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Oxytocin receptors modulate a social salience neural network in male prairie voles

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Abstract

Social behavior is regulated by conserved neural networks across vertebrates. Variation in the organization of neuropeptide systems across these networks is thought to contribute to individual and species diversity in network function during social contexts. For example, oxytocin (OT) is an ancient neuropeptide that binds to OT receptors (OTRs) in the brain and modulates social and reproductive behavior across vertebrate species, including humans. Central OTRs exhibit extraordinarily diverse expression patterns that are associated with individual and species differences in social behavior. In voles, OTR density in the nucleus accumbens (NAc)—a region important for social and reward learning—is associated with individual and species variation in social attachment behavior. Here we test whether OTRs in the NAc modulate a social salience network (SSN)—a network of interconnected brain nuclei thought to encode valence and incentive salience of sociosensory cues—during a social context in the socially monogamous male prairie vole. Using a selective OTR antagonist, we test whether activation of OTRs in the NAc during sociosexual interaction and mating modulates expression of the immediate early gene product Fos across nuclei of the SSN. We show that blockade of endogenous OTR signaling in the NAc during sociosexual interaction and mating does not strongly modulate levels of Fos expression in individual nodes of the network, but strongly modulates patterns of correlated Fos expression between the NAc and other SSN nuclei.

Keywords

Immediate early gene; Fos; Social behavior; Sexual behavior; Nucleus accumbens; Striatum; Functional connectivity; Functional coupling; Social decision making network; Social behavior network; Pair bonding network

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1. Introduction

Selective pressures favoring adaptive behavioral interaction with conspecifics have led to the evolution of diverse forms of social behavior within and across species (Hofmann et al., 2014). In vertebrates, social behavior is thought to be modulated by deeply conserved and neuro-peptide-sensitive neural networks. For example, the social decision-making network (SDMN) represents a synthesis of previously described social behavior and mesolimbic reward networks, and is thought to modulate social communication, parenting, sexual, and aggressive behaviors across vertebrates (Goodson, 2005; O'Connell and Hofmann, 2011, 2012).

Research efforts have aimed to identify organizing principles that describe both the anatomy and function of the SDMN. These efforts have led to models that emphasize distributed, interregional information processing among reciprocally interconnected nodes as a critical level of network function (Goodson, 2005; Goodson and Kabelik, 2009). It is explicitly hypothesized that distributed patterns of activity across the network more strongly reflect social context and behavior than activity within individual brain regions. Within this framework, individual nodes are hypothesized to serve dynamic functional roles depending on their functional “connectivity” or “coupling” with other nodes (Goodson and Kabelik, 2009; McIntosh, 2004; Teles et al., 2015). It is further hypothesized that organizational plasticity of steroid hormone and neuropeptide systems across the SDMN is an important mechanism contributing to diversity in distributed network function and social behavior within and across species (Barrett et al., 2013; Cardoso et al., 2015; Goodson, 2005; Goodson and Kabelik, 2009; Johnson and Young, 2015; Newman, 1999).

Although hypothesized links between neuropeptide signaling and distributed network function are consistent with a large body of literature, few studies have directly tested them. The oxytocin (OT) system is well poised for investigating these links. OT is a highly conserved neuro-peptide that modulates reproductive physiology and behavior across bilaterian animals, including nematode worms, rodents, and humans (Garrison et al., 2012; Grinevich et al., 2016; Lee et al., 2009). In vertebrates, the OT system innervates forebrain networks that modulate social behavior. Central distribution patterns and region-specific densities of OT receptors (OTRs) have undergone rapid evolutionary divergence between closely related species of birds and mammals that exhibit divergent social behavior (Anacker and Beery, 2013; Goodson, 2008; Johnson and Young, 2015). The critical functional roles of central and region-specific OTR populations in modulating social behavior have been demonstrated using selective OTR antagonists (OTAs), viral vector-mediated gene transfer, and/or RNAi knockdown in fishes (Goodson and Bass, 2000); birds (Goodson et al., 2009; Pedersen and Tomaszewski, 2012); and mammals (Bosch and Neumann, 2012; Burkett et al., 2016; D'Cunha et al., 2011; Donaldson and Young, 2008; Dumais et al., 2016; Johnson et al., 2016; Keebaugh et al., 2015; Nakajima et al., 2014; Ross et al., 2009b; Song et al., 2016; Young et al., 2001; Yu et al., 2016). Together these data suggest that evolutionary plasticity in region-specific OTR expression is a deeply conserved mechanism contributing to social behavioral diversity within and across species.

Previous experiments in non-mammalian species have used correlated expression of cytochrome oxidase (a metabolic marker) and immediate early genes (IEGs) to demonstrate that distributed patterns of transcriptional and metabolic activity across SDMN nuclei are strongly predictive of social context and behavior (Hoke et al., 2005; Sakata et al., 2000; Teles et al., 2015; Yang and Wilczynski, 2007). We recently used a similar approach to investigate how central OTR activation during a social context modulates IEG expression across a hypothesized social salience network (SSN) in male prairie voles. Briefly, the SSN is a neural network that is hypothesized to modulate selective social attachment and other forms of associative social learning by attaching valence and incentive salience to sociosensory cues. The SSN includes multiple SDMN nodes as well as multiple non-SDMN nodes, including the anterior olfactory nucleus (AON) and prefrontal cortex (PFC)—where OTR activation is critical for social learning and behavior in rodents (Nakajima et al., 2014; Oettl et al., 2016; Young et al., 2001)—and the paraventricular nucleus of the hypothalamus (PVN), a primary site of OT synthesis. We previously found that endogenous central OTR signaling during sociosexual interaction does not strongly modulate levels of Fos-immunoreactivity (Fos-ir) within individual SSN nuclei, but strongly modulates patterns of *correlated* Fos-ir across SSN nuclei, consistent with hypotheses that central OTRs modulate distributed neural network activity during social contexts (Johnson et al., 2016). These experiments, however, did not address the functional roles of region-specific OTR populations. To our knowledge, no experiments to date have tested whether endogenous activation of region-specific OTR populations during social contexts modulates activity-dependent markers (e.g. IEGs) across distributed neural networks. Here we aim to address this question by site-specifically manipulating endogenous OTR signaling in the NAc of male prairie voles during naturalistic sociosexual interaction with a female.

Briefly, the NAc is a SSN (and SDMN) nucleus in which OTRs (or homologous OT-like receptors) are variably expressed across distant vertebrate lineages (O'Connell and Hofmann, 2012). The NAc is anatomically positioned as an integration hub within the SSN, receiving direct projections from every other SSN nucleus—with the exception of the AON—including direct OTRergic axonal projections from the PVN (Ross et al., 2009a). OTR densities in the NAc correlate with socially monogamous mating strategies and other forms of social behavioral variation in prairie voles and other mammals (Insel and Shapiro, 1992; Mooney et al., 2015; Olazabal, 2014; Olazabal and Young, 2006b; Ophir et al., 2012; Ross et al., 2009b); and blockade of endogenous OTR (and other neuromodulatory receptor) signaling in the NAc inhibits pair bond formation and other forms of social behavior in prairie voles and other rodents (Bosch et al., 2016; Keebaugh et al., 2015; Olazabal and Young, 2006a; Ross and Young, 2009; Young et al., 2001; Yu et al., 2016). In humans, intranasal OT administration to romantically attached males modulates BOLD responses in the NAc during viewing of a partner's face, raising the possibility of conserved neural systems modulating selective social attachment in rodents and humans (Scheele et al., 2013). For these reasons, here we administer a selective OTA to test whether endogenous OTR activation in the NAc during sociosexual interaction and mating modulates Fos-ir across the SSN in male prairie voles.

2. Methods

2.1. Subjects

Sexually naïve male prairie voles were group housed until stereotaxic surgery during adulthood (60–180 days), after which they remained singly housed for 5–6 days until behavioral testing. Subjects were housed in ventilated 26 × 18 × 19 cm Plexiglass cages filled with Bedo'cobbs Laboratory Animal Bedding (The Andersons; Maumee, Ohio) kept at 22 °C under a 14:10 h light/dark cycle with ad libitum access to food (Lab Rabbit Diet HF #5326, LabDiet) and water. Subjects were drawn from our laboratory breeding colony originally derived from field captured voles in Champaign, Illinois. Stimulus animals were sexually experienced, ovariectomized, estrogen-primed (see below under “Cohabitation”), 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.

2.2. Cannulation

Adult male prairie voles were anesthetized by continuous administration of 1.5–2.5% isoflurane during stereotaxic implantation of a bilateral 26-gauge guide cannula (catalog no. C235G-2.0/SPC; Plastics One; Roanoke, VA) targeting the dorsal NAc shell with stereotaxic coordinates (from Bregma: A/P + 1.7 mm; M/L ± 1.0 mm; D/V – 4.5 mm; Fig. 1A). 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 5–6 days following surgery (Fig. 1B).

2.3. Antagonist administration

After recovery, and 3 h prior to cohabitation (Fig. 1B), subjects received 1–2% isoflurane anesthesia during bi-lateral microinjection of either 500 nL of control aCSF (n = 14 exposed; n = 6 unexposed) or 2.5 ng/μL OTA, peptidergic ornithine vasotocin analog desGly–NH₂,d(CH₂)⁵[Tyr(Me)², Thr⁴]OVT (Bachem, Torrance, CA), dissolved in 500 nL aCSF (n = 16 exposed) in accordance with previous experiments using site-specific OTA administration targeting forebrain nuclei (Burkett et al., 2016). Microinjections were performed using a 33-gauge internal cannula (C235I/SPC; Plastics One; Roanoke, VA) extending 0.5 mm beyond the end of the guide cannula. Injections were performed with a 10 μL Hamilton syringe (Hamilton; Reno, Nevada) connected to polyethylene-20 tubing (Plastics One), which in turn was connected to the internal cannula. Infusions were administered over 60 s, and the internal cannula was left in place for 3 min following infusion to prevent backflow. One subject (aCSF-treated, unexposed) was excluded due to significant backflow during infusion (final aCSF-unexposed group size, n = 5). All subjects received two consecutive infusions, one into each hemisphere. Larger group sizes were selected for socially exposed treatment groups to obtain sufficient samples of subjects that mated during cohabitation.

2.4. Cohabitation

As seen in Fig. 1B, 3 h after aCSF/OTA infusion, subjects underwent 30 min of video-recorded cohabitation with a sexually experienced, ovariectomized, estrogen-primed, adult stimulus female. In the three days preceding cohabitation, stimulus females were brought into behavioral estrus with daily subcutaneous injections of 4.0 μ g 17 β -estradiol benzoate dissolved in sesame oil (Sigma; St. Louis, MO; S3547). Cohabitation began with the introduction of the stimulus female into the male subject's home cage with food hoppers removed to facilitate video recording and behavioral analysis. Lab chow pellets and water bottles were left in the home cage to allow ad libitum access to food and water during the cohabitation. After 30 min of cohabitation, stimulus females were removed and subjects remained in their home cage for 45 min before transcardial perfusion (75 min following initial introduction of the stimulus female). For unexposed control subjects ($n = 5$), the cage was opened and a small amount 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 stimulus females.

2.5. Perfusion, post-fixation, and sectioning

75 min after the initiation of cohabitation (or control sessions), subjects were administered an overdose of isoflurane and were immediately perfused transcardially at a rate of approximately 4 mL/min with 40 mL of 1x phosphate buffered saline (PBS, pH = 7.4; diluted to 1x in distilled water from 10x PBS stock; Teknova; Hollister, CA; P0401) followed by 40 mL of 4% paraformaldehyde (Polysciences; Warrington, PA; 00380) in 1x PBS using an Easy-Load II MASTERFLEX pump (Cole-Palmer; Vernon Hills, IL). Immediately following perfusion, brains were extracted and post-fixed in 4% paraformaldehyde dissolved in 1X PBS overnight before storage in 30% sucrose in 1x PBS solution until sectioning. Brains were cut into 40 μ m sections using a Microm HM 440E freezing microtome and were stored in 1x PBS with 0.05% sodium azide until Nissl or immunohistochemical staining.

2.6. Behavioral scoring

Five mutually exclusive behaviors were quantified using Observer XT 10 behavioral scoring software (Noldus Information Technology Inc.; Leesburg, VA). These behaviors included non-interaction, social investigation, mounting, intromission, and huddling behavior. The operational definitions used for each behavior were as follows. Noninteraction included any male behavior not directed toward the female, including autogrooming, running, freezing, eating, drinking, sleeping, digging, and exploration. Investigation included social behavior directed toward the female, including physical pursuit, olfactory investigation, and allogrooming. Mounting was defined as placement of the forepaws on the female from the rear without intromission. Intromission was defined by stereotypical patterns of pelvic movement during copulation bouts with the female. Huddling behavior was defined as direct, side-by-side social contact between the male and female.

2.7. Nissl staining

Sections containing cannula implant tracks were mounted onto Superfrost Plus slides (Fisher Scientific; 12-550-150) and allowed to dry overnight before Nissl staining. Immediately prior to staining, sections were submerged in 100% ethanol (EtOH) for 2 min, 95% EtOH for 2 min, and 70% EtOH for 2 min. Sections were stained in 0.1% cresyl violet in distilled water for 2 min. After staining, sections were dehydrated in 95% EtOH twice for 2 min and then 100% EtOH twice for 2 min. Prior to coverslipping, slides were submerged in a 1:1 EtOH:HistoClear mixture for 2 min and were then submerged in fresh HistoClear twice for 2 min. Immediately after HistoClear treatment slides were coverslipped while still partially wet using Krystalon (EMD Chemicals Inc., Gibbstown, NJ).

2.8. Injection site validation

Anatomical placement of cannula implants was determined on Nissl-stained sections. Infusion sites were validated using a Nikon E800 microscope with a 10x objective. Subjects were included if the cannulation track terminated within 0.5 mm of the dorsal NAc. Two subjects were excluded; one (OTA-treated, exposed) due to an anterior miss, and a second (aCSF-treated, exposed) due to extensive unilateral leakage of blood through the cannula and into the NAc. Cannulation tracks in all remaining subjects terminated within 0.5 mm of the dorsal NAc and were included in subsequent behavioral analyses (Fig. 1A; n = 13 aCSF-treated, exposed; n = 15 OTA-treated, exposed; n = 5 aCSF-treated, unexposed).

2.9. Immunohistochemistry

Sections underwent three washes in 1x PBS, 10 min of incubation in 1% sodium hydroxide in 1x PBS, and three washes in 1x PBS with 0.5% Triton-X (PBST; Sigma) before treatment with 5% normal goat serum (Fitzgerald; Acton, MA) in PBST for 1 h at room temperature. Sections were then incubated for 48 h in primary rabbit polyclonal anti-Fos antibody (diluted in PBST to a final concentration of 1:20,000; Calbiochem ABE457) on an orbital shaker at 4 °C. Following primary incubation sections were washed five times in 1x PBS, once in 1x PBST, and were then incubated in secondary biotinylated goat anti-rabbit IgG antibody (diluted 1:500 in PBST; Vector Labs, BA-1000) for 2 h. After secondary incubation, sections were treated with an avidin-biotin peroxidase system (Vectastain Elite ABC System; Vector Labs: PK6100) for 1 h before staining with a Nickel-DAB peroxidase substrate kit (Vector Labs; SK4100). All sections were dehydrated in a series of increasingly concentrated ethanol solutions (5 min in 70% EtOH, 10 min in fresh 95% EtOH twice, 10 min in fresh 100% EtOH twice), bathed in Xylenes (15 min 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).

2.10. Selection of SSN nuclei

Fos-ir was analyzed in seven SSN nuclei: the NAc shell, AON, PFC, PVN, medial amygdala (MeA), basolateral amygdala (BLA), and ventral tegmental area (VTA). All of the analyzed regions are innervated by OTergic fibers, reciprocally interconnected with other SSN nuclei, and express OTRs in male prairie voles (see Fig. 2C for a simplified schematic of the SSN). The NAc shell was selectively targeted with OTA because OTR density and activation within

this nucleus are associated with social learning and behavior in prairie voles and other rodents (Barrett et al., 2015; Bosch et al., 2016; Dolen et al., 2013; Keebaugh et al., 2015; Mooney et al., 2015; Okuyama et al., 2016; Olazabal, 2014; Olazabal and Young, 2006a, 2006b; Ophir et al., 2012; Ophir et al., 2009; Ross et al., 2009b; Young et al., 2001; Yu et al., 2016; Zheng et al., 2013). Similarly, in the PFC, OTRs modulate multiple forms of social learning and behavior in rodents (Brill-Maoz and Maroun, 2016; Nakajima et al., 2014), including pair bonding in prairie voles (Young et al., 2001). The VTA also modulates pair bonding in male prairie voles (Curtis and Wang, 2005); projects to, releases dopamine (DA) within, and modulates IEG expression in multiple SSN nuclei (Argiolas and Melis, 2013); and contains OTR-expressing neurons that modulate both social behavior and dopamine release into the NAc shell (Peris et al., 2016; Shahrokh et al., 2010; Song et al., 2016; Stivers et al., 1988). The AON and MeA were selected due to their role in OTR-dependent social odor processing and social recognition (Ferguson et al., 2001; Gur et al., 2014; Oettl et al., 2016; Wacker and Ludwig, 2012). Direct projections from the BLA modulate both cue-evoked excitation of accumbal neurons and reward-seeking behavior; and this circuit is thought to relay sociosensory information from the amygdala to the NAc shell during formation of selective social attachments (Ambroggi et al., 2008; Numan and Young, 2016). Finally, the PVN is a major site of OT synthesis and somatodendritic OT release, and is the predominant source of OTergic fiber innervation to the NAc shell (Bosch et al., 2016; Ross et al., 2009a) and other SSN nuclei (Knobloch et al., 2012).

2.11. Imaging and analysis

Because the aim of these experiments was to investigate the effects of region-specific OTR signaling on Fos-ir, analyses of Fos-ir were restricted to subjects that mated during cohabitation (aCSF, $n = 11$; OTA, $n = 12$) to maximize divergence in presumed OT signaling (mating induces OT release) between socially-exposed treatment groups, consistent with previous experiments (Johnson et al., 2016). Images were captured through a Nikon E800 microscope and a 4x objective using MCID Core imaging software (InterFocus Ltd.; Cambridge, UK). The Mouse Brain (Franklin and Paxinos; 3rd Edition) atlas was used as a reference to determine anatomical boundaries. For the NAc, PFC, and MeA, images were captured from the shell, prelimbic, and posterodorsal divisions, respectively. For each brain region, Fos-ir was quantified in both left and right hemispheres across three consecutive sections for all subjects. Sections or hemispheres with damaged or absent tissue in the target region were excluded. Fos-positive nuclei were quantified for each brain region using the MCID grain count function with constant size, density, and shape thresholds applied across all subjects. For each subject, Fos-ir counts within each region were averaged, yielding a single average Fos-ir count per region per subject.

2.12. Statistics

2.12.1. Immunohistochemistry batch effects and data transformation—

Immunohistochemistry was performed in two batches for these experiments. To control for differences between batches, batch was included as a random effect in all linear mixed-effects regression analyses of raw Fos-ir count values. For multiple linear regression (MLR) and principle components analysis (PCA) of covariance, raw Fos-ir count values were

transformed into standardized Fos-ir z-scores within each batch in order to allow pooling of data across batches.

2.12.2. Sociosexual behaviors—Behavioral data from cohabitation were analyzed using a two-way repeated-measures ANOVA with treatment (OTA versus aCSF) as a between subjects factor and behavior (non-interaction, investigation, mounting, intromission, and huddling) as a within subjects factor. Poisson regression was used for analysis of count outcome variables (e.g. mating bout frequency). For all ANOVAs, effect size (Eta-squared) was calculated by dividing the individual effect sum of squares by the total sum of squares.

2.12.3. Fos-ir across the SSN—To test whether levels of Fos-ir across the SSN were influenced by treatment, we used a linear mixed-effects model (the “lme” function in the statistical software package R) with number of Fos-positive nuclei per mm² (hereafter referred to as “Fos-ir count”) as the dependent variable, treatment (aCSF-mated, OTA-mated, or aCSF-unexposed) and brain region (see “Selection of SSN nuclei” above) as fixed effects, and immunohistochemistry batch (see “Data transformation” above) and individual subject ID as random effects. To measure the effect of socio-sexual interaction and mating on Fos induction across the SSN, we first tested whether “treatment” was a significant predictor of Fos-ir count among only mated subjects. To do this, we excluded unexposed subjects and again used a linear mixed-effects model fitting Fos-ir count against treatment and brain region, with batch and individual subject ID included as random effects. Based on these results, treatment was removed as a predictor and Fos-ir count was fitted against “mating” (unexposed versus mated; with OTA-mated and aCSF-mated subjects pooled) and brain region (both fixed effects), with batch and individual subject ID included as random effects. We then measured the effect of sociosexual interaction and mating on Fos induction in each individual SSN nucleus using linear mixed-effect models that fit Fos-ir count in the region of interest against mating, with batch included as a random effect.

2.12.4. Effect of OTR antagonist administration on Fos-ir in the NAc shell—To test the hypothesis that site-specific manipulation of OTR signaling in the NAc influenced local Fos induction, we restricted analysis to mated subjects and used a linear mixed-effects model with Fos-ir count in the NAc shell as the dependent variable, treatment as a fixed effect, and batch as a random effect.

2.12.5. Fos-ir covariance between the NAc shell and other SSN nuclei—To investigate the hypothesis that blocking OTRs in the NAc specifically disrupts correlated Fos-ir between the NAc shell and the other SSN nodes, we transformed raw Fos-ir count values into standardized Fos-ir z-scores for each batch to enable pooling across batches (see “Data transformation” section above). We then used a multiple linear regression (MLR; the “lm” function in R) model with Fos-ir z-score in the NAc shell as the dependent variable and Fos-ir z-scores in the six other SSN nuclei as independent variables. Briefly, this analysis assessed the relationship between Fos-ir in the NAc shell and Fos-ir in the other SSN nuclei while simultaneously accounting for relationships among the other SSN nuclei. We extracted R^2 values from these analyses and compared how much of the variance of Fos-ir in the NAc shell was explained by Fos-ir in the other SSN nuclei between groups treated with OTA

versus aCSF. To test for statistical significance, we generated a permuted null distribution through multiple, random assignment of individuals to either of the investigated treatment groups, without replacement. For each permutation we calculated the multiple R^2 for both groups and the absolute value of the difference of this value between treatment groups. 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. We also extracted residuals from these analyses to test whether observed Fos-ir counts deviated from those predicted by the MLR model differentially by treatment (OTA versus aCSF). In order to test this, we first confirmed that these residuals did not significantly deviate from normality (Shapiro-Wilks test; $p = 0.5822$). We then used an F-test to determine whether variance of MLR residuals significantly differed between OTA- versus aCSF-treated groups.

2.12.6. Global Fos-ir covariance across the SSN—These analyses were performed as previously described and combined principal component analyses (PCA) to estimate effect size (difference in variance explained by the first principal component) and permutation testing to assess statistical significance of the effect (Johnson et al., 2016). For PCA analyses, we again used standardized Fos-ir z-scores to enable pooling across batches (see “Data transformation” section above).

3. Results

3.1. Mating frequency

We previously demonstrated that central OTR blockade reduces mating frequency in male prairie voles (Johnson et al., 2016), consistent with findings in rats (Argiolas et al., 1988). Here, we tested whether this effect is mediated specifically by OTRs within the NAc. aCSF-treated males ($n = 13$) engaged in an average frequency of 4.11 ± 1.05 intro-mission bouts per 30 min with the stimulus female compared to 5.56 ± 1.19 bouts per 30 min in OTA-treated males ($n = 15$). A regression analysis comparing Poisson distributions of mating bouts showed no significant differences in mating frequency between OTA- and aCSF-treated subjects ($z = 1.328$; $p = 0.184$), suggesting that OTRs in other nuclei modulate mating frequency in male prairie voles.

3.2. Sociosexual behaviors

We next tested whether OTR antagonism in the NAc was associated with differences in duration of sociosexual behavior (non-interaction, investigation, mounting, intromission, or huddling). Among subjects that mated with the stimulus female (aCSF, $n = 11$; OTA, $n = 12$), a two-way repeated-measures ANOVA revealed a significant main effect of behavior (non-interaction, investigation, mounting, intromission, or huddling; $F_{1,109} = 68.00$; $p < 0.001$; Eta-squared = 0.38), but not of treatment (aCSF versus OTA; $F_{1,109} = 0.01$; $p = 0.99$; Eta-squared < 0.01) or the interaction between behavior and treatment ($F_{1,109} = 0.01$; $p = 0.93$; Eta-squared < 0.01) on time engaged in the analyzed social behaviors during the 30 min period of cohabitation (Fig. 1C). Thus, among mated animals, OTA versus aCSF treatment had no detectable effect on any of the analyzed sociosexual behaviors, consistent with previous results following ICV administration (Johnson et al., 2016). Importantly, these

data suggest that any observed differences in Fos-ir between OTA- and aCSF-treated subjects in these experiments were not due to differences in performance of the analyzed social behaviors.

3.3. Fos-ir across the SSN

We previously found that sociosexual interaction and mating with a female is associated with a robust increase in Fos-ir across SSN nuclei in male prairie voles, regardless of central aCSF versus OTA administration (Johnson et al., 2016). As a positive control, we tested whether sociosexual interaction and mating with the stimulus female was associated with increased Fos-ir across SSN nuclei in the present experiments. To test this, we first fit a linear mixed-effects model with Fos-ir count as the dependent variable, treatment (aCSF-unexposed, aCSF-mated, OTA-mated) and brain region as fixed effects, and batch and individual subject ID as random effects. These analyses revealed significantly increased Fos-ir in aCSF-mated ($t_{2,24} = 3.74$, $p = 0.001$) and OTA-mated ($t_{2,24} = 4.47$, $p < 0.001$) subjects compared to unexposed controls. To test whether Fos-ir differed by aCSF- versus OTA-treatment among mated animals, we restricted analysis to mated subjects and fit a linear mixed-effects model with Fos-ir count as the dependent variable, treatment (aCSF or OTA) and brain region as fixed effects, and batch and individual subject ID as random effects. This test showed that Fos-ir did not significantly differ between aCSF-mated and OTA-mated animals across brain regions ($t_{1,20} = 0.87$, $p = 0.39$). These results suggest that sociosexual interaction and mating, but not OTR blockade in the NAc, strongly influenced levels of Fos-ir across SSN nuclei. To validate this interpretation, we excluded treatment as a predictor and fit a linear mixed-effects model of Fos-ir count against “mating” (OTA-mated and aCSF-mated subjects pooled versus unexposed controls) and brain region, with batch and individual subject ID included as random effects. This test confirmed that sociosexual interaction and mating with a female was associated with a significant increase in Fos-ir across the SSN ($t_{1,25} = 4.49$, $p < 0.001$), consistent with previous results (Johnson et al., 2016). To complement these analyses, we tested whether sociosexual interaction and mating was associated with increased Fos-ir in each SSN nucleus. For each individual brain region, we fit a linear mixed-effects model of Fos-ir count against mating (OTA-mated and aCSF-mated subjects pooled versus unexposed controls), with batch included as a random effect. These analyses confirmed that sociosexual interaction and mating with a female was associated with a significant ($p < 0.05$) increase in Fos-ir in all analyzed SSN nuclei with the exception of the VTA (Fig. 2A; Supplementary Table 1).

3.4. Fos-ir in the NAc shell

Because we used a selective OTR antagonist to manipulate OTR signaling specifically within the NAc, we tested whether this manipulation influenced local Fos-ir. To test this, we restricted analysis to mated animals and fit a linear mixed-effects model of Fos-ir count in the NAc shell against treatment (aCSF or OTA), with batch included as a random effect. This analysis revealed a trend toward increased Fos-ir in OTA-treated compared to aCSF-treated subjects, although this trend was not statistically significant ($t_{1,20} = 1.85$, $p = 0.08$).

3.5. Correlated Fos-ir between the NAc shell and other SSN nodes

We hypothesized that site-specific infusion of a selective OTA into the NAc would decrease correlated Fos-ir between the NAc shell and other SSN nodes. Consistent with this hypothesis, a MLR model fitting Fos-ir count in the NAc shell against Fos-ir count in every other SSN node revealed that Fos-ir count in non-accumbal SSN nodes explained a large amount of the variance in Fos-ir count in the NAc shell in aCSF-treated animals ($R^2 = 0.91$) but not in animals treated with OTA ($R^2 = 0.20$). In support of our hypothesis, permutation testing indicated that this difference in variance explained (71%) was statistically significant ($p = 0.02$). We also extracted residuals from the MLR model and tested whether the variance of the residuals differed between groups treated with OTA versus aCSF. In further support of our hypothesis, the variance of MLR residuals was significantly greater in OTA- ($\sigma^2 = 0.82$) compared to aCSF- ($\sigma^2 = 0.05$) treated subjects (F-test; $F_{1,20} = 17.25$, $p < 0.001$; Fig. 2B).

3.6. Global covariance of Fos-ir across the SSN

We previously found that blockade of central OTRs during sociosexual interaction and mating was associated with a “global” decrease in correlated Fos-ir across the SSN. We hypothesized that this effect was mediated by disruption of OTR signaling simultaneously across many interconnected forebrain nuclei during sociosexual interaction and mating. In these experiments, we hypothesized that blocking OTRs specifically in the NAc would result in disruption of covariance between the NAc shell and the other SSN nodes, in contrast to the more global effect we observed in our previous experiments following ICV administration of OTA (Johnson et al., 2016). To test this prediction, we combined PCA and permutation testing and tested whether OTR blockade in the NAc influenced global covariance of Fos-ir across the SSN as a whole. In line with our hypothesis, we found no effect of treatment on global covariance of Fos-ir across the SSN; the variance explained by the first principal component was virtually identical in animals treated with OTA and aCSF (difference in $R^2 < 0.01$, $p = 0.98$).

4. Discussion

Site-specific blockade of OTRs in the NAc had no effect on mating frequency or any of the analyzed sociosexual behaviors in male prairie voles during 30 min of sociosexual interaction with a female. We previously demonstrated that ICV administration of OTA reduces mating frequency in male prairie voles (Johnson et al., 2016). Taken together, these data suggest that OTRs in other nuclei modulate sexual behavior in male prairie voles, consistent with findings in rats (Argiolas and Melis, 2013; Gil et al., 2011). Importantly, these results suggest that any significant differences in Fos-ir between treatment groups are not due to differences in any of the analyzed sociosexual behaviors. Additionally, because most of the analyzed behaviors are active behaviors, it is unlikely that differences in Fos-ir between treatment groups are accounted for by differences in movement during the cohabitation, although we could not directly analyze locomotion per se.

Here we use Fos-ir as an indicator of transcriptional activity and synaptic plasticity across the SSN. Briefly, Fos is the protein product of the immediate early gene *C-fos*. In response to synaptic input, Ca^{2+} influx triggers Ca^{2+} -dependent kinase cascades that regulate

transcription of *C-fos* across many, but not all, neuronal phenotypes (Kawashima et al., 2014). Following translation, Fos protein heterodimerizes with JUN to form AP-1 transcription factor complexes that regulate downstream expression of genes involved in a variety of intracellular processes, including synaptic plasticity (Lyons and West, 2011). Thus, central Fos-ir is thought to reflect rapid adaptation of neuronal and synaptic physiology in response to synaptic input through robust transcriptional regulation, and has been linked with depolarization in many, but not all, neuronal phenotypes.

Fos-ir across SSN nuclei was significantly higher in mated versus un-exposed subjects, regardless of OTA- versus aCSF-treatment, confirming that sociosexual interaction and mating was associated with a robust induction of Fos-ir across the network. This pattern was observed in all brain regions analyzed, with no region-specific differences in Fos-ir distinguishing aCSF-treated versus OTA-treated animals. Notably, however, we observed a statistical trend toward increased Fos-ir in OTA-treated versus aCSF-treated subjects in the NAc shell, similar to previous results following ICV administration of OTA (Johnson et al., 2016). Together these data raise the possibility that endogenous activation of OTRs during sociosexual interaction and mating attenuates Fos induction in populations of accumbal neurons, and encourage future investigation of these relationships.

We hypothesized that endogenous activation of accumbal OTRs modulates correlated Fos-ir between the NAc shell and other SSN nuclei during sociosexual interaction and mating. In support of this hypothesis, we observed a robust and significant effect of treatment on correlated Fos-ir between the NAc shell and other SSN nuclei; Fos-ir counts in non-accumbal SSN nuclei collectively explained the vast majority of variance of Fos-ir counts in the NAc shell in aCSF-treated (91% of variance explained) but not OTA-treated (20% of variance explained) subjects. In further support of our hypothesis, we found that the variance of residuals extracted from the MLR model was significantly greater among OTA-treated compared to aCSF-treated subjects, indicating that observed Fos-ir count values deviated further from MLR-predicted Fos-ir count values in OTA- compared to aCSF-treated subjects. The effect of treatment on correlated Fos-ir across SSN nuclei was not generalizable to the network as a whole, as PCA combined with permutation testing suggested that global patterns of covariance of Fos-ir across the SSN did not differ between aCSF- and OTA-treated subjects. Taken together these data provide strong evidence that endogenous accumbal OTR signaling during sociosexual interaction and mating robustly modulates how synaptic plasticity and transcriptional activity in the NAc shell are correlated with synaptic plasticity and transcriptional activity in other nodes of the SSN.

Given the anatomical connectivity between the NAc shell and other SSN nuclei, these data raise the possibility that activation of accumbal OTRs during sociosexual interaction and mating modulates functional coupling between neuronal populations embedded within the NAc shell and neuronal populations embedded across SSN nuclei. Considering the trend toward elevated accumbal Fos-ir in OTA-mated subjects, it is intriguing to speculate that endogenous activation of OTRs in the NAc shell attenuates excitability of some neuronal subpopulations while simultaneously promoting a “state” of enhanced transmission across SSN projections to the NAc shell. Such a mechanism would be consistent with hippocampal slice recordings in rats in which selective OTR agonists greatly improve signal-to-noise ratio

and cortical information transfer by suppressing background noise (through inhibitory interneuronal networks) while increasing fidelity of spike transmission and spike timing onto CA1 pyramidal neurons (Owen et al., 2013).

The hypothesis that central OTR activation modulates correlated activity across distributed brain networks is consistent with continually emerging data from experiments in primates. For example, fMRI experiments in both rhesus macaques and humans have found that intranasal OT administration modulates correlated fluctuations in BOLD responses across limbic, striatal, cortical, and/or brainstem nuclei during a variety of social contexts (Bethlehem et al., 2013; Gorka et al., 2015; Kirsch et al., 2005; Liu et al., 2015; Riem et al., 2012; Rilling et al., 2012). However, investigations using intranasal OT should be interpreted with caution (Leng and Ludwig, 2016; Walum et al., 2016). Two studies have shown that naturally-occurring polymorphisms in human *OXTR* (the gene encoding OTR) are associated with correlated fluctuations in BOLD responses between hypothalamic, limbic, and/or cortical brain areas during social tasks (Tost et al., 2010; Tost et al., 2011), and one has shown that methylation of *OXTR* in peripheral blood cells is associated with decreased correlated fluctuations in BOLD responses among limbic and cortical regions while viewing fearful and angry faces (Puglia et al., 2015). However, it is unclear whether genetic/epigenetic effects are mediated directly (e.g. through differences in central OTR activation) or indirectly (e.g. through developmental differences in OTR expression or differences in peripheral OTR signaling). Additionally, in contrast to many non-primate species, hurdles in the development of specific ligands for primate OTRs have delayed mapping of central OTR distributions in the human brain (Freeman et al., 2014; Freeman and Young, 2016; Smith et al., 2012). It is therefore unclear whether the genetic and/or epigenetic polymorphisms investigated in these experiments reflect variation in OTR expression in the human brain. However, it is important to note that *OXTR* polymorphisms in the prairie vole robustly predict individual differences in OTR density in the NAc as well as pair bonding behavior (King et al., 2016).

There are several important limitations to our data. Firstly, the neuronal phenotypes and anatomical projections of Fos-positive neurons within each SSN nucleus are unknown, and therefore identifying anatomically interconnected neuronal subpopulations that become functionally connected during social contexts is a critical area for future investigation. Secondly, site-specific OTA administration targets all region-specific OTRs, obscuring any distinct functional roles that may depend on neuronal phenotype and cellular localization; future electrophysiological investigations are necessary to dissect the neuromodulatory roles of OTRs in different cellular contexts. Thirdly, these experiments focused on OTRs in a single SSN nucleus in male prairie voles. Future investigations of other steroid and neuropeptide systems, additional brain areas, female subjects, and alternative behavioral contexts and species are needed. Finally, site-specific OTR blockade does not likely reflect natural intra- and interspecific variation in levels of region-specific OTR expression and signaling (King et al., 2016). Future studies should investigate how natural variation in central OTR distribution influences distributed neural network function during social contexts within and across species.

Despite these limitations, these data advance our understanding of how neuropeptide systems modulate distributed network function. To our knowledge these are the first data to demonstrate that endogenous activation of a region-specific neuropeptide receptor population modulates distributed patterns of transcriptional activity across a neural network in a behaving vertebrate, linking OTR populations that are critical for social behavior with network properties that are hypothesized to be critical for social information processing. In contrast to previous experiments following central OTR blockade, here we show that site-specific blockade of OTRs in the NAc during sociosexual interaction does *not* modulate global patterns of correlated Fos-ir across nodes of the SSN. Instead, these data support the view that activation of accumbal OTRs selectively modulates coupling of the NAc shell with other SSN nodes. Taken together, these experiments are consistent with hypotheses that central and region-specific OTR signaling contribute to individual and species diversity in distributed neural network function during social contexts. This may occur in part through OTR-mediated promotion of transient network states that improve transmission from other nodes of the SSN to the NAc shell, facilitating attachment of salience and valence to sociosensory cues and thereby promoting social learning.

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

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Fig. 1.

Site-specific administration of OTA into the NAc has no effect on male sociosexual behaviors. Fig. 1A depicts cannula placement across all subjects, with cannula tracks represented by solid black vertical lines and the NAc (shell and core) represented by dotted boundary lines. Fig. 1B shows a schematic of the experimental timeline and design (D = day; m = male; f = female). Prior to testing, adult males received site-specific administration of either aCSF or OTA into the NAc. Fig. 1C shows box-and-whisker plots of the time engaged in non-interaction, social investigation, mounting, intromission, and huddling among mated males ($n = 11$ aCSF; $n = 12$ OTA) during 30 min of sociosexual interaction with a female. There was no main effect of treatment (aCSF versus OTA) on time engaged in any of these behaviors (two-way repeated measures ANOVA; $F_{1,109} < 0.01$, $p > 0.99$; Eta-squared < 0.01).

Fig. 2.

Effects of mating and accumbal OTR signaling on Fos-ir across the SSN. Fig. 2A shows Fos-ir, represented as least squared mean estimates of Fos-positive nuclei/mm², in each SSN nucleus across treatment groups (aCSF-unexposed, $n = 5$; aCSF-mated, $n = 11$; OTA-mated, $n = 12$). Sociosexual interaction and mating resulted in a robust induction of Fos-ir across the SSN (linear mixed-effects model; $t_{1,25} = 4.49$, $p < 0.001$). The direction of this effect was consistent in all SSN nuclei and was significant ($p < 0.05$) in every region except the VTA (see Supplementary Table 1). Among mated subjects, in the NAc shell, aCSF-treated males exhibited a trend toward decreased Fos-ir relative to OTA-treated males, although this effect was not statistically significant ($t_{1,20} = 1.85$; $p = 0.08$). A multiple linear regression (MLR) model revealed that Fos-ir across non-accumbal SSN nuclei explained a large amount of the variance of Fos-ir in the NAc shell in aCSF- ($R^2 = 0.91$) but not OTA- ($R^2 = 0.20$) treated subjects. A permutation test indicated that this difference in variance explained (71%) was statistically significant ($p = 0.02$). Fig. 2B illustrates the distribution of residuals

extracted from the MLR model. The variance of residuals was significantly greater in OTA- compared to aCSF-treated subjects (F-test; $F_{1,20} = 17.25$, $p < 0.001$). Fig. 2C illustrates neuroanatomical connectivity of the NAc shell with other SSN nuclei. In Fig. 2A, error bars indicate $\pm$ standard error of the mean, asterisks indicate $p < 0.05$, and daggers indicate $p < 0.10$. In Fig. 2C, dotted lines represent olfactory pathways; arrows represent excitatory input, perpendicular lines represent inhibitory input, and filled triangles represent innervation by OT fibers originating from the PVN. Abbreviations: AON = anterior olfactory nucleus; BLA = basolateral amygdala; MeA = medial amygdala; MOB = main olfactory bulb; NAc shell = nucleus accumbens shell; PFC = prefrontal cortex; Pir = piriform cortex; PVN = paraventricular nucleus of the hypothalamus; Tu = olfactory tubercle; VTA = ventral tegmental area.