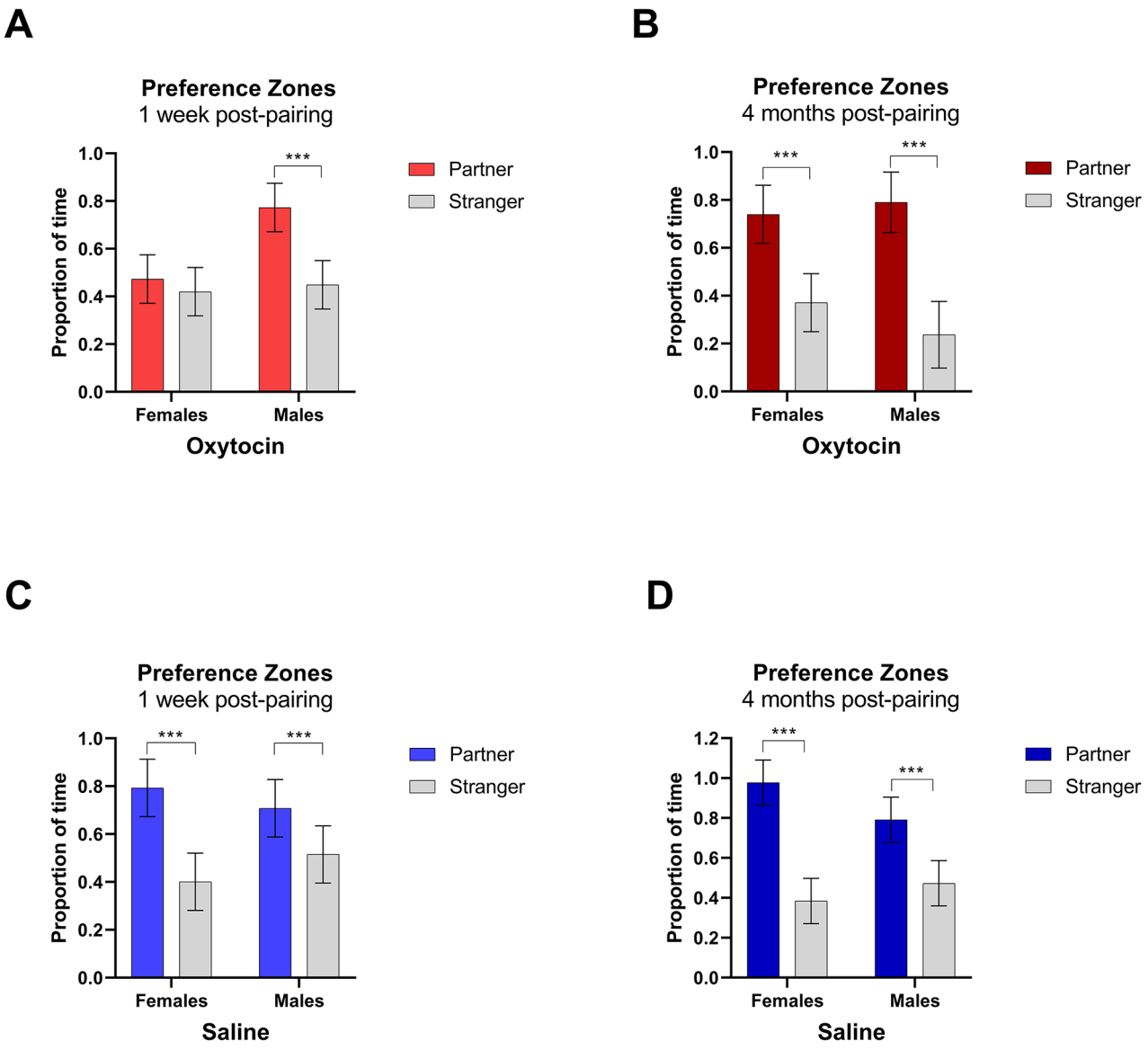

Figure 5.

A. At one week post-pairing, IN OXT treated males showed significant preference for their partner ($z=46.535$, $p<0.0001$) but not IN OXT females ($z=10.31$, $p=0.318$). B. At four months post-pairing, IN OXT males and females showed a significant preference for their partner compared to the stranger ($z=127.714$, $p<0.0001$; $z=60.654$, $p<0.0001$). C. At one week IN SAL females and males, showed a significant preference for their partners over the stranger ($z=27.081$, $p=0.0001$; $z=56.686$, $p<0.0001$). D. IN SAL males and females also showed a significant preference for their partner versus the stranger ($z=43.578$, $p=<0.0001$; $z=76.546$, $p<0.0001$).

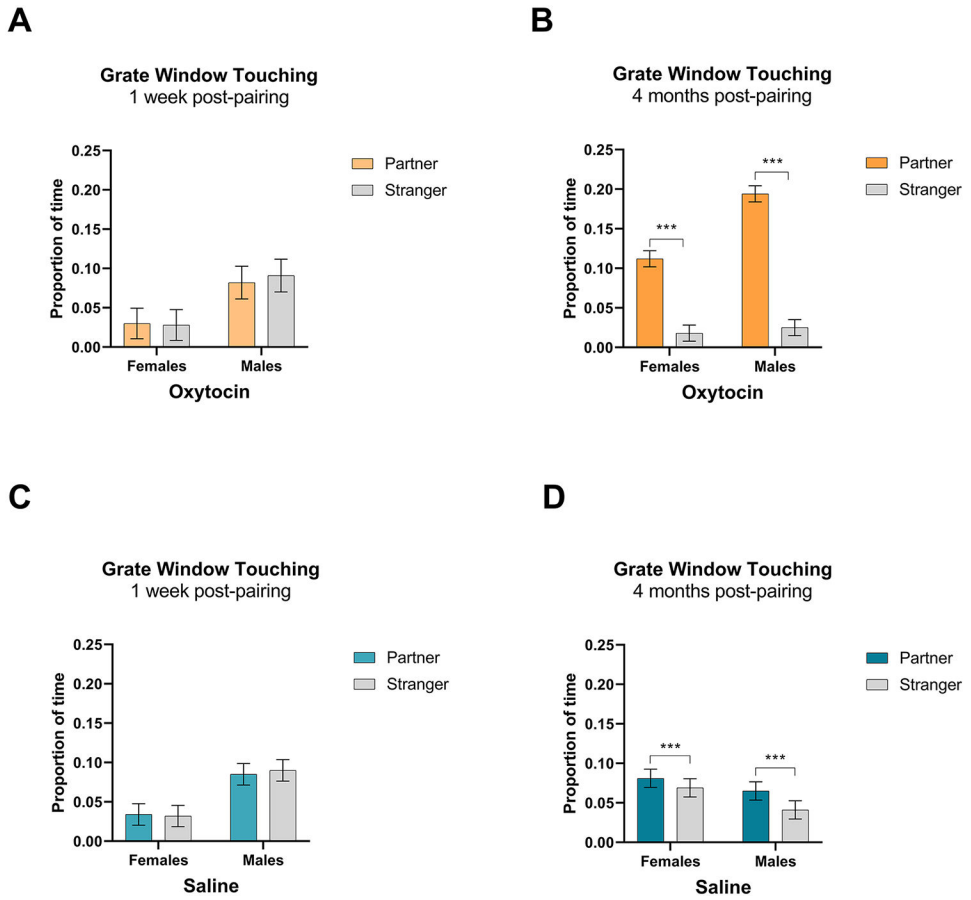

Figure 6.

A & C. At one week post-pairing neither IN OXT or SAL treated animals showed significant differences in the time spent touching the grated window of the partner or stranger. B & D. At four months post-pairing, both IN OXT and SAL females and males touched their partner's grated window more than the stranger's grated window (IN OXT females: z=69.71, p=<0.0001, males: z=83, p<0.0001; IN SAL females: z=7.964, p<.0001, males: z=17.804, p<0.0001).

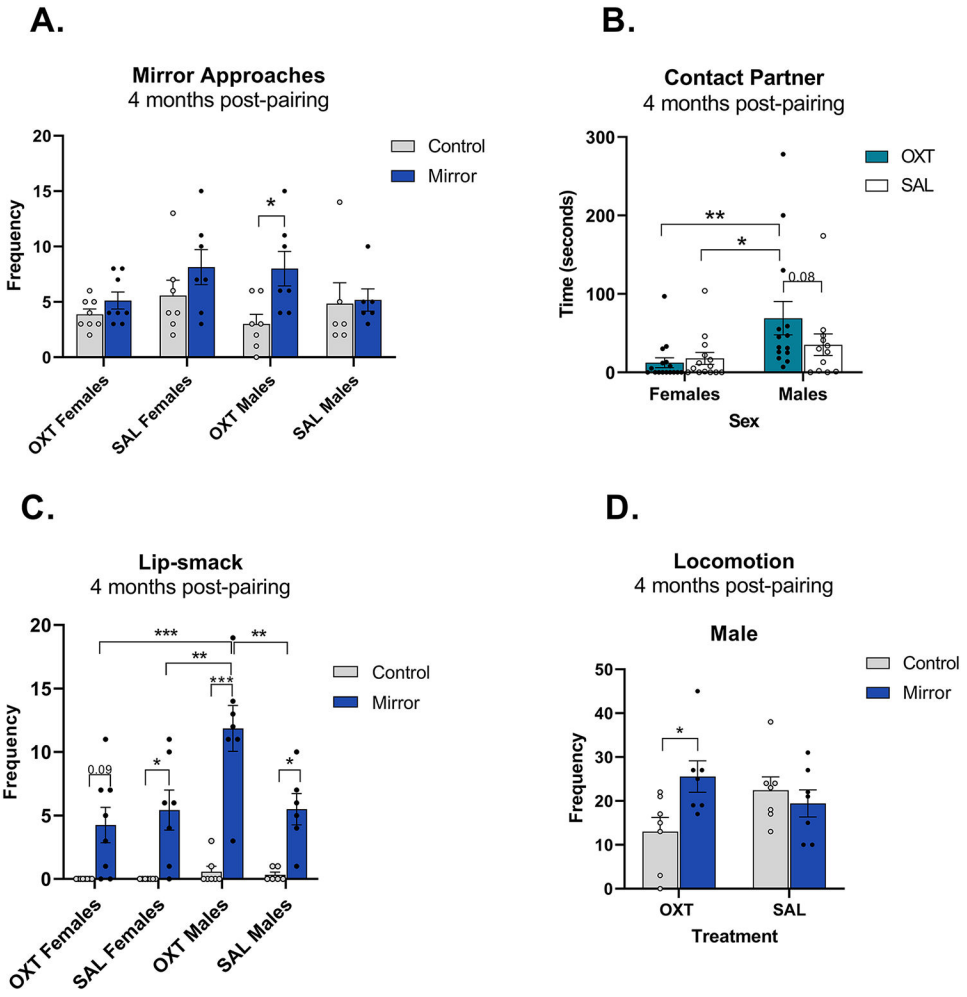

Figure 7. Mirror Study.

A. At four months post-pairing, OXT males approached the mirror more times when exposed to the reflective side compared to the control condition (t_{25}=4.088. p=0.008). Height of the bars indicate means; error bars indicate standard errors. B. At four months post-pairing, OXT males spent more time in contact with their partner compared to OXT females (t_{25}=4.60, p=0.0006) and SAL females (t_{25}=3.7, p= 0.0052), and showed a tendency to differ from SAL males (t_{25}=2.50, p=0.08). Height of the bars indicate means; error bars indicate standard errors. C. At four months post-pairing, OXT males lip-smacked more than SAL males (t_{25}=4.13, p=0.007), SAL females (t_{25}=4.22, p=.005) and OXT females (t_{25}=5.165, p=0.0006) when exposed to the mirror condition. All groups lip-smacked significantly more during the mirror condition compared to the control condition ($p \leq 0.05$), except OXT females (t_{25}=2.98, p=0.09). Height of the bars indicate means; error bars indicate standard errors. D. At four months, OXT males moved more times when exposed to the mirror condition compared to when they were exposed to the control condition (t_{25}=3.072, p=0.04). Height of the bars indicate means; error bars indicate standard errors.

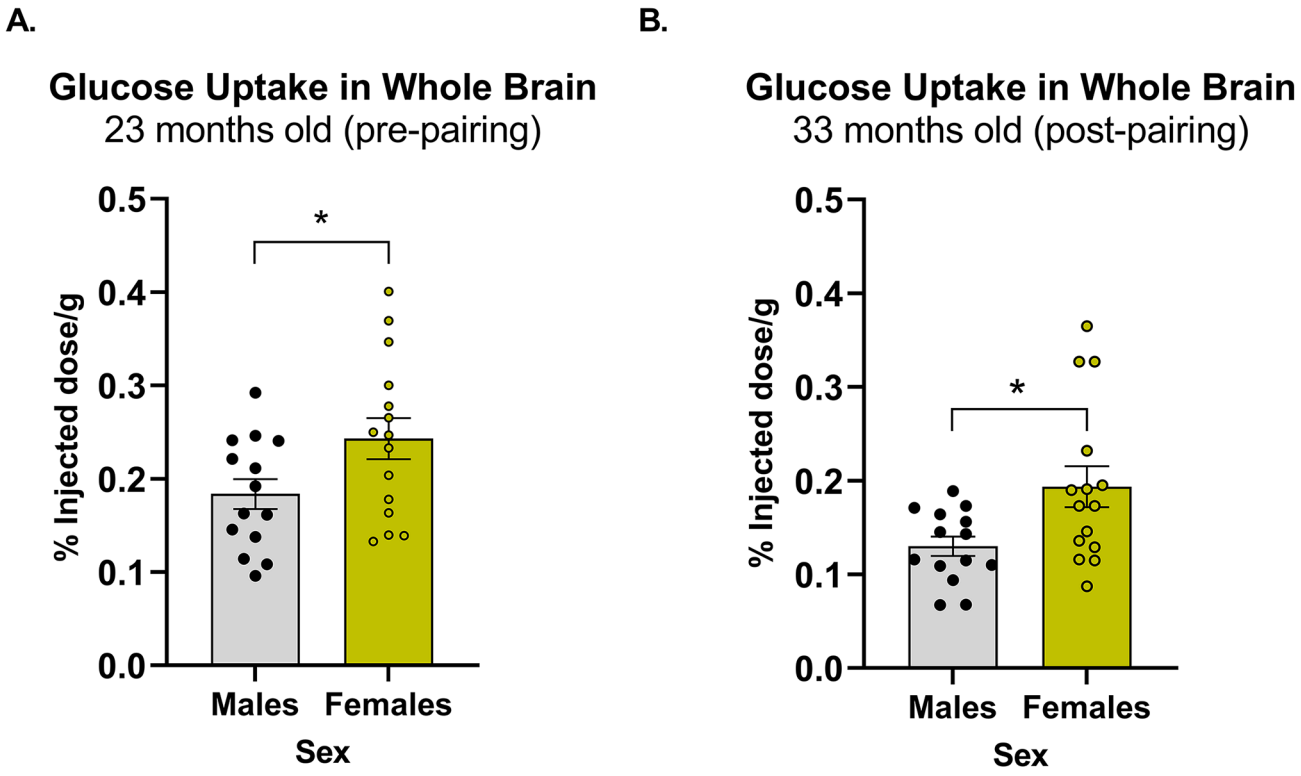

Figure 8. Imaging Study.

A. At 23 months old (pre-pairing), females had greater glucose uptake than males ($F_{1,25}$=4.25, p=0.0496). B. At 33 months old (3 months post-pairing), females also greater glucose uptake than males ($F_{1,25}$=6.11, p=.02). Data are presented in percent injected dose per gram of tissue (%ID/g). Height of the bars indicate means; error bars indicate standard errors.

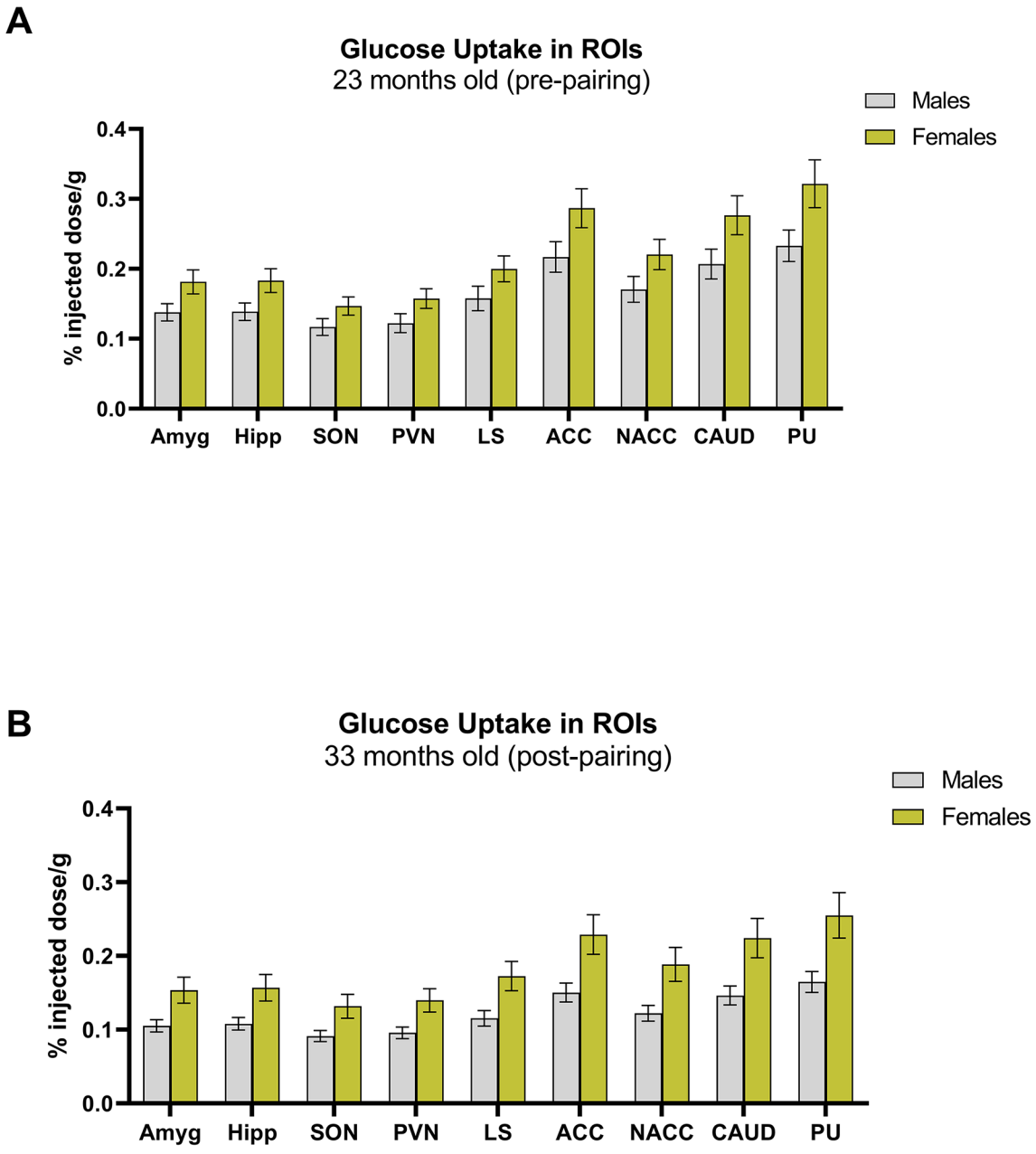

Figure 9. Imaging Study.

A. Females at 23 months old (pre-pairing) had a non-significant trend for greater glucose uptake compared to males ($F_{1,25}$=3.245, p=0.07) in the social salience network. B. Females at 33 months old (3 months post-pairing) had a greater glucose uptake compared to males in the social salience network ($F_{1,25}$=5.723, p=0.024).

Table 1.

Sequential Partner Preference.

Behavioral Ethogram.

Subject's behavior	**Description**	**Measure per trial**
Contact	Subject touches test cage (with any part of the body except tail)	Frequency
Latency to contact	Latency to first contact with the test cage or stimulus animal during each trial	Duration *
Location	Subject's location in the home cage	Frequency
Subject initiated behavior	**Description**	**Measure per trial**
Lipsmack	Lip movement with smacking sound	Presence/Absence
Chest rub	Rubbing chest area with hand or cage	Presence/Absence
Tail-lash/Arch back	Animal arches back, wags tail	Presence/Absence
Vocalization	Animal engages in calling behavior	Presence/Absence

* Time in seconds, approximation was obtained by multiplying interval number by 15

Table 2.

Simultaneous Partner Preference.

Behavioral ethogram for test live scoring

Simultaneous Partner Preference Test	
Behavior	**Description**
Partner proximity	Time the subject spent in the preference zone next to the partner
Stranger proximity	Time the subject spent in the preference zone next to the stranger
No proximity	Time the subject spent in neutral areas (any location in the test cage other than preference zones)
Touch partner grate	Time the subject intentionally touches the grate on the partner's side with hands or feet
Touch stranger grate	Time the subject intentionally touches the grate on the stranger's side with hands or feet

Table 3.

Mirror test.

Behavioral ethogram for reflective side and back side/control conditions.

Behavior	Description
Mirror approach latency	Time (seconds) from beginning of test session until subject has come within approx. 6 inches of the mirror stimulus
Proximity to mirror	Subject is within arm's length (approx. 6 in) of the mirror stimulus
Partner Approach	Subject moves within monkey arm's length of mate
Proximity to partner	Subject is within arm's length of mate and remains for at least 1 second
Partner contact	Subject and mate's bodies are in contact for at least 1 second.
Partner withdraw	Subject moves his body so that he is no longer in physical contact with mate
Movement	Bouts of movement where subject moves entire body at least one body length in continuous motion until immobile for at least 1 second
Lip smack	Subject makes rapid lip movement accompanied by smacking sound (bout)
Back Arch and tail lash	Arousal display that includes raising dorsal surface of the back and/or whipping tail side to side
Tail twine	Subject and mate tails are intertwined at least one full turn for at least 1 second.
Aggression	Subject grabs, hits, or bites mate as a low intensity display of aggression
Restraining	Subject reaches for, holds and/or pulls mate.

Table 4.

Pair Affiliation.

Summary of Multilevel Models with Treatment Effects on Home cage Pair mate Affiliation Across the First Four Months of Pairing. Subject ID nested within pair mate ID was included as a random effect in all models. To calculate p values for each estimate in Model 1, DF of 27 were used (29 subjects – 1 predictor – 1). To calculate the *p* value for each estimate in Model 2, DF of 25 were used (29 subjects – 3 predictors – 1). The Null AIC indicates the AIC of a model with the outcome variable predicted by only the intercept and random effects; for a full summary of the stepwise models tested, see Supplementary Table 1. * indicates $p < .05$.

	Null	**Model 1**				**Model 2**					
	AIC	**β**	**SE**	***t***	***p***	**AIC**	**β**	**SE**	***t***	***p***	**AIC**
Proximity	309.5	-	-	-	-	301.1	-	-	-	-	293.9
Treatment	-	−0.42	0.12	−3.46	0.002*	-	−0.42	0.12	−3.46	0.002*	-
Month	-	-	-	-	-	-	0.22	0.07	3.40	0.002*	-
Treatment x Month	-	-	-	-	-	-	−0.02	0.07	−0.36	0.72	-
Contact	311.5	-	-	-	-	303.7	-	-	-	-	296
Treatment	-	−0.39	0.12	−3.36	0.002*	-	−0.40	0.11	−3.46	0.002*	-
Month	-	-	-	-	-	-	0.20	0.07	3.02	0.006*	-
Treatment x Month	-	-	-	-	-	-	0.12	0.07	1.74	0.09	-
Tail Twining	304.2	-	-	-	-	301.7	-	-	-	-	305.4
Treatment	-	0.30	0.14	2.12	0.043*	-	0.30	0.14	2.12	0.044*	-
Month	-	-	-	-	-	-	−0.02	0.07	−0.25	0.81	-
Treatment x Month	-	-	-	-	-	-	−0.03	0.07	−0.47	0.64	-

Table 5.

Preference by time spent in preference zones.

GLMM analysis with Binomial distribution, treatment groups and fixed effects. Table shows estimates and standard error in parentheses. Results statistically significant at p≤0.05 two tailed. Signif. Codes: '***' 0.001 '**' 0.01 '*' 0.05.

Preference by time spent in preference zones				
	1 week post-pairing		**4 months post-pairing**	
Variables	**Oxytocin**	**Saline**	**Oxytocin**	**Saline**
Intercept	−0.748(0.008) ***	−0.232(0.09) *	−0.300(.108) **	−0.023(0.064)
Sex	0.491(0.011) ***	−0.112(.128)	0.536(0.158) ***	−0.212(0.011) ***
Preference	−0.118(0.011) ***	−0.683(0.120) ***	−0.690(0.011) ***	−0.935(0.012) ***
Preference × Sex	−0.426(0.016) ***	−0.364(0.017) ***	−0.985(0.017) ***	−0.421(0.016) ***

Table 6.

Preference by time spent touching grated windows.

GLMM with Binomial distribution, treatment groups and fixed effects. Table shows estimates and standard error in parentheses. Results statistically significant at p≤0.05 two tailed. Signif. Codes: '***' 0.001 '**' 0.01 '*' 0.05.

Preference by time spent touching grated windows				
	1 week post-pairing		**4 months post-pairing**	
Variables	**Oxytocin**	**Saline**	**Oxytocin**	**Saline**
Intercept	−3.500(0.195) ***	−3.377(0.136) ***	−2.192(0.331) ***	−2.507(0.152) ***
Sex	1(0.285) ***	0.909(0.026)***	0.556(0.017) ***	−0.235(0.022) ***
Preference	−0.078(0.0295)	−0.078(0.029)	−1.793(0.026) ***	−0.177(0.022) ***
Preference × Sex	0.140(0.036) ***	0.139(0.036) ***	−0.236(0.035) ***	−0.283(0.034) ***