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Central oxytocin receptors mediate mating-induced partner preferences and enhance correlated activation across forebrain nuclei in male prairie voles

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Abstract

Oxytocin (OT) is a deeply conserved nonapeptide that acts both peripherally and centrally to modulate reproductive physiology and sociosexual behavior across divergent taxa, including humans. In vertebrates, the distribution of the oxytocin receptor (OTR) in the brain is variable within and across species, and OTR signaling is critical for a variety of species-typical social and reproductive behaviors, including affiliative and pair bonding behaviors in multiple socially monogamous lineages of fishes, birds, and mammals. Early work in prairie voles suggested that the endogenous OT system modulates mating-induced partner preference formation in females but not males; however, there is significant evidence that central OTRs may modulate pair bonding behavior in both sexes. In addition, it remains unclear how transient windows of central OTR signaling during sociosexual interaction modulate neural activity to produce enduring shifts in sociobehavioral phenotypes, including the formation of selective social bonds. Here we re-examine the role of the central OT system in partner preference formation in male prairie voles using a selective OTR antagonist delivered intracranially. We then use the same antagonist to examine how central OTRs modulate behavior and immediate early gene (*Fos*) expression, a metric of neuronal activation, in males during brief sociosexual interaction with a female. Our results suggest that, as in females, OTR signaling is critical for partner preference formation in males and enhances correlated activation across sensory and reward processing brain areas during sociosexual interaction. These results are consistent with the hypothesis that central OTR signaling facilitates social bond formation by coordinating activity across a pair bonding neural network.

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Keywords

pair bonding; social attachment; monogamy; immediate-early gene; social decision-making network

Introduction

The oxytocin (OT) system is an evolutionarily conserved neuroendocrine mechanism that regulates reproductive and social behaviors across divergent taxa, spanning nematodes and humans (Donaldson and Young, 2008; Garrison et al., 2012; Grinevich et al., 2015). In mammals, OT modulates social recognition (Ferguson et al., 2001; Skuse et al., 2014), maternal responsiveness and mother-infant bonding (Numan and Young, 2015; Rilling and Young, 2014), pair bonding behaviors in monogamous species (Hurlemann and Scheele, 2015; Johnson and Young, 2015; Ross and Young, 2009), and even human-dog bonding (Nagasawa et al., 2015; Romero et al., 2014). It has been hypothesized that OT facilitates social bonding behaviors by modulating neural transmission and encoding of social information across sensory and reward processing brain areas (Johnson and Young, 2015; Numan and Young, 2015).

In mammals, OT is predominantly synthesized in the paraventricular, accessory, and supraoptic nuclei of the hypothalamus (Knobloch and Grinevich, 2014). OT is released within the brain in response to stimuli associated with parturition and nursing in females (Lee et al., 2009) and mating in both sexes (Ross et al., 2009a; Waldherr and Neumann, 2007), and OT neurons are activated following tactile stimulation in rats and voles (Barrett et al., 2015; Okabe et al., 2015). In humans, peripheral OT is increased in response to social vocalizations (Seltzer et al., 2010) and eye contact (Nagasawa et al., 2009; Nagasawa et al., 2015), although the relationship between peripheral and central OT remains unclear (Leng and Ludwig, 2015).

OT mediates its central effects by binding with high affinity to OT receptors (OTRs) and/or with lower affinity to vasopressin 1a (V1aR) receptors (Schorscher-Petcu et al., 2010; Song et al., 2014). The expression patterns of these receptors throughout the forebrain are extraordinarily diverse both within and across species, and are thought to have contributed to the evolution of diverse patterns of neural and sociobehavioral plasticity during species-typical social contexts (Goodson, 2008).

Microtine rodents, or voles, exhibit rich variation in social behaviors, and the socially monogamous prairie vole, *Microtus ochrogaster*, has been the subject of intense investigation of the neural mechanisms underlying diversity in social behaviors and mating strategies (McGraw and Young, 2010). Voles exhibit both intra- and interspecific variation in forebrain OTR and V1aR distribution (Phelps and Young, 2003; Young, 1999), and these patterns have been associated with variation in social investigation (Ophir et al., 2009), mating strategies and reproductive fitness within mating strategies (Ophir et al., 2012), alloparental care (Olazabal and Young, 2006a,b), sexual fidelity and space use (Ophir et al., 2008), and pair bonding (Insel and Shapiro, 1992; Insel et al., 1994; Lim et al., 2004a; Ross et al., 2009b). Recent investigations have implicated these systems in social affiliation and

bonding in additional vertebrate lineages spanning fishes (Oldfield and Hofmann, 2011), birds (Klatt and Goodson, 2013), and mammals (Cavanaugh et al., 2014; Romero et al., 2014; Smith et al., 2010), including humans (Hurlemann and Scheele, 2015; Walum et al., 2012; Walum et al., 2009).

In male and female prairie voles, mating facilitates pair bonding. In the laboratory, pair bond formation is reflected through formation of a robust and enduring preference for the mating partner relative to a novel opposite sex individual, referred to as a “partner preference” (Williams et al., 1992a). Early pharmacological studies revealed that OTR signaling in the brain is critical for partner preference formation in female prairie voles (Insel and Hulihan, 1995; Williams et al., 1994). More specifically, blocking OTR in the prefrontal cortex (PFC) or nucleus accumbens (NA) prevents mating-induced partner preferences in females (Young et al., 2001). Although exogenous central OT infusions facilitate partner preference formation in both sexes (Cho et al., 1999), a single seminal paper failed to show that OTR blockade inhibits mating induced partner preferences in males (Winslow and Insel, 1993). It appeared that endogenous vasopressin, and not OT, regulated pair bonding in male prairie voles by acting at V1aR receptors in the ventral pallidum and lateral septum (Lim et al., 2004b; Lim and Young, 2004; Liu and Wang, 2003). These sexually dimorphic roles for OT and AVP have pervaded the literature on pair bonding for the past 20 years.

In these experiments, we revisit the role of OTR in partner preference formation in male prairie voles, and demonstrate conclusively using a highly selective OTR antagonist (OTA) that, as in females, central OTR signaling is critical for pair bonding in males. We then introduce an abbreviated cohabitation paradigm to investigate the role of central OTR signaling in modulating sociosexual behavior and neural activity across a hypothesized pair bonding network (PBN), with the aim of identifying potential neural mechanisms by which central OTR signaling may modulate pair bond formation. To achieve this goal, we infuse OTA or aCSF intracerebroventricularly (ICV) into male prairie voles and measure sociosexual behaviors as well as induction of the immediate-early gene transcription factor, Fos, following cohabitation with a sexually receptive female. We first demonstrate that this paradigm is a useful tool for investigating sexual behavior and neural activity in male prairie voles by replicating findings from investigations in other rodents. Next, we restrict our analysis to nuclei within the PBN and find no evidence that central OTR signaling during sociosexual interaction modulates Fos expression within any of the analyzed brain nuclei. However, our data suggest that central OTR signaling during sociosexual interaction plays a critical role in modulating a robust pattern of correlated Fos expression *across* PBN nuclei. These results provide novel insights into how OTR modulates neural activity across the PBN, and are consistent with previously outlined hypotheses that OTR modulates functional connectivity (Goodson and Kabelik, 2009) and transmission of social information across conserved brain networks (Johnson and Young, 2015; Numan and Young, 2015) during species-typical social contexts.

Materials and Methods

Subjects

Male prairie voles were housed in groups of two or three until stereotaxic surgery during adulthood (60-200 days), after which they remained singly housed until behavioral testing. Housing consisted of a ventilated 26×18×19 cm Plexiglas cage filled with Bed-o-cobbs Laboratory Animal Bedding under a 14:10 h light/dark cycle at 22°C with *ad libitum* access to food (rabbit LabDiet) and water. Subjects were drawn from our laboratory breeding colony originally derived from field captured voles in Illinois. Stimulus animals were sexually experienced, ovariectomized, estrogen-primed (see below), adult female prairie voles. All procedures were conducted in accordance with the National Institutes of Health Guide for the Care and Use of Laboratory Animals and were approved by the Emory University Institutional Animal Care and Use Committee.

Experiment 1: Partner Preference

Intracranial Cannulation—Adult male prairie voles were anesthetized with 2.5% isoflurane inhalation and stereotaxically implanted unilaterally into the left hemisphere with a 22 gauge guide cannula (catalog no. C313GS-5/SPC; Plastics One; Roanoke, VA) aimed ICV using stereotaxic coordinates (A/P +0.6 mm; M/L ± 1.0 mm; D/V -3.0 mm). The guide cannula was fixed to the skull with a combination of Jet Acrylic Liquid and Jet Denture Repair Powder (Lang Dental Manufacturing Co., Inc.; Wheeling, IL). All subjects recovered for 4 days following surgery.

OTA administration—4 days following surgery, subjects received microinjections of either 2 µl of control artificial cerebral spinal fluid (aCSF, n=18) or 2.5 ng/µL of a selective oxytocin receptor antagonist (OTA), des Gly-NH₂d(CH₂)₅-[D-Tyr², Thr⁴]OVT (Manning, Miteva et al. 1995) dissolved in 2µl of aCSF (n=21) using a 28 gauge internal cannula (Plastics One) extending 0.5 mm beyond the end of the guide cannula into the lateral cerebral ventricle. Injections were performed with a 10 µl Hamilton syringe (Hamilton; Reno, Nevada) connected to polyethylene-20 tubing (Plastics One), which was in turn secured to the internal cannula. Infusions were administered over the course of 60 seconds, and the internal cannula was left in place for 3 minutes following infusion to prevent backflow. Two infusions were administered to each subject; one immediately prior to the 48-hour cohabitation, and the second 24 hours into the cohabitation.

Cohabitation and Behavioral Analysis—Immediately following OTA infusion, subjects were placed in a clean cage (26×18×19 cm) with a sexually experienced, ovariectomized, estrogen-primed, adult stimulus female for 48 hours. In the three days preceding cohabitation, stimulus females were brought into estrus with daily injections of 4.0 µg estradiol benzoate dissolved in sesame oil (Sigma; St. Louis, MO; S3547) injected subcutaneously. The first 3 hours of cohabitation were video recorded and analyzed for mating; only one pair did not mate during this period and was excluded from final analysis. After 24 hours of cohabitation, males were briefly removed from the home cage, anesthetized, infused with a second dose of OTA as described above, and immediately returned to cohabitation. Following 48 hours total of cohabitation male subjects and stimulus

females were separated overnight. The following day, all subjects underwent a 3-hour partner preference test. In this paradigm, the male subject is placed in a central “neutral” zone of a 3-chambered apparatus in which the familiar female “partner” is tethered in one far chamber and the novel female “stranger” is tethered in the opposite chamber (Williams et al., 1992b). The experimental animal is free to move throughout the chambers and the time spent in immobile social contact with each female is recorded using an automated tracking system (SocialScan 2.0, Clever Sys Inc., Reston, VA, USA; Ahern, Modi et al. 2009). All tests were video recorded and analyzed *post hoc* for time spent in immobile contact, or huddling, with the female partner.

Injection Site Validation—To verify placement of the ICV injection cannula in animals subjected to a partner preference test, 10% india ink was injected at the time of euthanization and staining of the entire ventricular system was required for designation as correct placement. Based on this criterion, subjects with missed cannulations (n=6) were excluded from the analysis, yielding final group sizes of n=16 for aCSF-treated and n=16 for OTA-treated groups.

Experiment 2: Sociosexual behavior and Fos expression

Intracranial cannulation—Following the same general procedure as above, guide cannula were unilaterally implanted targeting ICV (A/P -0.2 mm; M/L \pm 1.0 mm; D/V -3.0 mm), counterbalanced between left and right hemispheres across subjects. All subjects recovered for 5 days following surgery.

OTA administration—Following recovery, and three hours prior to the 30-minute cohabitation, subjects received microinjections of either 2 μ l of control aCSF (n=16 exposed; n=9 unexposed) or 2.5 ng/ μ L OTA dissolved in 2 μ l aCSF (n=20 exposed) following the same general procedure outlined above. Larger group sizes were selected for both socially exposed treatment groups to obtain sufficient numbers of subjects that mated with the stimulus female during cohabitation.

Cohabitation—3 hours following aCSF/OTA infusion, sexually naïve adult male subjects underwent a 30 minute cohabitation with a sexually experienced, ovariectomized, estrogen-primed (described above under “Experiment 1”), adult stimulus female and were video recorded. Cohabitation began with the introduction of the stimulus female into the male subject’s home cage (specifications described above) with food hoppers removed for optimal video analysis. Normal lab chow pellets and water bottles were left in the home cage for *ad libitum* access to food and water during the cohabitation. Following the 30 minute session, female subjects were removed and males remained in their home cage for 45 minutes before transcardial perfusion. For unexposed control subjects (n=9), the cage was opened and a small volume of bedding was scooped and immediately returned by an experimenter at the beginning and end of the 30 minute session to control for experimenter activity during introduction/removal of the stimulus female.

Behavioral scoring—Non-investigation, social investigation, mounting, and intromission behavior were quantified for male subjects using Observer XT 10 behavioral scoring

software (Noldus Information Technology Inc.; Leesburg, VA) by two independent scorers blind to treatment groups. Four different mutually exclusive behaviors performed by the male were scored: non-investigation, investigation, mounting, and intromission. The operational definitions used for each behavior were as follows. Non-investigation included any male behavior not directed towards the female, including autogrooming, running, freezing, eating, drinking, sleeping, digging, and exploration. Investigation comprised male behavior directed immediately towards the female, including physical pursuit, olfactory investigation, and allogrooming. Mounting was defined as placement of the forepaws on the female from the rear without stereotypical intromission behavior. Intromission was scored during copulation bouts with the female, including stereotypical patterns of thrusting.

Perfusion and post-fixation—75 minutes following initial introduction of the stimulus female or the control bedding manipulation, male subjects were administered an overdose of isoflurane and were immediately perfused transcardially at a rate of approximately 4 mL/minute with 40 mL of 1× phosphate buffered saline (pH=7.4; diluted to 1× with distilled, deionized water from 10× PBS stock; Teknova; Hollister, CA; P0401) followed by 40 mL of 4% paraformaldehyde (Polysciences; Warrington, PA; 00380) in 1× PBS using an Easy-Load II MASTERFLEX pump (Cole-Palmer; Vernon Hills, IL). Five subjects (n=2 aCSF-treated, n=3 OTA treated) were excluded from analysis of Fos expression due to low perfusion quality. Following perfusion, brains were extracted and post-fixed in 4% paraformaldehyde dissolved in 1× PBS overnight before being transferred to 30% sucrose in 1× PBS solution until sectioning.

Tissue Processing and Immunohistochemistry—40 μm brain sections were collected using a Microm HM 440E freezing microtome and were stored in 1× PBS with 0.05% sodium azide until immunohistochemical staining. Sections underwent 3 washes in 1× PBS, 10-minute incubation in 1% sodium hydroxide in 1× PBS, and 3 washes in 1× PBS with 0.5% Triton-X (Sigma) (PBST) before treatment with 5% normal goat serum (Fitzgerald; Acton, MA) in PBST for 1 hour at room temperature. Sections were then incubated for 48 hours in primary rabbit polyclonal anti-Fos antibody (1:1,000; Calbiochem PC38) on an orbital shaker at 4°C. Following primary incubation sections were washed 5 times in 1× PBS, once in 1× PBST, and were incubated in secondary biotinylated goat anti-rabbit IgG antibody (1:500; Vector Labs BA-1000) for 2 hours. After secondary incubation, sections were treated with an avidin-biotin peroxidase system (Vectastain Elite ABC System; Vector Labs: PK-6100) for 1 hour before staining with a Nickel-DAB peroxidase substrate kit (Vector Labs; SK-4100). All sections were dehydrated in a series of increasingly concentrated ethanol solutions (5 minutes in 70% EtOH, 10 minutes in fresh 95% EtOH twice, 10 minutes in fresh 100% EtOH twice), bathed in Xylenes (15 minutes in Xylenes twice), mounted onto Superfrost Plus slides (Fisher Scientific; 12-550-150) while still partially wet, and coverslipped using Krystalon (EMD Chemicals Inc., Gibbstown, NJ).

Injection Site Validation—To avoid interference with Nickel-DAB immunolabeling and confounding subsequent quantification of Fos protein expression, 10% india ink was not used for validation of cannula placement. Instead, location of the injection cannula was verified on anti-Fos-immunostained sections and subjects were included if the cannulation

track was continuous with the lateral intracerebral ventricles. All subjects were found to have cannulation tracks that were continuous with the lateral intracerebral ventricles and were included in behavioral analysis.

Imaging and FOS expression analysis—Images were captured using a Nikon E800 microscope and 10× objective using MCID Core imaging software (InterFocus Ltd; Cambridge, UK). Fos expression was analyzed within the medial preoptic area (MPOA) as a positive control, as previous studies in a wide range of species have demonstrated elevated Fos expression within this nucleus in males following mating. 7 additional OTR-expressing brain regions within the PBN (Fig. 4C) were imaged across subjects: anterior olfactory nucleus (AON), medial amygdala (MeA), basolateral amygdala (BLA), nucleus accumbens core (NAc), nucleus accumbens shell (NAs), medial prefrontal cortex (PFC), and paraventricular hypothalamic nucleus (PVN). The AON and MeA were analyzed because of their role in olfactory processing and social recognition (Ferguson et al., 2001). The BLA was analyzed because of its role in transmitting social information to the reward system (Numan and Young, 2015), and the demonstrated role of the amygdala in partner preference formation (Modi et al., 2015). The NAc, NAs, and PFC were analyzed because OTR signaling in these nuclei is critical for partner preference formation in female prairie voles (Young et al., 2001). Finally, the PVN was analyzed because it is the major source of OT innervation to the NA and other forebrain nuclei (Knobloch et al., 2012; Ross et al., 2009a). The Mouse Brain atlas (Franklin and Paxinos; 3rd Edition) was used as a reference to determine anatomical boundaries. For each brain region, Fos was quantified in both left and right hemispheres across 3 consecutive sections for all subjects; sections or hemispheres with damaged/absent tissue in the target region were excluded. Fos-positive nuclei were quantified for each region using the MCID grain count function with a constant threshold applied across all subjects. For each subject, Fos expression counts within each region were averaged, yielding a single average Fos count per brain region per subject.

Statistical analyses

All statistical analyses were performed in R 3.1.1.

Partner preference—Behavioral data from the partner preference test were analyzed using a two-way ANOVA with stimulus (partner versus stranger) and treatment (control versus OTA) as between subject factors. Planned Student's t-tests were used to compare time spent with the partner and stranger within each treatment group. Effect size (Cohen's *d*) was calculated by comparing the mean differences within the treatment groups, divided by the pooled standard deviation of the mean differences.

Sociosexual behaviors—Behavioral data from the 30 minute cohabitation period were analyzed using a two-way repeated-measures ANOVA with treatment (OTA versus aCSF) as a between subjects factor and behavior as a within subjects factor. Planned Student's t-tests were used to compare behavioral measures between treatment groups when appropriate. Poisson regression was used for analysis of count outcome variables (e.g. mating frequency). For all ANOVAs effect size (Eta Squared) was calculated by dividing the individual effects' sum of squares by the total sum of squares.

MPOA Fos expression—Fos expression within the MPOA was analyzed using a two-way ANOVA with treatment (OTA exposed versus aCSF exposed versus aCSF unexposed) and mating as between subjects factors. *Post-hoc* Student's t-tests were used to compare Fos expression within the MPOA between subjects that mated and subjects that did not. Effect size (Cohen's *d* was calculated by dividing the mean difference by the pooled standard deviation.

Fos expression within PBN nuclei—Fos expression within brain areas was analyzed across treatments using a two-way repeated measures ANOVA with treatment (OTA mated versus aCSF mated versus aCSF unexposed) as a between subjects factor and brain region as a within subjects factor. *Post-hoc* Student's t-tests were used to compare Fos expression between OTA- and aCSF-mated subjects. *Post-hoc* Student's t-tests were used to compare Fos expression between mated and unexposed subjects within each brain region. α was adjusted to 0.007 to correct for the multiple comparisons.

FOS expression covariance across the PBN—The correlation patterns across brain regions for the different treatment/exposure groups were first examined descriptively by visualizing the data in heat maps. These maps, showing Pearson's correlation coefficients, were generated using the data visualization package ggplot2 in R. Principal component analyses (PCA) were performed using the “PCA” function from the “FactoMineR” package to analyze differences in patterns of FOS expression between aCSF-mated and OTA-mated subjects. We generated a permuted null distribution through multiple, random assignment of individuals to one of the investigated treatment groups, without replacement. For each permutation we calculated the variance explained by the first principal component between groups and then the absolute value of the difference between these values. This procedure was repeated 10,000 times and the p-value for the difference in variance explained was calculated as the proportion of permuted differences exceeding the initial observed difference value.

Results

Effects of central OTA on male partner preference formation

A two-way ANOVA revealed a significant main effect of stimulus (partner versus stranger; $F_{1,60}=5.77$; $p=0.019$), and stimulus by treatment (aCSF versus OTA) interaction ($F_{1,60}=6.208$; $p=0.016$; $d=1.20$), but not of treatment ($F_{1,60}=0.429$; $p=0.51$) on duration of immobile social contact (e.g. huddling) during the partner preference test. *Post-hoc* two-tailed Student's t-tests showed that male prairie voles spent significantly more time in social contact with their partner versus the stranger following ICV administration of aCSF ($n=16$; $p=0.0028$) but not OTA ($n=16$; $p=0.95$; Figure 1).

Effects of central OTA on mating

We then used a 30 minute cohabitation paradigm to investigate the effect of central OTA administration on sexual behavior in male prairie voles. Central administration of OTA to adult male rats reduces intromissions during sociosexual interaction with a female (Argiolas et al., 1988), and therefore we hypothesized that central administration of OTA to adult male

prairie voles would reduce intromission frequency in this paradigm. Among all subjects that underwent cohabitation with a sexually experienced and receptive stimulus female (aCSF, n=16; OTA, n=20), intromission frequency was reduced in OTA-treated (2.49 ± 0.62 mating bouts per 30 minutes) relative to aCSF-treated (5.30 ± 1.14 mating bouts per 30 minutes) subjects. Consistent with findings in rats, a regression analysis comparing Poisson distributions of mating bouts by treatment (aCSF vs. OTA) revealed that frequency of mating bouts significantly differed by treatment ($z = -4.30$; $p = 2.35 \times 10^{-5}$; Figure 2A).

Mating and FOS expression in the MPOA

Previous work in other rodents has demonstrated that male sexual behavior is associated with elevated Fos expression within the MPOA (Dominguez and Hull, 2005; Heeb and Yahr, 1996; Kollack-Walker and Newman, 1997). Using this 30 minute cohabitation paradigm, we investigated whether the same effect could be detected in male prairie voles. Subjects with poor perfusion quality (n=5) were excluded from analysis. Among remaining subjects (aCSF, n=14; OTA, n=17), 3 aCSF- and 8 OTA-treated males did not mate, defined as intromission, during the 30 minute cohabitation. A two-way ANOVA revealed a significant main effect of mating ($F_{1,26} = 9.87$; $p = 0.0042$; Eta-squared=0.26), but not treatment (aCSF versus OTA; $F_{1,26} = 0.956$; $p = 0.34$; Eta-squared=0.03) or the interaction between treatment and mating ($F_{1,26} = 1.08$; $p = 0.31$; Eta-squared=0.03), on Fos expression within the MPOA. Treatment was collapsed as a factor for subsequent analyses and a Student's t-test revealed significantly elevated Fos expression in subjects that mated (n=20) versus subjects that were exposed to a female but that did not mate (n=11; $p = 0.0024$; Figure 2B; $d = 1.4$), consistent with findings in other rodent species (Dominguez and Hull, 2005; Heeb and Yahr, 1996; Kollack-Walker and Newman, 1997) and previous work from our laboratory comparing Fos expression within the MPOA in male prairie voles that were exposed to and mated with a novel female, were exposed to a familiar sibling, or were unexposed (Lim and Young, 2004).

Effects of OTA on male sociosexual behaviors in males that mated

Subsequent analyses of behavior and Fos expression were restricted to only subjects that mated to maximize divergence in central OTR signaling between aCSF- and OTA-treated groups, and to parallel the exclusion criterion of Experiment 1 (see “Materials and Methods”). Among mated subjects (aCSF, n=11; OTA, n=9), a two-way repeated-measures ANOVA revealed a significant main effect of behavior (non-investigation, investigation, mounting, or intromission; $F_{3,68} = 46.43$; $p < 2 \times 10^{-16}$; Eta-squared=0.22), but not of treatment (aCSF versus OTA; $F_{1,68} = 0.00$; $p = 0.99$; Eta-squared=0.00) or the interaction between behavior and treatment ($F_{3,68} = 0.35$; $p = 0.73$; Eta-squared=0.00) on time engaged in the analyzed social behaviors during the 30 minute period of cohabitation (Figure 3). Thus, among mated animals, OTA versus aCSF treatment had no detectable effect on any of the analyzed sociosexual behaviors.

OTR signaling during sociosexual interaction and Fos expression in PBN nuclei

Analysis of Fos expression in 7 OTR-expressing PBN nuclei (Fig. 4C; see “Materials and Methods”) was limited to subjects that mated during the 30 minute cohabitation to maximize

divergence of presumed central OTR signaling. aCSF-treated, unexposed subjects were also included for baseline comparison. A two-way repeated-measures ANOVA with treatment (aCSF-mated versus OTA-mated versus aCSF-unexposed subjects) as a between subjects factor and brain region as a within subjects factor revealed a significant main effect of treatment ($F_{2,167}=12.53$; $p=8.48\times 10^{-6}$; Eta-squared=0.08), but not brain region ($F_{6,167}=0.818$; $p=0.56$; Eta-squared=0.02) or the interaction between treatment and brain region ($F_{12,167}=1.46$; $p=0.15$; Eta-squared=0.05), on Fos expression in the analyzed brain nuclei. These results suggest a strong effect of treatment on Fos expression that did not differ significantly across brain regions; therefore, brain region was excluded as a predictor for subsequent ANOVA analyses of Fos expression across the PBN. When restricting analysis to subjects that mated, a *post-hoc* oneway repeated-measures ANOVA revealed that Fos expression across the PBN did not differ by treatment among mated animals ($F_{1,130}=1.24$; $p=0.27$; Eta-squared=0.01); therefore, OTA-treated and aCSF-treated mated subjects were pooled for subsequent *post-hoc* analyses. A one-way repeated measures ANOVA revealed a significant effect of sociosexual interaction and mating on Fos expression across the PBN between mated animals (pooled) and unexposed controls ($F_{1,191}=18.51$; $p=3.20\times 10^{-5}$; Eta-squared=0.07; Figure 4). Further post-hoc t-tests were performed to investigate Fos expression within individual brain nuclei between mated (pooled) and unexposed subjects, and Bonferroni correction for multiple comparisons resulted in an adjusted α of 0.007. Using this criterion, Fos expression was significantly ($p<0.007$) elevated in every analyzed region, except the PVN ($p=0.023$), in mated subjects relative to unexposed controls. Thus, sociosexual interaction and mating was associated with significant elevation in Fos expression across the PBN in mated animals relative to unexposed controls, and OTA did not significantly modulate Fos induction within any of the analyzed nuclei.

Effects of sociosexual interaction, mating, and OTR signaling on Fos expression covariance across the PBN

The MeA, AON, PFC, NA, PVN and BLA are part of a hypothesized neural network underlying mating induced pair bonds in prairie voles (Johnson and Young, 2015; Numan and Young, 2015). Based on our hypothesis that OTR facilitates transmission of social information across the PBN, and previously outlined hypotheses that OTR modulates functional connectivity across neural networks, we hypothesized that OTR signaling during sociosexual interaction and mating would modulate covariance in Fos expression across PBN nuclei. As seen in Figure 5a, under control conditions (no exposure to a female) Fos expression is weakly correlated across nodes, as measured by Pearson's correlation coefficients. Relative to unexposed controls, sociosexual interaction and mating results in a strong and relatively uniform increase in correlated Fos expression across the network (Figure 5b). In contrast, as seen in Figure 5c, relative to aCSF-treated mated animals, OTA injected animals exhibit a weaker, less uniform pattern of correlated FOS expression across the network. To test whether the difference between aCSF-versus OTA-treated animals is statistically significant, we used a combination of principal component analysis (PCA) and permutation testing. PCA converts a set of variables into linearly uncorrelated variables called principal components. In this transformation, the first principal component accounts for as much of the variance in the raw data variables as possible. In a situation where all

investigated variables are highly correlated, the first principal component will explain a large proportion of the variance. Consistent with the increase in correlated Fos expression across PBN nuclei in aCSF-treated mated animals (Figure 5b), PCA revealed that the first principal component accounts for 68.6% of the variance. In contrast, in OTA-treated mated animals, the first principal component accounts for 35.3% of the variance. We then used permutation testing to test whether this difference (33.3%) in variance explained by the first principal component between aCSF- and OTA-treated mated subjects exceeds what would be expected by chance (see “Materials and Methods”). This resulted in a p-value of 0.008, suggesting that OTA significantly disrupts the increase in correlated Fos expression across the PBN induced by sociosexual interaction and mating.

Discussion

Previous work has suggested that the central OT system may modulate pair bonding in prairie voles in a sex-specific manner (Insel and Hulihan, 1995; Winslow and Insel, 1993), with central OTRs modulating pair bonding in females but not in males. However, more recent investigations in both birds and mammals have suggested that the OT system may modulate bonding in both sexes. For example, investigations in estralid finches have found that the behavioral effects of central OT signaling on partner preference formation in males follows the same trend as in females, although it was not statistically significant within males only (Klatt and Goodson, 2013). In prairie voles, patterns of OTR expression across specific forebrain nuclei are associated with monogamous behavior and space use in males (Ophir et al., 2012), and central OT administration enhances partner preference formation in both females and males (Cho et al., 1999). Furthermore, a polymorphism in the OTR gene (*Oxtr*), which is robustly associated with patterns of OTR expression in the forebrain, predicts pair bonding behavior in male prairie voles (King, et al., *under review*). In humans, intranasal OT administration to romantically attached males increases activation of reward areas when viewing images of a partner's face, but not an unfamiliar stranger female's face, and increases preferred interpersonal distance from an unfamiliar female (Scheele et al., 2012; Scheele et al., 2013).

A single pharmacological study, using a less selective OTA and relatively small sample sizes for experimental groups (n=6-8 per group), failed to demonstrate an effect of OTA on male pair bonding (Winslow and Insel, 1993) and codified the notion that the endogenous OT system modulates pair bonding behavior exclusively in females. Here, we use a more highly selective OTA, administered ICV to larger samples of male prairie voles (n=16 per group) during cohabitation with a female, to investigate the role of central OTR signaling in male partner preference formation. In contrast to the previous study, OTA administration inhibited partner preference formation in mated male prairie voles (p=0.016). To our knowledge, this is the first conclusive, causal evidence demonstrating that endogenous OTR signaling modulates pair bonding in a male animal. In light of recent work in monogamous estralid finches (Klatt and Goodson, 2013) and monogamous cichlid fishes (Oldfield and Hofmann, 2011), work in additional vertebrates is required to investigate the hypothesis that the OT system is a deeply conserved neuroendocrine mechanism contributing to the evolution of social bonding in vertebrates, across sexes.

OT has deeply conserved roles in reproductive physiology and behavior across distant taxa spanning invertebrates and vertebrates (Garrison et al., 2012), including mammalian species such as mice, rats, rabbits, and squirrel monkeys (Argiolas and Melis, 2004). In rats, central administration of OTA reduces intromission frequency in males (Argiolas et al., 1988). We investigated whether this effect could be detected in male prairie voles using a brief 30 minute cohabitation paradigm. Consistent with these data, central OTR blockade reduced frequency of mating bouts (n=20) relative to aCSF-treatment (n=16) during sociosexual interaction with a female ($p=2.35 \times 10^{-5}$), suggesting that central OTR signaling modulates sexual behavior in male prairie voles. This result is consistent with findings in other mammals, but is intriguing in light of an earlier study demonstrating that central OT administration inhibits sexual behavior in male prairie voles (Mahalati et al., 1991). However, it is important to note that these data are not necessarily contradictory, as exogenous central OT delivery may not reflect endogenous central OT levels and can result in promiscuous signaling at both OTRs and V1aRs. In contrast, central delivery of an OTA specifically blocks endogenous signaling at central OTRs.

Neural investigations of sexual behavior have highlighted the hypothalamic MPOA as a deeply conserved neural locus regulating male sexual behavior across all vertebrate lineages (Dominguez and Hull, 2005). In male rats (Robertson et al., 1991), hamsters (Kollack-Walker and Newman, 1997), and gerbils (Heeb and Yahr, 1996), sexual behavior is associated with elevated Fos expression within this nucleus. Therefore, we hypothesized that intromission within this experimental paradigm would be associated with elevated MPOA Fos expression in male prairie voles. Indeed, intromission during the 30-minute period of sociosexual interaction was associated with a significant, non-OT-dependent elevation in MPOA Fos expression ($p=0.0024$), suggesting that, consistent with findings in other rodents, Fos induction within the MPOA reflects male sexual behavior in prairie voles. Collectively, these data are not surprising, but offer strong predictive validation of a simple experimental paradigm as a framework for conducting future behavioral and neural investigations in prairie voles.

In this study, Fos expression was used as a metric of neuronal activity and plasticity across OTR-expressing and -synthesizing PBN nuclei in response to sociosexual interaction with a female. Briefly, Fos protein is encoded by the immediate early gene (IEG) *c-fos* and can heterodimerize with JUN to form AP-1 transcription factor complexes that directly up- or down-regulate expression of AP-1 responsive genes involved in a variety of intracellular processes, including synaptic plasticity (Lyons and West, 2011). Therefore, Fos (and other IEG protein products, such as Arc and Egr-1) expression is thought to be one mechanism by which a cell can rapidly adapt its phenotype in response to a stimulus (e.g. neuronal depolarization) through robust transcriptional regulation. These qualities have positioned nuclear Fos expression as a powerful tool for measuring CNS-wide neuronal activity and plasticity during specific physiological, environmental, and behavioral contexts. Because translation of Fos protein typically peaks 60-120 minutes following stimulus presentation, Fos experiments often comprise an acute experience followed by brain fixation 60-120 minutes afterward.

Analysis of Fos expression across PBN nuclei was restricted to mated subjects to maximize divergence in central OTR signaling between treatment groups, and was limited to OTR-expressing forebrain nuclei that have been repeatedly implicated in a variety of processes (e.g. neuropeptide synthesis and release, social recognition, reward learning, and motivation; see “Materials and Methods”) that are thought to be integral to pair bond formation; these areas are nodes in a hypothesized PBN model (Johnson and Young, 2015). It is important to note that among those animals that mated, there were no observed differences in behavior (e.g. non-investigation, investigation, mounting, or intromission) between OTA- and aCSF-treated groups. Thus, differences in Fos expression are not likely due to differences in sensory or sexual stimulation between treatment groups, but more likely reflect differences in function and/or modulation of the PBN during equivalent sociosexual contexts.

Sociosexual interaction and mating with a female was associated with significantly elevated Fos expression across the analyzed PBN nuclei ($p=1.03\times 10^{-6}$), but no significant differences were observed in Fos expression within any brain region between OTA- and aCSF-mated animals. These results suggest that sociosexual interaction and mating in male prairie voles is associated with robust, non-OT-dependent increases in neuronal activity and plasticity across the PBN. However, these data should be interpreted with caution. Experiments employing alternative behavioral paradigms and analyses, larger sample sizes, alternative markers for neuronal activity and plasticity, and co-labeling of specific neuronal populations may reveal important patterns of OT-mediated neuronal activity and plasticity within these nuclei during sociosexual interaction that were not detected in this study.

Correlated IEG expression across brain nuclei has been previously used as a tool to investigate functional connectivity (broadly defined as correlated activation of spatially distinct neural loci) between or among those regions during specific physiological, behavioral or environmental contexts (Hoke et al., 2005; Schiltz et al., 2007; Wheeler et al., 2014; Yang and Wilczynski, 2007), with increases in correlated IEG expression between regions indicating increased functional connectivity. Here, we investigate whether central OTR signaling during sociosexual interaction with a female modulates correlated patterns of Fos expression across PBN nuclei in male prairie voles. These analyses reveal an increase in correlated Fos expression across PBN nuclei in aCSF-treated subjects relative to both unexposed controls and OTA-treated subjects, consistent with the hypothesis that central OTR signaling during sociosexual interaction and mating with a female enhances functional connectivity across the PBN in male prairie voles. These data are interesting in light of recent human fMRI studies showing that intranasal OT administration increases functional connectivity across a variety of striatal and limbic nuclei during various social contexts (Fan et al., 2015; Gorka et al., 2015; Hu et al., 2015; Riem et al., 2012); however, studies using intranasal OT should be interpreted with caution (Walum et al., 2015).

In light of these results, one hypothesis is that patterns of OTR (and V1aR) distribution across cortical, striatal, and limbic networks reflect patterns of functional connectivity that can be recruited during contexts that elicit OT release. More specifically, within the context of pair bonding in male prairie voles, it may be that sociosexual interaction triggers OT release and initiates patterns of functional connectivity across PBN nuclei that facilitate

transmission of the partner's unique sociosensory signature from sensory to reward processing nuclei, where it is encoded as a positive stimulus.

There are important limitations to these data and interpretations. First, differences in correlated patterns of Fos expression do not reveal how central OTR signaling modulates activity of specific neuronal phenotypes or dynamic electrophysiological network properties. We are currently performing electrophysiological recordings in multiple brain regions simultaneously in behaving voles to better understand how central OTR signaling modulates functional connectivity in the PBN during mating with real-time temporal resolution. Second, relatively small sample sizes, a high volume of pairwise comparisons, and brain-wide OTR blockade preclude conclusions about OTR-mediated disruptions in the functional relationships between specific PBN nuclei; instead, the data are consistent with a broader interpretation that central OTR signaling during sociosexual interaction and mating promotes increased functional connectivity across the network as a whole. Thirdly, the resolution of the present study is limited to whole brain nuclei, each of which is composed of heterogeneous cell populations with diverse connectivities, properties, and functions. Fourthly, this behavioral paradigm included a diverse range of complex social interactions between the subject male and a unique stimulus female (e.g. antagonistic interaction to intromission) that varied across subjects; therefore, the behavioral specificity of these results is limited. Finally, the present study does not address whether or how social context is related to the neuromodulatory effects of increased central OTR signaling. Alternative paradigms are required to dissect how central OTR signaling modulates these circuits during specific behavioral states and more precise social contexts.

Despite these limitations, these data are consistent with previously outlined hypotheses (Goodson and Kabelik, 2009) that neuropeptide signaling can modulate adaptive forms of sociobehavioral plasticity in part by modulating patterns of functional connectivity across evolutionarily conserved neural networks, such as the social decision making network in vertebrates (O'Connell and Hofmann, 2011, 2012), during social and reproductive contexts that elicit OT and AVP release. Within this framework, unique OTR (and V1aR) expression patterns across the prairie vole forebrain may allow OT release during sociosexual interaction to trigger distinct, transient patterns of functional connectivity across the PBN that facilitate pair bond formation.

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Highlights

- Brain oxytocin receptors are critical for partner preferences in male prairie voles.
- Mating induces neural activation in nuclei processing social information and reward.
- Mating enhances correlated activation across a pair bonding network.
- Blocking oxytocin receptors during mating disrupts correlated neural activation.

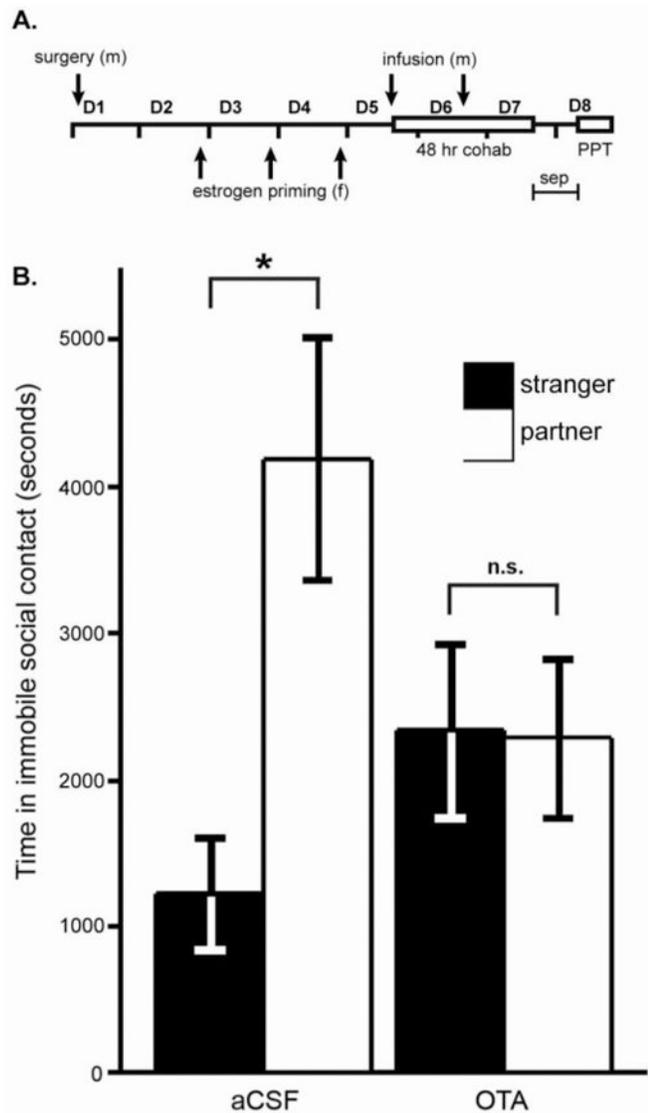


Figure 1. Central OTR signaling modulates pair bond formation in male prairie voles

Figure 1A represents a schematic of the experimental timeline and design (D=day; m=male; f=female; sep=overnight separation; PPT=partner preference test). Prior to testing, adult males received either aCSF or OTA (administered ICV) throughout a 48-hour cohabitation period with an ovariectomized, estrogen-primed, sexually experienced adult female. Figure 1B depicts time spent in immobile social contact (e.g. huddling) with the partner versus the stranger in a 3-hour partner preference test in aCSF- and OTA-treated animals. There was a significant interaction effect of treatment (OTA versus aCSF) by stimulus (partner versus stranger) on time spent in immobile social contact ($F_{1,60}=6.208$, $p=0.016$; two-way repeated measures ANOVA). aCSF-treated males ($n=16$) spent significantly more time in immobile social contact with the partner (4193 ± 826 seconds) compared to the stranger (1226 ± 387 seconds; $p=0.0028$; Student's *t*-test). In contrast, time spent in immobile social contact with the partner (2285 ± 541 seconds) versus the stranger (2339 ± 588 seconds) did not significantly

differ within OTA-treated subjects ($p=0.95$; Student's t-test). Asterisk indicates a p-value below 0.05.

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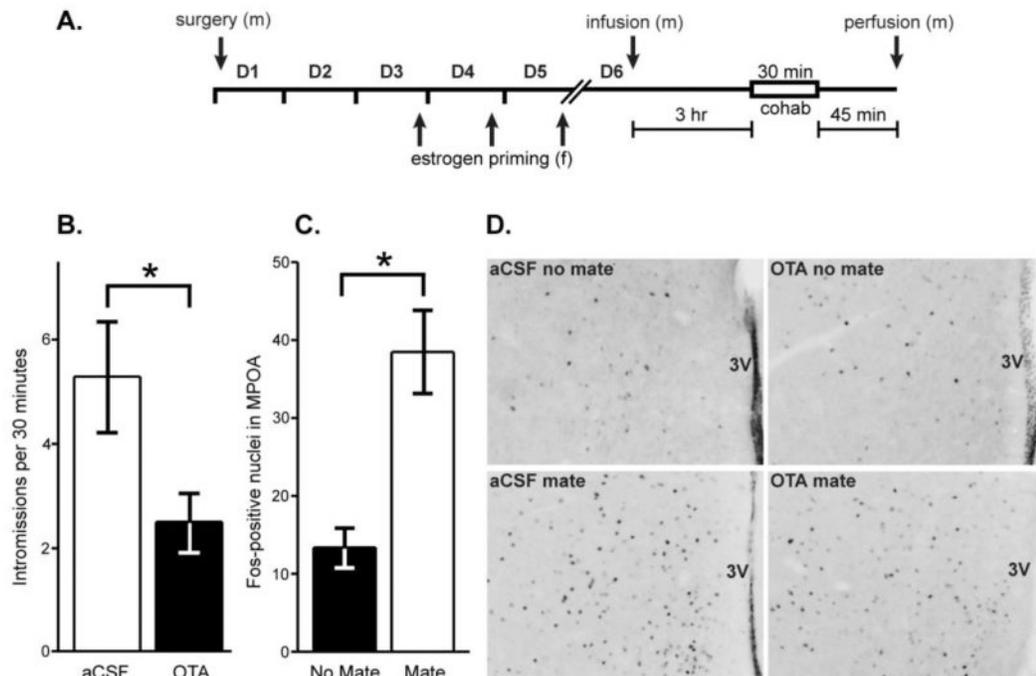


Figure 2. Central administration of OTA reduces frequency of intromission bouts and mating is associated with elevated MPOA Fos expression

Figure 2A shows a schematic of the experimental timeline and design for Fos experiments (D=day; m=male; f=female). Prior to testing, adult males received either aCSF or OTA (administered ICV). Figure 2B shows the number of intromission bouts exhibited during a 30-minute period of sociosexual interaction with an ovariectomized, estrogen-primed, sexually experienced adult female. aCSF-treated subjects ($n=14$) engaged in an average frequency of 5.30 ± 1.14 intromission bouts with the stimulus female per 30 minutes, which differed significantly relative to OTA-treated males (2.49 ± 0.62 bouts per 30 min; $z=-4.30$; $p=2.35 \times 10^{-5}$; Poisson regression). Figure 2C shows the number of Fos positive nuclei within the medial preoptic area between subjects that did not mate ($n=11$) compared to those that mated ($n=20$; pooled across OTA and aCSF treatment) during the 30-minute period of sociosexual interaction. Subjects that did not mate exhibited significantly fewer Fos-positive nuclei (13.49 ± 2.51) compared to subjects that mated (38.54 ± 5.34 ; $t_{28}=-3.34$, $p=0.0024$; Student's t-test). Asterisks indicate p-values below 0.05. Figure 2D shows representative images of Fos expression within the MPOA across all treatment groups.

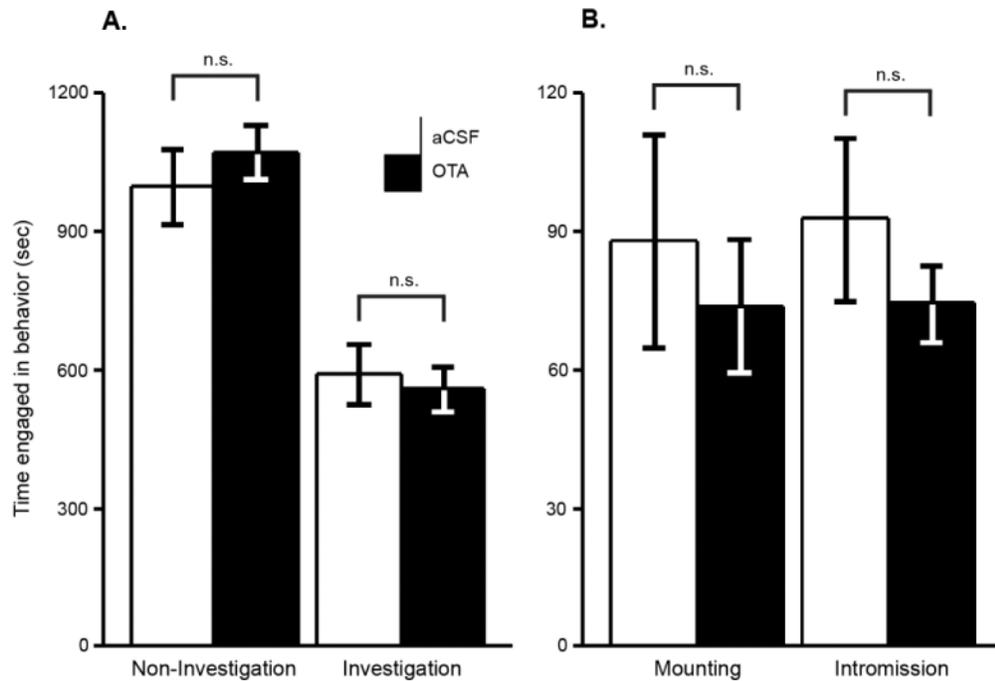


Figure 3. OTA- and aCSF-mated subjects do not differ in time engaged in non-investigation, social investigation, mounting, or intromission

Figure 3 shows the time engaged in (A) non-investigation and social investigation and (B) mounting and intromission between aCSF- (n=11) and OTA-mated (n=9) males during a 30-minute period of sociosexual interaction with a female. There was no main effect of treatment on time engaged in any of these behaviors ($F_{1,68}=0.00$, $p=0.99$, two-way repeated measures ANOVA). “n.s.” indicates that the difference is not statistically significant.

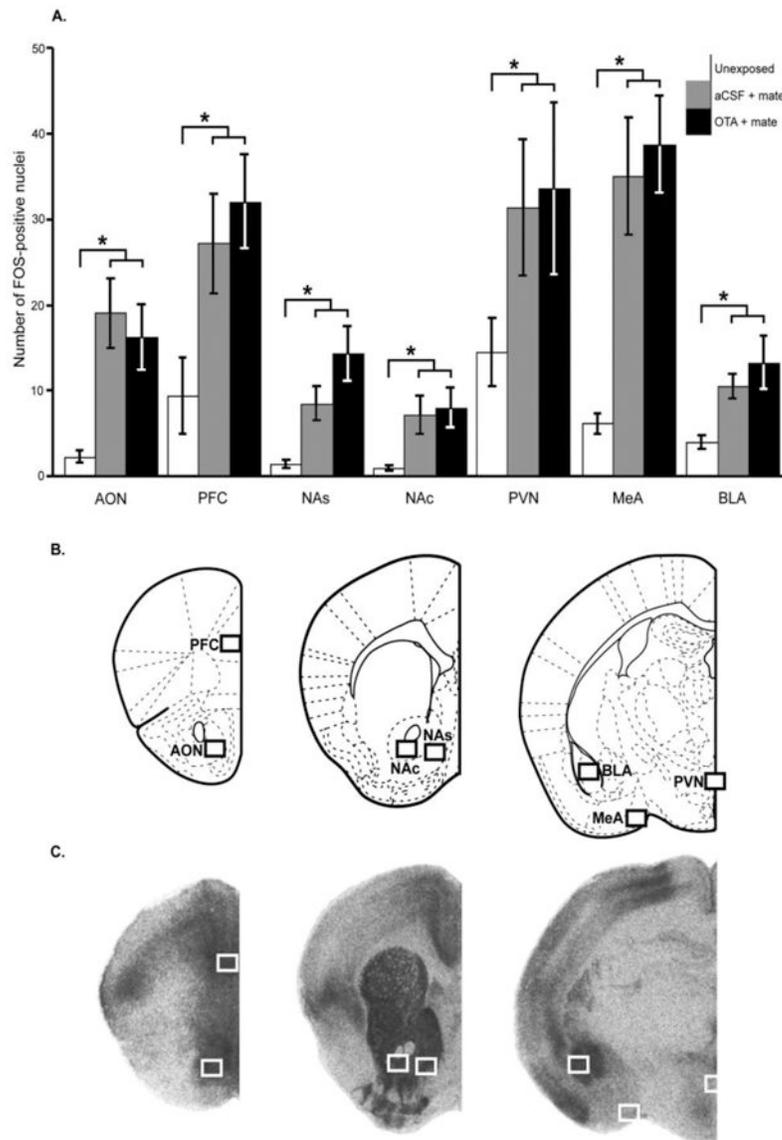


Figure 4. Sociosexual interaction is associated with elevated FOS expression across OTR-dense PBN nodes

Figure 4A shows measures of FOS-positive nuclei across seven PBN nodes in aCSF-unexposed, aCSF-mated, and OTA-mated male prairie voles. There was a significant main effect of treatment on FOS expression across these areas ($F_{2,167}=12.53$; $p=8.48\times 10^{-6}$; two-way repeated measures ANOVA). Within mated subjects, there was no main effect of treatment (OTA versus aCSF) on FOS expression across these areas ($F_{1,130}=1.24$; $p=0.27$). Collapsed across treatments, mated subjects exhibited elevated FOS expression in all of the analyzed brain areas relative to unexposed controls. Asterisks indicate p-values below 0.05. Figure 4B shows the neuroanatomical positions (rectangular frames) at which images were captured for FOS expression analysis in each of these regions using illustrations from The Mouse Brain atlas (Franklin and Paxinos Third Edition). Figure 4C highlights these regions (rectangular frames) on representative autoradiogram films depicting OTR densities (black) in a male prairie vole. Abbreviations: AON=anterior olfactory nucleus; PFC=prefrontal

cortex; NAs=nucleus accumbens shell; NAc=nucleus accumbens core; PVN=paraventricular hypothalamic nucleus; MeA=medial amygdala; BLA=basolateral amygdala.

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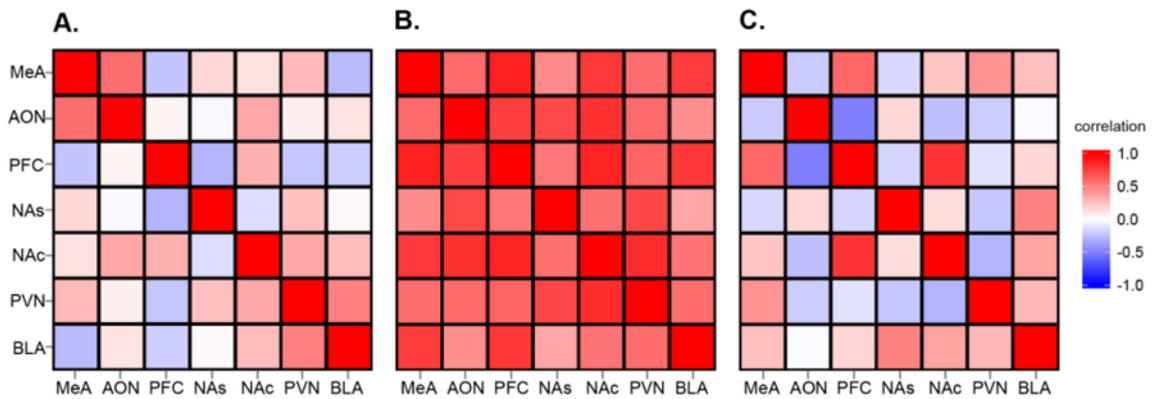


Figure 5. Patterns of covariation between brain regions within the pair-bonding network

Figure 5 shows pair-wise comparisons (Pearson's correlation coefficients) of brain regions involved in pair bonding. The heatmaps are symmetrical, meaning that the upper and lower triangle in each map is showing the same data. Figure 5A shows animals unexposed to individual of the opposite sex and not given any drug treatment. Figure 5B shows mated animals given a central injection of aCSF. Figure 5C shows mated animals given a central injection of OTA. Abbreviations: MeA=medial amygdala; AON=anterior olfactory nucleus; PFC=prefrontal cortex; NAs=nucleus accumbens shell; NAc=nucleus accumbens core; PVN=paraventricular hypothalamic nucleus; BLA=basolateral amygdala.