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Fuad Haddad April 12th, 2016

Natural Variations within the Oxytocin Receptor Gene Modulate Neural Network Activity Associated with Pair Bonding Behavior in Male Prairie Voles

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Oxytocin (OT), a hormonal peptide, has been evolutionarily conserved across a variety of species to regulate reproductive and social behavior. OT acts by binding to oxytocin receptors (OTR) which have been suggested to play a critical role in mating, pair bonding, and maternal behavior. Furthermore, natural OTR variations influence pair bonding behavior. While OTR has been found to modulate functional connectivity across a hypothesized neural network of pair bonding nuclei, the question as to whether naturally occurring variations in OTR expression are associated with differences in functional connectivity remains unclear. Understanding the relationships between natural *Oxtr* variation (the gene encoding oxytocin receptor) and differences in neural activity during social contexts will provide insights for human social cognition and therapeutic applications for social deficits in autism, depression, schizophrenia, and psychopathy.

METHODS:

The prairie vole was used as an experimental model due to its well-researched application as a translational model for pair bonding. Male prairie voles were subjected to a 30 minute cohabitation paradigm with treatment being exposure to a female. Fos, an early immediate gene protein product, was used to quantify neuronal activation and correlated activity within regions of interest (ROIs) associated with pair bonding. Males were genotyped at a previously identified single nucleotide polymorphism within *Oxtr* to predict levels of central OTR expression.

RESULTS:

Analysis of Fos expression demonstrated that natural OTR variation within the nucleus accumbens (NAc) modulates functional connectivity between hypothesized ROIs such that males carrying the high expressing *Oxtr* genotype displayed decreased correlated activity between the ventral pallidum (VP) and prefrontal cortex (PFC) and anterior olfactory nucleus compared to those with the low expressing *Oxtr* genotype.

CONCLUSION:

Natural variations in OTR lead to modulations in functional connectivity between pair bonding brain nuclei in male prairie voles during sociosexual interaction with a female and provide important insights for understanding how natural OTR variation may affect pair bonding and other complex social behavior.

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Introduction

The Phenomenon of Monogamy

Comparing the endearing relationships between family, friends, or lovers, the ability to create social bonds is a central function of human development and propagation. Deficits in this ability manifest within several psychological disorders such as autism, depression, schizophrenia, and psychopathy (Volkmar, 2001, Feldman et al., 2016). Contrastingly, strong relationships with sexual partners, or pair bonds, serve as the foundations of affiliative and nurturing behavior. These relationships are prevalent within monogamous social organizations, which has been hypothesized to be the core of human social structure (Chapais, 2013).

While the popular conception of monogamy refers to genetic monogamy, the exclusive association and mating between conspecifics, the prevalence of extra-pair copulations in nature suggests that social rather than genetic monogamy more accurately reflects the behavior of many monogamous species. Among non-human mammals, a socially monogamous relationship does not require sexual exclusivity but usually includes preferential mating, cooperative nesting, and bi-parental offspring care. With only 3-5% of mammals satisfying these criteria, the prairie vole has been targeted as a candidate for translational research to study pair bonding and other complex social behaviors not observed in more common laboratory rodents like mice and rats. By manipulating this model, researchers are able to understand the neurobiology and mechanisms associated with monogamous behavior, which may have implications for understanding human social cognition, behavior, and pathology (Young and Wang, 2004).

The Prairie Vole as a Translational Social Model for Monogamy

The prairie vole (*Microtus ochrogaster),* a brown rodent species weighing approximately 40 g, lives indigenously in a grassland environment. The hypothesis for the evolution of monogamous behavior is that due to selective pressure from limited resources, offspring survival is maximized by bi-parental behavior. In a natural environment, nests generally hold a mating pair and their progeny. The male takes the unusual role within the rodent community of exhibiting paternal care through pup retrieval, nest building, nest guarding, etc (Aragona and Wang, 2004). When prairie voles lose their mate the majority never take on another partner. While prairie voles exhibit monogamous behavior, other vole species such as the montane (*Microtus montanus)* and meadow (*Microtus pennsylvanicus)* vole do not (Young and Wang, 2004). These findings attest to the diversity of social organization present across *Microtine* species, and have led to comparative studies and laboratory adaptation of the prairie vole as a translational model for social cognition and behavior.

In a laboratory environment, specific experimental paradigms have been developed in which prairie voles exhibit a similar behavioral profile as they do in the wild. One action that is indicative of such monogamous behavior is pair bonding with an opposing gendered conspecific. A laboratory metric for pair bond formation has been described as a "partner preference" and is tested using a partner preference test (PPT; Figure 1). In this paradigm, the subject is placed freely in the middle of a three chamber apparatus. Within one of the adjacent chambers is a tethered novel ("stranger") while in the other is a tethered familiar conspecific ("partner"). The creation of a partner preference is inferred if the subject spends a significant amount of time in close proximity to the partner compared to the stranger. While mating is not required for the production of a partner preference, the act of mating enhances its development in females

(Williams et al., 1992). The partner preference test and other paradigms have been used to advance our understanding of the biological mechanisms underlying pair bonding and other complex social behaviors.

Oxytocin Influence on Mammalian Behavior

Oxytocin (OT) is a peptide hormone comprised of nine amino acids primarily synthesized by two nuclei within the hypothalamus, the paraventricular and supraoptic nuclei. While OT may travel through the neurohypophysis to exert is peripheral effects on a body, the paraventricular nucleus is primarily responsible for central OT transmission with projections to the hippocampus, hypothalamus, nucleus accumbens, and amygdala (Ishak et al., 2011).

Regarding behavioral influences, the hormone has been associated with several maternal and prosocial effects. OT has been linked to catalyzing uterine contractions during childbirth, maternal nurturing via the letdown reflex of lactation, and maternal bonding (Ross et al., 2009a). Low doses of subcutaneous OT injections correlate with increased social recognition in male rats, while other hormonal candidates such as vasopressin do not (Popik et al., 1992). Furthermore, transgenic OT knockout models show increased adult aggression and social recognition deficits which can be rescued with 1.0 ng OT infusion to the medial amygdala before social exposure (Ferguson et al., 2000, Ferguson et al., 2001, Winslow and Insel, 2002).

Regarding pair bonding behavior, centrally administered treatment of OT leads to increased social contact and partner preference formation (PPF), while blockage of OT transmission via centrally administered OT antagonist (OTA) inhibits PPF (Williams et al., 1994, Cho et al., 1999). OT could have a developmental role as neonatal oxytocin exposure facilitates successful PPF in adult male prairie voles (Bales and Carter, 2003). Contrastingly however,

prolonged developmental exposure to intranasal OT in male prairie voles has been shown to inhibit PPF as adults (Bales et al., 2013).

Human studies suggest that oxytocin is a crucial component of social cognition. After compiling several oxytocin nasal spray findings, researchers have concluded that intranasal administration of oxytocin improves early emotional perception and assessment from social cues, particularly those associated with positive valence (Guastella and MacLeod, 2012). Additionally, intranasal administration of oxytocin has been shown to provide symptomatic relief for a variety of conditions such as autism, insecure attachment, social anxiety disorder, posttraumatic stress disorder, schizophrenia, and anorgasmia (Macdonald and Macdonald, 2010).

Variant oxytocin receptor gene expression, whether through single nucleotide polymorphisms (SNPs) or methylation, has been associated with variation in pair bonding (Lee et al., 2009, Walum et al., 2012), social motivation (Lee et al., 2009), general sociality in ADHD patients (Li et al., 2015), prosociality (Kogan et al., 2011), empathetic concern (Smith et al., 2014), infantile attachment (Chen et al., 2011), perceptions of trustworthiness (Tabak et al., 2014), and facial recognition (Skuse et al., 2014). These findings indicate that OT is a deeply conserved biological mechanism that regulates social behavior across species, including humans.

The Pair Bonding Pathway

Synthesizing several of the previous pair bonding and maternal bonding studies, Drs. Numan and Young have developed a hypothesized pair bond network (Figure 2). The hypothesis states that during mating, the ventral tegmental area (VTA) sends its dopaminergic input to the paraventricular nucleus of the hypothalamus (PVN), the nucleus accumbens shell (NAs), and prefrontal cortex (PFC). Reciprocally, the PVN sends its oxytocin input to the VTA, NAs, and

PFC. A simplified model highlighting projections thought to modulate pair bonding behavior are depicted in figure 3 (Young and Wang, 2004).

During the initial phases of pair bond formation, it is hypothesized that OT modulates olfactory processing by binding to oxytocin receptors (OTRs) that densely populate primary olfactory processing pathways. The olfactory bulb and the anterior olfactory nucleus (AON) project mainly glutamatergic input to strengthen synaptic modulation of specific medial amygdala (MeA) neurons. Consequently such MeA neurons modulate activity within the NAs and ventral pallidum (VP), which in male prairie voles has been identified as a critical structure for pair bonding (Lim et al., 2004).

In order to solidify the latter stage of pair bonding, it is hypothesized that both VTA dopaminergic projections (Vanderschuren et al., 1999) and PVN OTergic projections into the NAs facilitate inhibition of inhibitory GABAergic medium spiny neurons (MSNs). The projections of these MSNs innervate the VP, with the overall result being a disinhibition of the VP. The VP is now free to be strongly stimulated by the previous glutamatergic input from the MeA arising from olfactory stimuli. Over time, olfactory stimuli from the mated partner will strongly activate the VP, while olfactory stimuli from strangers do not, instead stimulating vasopressin release to the anterior hypothalamus which is involved in regulating hostility and dismissal (Numan and Young, 2016).

Despite hypothesized models of OTergic modulation of this pair bonding neural network, many questions remain unanswered. Specifically, such questions are investigating the relationships between variation in oxytocin receptor gene, OTR distribution across the network, neural network function, and ultimately social behavior are critical for advancing the field.

Oxytocin Receptor, Oxytocin Receptor Gene, and NT213739 Polymorphisms

Comparative analyses of central receptor densities were some of the first approaches taken by scientists seeking to explain discrepancies in behavior across vole species. In one early study, oxytocin receptor densities were compared between monogamous and non-monogamous vole species. After analysis, monogamous voles were found to have significantly increased OTR within the nucleus accumbens (NAc) and caudate putamen (Figure 4) (Insel and Shapiro, 1992). Contrastingly, non-monogamous animals have lowered NAc OTR binding which, compared to monogamous species, may contribute to their differences in behavior (Insel and Young, 2001). Increasing NAc OTR density via viral vectors facilitates PPF while OTA administered within this region inhibits PPF (Young et al., 2001, Ross et al., 2009b). Interestingly, there is significant individual variation in OTR binding density in the NAc of prairie voles. Building upon monogamous behavior, the NAc OTR has been implicated in parental behavior as well. Sexually naive females are more likely to take care of another's offspring if they have high NAc density and are less likely to exhibit this behavior when OTA is administered to the NAc (Olazabal and Young, 2006).

Central OTR density variation has been attributed to variation in the expression of the oxytocin receptor gene (*Oxtr*). A specific SNP at nucleotide 213739 (NT213739) has been targeted for additional research due to its relationship with pair bonding behavior and central OTR expression in prairie voles (King et al., 2015). This C/T polymorphism predicts 70-80% of variation in NAc OTR density. At this genetic locus, homozygous T/T individuals display low, heterozygous C/T individuals display intermediate, and homozygous C/C individuals display high NAc OTR density (Figure 5). Furthermore, C/C individuals are significantly more likely to develop a PPF than T/T genotyped animals. These results indicate that genetic variation in *Oxtr*

modulates both pair bonding behavior as well as patterns of central OTR expression across the pair bonding network.

Experimental Aim

While previous research has demonstrated how natural variation in *Oxtr* affects OTR density and pair bonding behavior, our understanding of how OTRs modulate patterns of the neuronal activation across the pair bonding network that regulate this behavior is limited. To address this question, researchers have been utilizing Fos expression as a metric of neuronal activation and plasticity (Hoffman et al., 1994). Recent studies have found that central OTR modulates correlated Fos activity across pair bonding nuclei in male prairie voles (Johnson et al., 2016). However, the question regarding how natural variation in *Oxtr* modulates neural activity across forebrain nuclei has yet to be explored. To answer this question we proposed the following experiment. We used an abbreviated cohabitation paradigm with mating to investigate the effect of the NT213739 genotype on correlated Fos expression across our regions of interest (ROIs): the nucleus accumbens shell (NAs), the anterior olfactory nucleus (AON), medial prefrontal cortex (PFC), and the ventral pallidum (VP).

The medial PFC was selected due to its neuronal projections to the NAs and its central role in the reward system of the brain. The NAs was selected due to the crucial role of local OT transmission in PPF and because NT213739 SNP variation is associated with differences in OTR expression in this nucleus (Olazabal and Young, 2006, Smeltzer et al., 2006, Dolen and Malenka, 2014, Moaddab et al., 2015). The NAs consequently projects to the VP, a nucleus that is crucial for PPF in male prairie voles (Lim et al., 2004). Lastly the AON was selected due to its role in

olfaction and its connections with the olfactory bulb and other brain structures such as the hypothalamus (Ferrer, 1969).

Our hypothesis is that natural variations in *Oxtr* modulate neuronal activity between pair bonding nuclei. We hypothesis that in low expressing NAs OTR (T/T) subjects, there exist a robust Fos expression covariance between the PFC and the VP. However, high expressing NAs OTR (C/C) subjects will display a functional uncoupling by disrupting the positive Fos activity correlation between these two nuclei. This will result in decreased activation of the medium spiny neurons. The effect will be a disinhibition and increased firing of the VP and lead to increased attraction towards the mating partner (Figure 6).

Materials and Methods

Animal Subjects

Animal subject protocol was adapted from Johnson et al. 2016, pg. 8. All animal subjects in the study were raised within Emory University's breeding colony at the Yerkes Primate Research Center, accredited by the Association for Assessment and Accreditation of Laboratory Animal Care (AAALAC). Subjects were the progeny of Illinois field netted prairie voles. Testing occurred between the ages of ten weeks and nine months.

Prairie voles were regularly weaned at post-natal 19–21 days at the Yerkes Primate Research Center, and housed in 28x17x13 cm translucent Plexiglas cages in two to three same-sex groups. Subjects were placed within a 12-h dark/light cycle and a steady environmental temperature of 22 °C with access to water and food (LabDiet® rabbit, Purina, Richmond, IN, USA) ad libitum. Bedding supplemented in the cages came from Bed-ócobs® Laboratory Animal Bedding (OH, USA).

All procedures and techniques used in this experiment have been performed in agreement with the National Institutes of Health Guide for the Care and Use of Laboratory Animals (NIH Publications No. 80-23) and approved by the Institutional Animal Care and Use Committee of Emory University (IACUC). Every exertion was made to diminish the quantity of animals used and their suffering. Mating conspecifics were sexually experienced adult female prairie voles who were overectomized and estrogen primed.

Cohabitation Protocol

Cohabitation protocol was adapted from Johnson et al. 2016, pg. 9. In the three days leading to cohabitation, female prairie voles were injected subcutaneously, once daily with 4.0 μg estradiol benzoate dissolved in sesame oil (Sigma; St. Louis, MO; S3547). After being brought into estrus, each female was placed into a male subject's cage (26 x 18 x 19 cm) for 30 minutes. After this time, each female was removed from the male's cage and returned to their native cage.

During the 30 minutes, administered lab food capsules and water dispensers remained in the cage for the voles' leisure. In order to account for the added stimulus of the researcher's presence when transporting the female to and from the cage, researchers opened the cage of unexposed males (n = 9) and removed a handful of bedding at the beginning which they return at the end of the 30 minute period.

Mating Analysis

The mating behavior analysis protocol was adapted from Johnson et al. 2016, pg. 9. Male and female prairie voles were recorded using a Canon HD camera during the 30 minute

cohabitation. Two independent scorers blind to treatment groups used Observer XT 10 behavioral scoring software (Noldus Information Technology Inc.; Leesburg, VA) to score mating behavior. Male mating was said to occur if any intromission occurred during the cohabitation paradigm. Intromission was quantified as the duration of total copulating sessions characterized by the conventional thrusting forms.

Perfusion and Post-Fixation

Males were overdosed with isoflurane 45 minutes after the 30 minute cohabitation period and then cardially perfused with phosphate buffered saline (pH = 7.4; diluted to 1× with distilled, deionized water from 10× PBS stock; Teknova; Hollister, CA; P0401) at an approximate rate of 4 mL/min. Using an Easy-Load II MASTERFLEX device (Cole-Palmer; Vernon Hills, IL), males were then perfused by 40 ml of 4% paraformaldehyde (Polysciences; Warrington, PA; 00,380) in 1x PBS. Any subjects that did not receive adequate perfusion were removed from further analysis.

After this procedure, researchers dissected skulls and extracted brains. Using the same paraformaldehyde solution as before (4% in 1x PBS), extracted brains were placed initially overnight before transferring the next day to a pre-sectioning solution of 30% sucrose in 1x PBS. Figure 7 represents a timeline of the cohabitation and perfusion protocols.

DNA Extraction and Genotyping

DNA extraction and genotyping protocol was derived from King et al. 2015, supplemental information pg. 3. Tail endings were removed from each male subject postmortem and stored at -80 C. Qiagen DNeasy kit was used to extract the DNA which was later amplified

by the use of a 140 bp sequence which contained NT213739 undergoing a polymerase chain reaction (PCR). The PCR cycling protocol was the following: denaturing at 94˚C for 30 seconds, followed by annealing at 53˚C for 30 seconds, followed by elongation at 72˚C for 30 seconds, followed by an additional elongation step at 72˚C for 10 min. The primers used for this reaction were the following:

forward: 5'-CTAGGCTTTGGTTGGGGAAATAAC-3'

reverse: 5'- TTGGGTCTTGTTATGGTCCTGAC-3'

Amplified DNA was digested for 1.5 hours at 3˚C by BsiHKAI (New England Biolabs, Ipswich, MA), a restriction enzyme which cleaves selectively at the C allele of NT213739. Because BsiHKAI will not cleave the T allele, the resulting location and number of bands from gel electrophoresis would determine the genotype of each subject (Figure 8).

After analysis, the following subjects went on for further review: For exposed to female (n=30): n = 9 for T/T, n = 10 for C/T, n = 11 for C/C. For unexposed (n = 9): n = 3 for T/T, n = 3 for C/T, n = 3 for C/C (Table 1).

Tissue Processing, Section Staining, and Slide Preparation

Tissue processing, section staining, and slide preparation protocols were derived from Johnson et al. 2016, pg. 10. Extracted brains were sliced using a Microm HM 440E to create 40 µm sections and stored in 0.05% sodium azide in 1x PBS solution while waiting to be stained. To begin the staining process, sections were washed 3 times in 1× PBS, followed by a 10-minute incubation in 1% sodium hydroxide in 1× PBS, and 3 washes in 1× PBS with 0.5% Triton-X (Sigma) (PBST). Sections were then immersed for 1 hour at room temperature in 5% normal goat serum (Fitzgerald; Acton, MA) in PBST. Sections were then treated with a 48 hour

incubation (orbital shaker at 4 °C) of primary rabbit polyclonal anti-Fos anti-body (1:1000; Calbiochem PC38). Following the polyclonal treatment, sections went through an additional 5 washes of 1x PBS, one wash of 1x PBST, followed lastly by a 2 hour stain of biotinylated goat anti-rabbit IgG (1:500; Vector Labs BA-1000), a secondary antibody.

After the secondary stain, sections were stained for 1 hour with an avidin-biotin peroxidase system (Vectastain Elite ABC System; Vector Labs: PK-6100) with a Nickel-DAB peroxidase substrate kit (Vector Labs; SK- 4100) treatment occurring afterwards.

In order to prepare sections for mounting, in a step wise fashion, increasingly concentrated ethanol solutions were used for dehydration (5 min in 70% EtOH, 10 min in fresh 95% EtOH twice, 10 min in fresh 100% EtOH twice). Sections were then immersed in Xylenes (15 min in Xylenes twice). Sections were then transferred to a .1x PBS well where they would be rostrally to caudally mounted onto Superfrost Plus slides (Fisher Scientific; 12-550-150) and treated with Krystalon (EMD Chemicals Inc., Gibbstown, NJ) for cover slipping.

Imaging and Fos Quantification

Imaging and Fos quantification protocols were adapted from Johnson et al. 2016, pg. 10-11. A Nikon E800 microscope under a 10x magnifying objective through MCID Core imaging software (InterFocus Ltd., Cambridge, UK) was used to capture images of ROIs across all male subjects. ROIs (Figure 9) include the NAs, PFC, AON, and VP. To determine region location and boundaries, images were framed by referencing the *The Mouse Brain* atlas (Franklin and Paxinos; 3rd Edition). NAs and AON images were taken from the both the left and right hemispheres while the PFC and VP, due to their medial location, were taken on one image. Any

interfered images from aberrated cover slipping or damaged tissue were excluded from further analysis.

After imaging the ROIs, manual cropping within Windows Paint or Adobe Photoshop produced the area to be quantified. The MCID grain count function under a discriminating threshold ($0.001 < \text{area} < 1000$) was used to determine the number of positive Fos nuclei within each image. Fos expression per ROI was then determined by taking the average of all of the Fos counts per image within the subject. The process of initial imaging to mean number of Fos positive nuclei per ROI was performed twice for the AON. For each subject, the two means were averaged once more to determine the final AON Fos expression.

Statistical Analysis

All statistical analysis was performed in RStudio version 3.2.4, Microsoft Excel, or Vassar Stats (http://vassarstats.net/rdiff.html).

FOS Expression within ROI

A series of Welch's two sample t-tests were used to examine mean Fos expression between: 1) exposed and mated animals (T/T n = 7, C/T n = 7, C/C n = 8) vs unexposed animals pooled across all brain regions, 2) exposed and mated animals vs unexposed animals within each ROI, and 3) mated vs unmated animals in the exposed group within each ROI. A series of one-way ANOVA tests were then used to test the variance of mean Fos expression across the three genotypes within each ROI. Lastly, a *post-hoc* t-test was used to compare mean Fos expression in the NAs between C/C and T/T animals in the mated and exposed group.

Fos Expression Covariance Analysis across ROIs

Correlation of Fos expression across ROIs was analyzed by first creating a covariance matrix for Fos expression across ROIs. The correlation coefficients were then expressed visually by using a package within RStudio called ggplot2. This package translated the calculated Pearson's correlation coefficients into visualized heat maps. Three heat maps were generated to represent the C/C, C/T, and T/T populations.

A test using the Fisher r-to-z transformation was used to analyze the significance of the differences between each pair of correlation coefficients across the C/C and T/T populations. A final post-hoc t-test was then used to compare the means of the correlation coefficients between the C/C and T/T groups.

Results

Effects of NT213739 Genotype on Number of Positive Fos Nuclei

Male subjects that copulated during the 30 minute cohabitation were used to visually represent the Fos cell count density of the four ROIs (Figure 10). A Welch's sample t-test between exposed and mated vs unexposed animals pooled across all ROIs showed that Fos expression was significantly higher in the mated and exposed group ($n = 40$, $p = 4.089e\text{-}11$). This result is also consistent within each ROI, with the Fos expression in the exposed and mated group being significantly higher (Table 2). Furthermore, a series of t-tests showed that Fos expression in mated groups did not significantly differ from that of exposed non-mating group (Table 3).

A series of *post-hoc* one-way ANOVA tests revealed that Fos expressions within each ROI did not differ by genotype (T/T, C/T, C/C) among mated animals (Table 4). Additionally, a

further *post-hoc* t test showed that Fos expression within the NAs did not differ between low expression (C/C) and high expression (T/T) mated subjects (n = 11 for C/C, n = 9 for T/T, p = 0.1417).

Effects of SNP Genotype on Covariance of Neuronal Activation

We hypothesized that NAc OTR density due to NT213739 genotype modulates the covariance of neuronal activation across ROIs. Derived by Pearson's correlation coefficients, subjects with low expression of NAc OTR (C/C) displayed positive correlations across nodes (Figure 11). Contrastingly, subjects with high expression of NAc OTR (T/T) displayed a weaker and sporadic set of correlations across nodes (Figure 13) with heterozygous subjects projecting an intermediate result between the high and low groups statistics (Figure 12).

To test quantitatively whether correlations between T/T and C/C subjects were significant, we performed a significance test which employs the Fisher's r to z transformation. After comparing every set of correlations, the only significant difference was between the VP and PFC (z = 2.06, p = 0.0394) and a trending difference between the VP and AON (z = 1.87, p = 0.0615). All other results are presented in table 5. A final *post-hoc* t-test revealed that there was a trending difference between the mean of all correlation coefficients of C/C subjects and the mean of all correlation coefficients of T/T subjects (n = 6, p = 0.07896).

Discussion

Previous findings within the pair bonding field have implicated OT as a critical neurotransmitter for the creation of PPF in female but not male prairie voles (Insel and Hulihan, 1995). Despite these results, recent findings have suggested that in male prairie voles OT

transmission modulates pair bonding behavior and activity across a network of nuclei hypothesized to be critical for pair bonding (Johnson et al., 2016). The pair bonding network has been hypothesized to involve the synthesis of two primary pathways: the reward system and the olfactory transduction network. Both pathways converge on the NAs which been suggested to have the responsibility of providing inhibitory GABAergic transmission to the VP (Numan and Young, 2016). Consequently, the VP has been implicated in male prairie voles to be a critical structure for pair bonding (Insel and Hulihan, 1995). OTA administration throughout this pair bond network has been shown to decrease the correlated activity or "functional connectivity" in addition to inhibiting PPF (Johnson et al., 2016).

Focusing on NAs as a regulatory mechanism for pair bonding, previous research has additionally shown that genetic mutations within the *Oxtr* gene modulate patterns of central *Oxtr* expression across olfactory and reward processing brain nuclei. Furthermore, this genetic variation has been associated with accelerated PPF formation in high expressing compared to low expressing subjects (King et al., 2015). As central OTR expression is such a crucial regulatory mechanism for pair bonding behavior, we investigated whether naturally occurring genetic variation in *Oxtr* could influence Fos expression and covariation between nuclei that are critical for pair bonding.

In this study, Fos expression was used as a quantification of neuronal activation across OTR-implicated ROIs. Fos protein is transcribed by the immediate early gene *c-fos* which combines with JUN to create AP-1 transcription factor complexes. These complexes modulate AP-responsive genes which are involved in neurophysiological changes such as synaptic plasticity. Consequently, Fos expression is considered to be a pathway in which neurons are able to respond to a stimulating depolarization by strongly modulating AP-1 responsive genes to elicit

a quick phenotypical change. For this reason Fos expression is used to measure neuronal activity. As the translation of Fos protein typically maximizes at 1-2 hours after neural activation, Fos experiments generally involve brain fixation 1-2 hours after stimulus presentation (Sheng and Greenberg, 1990, Lyons and West, 2011).

Another application of immediate early gene analysis is determining "functional connectivity" (or covariance in Fos expression) between brain regions. Expression values within each region can be compared to derive correlations across regions. Such correlations are said to be a measure of functional connectivity and have been implemented in several experimental contexts (Kaufman et al., 1993, Petrov et al., 1996, Schiltz et al., 2007).

In our study, we were interested in how natural variation in central OTR expression modulates Fos expression and covariance within and across ROIs. NT213739 genotype was determined to have no significant effect on Fos expression within any ROI for mated subjects. These results suggest that variation in NT213739, and consequently NAc OTR density, do not play a critical role in modulating the total activation within these brain regions. However, it is important to consider that the null result could be due to the low power of the t-test as a consequence of the small sample sizes (n = 11 for C/C, n = 9 for T/T). There is the possibility that a subject group with larger statistical power could detect a significant difference in Fos expression between these two groups.

Interestingly for mated subjects, NT213739 genotype was determined to have a trending effect on Fos expression covariance across ROI, with low NAc OTR (T/T) subjects displaying a stronger averaged correlation than high NAc OTR (C/C) subjects (n = 6 p = 0.07896). When comparing individual correlations between high and low mated groups, we see the strongest

effect when comparing the correlation values between the PFC and VP ($z = 2.06$, $p = 0.0394$) (Figure 14).

One hypothesis to explain these results is that such changes in functional connectivity are the result of variation in central OTR expression. Oxytocin transmission involves the activation of a G protein cascade. The resulting transmission could have a multitude of effects, although it has been classically identified as having excitatory effects on neurons (Terenzi and Ingram, 2005). It is possible that OT transmission could be potentiating the NAs's GABAergic medium spiny neurons. Increases in NAs's inhibitory control on the VP would lead to a decrease in VP activation. Therefore, regarding the functional connectivity between the PFC and the VP, increases in NAc OTR density could be causing an inverse relationship regarding their functional connectivity. This would correspond with why we see a positive PFC to VP correlation in T/T subjects switch to a negative correlation in C/C subjects.

Studies have shown that the VP provides inhibitory GABAergic transmission to the thalamus, primarily to the mediodorsal nucleus. The VP can then regulate sociosexual behavior through its thalamic loop to the frontal cortex or to the brainstem directly (Vives and Mogenson, 1985, Nakano, 2000, Heimer, 2003). Using this model, a decreased VP firing would then lead to a disinhibition of sociosexual behavior.

In light of our results, one interpretation is that variation in oxytocin NAs transmission as a result of NT213739 genotype is modulating PFC to VP activation. While there would be little resulting inhibition of the VP in "low" (T/T) expressing subjects, "high" (C/C) expressing subjects would see an increased VP inhibition. The resulting inhibition would lead to a disinhibition of nuclei, such as the brainstem, associated with sociosexual behavior (Figure 15).

Limitations

There are important limitations to this study which provide critical context for its interpretation. First, Fos use is restricted because Fos staining does not differentiate between neuronal phenotypes. Second, without an identifying factor between neurons, differences in correlated patterns of Fos expression cannot reveal how central OTR signaling modulates activity of specific types of neurons, which is ultimately necessary for a comprehensive understanding of neural circuits. Third, using Fos provides an accurate representation of robust transcriptional activity, but it fails to adequately address the amount and strength of neurotransmitter release. Fourth, within the experimental protocol there was not a procedure to stain OTR in the NAc. Therefore, variation in subjects' OTR expression can only be inferred from their genotypes. Lastly, the experimental protocol did not account for specific types of behavior other than intromission and mating. There could exist the possibility of differences in aspects of sociosexual behavior between genotype groups which could account for differences in Fos expression.

Future Directions

In order to provide a more definitive conclusion as to how NT213739 genotype influences Fos expression, future studies should direct their attention to the mechanisms associated with OT transmission within the NAc. Without a proper understanding as to the molecular cascades of OT binding, conclusions about how NAc OTR density modulates Fos covariance across the ROI (specifically the PVN to VP) cannot be definitely made.

Conclusion

Despite the previous limitations, we were able to use the prairie vole model to demonstrate for the first time that a naturally-occurring SNP in *Oxtr* influences functional connectivity between sensory and reward processing brain nuclei that are important for complex social behavior. Our observations have led to a new model for explaining how variation in OTR density in the NAc could enhance PPF by leading to increased inhibition of the GABAergic ventral pallidum, which is in contrast to the initial model proposed by Numan and Young, 2016.

Appendix A

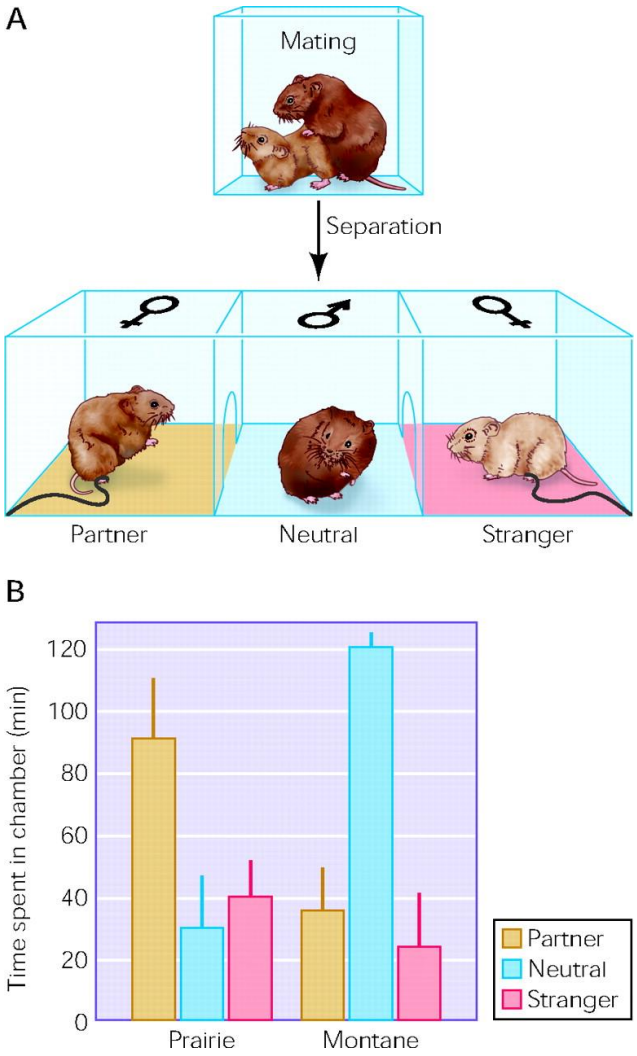

Figure 1: Partner Preference Test

During a temporal protocol of frequency and duration, a conspecific is brought in the presence of an opposing gender conspecific and is allowed to sociosexually interact. After this protocol, the experimental subject is placed in a three compartmentalized apparatus with the previously interacted conspecific ("partner") and the novel conspecific ("stranger") tethered to opposing ends (A). During this time, the experimental subject is recorded for time spent in close proximity to either conspecific or in a neutral middle area. When this paradigm is presented to prairie and montane voles, prairie voles show the creation of a pair bond by significantly spending more time with the partner while the montane voles do not (B).

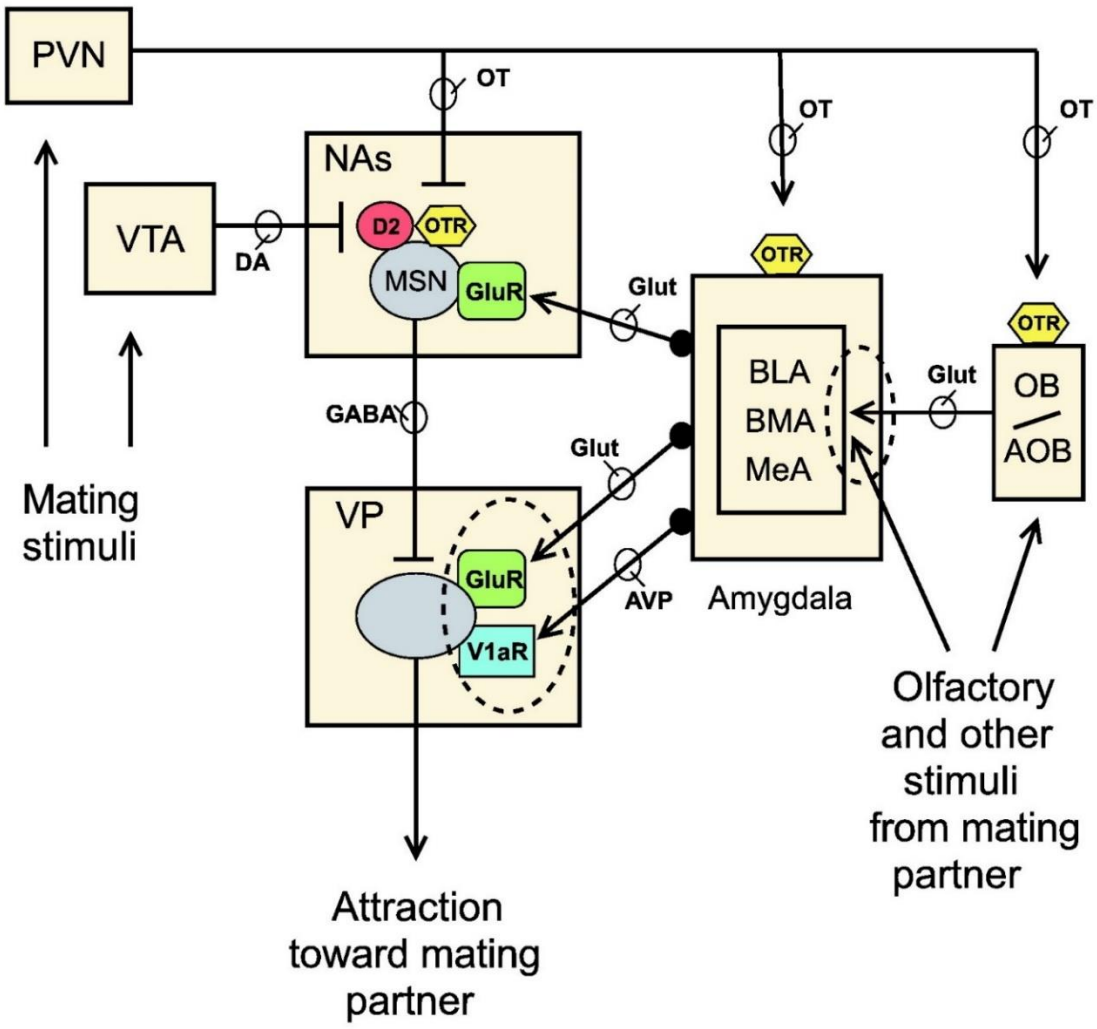

Figure 2: Hypothetical neuronal model for pair bond formation in prairie voles

When a prairie vole mates, dopaminergic input from the ventral tegmental area (VTA) and oxytocin input from the paraventricular nucleus (PVN) converge onto the nucleus accumbens shell's (NAs) inhibitory GABAergic medium spiny neurons (MSNs). The mate-induced olfactory cues stimulate nuclei within the olfactory bulb (OB) and anterior olfactory bulb (AON) along with strengthening amygdala synapses. A long-lasting partner attraction is formed when D2 and oxytocin receptor (OTR) input suppress the NAc MSNs while the glutamate (GluR) and vasopressin (V1ar) receptor input activate the VP. The disinhibition followed by the activation of the VP for prolonged periods of time results in the creation of a pair bond.

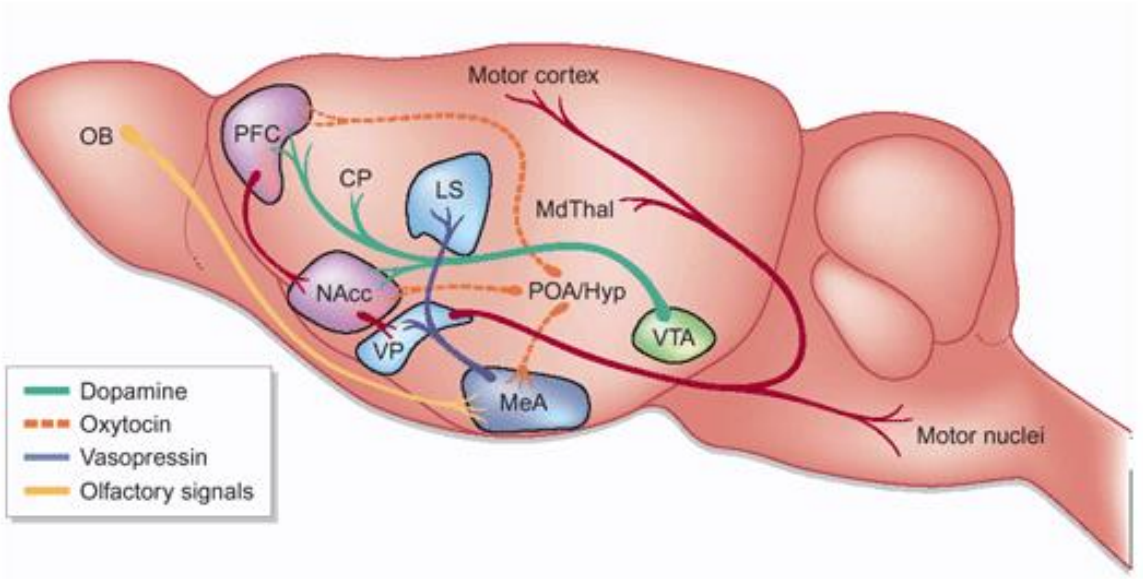

Figure 3: Sagittal view of hypothesized pair bonding network in prairie voles

Abbreviations: olfactory bulb (OB); medial amygdala (MeA); ventral pallidum (VP); nucleus accumbens (NAcc); prefrontal cortex (PFC); caudate putamen (CP); lateral septum (LS); preoptic or hypothalamic area (POA/Hyp); ventral tegmental area (VTA); mediodorsal thalamus (MdThai)

Mating increases VTA activation which consequently stimulates the PFC and NAcc with dopamine. At the same time, the OB is transducing olfactory cues and relaying its stimulation to the MeA. Adding to the model hypothesized in Figure 2, the PFC projects primarily glutamatergic input to the NAcc which in turn has its effects down the cascade in the VP to influence partner preference formation.

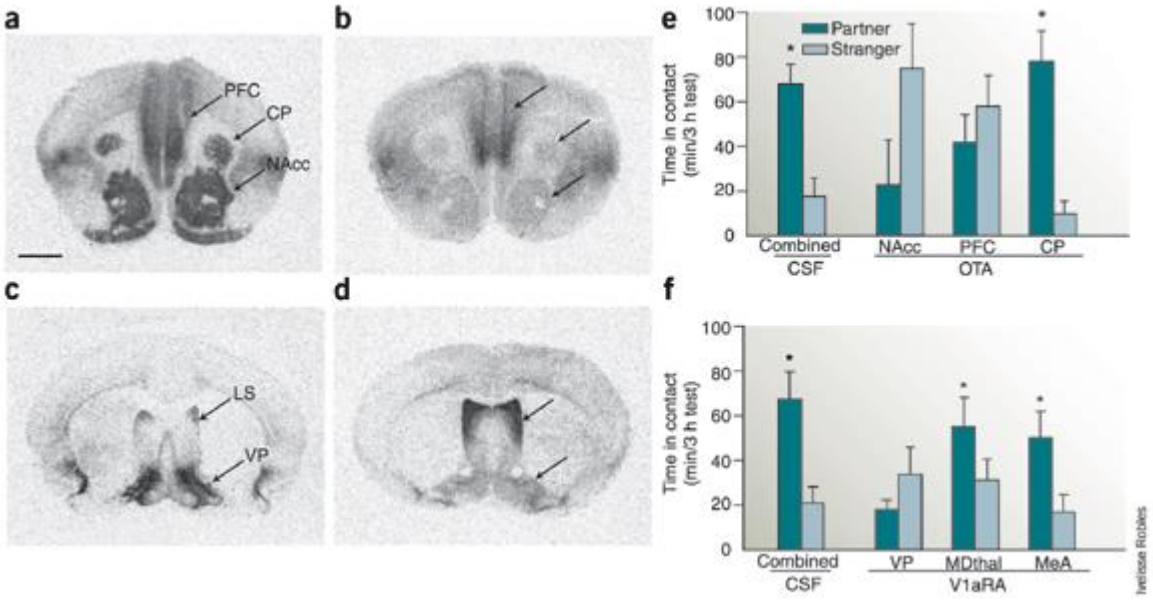

Figure 4: A comparison between oxytocin and vasopressin receptor density between the prairie and montane vole and its consequence on pair bonding behavior

Prairie voles (a) are shown having a higher oxytocin receptor density in the nucleus accumbens (NAcc) and caudate putamen (CP) although not the prefrontal cortex (PFC) when compared to montane prairie voles (b). Male prairie voles (c) display a higher amount of vasopressin receptors within their ventral pallidum (VP) compared to male montane voles (d). In female prairie voles, a bilateral infusion of oxytocin antagonist to the NAcc and PFC but not the CP blocks partner preference formation (e). In male prairie voles, an infusion of a vasopressin antagonist to the VP but not the mediodorsal thalamus (MDthal) or medial amygdala (MeA) blocks partner preference formation (f).

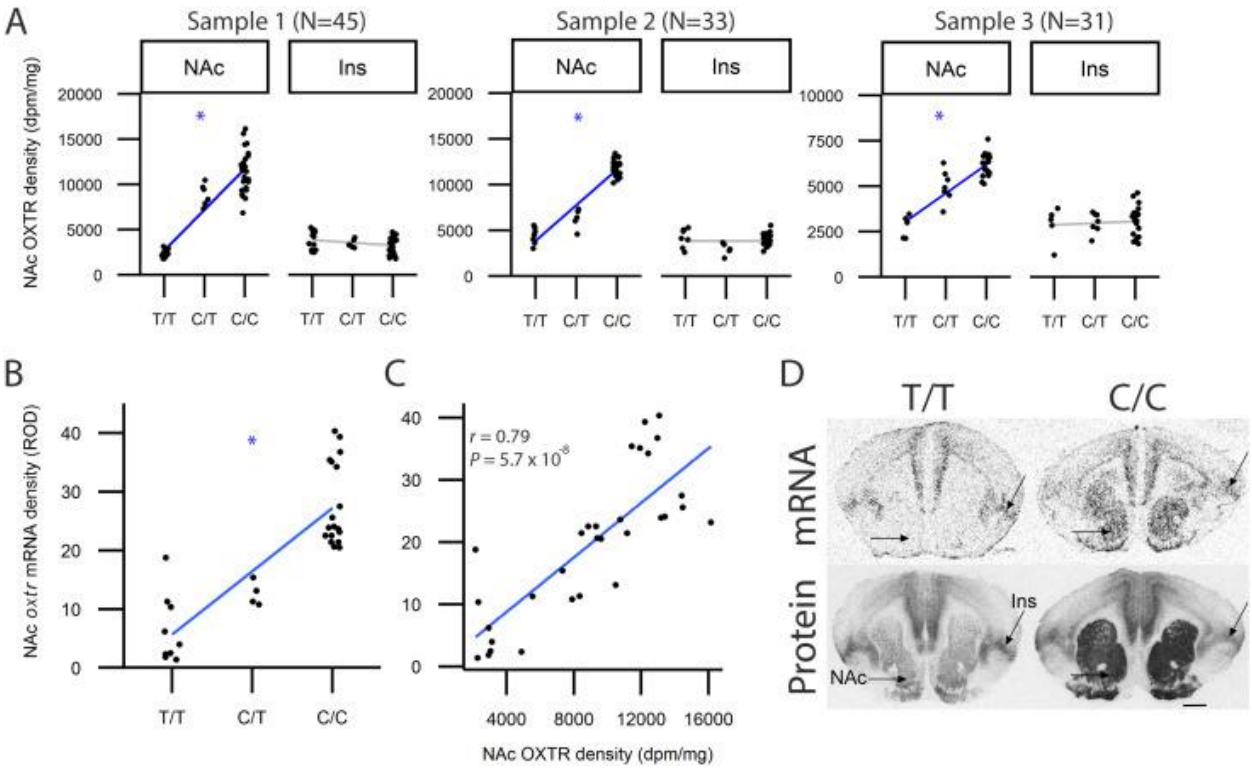

Figure 5: NT213739 genotype is a strong predictor of nucleus accumbens (NAc) oxytocin receptor density (OTR) in prairie voles

NAc OTR correlates with NT213739 genotype for three independent populations but not within a control region, the insular (Ins; A). NAc oxytocin receptor gene mRNA density correlates with NT213739 genotype (B). NAc oxytocin receptor gene mRNA density positively correlates with NAc oxytocin receptor gene protein binding density (C). Visual representations of differences between "low" (T/T) and "high" (C/C) oxytocin receptor gene mRNA and protein expression (D).

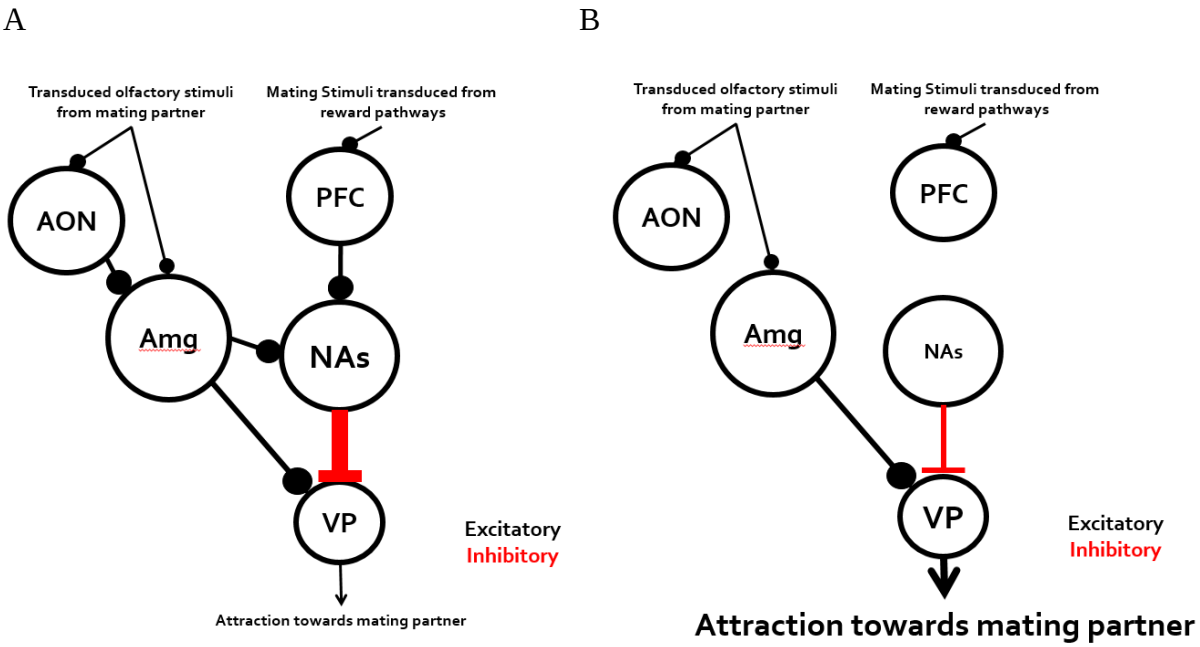

Figure 6: Hypothesis for NT213739 genotype's effect on hypothesized pair bonding nuclei

Within this hypothesized pair bonding network, olfactory information travels to the anterior olfactory nucleus (AON) and the amygdala (Amg) to provide excitatory stimulation of both the nucleus accumbens shell (NAs) and the ventral pallidum (VP). Consequently, mating stimuli is transduced from reward pathway nuclei is relayed to the prefrontal cortex (PFC). The PFC provides excitatory stimulation to the NAs which through the medium spinal neurons provides inhibition to the VP.

One hypothesis to explain how NT213739 genotype effects attraction towards a mating partner is that genotype modulates Fos covariance across pair bonding nuclei. Subjects with low expression of NAc OTR (T/T) would display a functional coupling within the AON and PFC to the VP (A). The consequence would be increased NAs inhibitory activity to the VP and a decreased attraction towards a mating partner. Contrastingly, subjects with high expression of NAc OTR (C/C) would display a functional decoupling within the AON and PFC to the VP (B). The consequence would be decreased NAs inhibitory activity to the VP and increased attraction towards a mating partner.

Appendix B

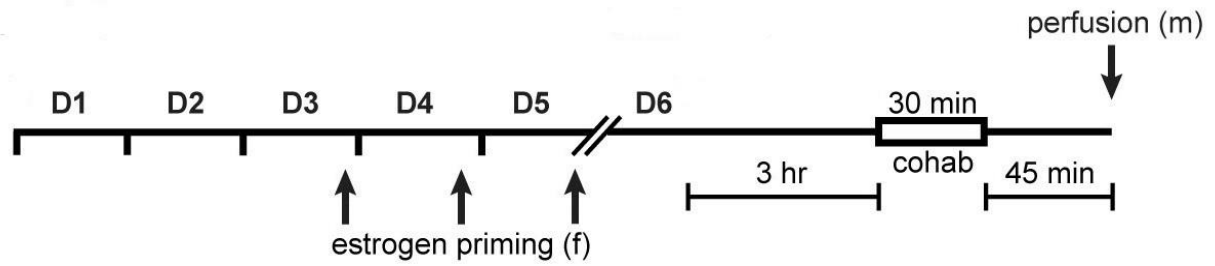

Figure 7: Cohabitation and perfusion protocol

(f) stands for female; (m) stands for male; (cohab) stands for cohabitation

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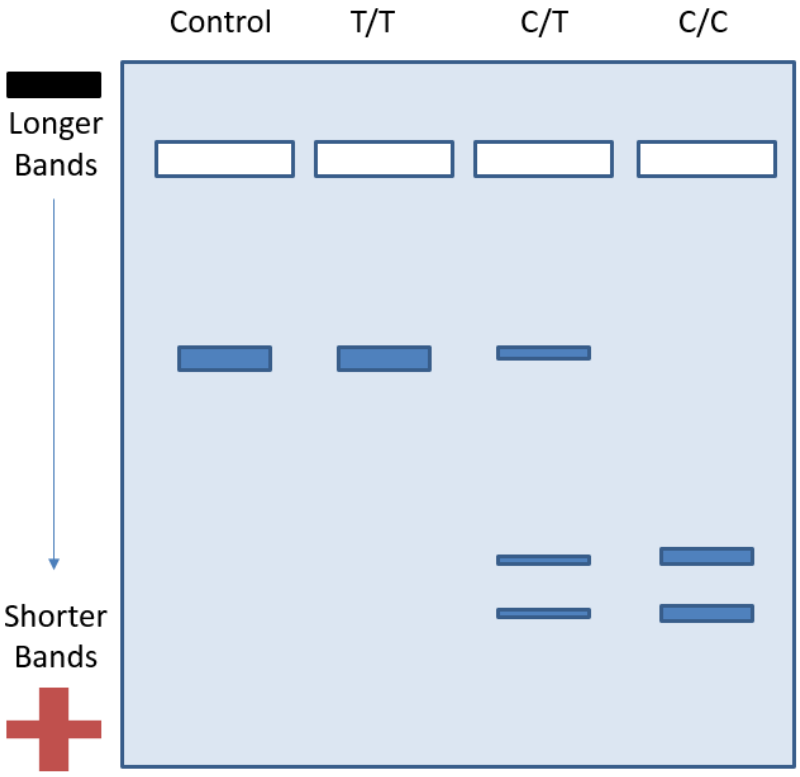
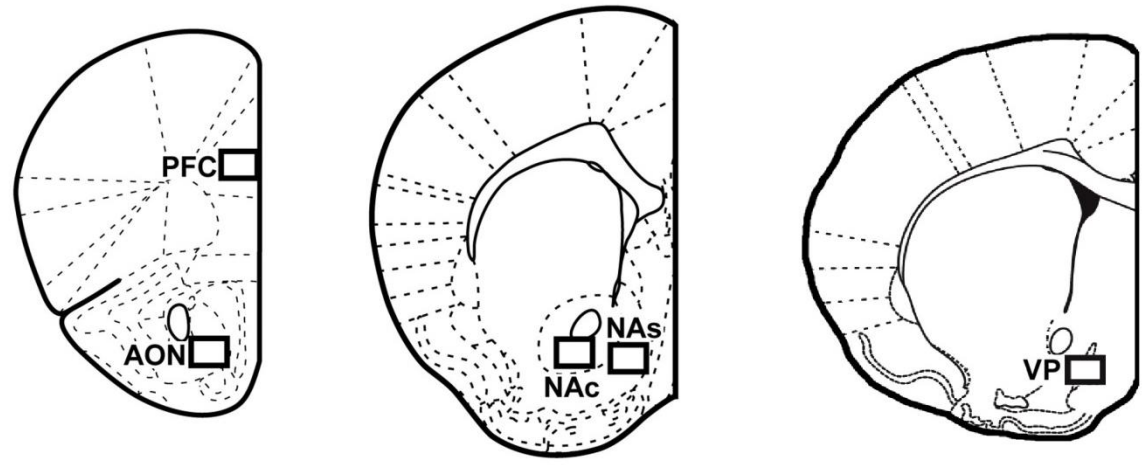


Figure 8: Gel electrophoresis after oxytocin receptor gene is processed with BsiHKAI

BsiHKAI is an enzyme that cleaves at the C allele of NT213739. The resulting density and position of the bands within the agarose gel depict what each genotypical array would appear like.

A.



B. **C.**

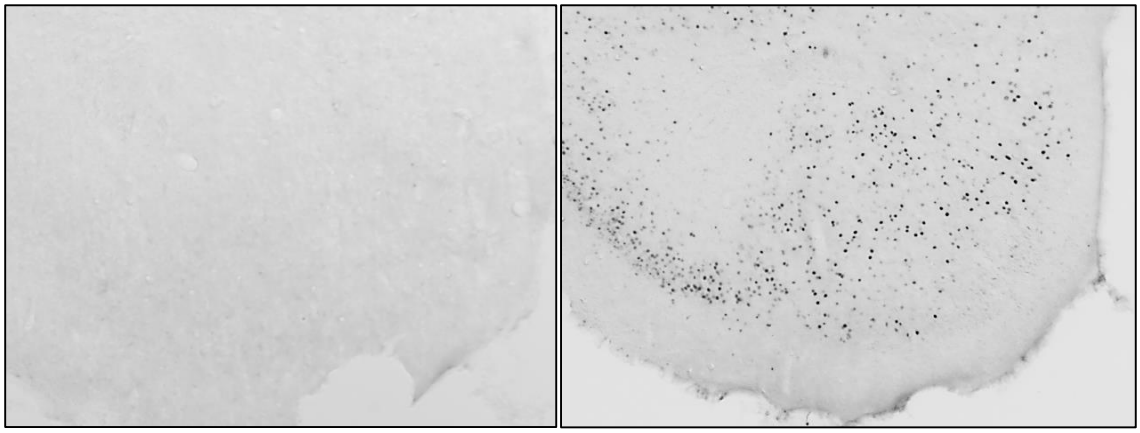


Figure 9: An anatomical representation of ROIs and Fos expression

Panel A provides an anatomical representation using the *The Mouse Brain* atlas to locate the anterior olfactory nucleus (AON), prefrontal cortex (PFC), nucleus accumbens shell (NAs), and ventral pallidum (VP). An example of how Fos expression appears during imaging is evident through a comparison between unexposed and exposed mated subjects. Panel B is an AON image of an unexposed subject, which appears to have little amounts of Fos positive nuclei. Panel C is an AON image of an exposed and mated subject, which appears to have a robust amount of Fos positive nuclei.

Appendix C

	Unexposed	Exposed	Exposed + Mated	Exposed Not Mated
"Low" (T/T) Expression	3	9	7	2
Heterozygous (C/T)	3	10	7	3
"High" (C/C) Expression	3	11	8	3

Table 1: Male prairie vole groups comparing treatment (unexposed vs. exposed to female) and mated (mated or did not mate with female) to NT213739 genotype.

Area	Significance (P value)
NAs	4.887e-06*
PFC	0.00163*
AON	6.13e-09*
VP	0.0488*

Table 2: Exposure and mating with a female increases Fos expression in every ROI

* signifies $p < 0.05$

Area	Significance (P value)
NAs	0.2757
PFC	0.1845
AON	0.3481
VP	0.07459

Table 3: Mating does not affect Fos expression within exposed animals per ROI

Area	F Value	Significance (P value)
NAs	1.653	0.210
PFC	0.000	0.999
AON	0.778	0.385
VP	0.129	0.722

Table 4: For mated animals, mean Fos expression does not differ between genotypes in each ROI

VP	P = 0.2757	P = 0.0394*	P = 0.0615
AON	P = 0.2005	P = 0.8493	
PFC	P = 0.2891		
	NAs	**PFC**	**AON**

Table 5: Fisher's z to r transformation shows significant interactions between the VP and PFC and trending interactions between the VP and AON.

* signifies $p < 0.05$

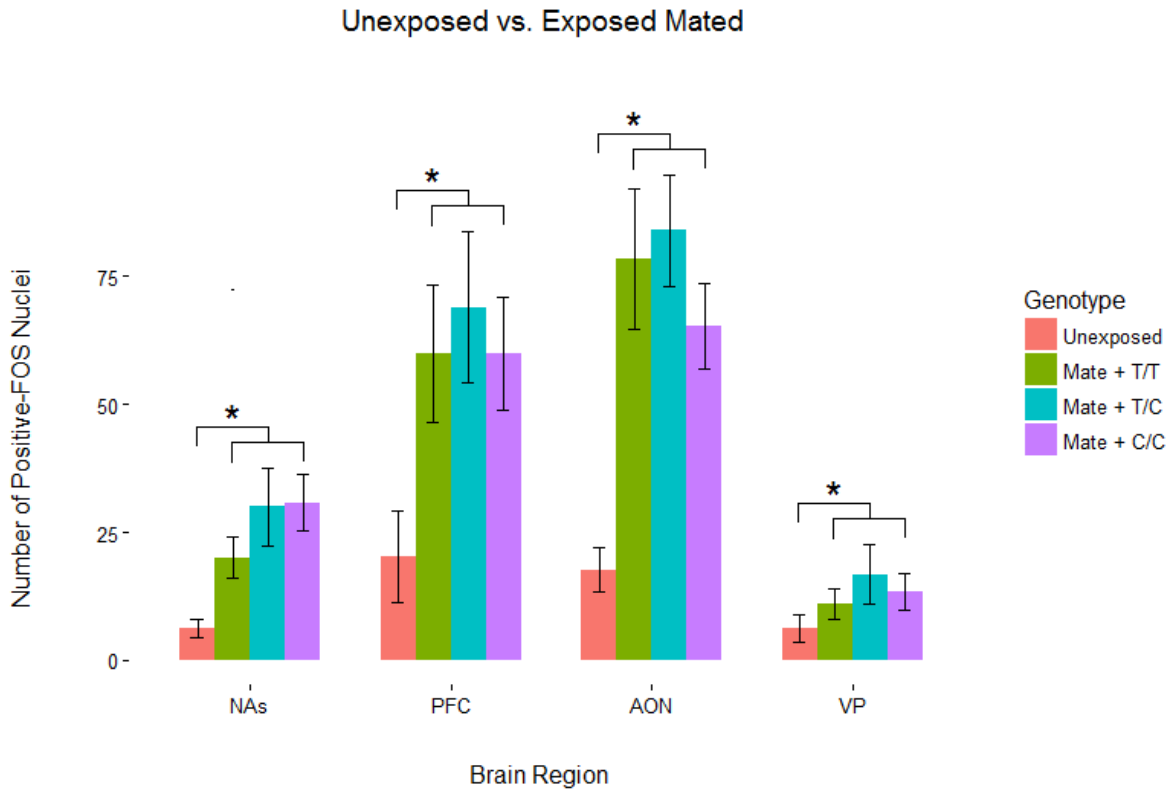


Figure 10: Increases in Fos expression are associated with exposure to female in male prairie voles within regions of interest (ROIs)

Abbreviations: nucleus accumbens (NAs); prefrontal cortex (PFC); anterior olfactory nucleus (AON); ventral pallidum (VP)

Fos expression was quantified through the amount of positive Fos nuclei within each region of interest. Within each ROI, no significant effect of genotype occurred. When genotype is pooled and compared to unexposed subjects, however, there exists a significant increase in Fos expression for mated subjects. All black brackets represent the standard error of the mean.

For more information on such statistical analysis, turn to tables 2, 3, and 4.

* signifies $p < 0.05$

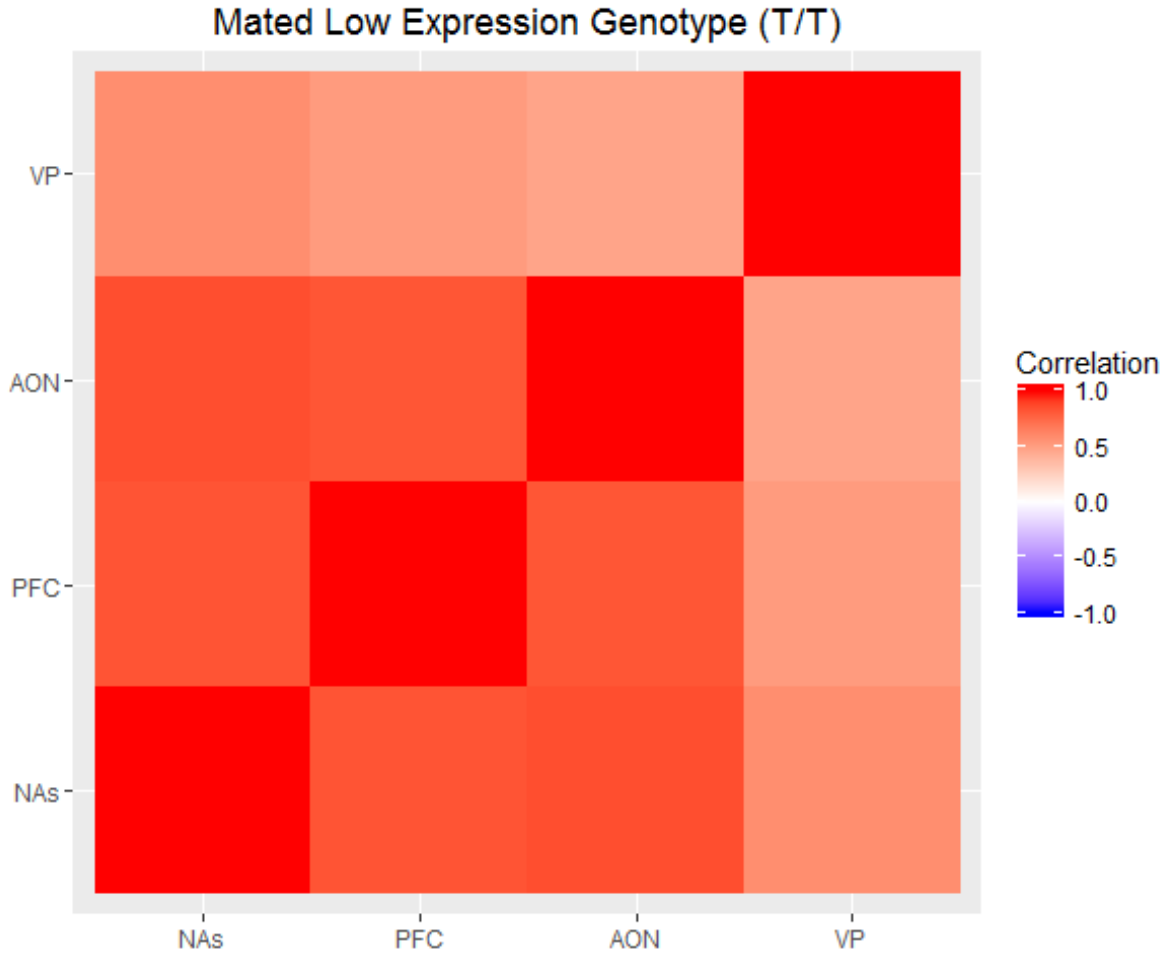


Figure 11: Fos covariance heat map for mated low expression (T/T) subjects

Abbreviations: nucleus accumbens shell (NAs); prefrontal cortex (PFC); anterior olfactory nucleus (AON); ventral pallidum (VP).

Heat maps were visualized through a ggplot2 package in RStudio which converts calculated Pearson's correlation coefficients. Such correlation coefficients are presented in the table below.

VP	0.5763544	0.5135623	0.4693321	1.0000000
AON	0.8484352	0.8214302	1.0000000	0.4693321
PFC	0.8274204	1.0000000	0.8214302	0.5135623
NAs	1.0000000	0.8274204	0.8484352	0.5763544
	NAs	**PFC**	**AON**	**VP**

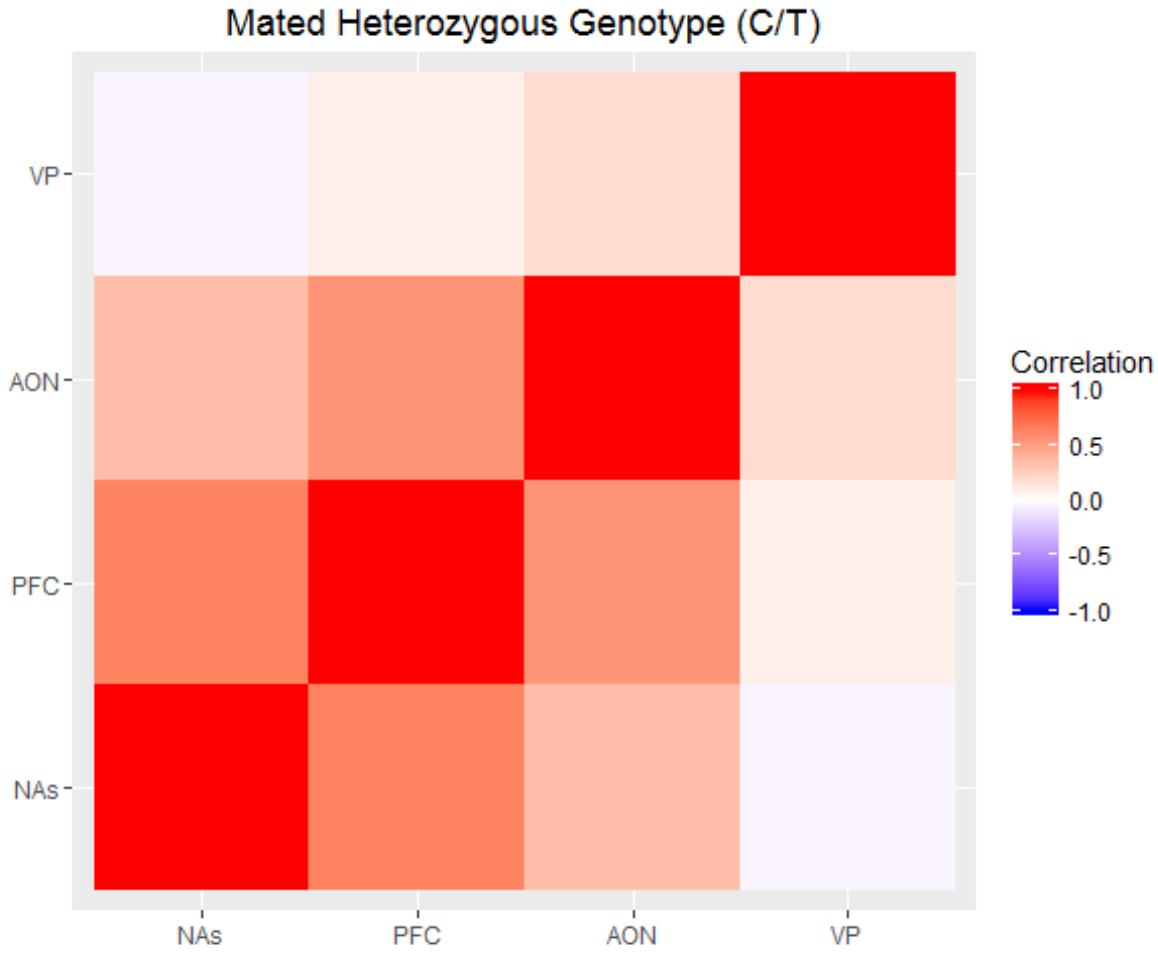


Figure 12: Fos covariance heat map for mated NT213739 heterozygous (C/T) subjects

Abbreviations: nucleus accumbens shell (NAs); prefrontal cortex (PFC); anterior olfactory nucleus (AON); ventral pallidum (VP).

Heat maps were visualized through a ggplot2 package in RStudio which converts calculated Pearson's correlation coefficients. Such correlation coefficients are presented in the table below.

VP	-0.0463105	0.08043696	0.1828121	1.0000000
AON	0.33810428	0.55314372	1.0000000	0.18281206
PFC	0.63502714	1.0000000	0.5531437	0.08043696
NAs	1.0000000	0.63502714	0.3381043	-0.0463105
	NAs	**PFC**	**AON**	**VP**

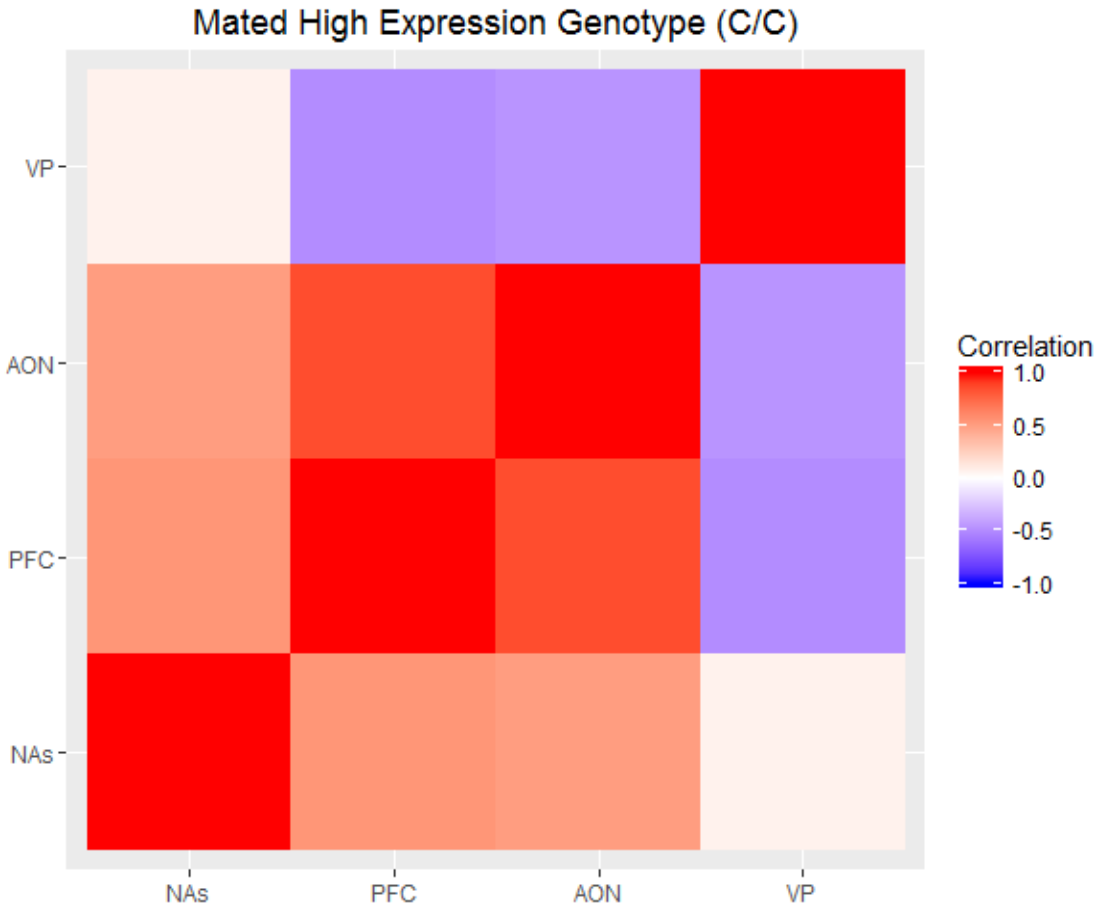


Figure 13: Fos covariance heat map for mated high expression (C/C) subjects

Abbreviations: nucleus accumbens shell (NAs); prefrontal cortex (PFC); anterior olfactory nucleus (AON); ventral pallidum (VP).

Heat maps were visualized through a ggplot2 package in RStudio which converts calculated Pearson's correlation coefficients. Such correlation coefficients are presented in the table below.

VP	0.0703156	-0.4962072	-0.4621163	1.0000000
AON	0.5070824	0.8519812	1.0000000	-0.4621163
PFC	0.8274204	1.0000000	0.8519812	-0.4962072
NAs	1.0000000	0.5420881	0.5070824	0.0703156
	NAs	**PFC**	**AON**	**VP**

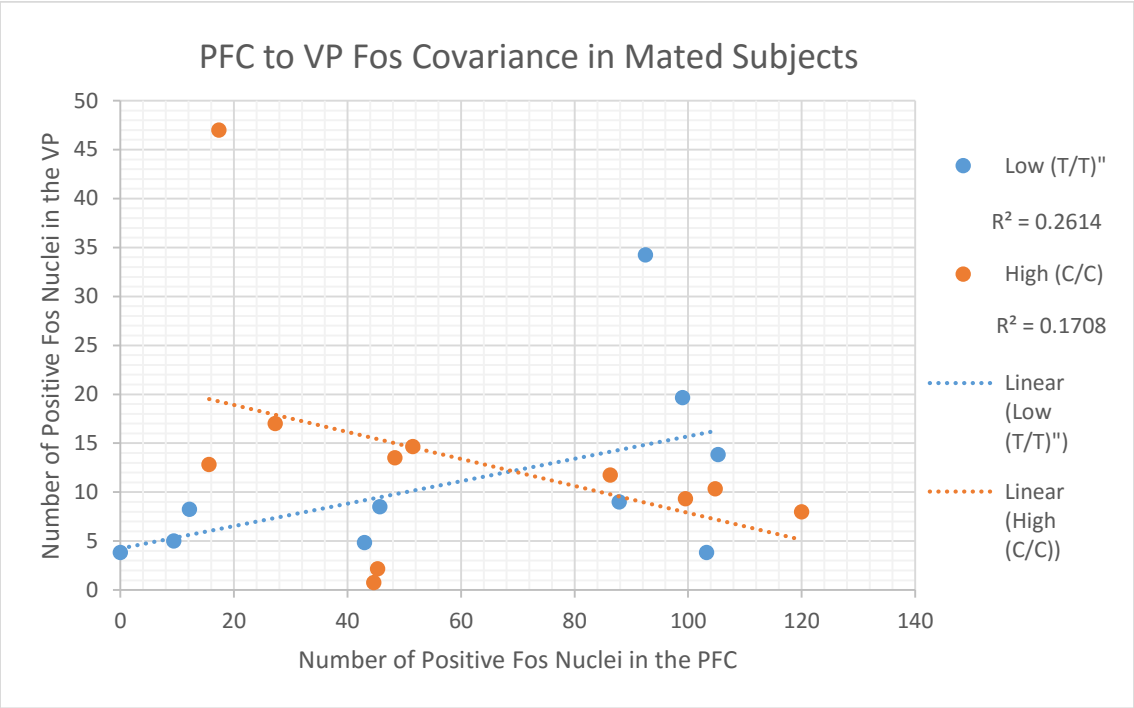


Figure 14: Comparison of prefrontal cortex (PFC) and ventral pallidum (VP) Fos covariance between "high" (C/C) and "low" (T/T) expressing oxytocin receptor density mated groups

This graph is comparing the number of positive Fos nuclei within the VP to the PFC per mated subject. There exists a shift from a positive correlation among the "low" (T/T) expressing group towards a negative correlation among the "high" (C/C) expressing group.

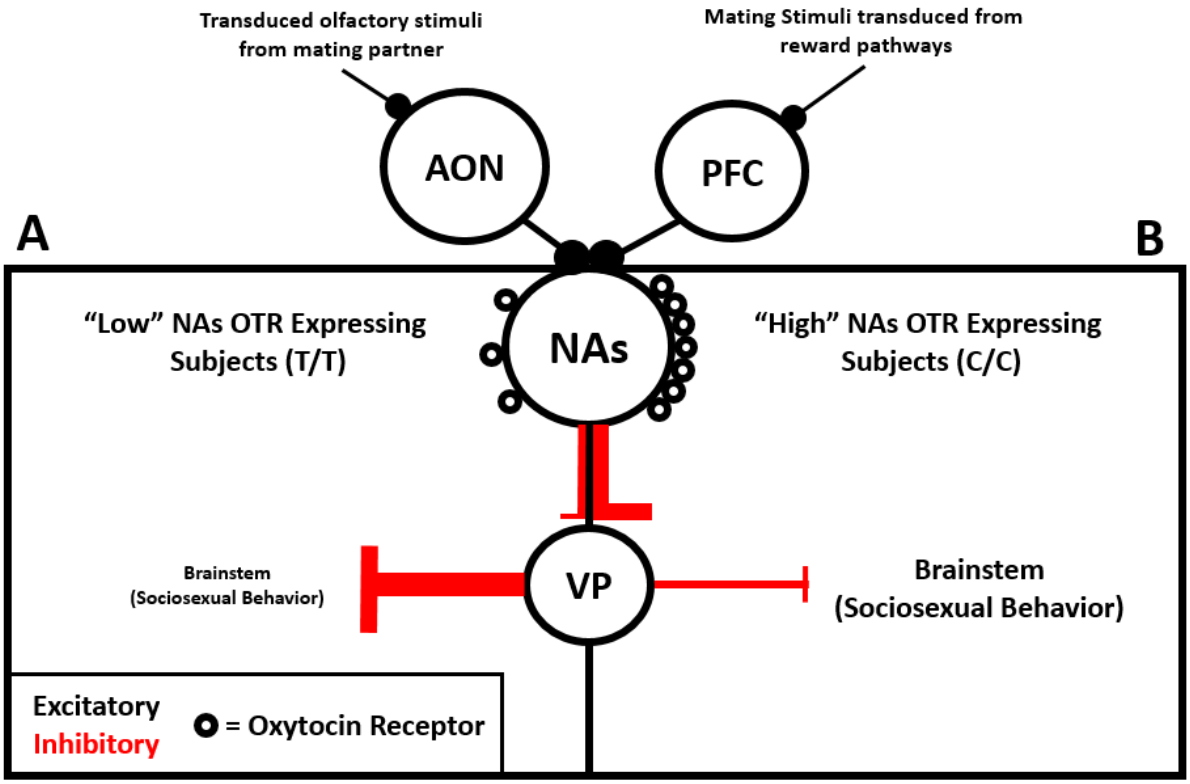


Figure 15: Revised hypothesis for how NT213739 genotype modulates sociosexual behavior

Adapted and simplified from Figure 2 and 3, the general pathway of pair bonding behavior originates from mating stimuli information coming into the prefrontal cortex (PFC) and olfactory information coming into the anterior olfactory nucleus (AON). Consequently these two nuclei, through intermediates or not, synapse at the nucleus accumbens shell (NAs). The medium spiny neurons (MSN) within the NAs provide inhibitory input to the ventral pallidum (VP). The VP then send GABAergic input to the mediodorsal thalamus and to the brainstem to regulate sociosexual behavior.

Regarding NT213739 genotype's influence on this hypothesized neuronal network, we have proposed that oxytocin receptors are producing an excitatory effect on NAs MSN. In panel A, which represents T/T genotype subjects, NAs OTR is low resulting in a decrease in MSN firing. This leads to a disinhibition of the VP which allows the increased VP activation to suppress sociosexual behavior. In panel B, which represents C/C genotype subjects, NAs OTR is high resulting in an increase in MSN firing. This leads to an inhibition of the VP which allows for a disinhibition to the brainstem resulting in increased sociosexual behavior.

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