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Oxytocin modulates distributed immediate early gene expression across forebrain nuclei in male prairie voles

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# Oxytocin modulates distributed immediate early gene expression across forebrain nuclei in male prairie voles

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B.S., University of Illinois at Urbana-Champaign, 2009

Advisor: Larry Young, Ph.D.

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**Abstract**

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By Zachary V Johnson

Oxytocin receptors (OXTRs) modulate vertebrate social behavior and exhibit diverse patterns of central expression both within and across species. Neural network models hypothesize that social behavior is modulated in a fundamentally distributed and interregional manner across reciprocally interconnected limbic and mesolimbic reward nuclei; and further that diverse patterns of neuropeptide receptor (e.g. OXTR) expression across these networks allow for diverse and species-specific network functions during social contexts. Previous experiments have not tied these hypothesized functional principles with specific neuromodulatory systems. Here, we first establish that central OXTRs modulate pair bonding behavior in socially monogamous male prairie voles, reversing a narrative that has pervaded the literature for decades. Then, using Fos immunoreactivity as a proxy of neural activation, we apply pharmacological and genetic approaches to investigate how multiple parameters of OXTR organization and signaling are linked to distributed patterns of Fos expression across a neural network that modulates social olfactory learning in rodents. We show that central OXTR signaling during sociosexual interaction modulates global covariance in Fos expression across this network in male prairie voles ($p=0.008$). We then restrict our focus to the nucleus accumbens (NAcc), a region in which OXTR density and signaling are important for social learning and behavior in prairie voles and other rodents. We hypothesized that variation in OXTR signaling in the NAcc of prairie voles would result in variation in distributed patterns of Fos expression across the network. We demonstrated that site-specific blockade of OXTRs in the NAcc decreases coupling (i.e. correlated Fos expression) of the NAcc shell with other nuclei in the network ($p=0.02$) during sociosexual interaction in male prairie voles. Next, we used a naturally occurring genetic polymorphism in the prairie vole OXTR gene (*Oxtr;* NT213739) that strongly predicts individual variation in OXTR binding density in the NAcc and other regions, and showed that genotype at this locus is associated with differences in global covariance in Fos expression across the network during sociosexual interaction ($p=0.02$). Collectively, these data support previously hypothesized links between central neuropeptide systems and distributed network function during social contexts, and suggest mechanisms by which OXTR signaling may modulate salience and reinforcing value of social stimuli during the formation of selective social attachments.

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# PROLOGUE

Human social cognition and behavior are critical factors in the structure and function of our societies. Decades of research have revealed deeply conserved neural systems regulating social behavior across distantly related vertebrates, including humans. For example, central oxytocin-like systems have emerged as deeply conserved neuroendocrine mechanisms regulating reproductive and social behavior across distantly related bilaterian animals; and oxytocin has been implicated in social behavior, social cognition, neural activity during social contexts, and autism spectrum phenotypes in humans.

This dissertation provides an overview and three independent experimental investigations of the central oxytocin system and its role in regulating neural function and behavior during social contexts. This dissertation is organized into five chapters. Chapter 1 provides a brief introduction to the central oxytocin system and emerging hypotheses regarding its modulation of behavior and neural function during social contexts. Chapters 2, 3, and 4 describe three independent experimental approaches that we adopted to address these hypotheses in socially monogamous rodents. Chapter 5 synthesizes these findings and offers additional context for interpreting experimental results.

# CHAPTER 1

## Introduction

**Adapted from:**

**Johnson, Z.V., Young, L.J., 2015. Neurobiological mechanisms of social attachment and pair bonding. Current Opinion in Behavioral Sciences 3, 38-44.**

## Introduction

Selective pressures favoring adaptive behavioral interaction with conspecifics have led to the evolution of extraordinarily diverse forms of social behavior both within and across species. In humans, social behavior and cognition have enormous influence on the structure and function of our societies. Though they are undoubtedly shaped by environmental factors (e.g. culture, early life experience), specific human social behavioral capacities appear across cultures, through our fossil record, in our closest evolutionary relatives, and in distantly related animals (e.g. social recognition, selective social attachment, bi-parental care, cooperative foraging and hunting, group living, etc.), suggesting they have biological roots. Understanding our behavior and cognition (and their dysfunction) as evolved social organisms requires dissection of their biological substrates.

Among the most powerful opportunities for investigating the biology of behavior are 1) phylogenetically distant species exhibiting convergent behavior, 2) closely related species exhibiting strikingly divergent behavior, and 3) individual species exhibiting high intraspecific variation in behavior. Within these contexts, comparative approaches can accelerate the identification of neurobiological, genetic, epigenetic, developmental, ecological, and evolutionary factors underlying the behaviors of interest [1]. In this chapter, we highlight the capacity to form enduring and selective social attachments with mating partners, or pair bonds, as an example in which these comparative approaches have identified deeply conserved biological mechanisms regulating complex social behavior across distantly related species, including humans. We then provide a brief review of neurobiological systems mediating selective social attachment (and other social behaviors) and their historical integration into functional models of neural networks that regulate social behavior across vertebrates. Subsequent chapters will focus on one of these

systems, the oxytocin (OT) system, and its modulation of social behavior and neural network function.

## Investigating pair bonding behavior

The capacity to form selective social attachments is an integral component of human sociality. One excellent opportunity for investigating the biological basis of selective social attachments is the independent evolution of pair bonding behavior across distant taxa. While sexual promiscuity is the dominant mating strategy in animals, socially monogamous mating strategies have evolved in diverse lineages spanning invertebrates, fishes, amphibians, reptiles, birds, and mammals. These systems are characterized by enduring, often lifelong, selective social attachments between mating partners (though typically not sexual exclusivity). Much of our understanding of the neurobiology of pair bonding has come from the comparative approaches mentioned above, particularly through investigations of microtine rodents. The neurobiology of pair bonding behavior in microtines, particularly the prairie vole (*Microtus ochrogaster*), will be a major, but not exclusive, focus of this chapter.

Microtine rodents exhibit extraordinary diversity in mating strategies, social structures, and social behavioral plasticity. The biological mechanisms of complex social behaviors in microtines have been most rigorously investigated in prairie voles, which exhibit socially monogamous mating behavior, alloparental and bi-parental care of offspring, selective aggression toward unfamiliar opposite-sex conspecifics (after pair bonding), empathy-based consoling behavior toward distressed mating partners and siblings, and both anxiety- and depressive-like behavior during separation from the mating partner [2-5]. Prairie voles also exhibit a high degree of intraspecific variation in these behaviors, including pair bonding behavior; both males and females can exhibit promiscuous “wandering” phenotypes [6].

Laboratory investigations of pair bonding behavior were initiated with the "partner preference" paradigm, in which a subject can freely spend its time with its familiar mating partner, a novel sexually receptive individual, or in isolation in a socially "neutral" zone [7]. This paradigm has been used to demonstrate that within 48 hours of co-habitation with a mate, socially monogamous prairie voles, pine voles, and mandarin voles—unlike promiscuous montane and meadow voles—tend to preferentially affiliate with the familiar mating partner. These selective partner preferences have become a laboratory metric for pair bond formation, and manipulations within the partner preference paradigm are used to investigate developmental, genetic, endocrine, and neurobiological factors modulating selective social attachment.

It is important to note that formation, maintenance, and expression of pair bonds consists of numerous component processes that can be investigated across many vertebrates species (including many that do not exhibit socially monogamous behavior), such as social recognition, social tolerance, affiliative behavior, reward learning, paternal behavior, social buffering, aggression toward opposite-sex conspecifics, negative affect during separation, and sociospatial memory. Investigations of these processes in microtines and other species have simultaneously advanced our understanding of biological systems modulating selective social attachment and other forms of social behavior and cognition across vertebrates.

## Oxytocin and vasopressin in pair bonding behavior

Early studies in microtines were focused on the neurohypophyseal nonapeptides oxytocin (OT) and vasopressin (AVP), which were known to regulate multiple aspects of social and reproductive behavior in other species. Comparative analyses revealed that distribution patterns of oxytocin receptors (OTRs) and vasopressin 1a receptors (V1aRs) differ markedly among closely-related microtine species exhibiting socially monogamous versus promiscuous mating systems.

Specifically, differences in OTR and V1aR densities are especially prominent in multiple mesolimbic reward nuclei including the prefrontal cortex (PFC), nucleus accumbens (NA), ventral pallidum (VP), and lateral septum (LS) [8, 9]. Blockade of OTR or V1aR in these areas during co-habitation—specifically OTR blockade in the NA or PFC or V1aR blockade in the LS or VP—inhibits partner preference formation in prairie voles, demonstrating the functional significance of these receptors in mesolimbic reward networks [8]. It is important to note that effects of central OTR manipulations on pair bonding behavior have been predominantly demonstrated in female prairie voles, while the effects of central V1aR manipulations on pair bonding behavior have been predominantly demonstrated in male prairie voles, a topic that will be discussed in more detail in Chapter 2.

Socially monogamous prairie, pine, and Taiwan voles exhibit higher V1aR expression in the VP compared to promiscuous montane and meadow voles, suggesting that increased V1aR expression in the VP may be a conserved mechanism contributing to the convergent evolution of pair bonding behavior across microtine species [8, 9]. In support of this hypothesis, RNAi knockdown and selective antagonism of V1aRs in the VP of socially monogamous prairie voles inhibits partner preference formation, and overexpression of V1aR in the VP of promiscuous meadow voles induces partner preference formation [10, 11]. Multiple species of socially monogamous and non-monogamous deer mice exhibit high V1aR expression in the VP [12], suggesting that high levels of V1aR in the VP are not sufficient, but may be necessary, for the evolution of socially monogamous mating behavior across rodents.

The VP is the principal target of efferent projections from the NA, a mesolimbic reward area in which socially monogamous prairie voles and Taiwan voles exhibit higher OTR densities relative to non-monogamous meadow voles, montane voles, mice, and rats. Like prairie voles, socially

monogamous marmosets exhibit high OTR density in the NA, while coppery titi monkeys have high V1aR in this area [13, 14]. Humans also exhibit a capacity to form selective social attachments with mating partners; ligands promiscuous for both OTRs and V1aRs have been used to investigate human brain tissue and have revealed dense binding in the NA [15, 16]. To the best of our knowledge, every socially monogamous mammalian species examined to date exhibits high OTR and/or V1aR expression in the NA-VP circuit, encouraging further research on neuropeptidergic regulation of this circuit in the evolution of pair bonding behavior.

Growing evidence suggests that OT and AVP modulate formation of selective social attachments in diverse vertebrate lineages [17]. Blockade of both OTR and V1aR homologs in monogamous cichlid fishes reduces affiliative behavior during bond formation [18]. Exogenous OT delivery promotes affiliative behavior toward conspecific and human partners in dogs [19]. The OTR homolog (mesotocin receptor) mediates pair bonding behavior in monogamous zebra finches [20-22]. OT and AVP regulate pair bonding behavior in socially monogamous marmosets and coppery titi monkeys, respectively [23-25]. Humans exhibit variation in selective social attachment to mating partners, and *OXTR* and *V1AR* gene variants are associated with relationship status [26, 27]; plasma OT levels are correlated with duration of romantic relationships [28]; and in romantically attached heterosexual males, intranasal OT increases NA activity while viewing their partner's face and increases preferred interpersonal distance from unfamiliar females [29, 30].

## Mesolimbic dopamine systems in pair bonding behavior

All nervous systems face the challenge of filtering, encoding, and updating a continuous barrage of multi-modal sensory information into learned associations that guide adaptive behavior [31]. In vertebrates, the mesolimbic reward network—in part comprising connections between the ventral tegmental area (VTA), PFC, NA, VP, and LS—is an evolutionarily conserved neural network that

facilitates this process by generating motivation to seek reward and avoid aversion, and by assigning salience to cues associated with these outcomes. The action of dopamine (DA) within this pathway is critical for these functions, predominantly by encoding reward prediction error [32, 33].

Mesolimbic DA networks modulate pair bonding behavior. Partner preference formation is facilitated by mating, which in prairie voles, like mice and rats, increases DA release and turnover in the NA [8]. DA action at one of its target receptors, D2R, in the NA is necessary for bond formation [8]. Interestingly, concurrent signaling at both OTRs and D2Rs is necessary for pair bond formation, and it has recently been hypothesized that these two receptors heterodimerize to mediate their effects [34, 35].

After bonding, a second DA receptor, D1R, is upregulated in the NA and is critical for bond maintenance [36]. The balance of D1R:D2R signaling in the ventral striatum has also been implicated in drug addiction and abuse [37]. In mice, repeated administration of cocaine increases the ratio of D1R:D2R signaling in the NA, and this reorganization is necessary for subsequent behavioral plasticity [38]. In prairie voles, the ratio of D1R:D2R expression in the NA increases following pair bond formation, and this reorganization is necessary for subsequent behavioral plasticity (increased aggression toward unfamiliar opposite-sex conspecifics) [36]. Drugs of abuse impair social attachment in prairie voles (like humans) due to plastic changes in mesolimbic DA circuits. In prairie voles, amphetamine-induced upregulation of D1R inhibits bonding; in contrast, after bonding, D1R is upregulated and mediates a decrease in the reward value of amphetamines [39, 40]. Interestingly, manipulations of both central OT and AVP signaling have been repeatedly shown to modulate responses to cocaine and other drugs of abuse in rats and mice [41, 42]; and in prairie voles, manipulations of central OT signaling modulate accelerated partner preference

formation induced by selective D2R activation in the NA [34]. Collectively these data suggest overlap in the neural mechanisms modulating pair bonding and drug reinforcement, and have contributed to the conceptualization of pair bonds as social addictions, mediated by plasticity in mesolimbic DA networks that regulate numerous forms of reward error prediction, incentive motivation, and appetitive behavior [37, 43]. Studies in socially monogamous zebra finches and coppery titi monkeys have also reported reorganization in mesolimbic DA regions following pair bond formation, suggesting that neural plasticity in these networks may be an evolutionarily conserved feature of pair bonding behavior across vertebrates [44, 45].

**Component processes and other systems in pair bond formation**

Formation, expression, and maintenance of pair bonds involves a variety of component processes that often exhibited by species that do *not* exhibit social monogamy. For example, pair bonding depends on social recognition, a neural process that, in mice and other rodents, is olfactory and dependent on OT and AVP signaling [46]. OTRs are densely expressed in olfactory processing networks in rodents, while in primates—in which audition and vision are more dominant sensory modalities—OTRs are expressed in areas critical for visual and auditory attention and processing, suggesting that OT signaling may modulate sensory processing across species, though the particular modalities may differ [13, 14, 47]. Consistent with this hypothesis, human polymorphisms in *OXTR* predict performance in facial recognition tasks, suggesting that OT's role in social recognition may be conserved between rodents and humans, across sensory modalities [48].

Pair bonding typically involves enduring social tolerance and affiliative behavior toward the mating partner. Microtine species vary in social tolerance and affiliative behavior toward opposite-sex conspecifics, perhaps in part due to differences in D1R signaling in the NA. In prairie voles,

bond-induced upregulation of accumbal D1R expression mediates a drastic increase in both avoidance and aggressive behavior toward unfamiliar conspecifics, and selective D1R activation in the NA inhibits pair bond formation [8]. Meadow voles, which are less affiliative in the laboratory (though exhibit seasonal plasticity in the wild), have higher baseline D1R densities in the NA, and selective D1R *blockade* in the NA increases affiliative behavior, consistent with findings in mice [49, 50]. Studies in socially monogamous zebra finches suggest that mesolimbic DA networks may play a critical role in social tolerance, avoidance, and affiliative behavior across vertebrate lineages [51-53].

Formation, maintenance, and expression of selective social bonds depend on numerous additional component processes, including social motivation, reward learning, mate guarding, social buffering, negative affect during separation, and sociospatial memory. In addition to DA, opioid signaling is important during reward learning and regulates formation and maintenance of pair bonds in prairie voles, perhaps by mediating positive hedonics during formation and negative hedonics during maintenance [54, 55]. The corticotrophin releasing factor (CRF) system, which mediates anxiety-like behavior during maternal separation, mediates pair bond formation and depressive-like behavior during separation from the mating partner [4, 56]. Social buffering is a common feature of social bonds; in prairie voles, following a stressful experience, OT release in the presence of the partner reduces peripheral stress hormone levels and anxiety-like behavior [57]. In nature, pair bond maintenance depends on accurate recall of the mating partner's geographical territory. Recent field studies have shown that central OTR and V1aR binding density in spatial navigation/memory areas predicts space use, mating strategy ("resident" or "wanderer"), and reproductive success in prairie voles, possibly by facilitating integration of social information (e.g.

territories of partner, reproductive competitors, and/or reproductive opportunities) into spatial maps [6, 58].

Other forms of selective social attachments (e.g. maternal bonds) require many of the same component processes and neural substrates as pair bonds between mates, including social recognition and central OT signaling [17]. It is hypothesized that the same neural systems underlying maternal attachment to offspring serve as the primary neurobiological substrates facilitating evolution of selective attachment to mating partners [59, 60]. This general hypothesis may extend more broadly to additional forms of maternal behavioral plasticity, as males in socially monogamous species often exhibit a suite of maternal-like behaviors in addition to selective social attachment to the mating partner, including parental care of offspring, aggression toward unfamiliar opposite-sex conspecifics, and anxiety-like behavior during periods of separation from the mating partner.

## Variation in nonapeptide systems and social behavioral diversity

OT- and AVP-synthesizing neurons (and their homologues) vary in cellular morphology, neuroanatomical distribution, projection targets, and context-dependent activation patterns across vertebrate lineages [31, 61, 62]. It is important to note that greater evolutionary plasticity in these parameters has been observed in AVP compared to OT neurons; AVP neurons exhibit species diversity in extra-hypothalamic forebrain distribution and therefore in central projection targets and context-dependent activation patterns [63, 64]. In contrast, OT soma are localized in the hypothalamus across vertebrates, and evolutionary variation in the parameters mentioned above largely aligns with deep evolutionary divergence between fishes, amphibians, reptiles, birds, and mammals [62, 65]. Although the evolution of OT and AVP neurons has undoubtedly shaped the evolution of vertebrate social behavior, an extensive review of this important topic is beyond the

scope of this chapter (but can be found in the references above). Here, we will shift focus to variation in central expression patterns of OTRs and V1aRs (and homologous receptors).

Experiments in fishes [66, 67], amphibians [68, 69], birds [70, 71], and mammals [9, 72-75]—including primates [13, 14]—have revealed diverse central expression patterns and region-specific densities of OTRs and V1aRs across distant vertebrate lineages. Comparative studies in microtines were among the first to demonstrate the remarkable divergence in neuroanatomical distribution and region-specific densities of OTRs and V1aRs between closely-related species of the same genus [76, 77]. These were followed by additional comparative experiments in closely related species of estrildid finches [78, 79], bathyergid rodents [80], scotinomys mice [81], peromyscus mice [12, 82], and ctenomys tuco-tucos [83], collectively revealing that central expression patterns of OTRs and V1aRs have undergone unusually rapid evolutionary divergence in tandem with social behavioral divergence in multiple independent lineages of vertebrates. This remarkable evolutionary plasticity in central organization of OTRs and V1aRs is thought to be a major neurobiological mechanism underlying species diversity in social behavior [31, 62, 84]. Consistent with this hypothesis, interspecific variation in region-specific OTR and/or V1aR expression is correlated with gregariousness in estrildid finches [78, 79], group living and parental care in some rodents [85, 86], and mating systems in microtine (but not peromyscus) rodents [76, 77] and possibly in primates [14, 60, 87, 88].

Many species also exhibit *intra*specific variation in central OTR and V1aR expression that correlates with individual variation in social behavior. In rodents, for example, intraspecific variation in region-specific expression of V1aR is associated with variation in social investigation, pair bonding, selective aggression, space use, mating tactics, sexual fidelity, parental behavior, and reproductive success in prairie voles [89-93]; paternal behavior in meadow voles [94];

dominance behavior in hamsters [95]; and maternal behavior in mice and rats [96, 97]. Individual variation in region-specific expression of OTR is associated with variation in social investigation, alloparental behavior, space use, and social monogamy in prairie voles [85, 98, 99]; social contact and paternal behavior in meadow voles [94, 100]; social investigation, anxiety-like behavior during social isolation, aggression, sexual behavior, and maternal behavior in rats [101-104]; maternal behavior in mice [96]; and social status in eusocial naked mole rats [105]. Selective receptor antagonists and RNAi knockdown have been used to demonstrate the functional roles of region-specific receptor populations in modulating social behavior in fishes, birds, and mammals [61, 79, 101, 106-116].

Interestingly, central patterns of OTR and V1aR expression in adult prairie voles do not appear to differ by sex and are almost entirely resilient to robust hormonal and environmental manipulations. In contrast, individual differences in region-specific expression have been linked to genetic variation in and around OXTR and AVPR1A [117-120]. However, it is important to note that patterns of central OTR and V1aR expression vary throughout postnatal development in microtines and other rodents [121-126]; exhibit region-specific epigenetic modification and steroid hormone regulation in some rodents [127-129]; and vary seasonally, between sexes, and across reproductive cycles in some rodents, although no clear patterns have emerged [129]. These data suggest OXTR and AVPR1A expression can be modulated by diverse gene regulatory mechanisms across species; it may be that evolutionary plasticity in OXTR and AVPR1A gene regulatory regions allows for diverse forms of region-specific transcriptional regulation. Interestingly, polymorphisms in OXTR and AVPR1A are associated with individual differences in social behavior and cognition in chimpanzees and humans [26, 27, 130]; as well as neural activity during social tasks and autism

spectrum phenotypes in humans [131-136], although forebrain receptor distributions have not yet been conclusively mapped in these species with selective ligands [16].

## Neural network models

Significant progress has been made toward integrating our knowledge of neuropeptide (and steroid hormone) systems into functional models of neural networks that regulate social behavior. Perhaps the most explicit first leap toward synthesizing these domains was the initial conceptualization of a conserved mammalian "social behavior network" (SBN)—comprising reciprocally interconnected, steroid-sensitive hypothalamic, amygdalar, and midbrain nuclei [137, 138]—in the late 1990's. Decades of behavioral and neuroanatomical experiments had presented new challenges for modeling neural network regulation of social behavior, namely by revealing that 1) many of the same brain nuclei, neuronal populations, and steroid hormone systems modulate a wide range of male and female social behaviors (e.g. sexual behavior, scent-marking, maternal behavior, and aggression) across multiple rodent species, 2) similar behavioral effects (e.g. rescue of sexual behavior in castrated males) can be induced by similar steroid hormonal manipulations in different limbic nuclei, and 3) anatomically, many behaviorally critical brain nuclei and neuronal populations are reciprocally interconnected. With the aim of providing an intentionally simplified but useful framework for integrating these findings, the SBN was characterized as an evolutionarily conserved, steroid-sensitive, reciprocally interconnected network of limbic nuclei that modulates all mammalian social behavior (in both sexes) in a distributed, non-linear fashion. Within this framework, it was thought that developmental, sexual, seasonal, and evolutionary variation in steroid hormone release and receptor expression across the network could allow for extensive functional variation in similar social contexts. In addition to information processing and storage at the level of synapses and intraregional neural circuits, emphasis was placed on

distributed, interregional activity and plasticity across the network as an another critical yet poorly understood functional layer of sensory/hormonal integration and behavioral modulation that should be subjected to experimental investigation.

Important advances have built upon these principles and have been incorporated into functional models of neural networks that modulate social behavior. Comparative neuroanatomical experiments suggest that the SBN is deeply conserved across all vertebrates, facilitating integration of findings from fishes, amphibians, reptiles, and birds [61]. Mesolimbic dopamine networks are also deeply conserved across vertebrates and reciprocally interact with neuropeptide and steroid hormone systems to modulate social learning and behavior; these networks have been combined with the SBN into an anatomically expanded vertebrate "social decision making" network (SDMN) [31, 139]. Diverse neuroanatomical organization of deeply conserved neuropeptide systems—particularly OT, AVP, and their respective receptors (and evolutionary homologues)—across the SDMN have also been explicitly hypothesized (in addition to steroid hormone systems) to serve as additional neuromodulatory layers facilitating diverse network functions during similar social contexts [87]. Finally, conceptualizations of distributed, interregional information processing across these networks have also developed; in addition to the overall pattern of relative activity or "weighting" across nodes, patterns of functional "coupling" or "connectivity" across nodes (i.e. correlated electrophysiological activity, gene expression, metabolic activity, etc.) are emphasized as important reflections of network function and are hypothesized to be modulated by steroid hormone and neuropeptide systems [140]. This functional principle has been tested using expression of immediately early genes (e.g. Fos, egr-1) and metabolic markers (e.g. cytochrome oxidase) to show that distinct social contexts are associated with distinct patterns of correlated expression across SDMN nuclei in fishes, amphibians, reptiles, and birds [53, 141-144]; however,

previous experiments have not tested whether these distributed functional properties are indeed tied to specific neuromodulatory systems.

It is important to note that like the SBN, the SDMN is not intended to be a comprehensive model of all brain nuclei that are critical for social behavior. Instead, it is intended to provide a useful evolutionary framework for integrating advances across species and experimental paradigms. Similarly, intentionally simplified neural network models of associative social olfactory learning (which include multiple SBN and SDMN nodes) have emerged from investigations of social odor processing, social recognition, and formation of selective social attachments. Like the SDM and SDMN, these networks are also thought to comprise deeply conserved, steroid- and neuropeptide-sensitive, anatomically interconnected nuclei; and like behavioral modulation by the SBN and SDMN, associative learning of social olfactory profiles in these networks is thought to occur at multiple anatomically-defined levels, including distributed interregional processing that is modulated by mesolimbic dopamine, steroid hormone, and neuropeptide systems. In prairie voles, for example, it is thought that unique organization of OTR, V1aR, and D1R/D2R (and other systems) across associative social olfactory learning networks facilitates unique and distributed encoding of partner-associated cues during sociosexual interaction. Our current model is that during sociosexual interaction, OT and AVP release facilitates transmission and neural encoding of the partner's sensory profile across interconnected olfactory, limbic, and mesolimbic reward nuclei; and simultaneous DA release modulates reward learning and integration of partner- and mating-associated cues. Neuroplastic changes associated with these processes, including upregulation of D1R in the NA, bias the network toward encoding subsequent unfamiliar opposite-sex olfactory signatures as aversive and facilitate plasticity in a suite of SDMN-regulated social behaviors associated with pair bond formation, expression, and maintenance.

## Conclusions

Evolutionarily conserved neural networks and neuromodulatory systems regulate selective social attachment and other forms of social learning and behavior across species. For example, the OT and AVP systems are deeply conserved neuroendocrine systems that modulate social behavior across vertebrates. Central distribution patterns of OTRs and V1aRs exhibit rapid evolutionary plasticity and anatomical variation that are functionally important for social behavioral diversity. These anatomical and functional properties have been integrated into continuously advancing models of neural networks that regulate social learning and behavior. It is hypothesized that anatomical variation in central steroid hormone and neuropeptide systems reflects functional variation across neural networks at multiple levels, including a distributed interregional level of processing where variation is tied to differences in functional connectivity across neural networks during social contexts. This hypothesis has not been directly tested or tied to specific neuromodulatory systems and therefore represents a significant gap between hypothesized models and experimental evidence. In the following chapters we address this gap by investigating the OT system—long thought to modulate pair bonding behavior exclusively in females—in male prairie voles. We use both pharmacological and genetic approaches to investigate the role of this system in modulating selective social attachment, sociosexual behavior, and distributed activity across a hypothesized pair bonding neural network (PBN) during sociosexual interaction with a female.

# CHAPTER 2

## Central oxytocin receptors modulate sexual behavior, partner preference formation, and correlated Fos expression across a pair bonding neural network in male prairie voles

**Adapted from:**

**Johnson, Z.V., Walum, H., Jamal, Y.A., Xiao, Y., Keebaugh, A.C., Inoue, K., Young, L.J., 2016. Central oxytocin receptors mediate mating-induced partner preferences and enhance correlated activation across forebrain nuclei in male prairie voles. Hormones and Behavior 79, 8-17.**

**Abstract**

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

**Keywords:** pair bonding, social attachment, monogamy, immediate-early gene, social decision-making network

## Introduction

The oxytocin (OT) system is an evolutionarily conserved neuroendocrine mechanism that regulates reproductive and social behaviors across divergent taxa, spanning nematodes and humans [65, 145, 146]. In mammals, OT modulates social recognition [107, 130], maternal responsiveness and mother-infant bonding [59, 147], pair bonding behaviors in monogamous species [87, 148, 149], and even human-dog bonding [150, 151]. It has been hypothesized that OT facilitates social bonding behaviors by modulating neural transmission and encoding of social information across sensory and reward processing brain areas [87, 147].

In mammals, OT is predominantly synthesized in the paraventricular, accessory, and supraoptic nuclei of the hypothalamus [62]. OT is released within the brain in response to stimuli associated with parturition and nursing in females [152] and mating in both sexes [153, 154], and OT neurons are activated following tactile stimulation in rats and voles [155, 156]. In humans, peripheral OT is increased in response to social vocalizations [157] and eye contact [151, 158], although the relationship between peripheral and central OT remains unclear [159].

OT mediates its central effects by binding with high affinity to OT receptors (OTRs) and/or with lower affinity to vasopressin 1a (V1aR) receptors [160, 161]. The expression patterns of these receptors throughout the forebrain are extraordinarily diverse both within and across species, and are thought to have contributed to the evolution of diverse patterns of neural and sociobehavioral plasticity during species-typical social contexts [162].

*Microtine* rodents, or voles, exhibit rich variation in social behaviors, and the socially monogamous prairie vole, *Microtus ochrogaster*, has been the subject of intense investigation of the neural mechanisms underlying diversity in social behaviors and mating strategies [7]. Voles exhibit both intra- and interspecific variation in forebrain OTR and V1aR distribution [163, 164],

and these patterns have been associated with variation in social investigation [99], mating strategies and reproductive fitness within mating strategies [98], alloparental care [85, 111], sexual fidelity and space use [6], and pair bonding [10, 76, 77, 165]. Recent investigations have implicated these systems in social affiliation and bonding in additional vertebrate lineages spanning fishes [18], birds [166], and mammals [23, 150, 167], including humans [149, 168, 169].

In male and female prairie voles, mating facilitates pair bonding. In the laboratory, pair bond formation is reflected through formation of a robust and enduring preference for the mating partner relative to a novel opposite sex individual, referred to as a "partner preference" [170]. Early pharmacological studies revealed that OTR signaling in the brain is critical for partner preference formation in female prairie voles [171, 172]. More specifically, blocking OTR in the prefrontal cortex (PFC) or nucleus accumbens (NA) prevents mating-induced partner preferences in females [108]. Although exogenous central OT infusions facilitate partner preference formation in both sexes, a single seminal paper failed to show that OTR blockade inhibits mating induced partner preferences in males [173, 174]. It appeared that endogenous vasopressin, and not OT, regulated pair bonding in male prairie voles by acting at V1aR receptors in the ventral pallidum and lateral septum [34, 109, 175]. These sexually dimorphic roles for OT and AVP have pervaded the literature on pair bonding for the past 20 years.

In these experiments, we revisit the role of OTR in partner preference formation in male prairie voles, and demonstrate conclusively using a highly selective OTR antagonist (OTA) that, as in females, central OTR signaling is critical for pair bonding in males. We then introduce an abbreviated cohabitation paradigm to investigate the role of central OTR signaling in modulating sociosexual behavior and neural activity across a hypothesized pair bonding network (PBN), with the aim of identifying potential neural mechanisms by which central OTR signaling may modulate

pair bond formation. To achieve this goal, we infuse OTA or aCSF intracerebroventricularly (ICV) into male prairie voles and measure sociosexual behaviors as well as induction of the immediate-early gene transcription factor, Fos, following cohabitation with a sexually receptive female. We first demonstrate that this paradigm is a useful tool for investigating sexual behavior and neural activity in male prairie voles by replicating findings from investigations in other rodents. Next, we restrict our analysis to nuclei within the PBN and find no evidence that central OTR signaling during sociosexual interaction modulates Fos expression within any of the analyzed brain nuclei. However, our data suggest that central OTR signaling during sociosexual interaction plays a critical role in modulating a robust pattern of correlated Fos expression *across* PBN nuclei. These results provide novel insights into how OTR modulates neural activity across the PBN, and are consistent with previously outlined hypotheses that OTR modulates functional connectivity [140] and transmission of social information across conserved brain networks [87, 147] during species-typical social contexts.

## Materials and Methods

### Subjects

Male prairie voles were housed in groups of two or three until stereotaxic surgery during adulthood (60-200 days), after which they remained singly housed until behavioral testing. Housing consisted of a ventilated 26x18x19 cm Plexiglas cage filled with Bed-o-cobbs Laboratory Animal Bedding under a 14:10 h light/dark cycle at 22°C with *ad libitum* access to food (rabbit LabDiet) and water. Subjects were drawn from our laboratory breeding colony originally derived from field captured voles in Illinois. Stimulus animals were sexually experienced, ovariectomized, estrogen-primed (see below), adult female prairie voles. All procedures were conducted in accordance with the

National Institutes of Health Guide for the Care and Use of Laboratory Animals and were approved by the Emory University Institutional Animal Care and Use Committee.

**Experiment 1: Partner Preference**

*Intracranial Cannulation*

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

*OTA administration*

4 days following surgery, subjects received microinjections of either 2 µl of control artificial cerebral spinal fluid (aCSF, n=18) or 2.5 ng/µL of a selective oxytocin receptor antagonist (OTA), des Gly-$NH_2$d$(CH_2)_5$-[D-$Tyr^2$,$Thr^4$]OVT [176] dissolved in 2µl of aCSF (n=21) using a 28 gauge internal cannula (Plastics One) extending 0.5 mm beyond the end of the guide cannula into the lateral cerebral ventricle. Injections were performed with a 10 µl Hamilton syringe (Hamilton; Reno, Nevada) connected to polyethylene-20 tubing (Plastics One), which was in turn secured to the internal cannula. Infusions were administered over the course of 60 seconds, and the internal cannula was left in place for 3 minutes following infusion to prevent backflow. Two infusions were administered to each subject; one immediately prior to the 48-hour cohabitation, and the second 24 hours into the cohabitation.

*Cohabitation and Behavioral Analysis*

Immediately following OTA infusion, subjects were placed in a clean cage (26x18x19 cm) with a sexually experienced, ovariectomized, estrogen-primed, adult stimulus female for 48 hours. In the three days preceding cohabitation, stimulus females were brought into estrus with daily injections of 4.0 µg estradiol benzoate dissolved in sesame oil (Sigma; St. Louis, MO; S3547) injected subcutaneously. The first 3 hours of cohabitation were video recorded and analyzed for mating; only one pair did not mate during this period and was excluded from final analysis. After 24 hours of cohabitation, males were briefly removed from the home cage, anesthetized, infused with a second dose of OTA as described above, and immediately returned to cohabitation. Following 48 hours total of cohabitation male subjects and stimulus females were separated overnight. The following day, all subjects underwent a 3-hour partner preference test. In this paradigm, the male subject is placed in a central "neutral" zone of a 3-chambered apparatus in which the familiar female "partner" is tethered in one far chamber and the novel female "stranger" is tethered in the opposite chamber [177]. The experimental animal is free to move throughout the chambers and the time spent in immobile social contact with each female is recorded using an automated tracking system (SocialScan 2.0, Clever Sys Inc., Reston, VA, USA) [178]. All tests were video recorded and analyzed *post hoc* for time spent in immobile contact, or huddling, with the female partner.

*Injection Site Validation*

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

**Experiment 2: Sociosexual behavior and Fos expression**

*Intracranial cannulation*

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

*OTA administration*

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

*Cohabitation*

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

*Behavioral scoring*

Non-interaction, social investigation, mounting, and intromission behavior were quantified for male subjects using Observer XT 10 behavioral scoring software (Noldus Information Technology Inc.; Leesburg, VA) by two independent scorers blind to treatment groups. Four different mutually exclusive behaviors performed by the male were scored: non-interaction, investigation, mounting, and intromission. The operational definitions used for each behavior were as follows. Non-interaction included any male behavior not directed towards the female, including autogrooming, running, freezing, eating, drinking, sleeping, digging, and exploration. Investigation comprised male behavior directed immediately towards the female, including physical pursuit, olfactory investigation, and allogrooming. Mounting was defined as placement of the forepaws on the female from the rear without stereotypical intromission behavior. Intromission was scored during copulation bouts with the female, including stereotypical patterns of thrusting.

*Perfusion and post-fixation*

Seventy-five minutes following initial introduction of the stimulus female or the control bedding manipulation, male subjects were administered an overdose of isofluorane and were immediately perfused transcardially at a rate of approximately 4 mL/minute with 40 mL of 1x phosphate buffered saline (pH=7.4; diluted to 1x with distilled, deionized 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). Five subjects (n=2 aCSF-treated, n=3 OTA treated) were excluded from analysis of Fos expression due to low perfusion quality. Following perfusion, brains were extracted and post-fixed in 4% paraformaldehyde dissolved in 1X PBS overnight before being transferred to 30% sucrose in 1x PBS solution until sectioning.

*Tissue Processing and Immunohistochemistry*

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

*Injection Site Validation*

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

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

*Imaging and Fos expression analysis*

Images were captured using a Nikon E800 microscope and 10x objective using MCID Core imaging software (InterFocus Ltd; Cambridge, UK). Fos expression was analyzed within the medial preoptic area (MPOA) as a positive control, as previous studies in a wide range of species have demonstrated elevated Fos expression within this nucleus in males following mating. 7 additional OTR-expressing brain regions within the PBN (Fig. 4C) were imaged across subjects: anterior olfactory nucleus (AON), medial amygdala (MeA), basolateral amygdala (BLA), nucleus accumbens core (NAc), nucleus accumbens shell (NAs), medial prefrontal cortex (PFC), and paraventricular hypothalamic nucleus (PVN). The AON and MeA were analyzed because of their role in olfactory processing and social recognition [107]. The BLA was analyzed because of its role in transmitting social information to the reward system [147], and the demonstrated role of the amygdala in partner preference formation [179]. The NAc, NAs, and PFC were analyzed because OTR signaling in these nuclei is critical for partner preference formation in female prairie voles [108]. Finally, the PVN was analyzed because it is the major source of OT innervation to the NA and other forebrain nuclei [153, 180]. The Mouse Brain atlas (Franklin and Paxinos; 3rd Edition) was used as a reference to determine anatomical boundaries. For each brain region, Fos was quantified in both left and right hemispheres across 3 consecutive sections for all subjects; sections or hemispheres with damaged/absent tissue in the target region were excluded. Fos-positive nuclei were quantified for each region using the MCID grain count function with a constant threshold applied across all subjects. For each subject, Fos expression counts within each region were averaged, yielding a single average Fos count per brain region per subject.

**Statistical analyses**

All statistical analyses were performed in R 3.1.1.

*Partner preference*

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

*Sociosexual behaviors*

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

*MPOA Fos expression*

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

*Fos expression within PBN nuclei*

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

*Fos expression covariance across the PBN*

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

## Results

*Effects of central OTA on male partner preference formation*

A two-way ANOVA revealed a significant main effect of stimulus (partner versus stranger; $F_{1,60}$=5.77; p=0.019), and stimulus by treatment (aCSF versus OTA) interaction ($F_{1,60}$=6.208;

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

*Effects of central OTA on mating*

We then used a 30 minute cohabitation paradigm to investigate the effect of central OTA administration on sexual behavior in male prairie voles. Central administration of OTA to adult male rats reduces intromissions during sociosexual interaction with a female [181], and therefore we hypothesized that central administration of OTA to adult male prairie voles would reduce intromission frequency in this paradigm. Among all subjects that underwent cohabitation with a sexually experienced and receptive stimulus female (aCSF, n=16; OTA, n=20), intromission frequency was reduced in OTA-treated (2.49±0.62 mating bouts per 30 minutes) relative to aCSF-treated (5.30±1.14 mating bouts per 30 minutes) subjects. Consistent with findings in rats, a regression analysis comparing Poisson distributions of mating bouts by treatment (aCSF vs. OTA) revealed that frequency of mating bouts significantly differed by treatment (z=-4.30; $p=2.35x10^{-5}$; Figure 2A).

*Mating and Fos expression in the MPOA*

Previous work in other rodents has demonstrated that male sexual behavior is associated with elevated Fos expression within the MPOA [182-184]. Using this 30 minute cohabitation paradigm, we investigated whether the same effect could be detected in male prairie voles. Subjects with poor perfusion quality (n=5) were excluded from analysis. Among remaining subjects (aCSF, n=14; OTA, n=17), 3 aCSF- and 8 OTA-treated males did not mate, defined as intromission, during the

30 minute cohabitation. A two-way ANOVA revealed a significant main effect of mating ($F_{1,26}$=9.87; p=0.0042; Partial Eta-squared=0.28), but not treatment (aCSF versus OTA; $F_{1,26}$=0.956; p=0.34; Partial Eta-squared=0.04) or the interaction between treatment and mating ($F_{1,26}$=1.08; p=0.31; Partial Eta-squared=0.04), on Fos expression within the MPOA. Treatment was collapsed as a factor for subsequent analyses and a Student's t-test revealed significantly elevated Fos expression in subjects that mated (n=20) versus subjects that were exposed to a female but that did not mate (n=11; p=0.0024; Figure 2B; *d*=1.4), consistent with findings in other rodent species [182-184] and previous work from our laboratory comparing Fos expression within the MPOA in male prairie voles that were exposed to and mated with a novel female, were exposed to a familiar sibling, or were unexposed [109].

*Effects of OTA on male sociosexual behaviors in males that mated*

Subsequent analyses of behavior and Fos expression were restricted to only subjects that mated to maximize divergence in central OTR signaling between aCSF- and OTA-treated groups, and to parallel the exclusion criterion of Experiment 1 (see "Materials and Methods"). Among mated subjects (aCSF, n=11; OTA, n=9), a two-way repeated-measures ANOVA revealed a significant main effect of behavior (non-interaction, investigation, mounting, or intromission; $F_{3,68}$=46.43; $p<2x10^{-16}$; Partial Eta-squared=0.67), but not of treatment (aCSF versus OTA; $F_{1,68}$=0.00; p=0.99; Partial Eta-squared=0.00) or the interaction between behavior and treatment ($F_{3,68}$=0.35; p=0.73; Partial Eta-squared=0.02) on time engaged in the analyzed social behaviors during the 30 minute period of cohabitation (Figure 3). Thus, among mated animals, OTA versus aCSF treatment had no detectable effect on any of the analyzed sociosexual behaviors.

*OTR signaling during sociosexual interaction and Fos expression in PBN nuclei*

Analysis of Fos expression in 7 OTR-expressing PBN nuclei (Fig. 4C; see "Materials and Methods") was limited to subjects that mated during the 30 minute cohabitation to maximize divergence of presumed central OTR signaling. aCSF-treated, unexposed subjects were also included for baseline comparison. A two-way repeated-measures ANOVA with treatment (aCSF-mated versus OTA-mated versus aCSF-unexposed subjects) as a between subjects factor and brain region as a within subjects factor revealed a significant main effect of treatment ($F_{2,167}$=12.53; p=8.48x$10^{-6}$; Partial Eta-squared=0.13), but not brain region ($F_{6,167}$=0.818; p=0.56; Partial Eta-squared=0.03) or the interaction between treatment and brain region ($F_{12,167}$=1.46; p=0.15; Partial Eta-squared=0.09), on Fos expression in the analyzed brain nuclei. These results suggest a strong effect of treatment on Fos expression that did not differ significantly across brain regions; therefore, brain region was excluded as a predictor for subsequent ANOVA analyses. When restricting analysis to subjects that mated, a *post-hoc* one-way repeated-measures ANOVA revealed that Fos expression across the PBN did not differ by treatment among mated animals ($F_{1,130}$=1.24; p=0.27; Partial Eta-squared=0.01); therefore, OTA-treated and aCSF-treated mated subjects were pooled for subsequent *post-hoc* analyses. A one-way repeated measures ANOVA revealed a significant effect of sociosexual interaction and mating on Fos expression across the PBN between mated animals (pooled) and unexposed controls ($F_{1,191}$=18.51; p=3.20x$10^{-5}$; Partial Eta-squared=0.09; Figure 4). Further post-hoc t-tests were performed to investigate Fos expression within individual brain nuclei between mated (pooled) and unexposed subjects, and Bonferroni correction for multiple comparisons resulted in an adjusted α of 0.007. Using this criterion, Fos expression was significantly ($p<0.007$) elevated in every analyzed region, except the PVN (p=0.023), in mated subjects relative to unexposed controls. Thus, sociosexual interaction and mating was associated with significant elevation in Fos expression across the PBN in mated

animals relative to unexposed controls, and OTA did not significantly modulate Fos induction within any of the analyzed nuclei.

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

The MeA, AON, PFC, NA, PVN and BLA are part of a hypothesized neural network underlying mating induced pair bonds in prairie voles [87, 147]. Based on our hypothesis that OTR facilitates transmission of social information across the PBN, and previously outlined hypotheses that OTR modulates functional connectivity across neural networks, we hypothesized that OTR signaling during sociosexual interaction and mating would modulate covariance in Fos expression across PBN nuclei. As seen in Figure 5a, under control conditions (no exposure to a female) Fos expression is weakly correlated across nodes, as measured by Pearson's correlation coefficients. Relative to unexposed controls, sociosexual interaction and mating results in a strong and relatively uniform increase in correlated Fos expression across the network (Figure 5b). In contrast, as seen in Figure 5c, relative to aCSF-treated mated animals, OTA injected animals exhibit a weaker, less uniform pattern of correlated Fos expression across the network. To test whether the difference between aCSF- versus OTA-treated animals is statistically significant, we used a combination of principal component analysis (PCA) and permutation testing. PCA converts a set of variables into linearly uncorrelated variables called principal components. In this transformation, the first principal component accounts for as much of the variance in the raw data variables as possible. In a situation where all investigated variables are highly correlated, the first principal component will explain a large proportion of the variance. Consistent with the increase in correlated Fos expression across PBN nuclei in aCSF-treated mated animals (Figure 5b), PCA revealed that the first principal component accounts for 68.6% of the variance. In contrast, in OTA-

treated mated animals, the first principal component accounts for 35.3% of the variance. We then used permutation testing to test whether this difference (33.3%) in variance explained by the first principal component between aCSF- and OTA-treated mated subjects exceeds what would be expected by chance (see "Materials and Methods"). This resulted in a p-value of 0.008, suggesting that OTA significantly disrupts the increase in correlated Fos expression across the PBN induced by sociosexual interaction and mating.

## Discussion

Previous work has suggested that the central OT system may modulate pair bonding in prairie voles in a sex-specific manner [172, 173], with central OTRs modulating pair bonding in females but not in males. However, more recent investigations in both birds and mammals have suggested that the OT system may modulate bonding in both sexes. For example, investigations in estralid finches have found that the behavioral effects of central OT signaling on partner preference formation in males follows the same trend as in females, although it was not statistically significant within males only [166]. In prairie voles, patterns of OTR expression across specific forebrain nuclei are associated with monogamous behavior and space use in males [98], and central OT administration enhances partner preference formation in both females and males [174]. Furthermore, a polymorphism in the OTR gene (*Oxtr*), which is robustly associated with patterns of OTR expression in the forebrain, predicts pair bonding behavior in male prairie voles (King, et al., *under review*). In humans, intranasal OT administration to romantically attached males increases activation of reward areas when viewing images of a partner's face, but not an unfamiliar stranger female's face, and increases preferred interpersonal distance from an unfamiliar female [29, 185].

A single pharmacological study, using a less selective OTA and relatively small sample sizes for experimental groups (n=6-8 per group), failed to demonstrate an effect of OTA on male pair bonding [173] and codified the notion that the endogenous OT system modulates pair bonding behavior exclusively in females. Here, we use a more highly selective OTA, administered ICV to larger samples of male prairie voles (n=16 per group) during cohabitation with a female, to investigate the role of central OTR signaling in male partner preference formation. In contrast to the previous study, OTA administration inhibited partner preference formation in mated male prairie voles ($p=0.016$). To our knowledge, this is the first conclusive, causal evidence demonstrating that endogenous OTR signaling modulates pair bonding in a male animal. In light of recent work in monogamous estralid finches [166] and monogamous cichlid fishes [18], work in additional vertebrates is required to investigate the hypothesis that the OT system is a deeply conserved neuroendocrine mechanism contributing to the evolution of social bonding in vertebrates, across sexes.

OT has deeply conserved roles in reproductive physiology and behavior across distant taxa spanning invertebrates and vertebrates [146], including mammalian species such as mice, rats, rabbits, and squirrel monkeys [186]. In rats, central administration of OTA reduces intromission frequency in males [181]. We investigated whether this effect could be detected in male prairie voles using a brief 30 minute cohabitation paradigm. Consistent with these data, central OTR blockade reduced frequency of mating bouts (n=20) relative to aCSF-treatment (n=16) during sociosexual interaction with a female ($p=2.35x10^{-5}$), suggesting that central OTR signaling modulates sexual behavior in male prairie voles. This result is consistent with findings in other mammals, but is intriguing in light of an earlier study demonstrating that central OT administration inhibits sexual behavior in male prairie voles [187]. However, it is important to note that these data

are not necessarily contradictory, as exogenous central OT delivery may not reflect endogenous central OT levels and can result in promiscuous signaling at both OTRs and V1aRs. In contrast, central delivery of an OTA specifically blocks endogenous signaling at central OTRs.

Neural investigations of sexual behavior have highlighted the hypothalamic MPOA as a deeply conserved neural locus regulating male sexual behavior across all vertebrate lineages [184]. In male rats [188], hamsters [183], and gerbils [182], sexual behavior is associated with elevated Fos expression within this nucleus. Therefore, we hypothesized that intromission within this experimental paradigm would be associated with elevated MPOA Fos expression in male prairie voles. Indeed, intromission during the 30-minute period of sociosexual interaction was associated with a significant, non-OT-dependent elevation in MPOA Fos expression ($p=0.0024$), suggesting that, consistent with findings in other rodents, Fos induction within the MPOA reflects male sexual behavior in prairie voles. Collectively, these data are not surprising, but offer strong predictive validation of a simple experimental paradigm as a framework for conducting future behavioral and neural investigations in prairie voles.

In this study, Fos expression was used as a metric of neuronal activity and plasticity across OTR-expressing and -synthesizing PBN nuclei in response to sociosexual interaction with a female. Briefly, Fos protein is encoded by the immediate early gene (IEG) *c-fos* and can heterodimerize with JUN to form AP-1 transcription factor complexes that directly up- or down-regulate expression of AP-1 responsive genes involved in a variety of intracellular processes, including synaptic plasticity [189]. Therefore, Fos (and other IEG protein products, such as Arc and Egr-1) expression is thought to be one mechanism by which a cell can rapidly adapt its phenotype in response to a stimulus (e.g. neuronal depolarization) through robust transcriptional regulation. These qualities have positioned nuclear Fos expression as a powerful tool for measuring CNS-

wide neuronal activity and plasticity during specific physiological, environmental, and behavioral contexts. Because translation of Fos protein typically peaks 60-120 minutes following stimulus presentation, Fos experiments often comprise an acute experience followed by brain fixation 60-120 minutes afterward.

Analysis of Fos expression across PBN nuclei was restricted to mated subjects to maximize divergence in central OTR signaling between treatment groups, and was limited to OTR-expressing forebrain nuclei that have been repeatedly implicated in a variety of processes (e.g. neuropeptide synthesis and release, social recognition, reward learning, and motivation; see "Materials and Methods") that are thought to be integral to pair bond formation; these areas are nodes in a hypothesized PBN model [87]. It is important to note that among those animals that mated, there were no observed differences in behavior (e.g. non-interaction, investigation, mounting, or intromission) between OTA- and aCSF-treated groups. Thus, differences in Fos expression are not likely due to differences in sensory or sexual stimulation between treatment groups, but more likely reflect differences in function and/or modulation of the PBN during equivalent sociosexual contexts.

Sociosexual interaction and mating with a female was associated with significantly elevated Fos expression across the analyzed PBN nuclei ($p=1.03x10^{-6}$), but no significant differences were observed in Fos expression within any brain region between OTA- and aCSF-mated animals. These results suggest that sociosexual interaction and mating in male prairie voles is associated with robust, non-OT-dependent increases in neuronal activity and plasticity across the PBN. However, these data should be interpreted with caution. Experiments employing alternative behavioral paradigms and analyses, larger sample sizes, alternative markers for neuronal activity and plasticity, and co-labeling of specific neuronal populations may reveal important patterns of

OT-mediated neuronal activity and plasticity within these nuclei during sociosexual interaction that were not detected in this study.

Correlated IEG expression across brain nuclei has been previously used as a tool to investigate functional connectivity (broadly defined as correlated activation of spatially distinct neural loci) between or among those regions during specific physiological, behavioral or environmental contexts [141, 144, 190, 191], with increases in correlated IEG expression between regions indicating increased functional connectivity. Here, we investigate whether central OTR signaling during sociosexual interaction with a female modulates correlated patterns of Fos expression across PBN nuclei in male prairie voles. These analyses reveal an increase in correlated Fos expression across PBN nuclei in aCSF-treated subjects relative to both unexposed controls and OTA-treated subjects, consistent with the hypothesis that central OTR signaling during sociosexual interaction and mating with a female enhances functional connectivity across the PBN in male prairie voles. These data are interesting in light of recent human fMRI studies showing that intranasal OT administration increases functional connectivity across a variety of striatal and limbic nuclei during various social contexts [192-195]; however, studies using intranasal OT should be interpreted with caution [196].

In light of these results, one hypothesis is that patterns of OTR (and V1aR) distribution across cortical, striatal, and limbic networks reflect patterns of functional connectivity that can be recruited during contexts that elicit OT release. More specifically, within the context of pair bonding in male prairie voles, it may be that sociosexual interaction triggers OT release and initiates patterns of functional connectivity across PBN nuclei that facilitate transmission of the partner's unique sociosensory signature from sensory to reward processing nuclei, where it is encoded as a positive stimulus.

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

Despite these limitations, these data are consistent with previously outlined hypotheses [140] that neuropeptide signaling can modulate adaptive forms of sociobehavioral plasticity in part by modulating patterns of functional connectivity across evolutionarily conserved neural networks, such as the social decision making network in vertebrates [31, 139], during social and reproductive

contexts that elicit OT and AVP release. Within this framework, unique OTR (and V1aR) expression patterns across the prairie vole forebrain may allow OT release during sociosexual interaction to trigger distinct, transient patterns of functional connectivity across the PBN that facilitate pair bond formation.

# Chapter 3

## Endogenous activation of accumbal oxytocin receptors during sociosexual interaction modulates correlated immediate early gene expression across forebrain nuclei in male prairie voles

**Abstract**

Oxytocin is a deeply conserved neuropeptide that modulates social and reproductive behavior across invertebrates and mammals, including humans. Oxytocin receptors (OTRs) in the brain exhibit extraordinarily diverse expression patterns and region-specific densities that are associated with divergence in species-typical social behaviors. Experiments spanning a wide range of vertebrate species have demonstrated that OTRs in specific brain regions are critical modulators of social learning and behavior. These findings have been integrated into functional models of neural networks that posit that central OTRs and other neuromodulatory receptors shift patterns of functional network connectivity during social contexts to shape sensory perception, associative learning, and social behavior; however, relatively few experiments have linked endogenous function of specific neuromodulatory systems during social contexts with these hypothesized principles. Here we use Fos expression to investigate whether region-specific OTRs modulate patterns of functional coupling during social contexts across a neural network that is thought to modulate social olfactory learning in male prairie voles. We use site-specific administration of a highly selective OTR antagonist into the nucleus accumbens—a mesolimbic reward region where OTRs are critical for social learning and behavior across species—to show that endogenous OTR activation is necessary for functional coupling with other mesolimbic reward and social olfactory nuclei during sociosexual interaction.

**Keywords:** oxytocin, oxytocin receptor, social behavior, functional connectivity, pair bonding network, mesolimbic reward system, social decision making network

## Introduction

Oxytocin (OT) is a deeply conserved peptide that modulates reproductive physiology and behavior in species as distantly related as nematode worms and humans [146, 152]. Interest in OT has surged in recent decades in response to a growing body of work demonstrating its role in regulating vertebrate social behavior.

In mammals, OT is released centrally and/or peripherally in a variety of reproductive contexts, including parturition, lactation, and mating [152]. Recent work has shown that OT release can also be triggered by more subtle social stimuli, such as social vocalizations [157], eye contact [151], and touch [156].

The sites of OT synthesis and release are deeply conserved across mammals; OT is synthesized in hypothalamic neurons that can project peripherally to the pituitary and/or centrally to a variety of brain nuclei [62]. In contrast, there is extraordinary variation in the distribution patterns and region-specific densities of OT's target receptors both within and across species [86, 87, 118, 162]. For example, in *Microtine* rodents, monogamous prairie voles exhibit high oxytocin receptor (OTR) densities in multiple forebrain nuclei relative to non-monogamous meadow and montane voles [76]. OTR densities in specific nuclei are correlated with variation in social behavior in multiple vertebrate lineages, including social investigation, alloparental care, patterns of space use, and social monogamy in prairie voles [85, 98, 99]; social contact in meadow voles [100]; sexual and maternal behavior in rats [101, 197]; and flocking behavior across five species of estrildid finches [79]. Selective OTR antagonists (OTAs) and RNAi knockdown have been used to demonstrate that central OTR populations modulate specific social behaviors in a variety of vertebrate lineages spanning fishes [106]; birds [22, 79]; and mammals [5, 108, 112, 114, 116,

198-200]. These data suggest that evolutionary plasticity in central OTR expression is a deeply conserved neuroendocrine mechanism underlying social behavioral diversity.

The enormous body of work characterizing central OTergic modulation of behavior has been integrated with functional models of deeply conserved neural networks that regulate numerous dimensions of vertebrate sociality [87, 140]. For example, a recently outlined social decision making network (SDMN) is evolutionarily conserved across jawed vertebrates. This network is a synthesis of the mesolimbic reward network and social behavior network, and is thought to modulate social communication, parenting, sexual, and aggressive behaviors in a context-dependent manner across species [31, 61, 139]. Recent experiments and models have focused on identifying and characterizing underlying functional properties of the SDMN in relation to specific social contexts and behaviors. Although these models are oversimplified and not mutually exclusive, each places emphasis on testable network properties. For example, many experiments have suggested that activity within individual nodes of the SDMN is a weaker predictor of behavioral output than the overall *pattern* of activity across the network [140, 143]. These experiments offer support for "neural context" models of SDMN function, in which individual nodes of the network can serve dynamic functional roles depending on how their activity is correlated (i.e. their functional "connectivity" or "coupling") with other nodes [201]. Within this framework, it is hypothesized that variation in distribution patterns and region-specific densities of steroid and neuropeptide receptors across the SDMN is a core mechanism driving diverse network functions during social contexts [140].

Evolutionary plasticity in central distribution of OTRs may allow for diverse patterns of functional connectivity across conserved neural networks during social contexts that trigger OT release. Unfortunately, studies investigating OTergic modulation of network connectivity are severely

lacking and collectively offer limited support. Previous experiments in a variety of non-mammalian species have used correlated expression of cytochrome oxidase (a metabolic marker) and immediate early genes (IEGs) to measure patterns of functional connectivity across neural networks during specific social contexts [141-144]. We previously combined this approach with central administration of a highly selective OTA to show that central OTRs modulate patterns of correlated Fos expression across a hypothesized pair bonding network (PBN) during sociosexual interaction in male prairie voles [198], consistent with predictions that central OTR signaling modulates functional connectivity during specific social contexts. However, these experiments do not address the functional roles of region-specific OTR populations. To our knowledge, no experiments to date have demonstrated that endogenous, region-specific neuropeptide signaling during social contexts modulates functional coupling across neural networks. Here we use site-specific administration of a highly selective OTA to test whether endogenous activation of OTRs in the NA—a PBN (and SDMN) nucleus where OTR signaling is critical for social learning and behavior across species—modulates correlated expression of Fos across the PBN during sociosexual interaction.

## Methods

### *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 26x18x19 cm Plexiglass cages filled with Bed-o'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 (rabbit 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), 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.

*Cannulation*

Adult male prairie voles were anesthetized through continuous administration of 1.5-2.5% isofluorane during stereotaxic implantation of a bilateral 26-gauge guide cannula (catalog no. C235G-2.0/SPC; Plastics One; Roanoke, VA) targeting the NA using stereotaxic coordinates (A/P +1.7 mm; M/L ± 1.0 mm; D/V -4.5 mm). The guide cannula was fixed to the skull with a combination of Jet Acrylic Liquid and Jet Denture Repair Powder (Lang Dental Manufacturing Co., Inc.; Wheeling, IL). All subjects recovered for 5-6 days following surgery.

*Antagonist administration*

After recovery, and 3 hours prior to the 30-minute cohabitation, subjects received microinjections of either 500 nl of control aCSF (n=14 exposed; n=6 unexposed) or 2.5 ng/µL OTA peptidergic ornithine vasotocin analog desGly–$NH_2$,d$(CH_2)^5$[Tyr(Me)$^2$, Thr$^4$]OVT (Bachem, Torrance, CA) dissolved in 500 nl aCSF (n=16 exposed) using a 32 gauge internal cannula (Plastics One) extending 0.5 mm beyond the end of the guide cannula into the dorsal NA shell. Injections were performed with a 10 µl Hamilton syringe (Hamilton; Reno, Nevada) connected to polyethylene-20 tubing (Plastics One), which was connected to the internal cannula. Infusions were administered over 60 seconds, and the internal cannula was left in place for 3 minutes following infusion to prevent backflow. One subject (aCSF-treated, unexposed) was excluded due to backflow during infusion. Subjects received two consecutive infusions; one into the left hemisphere and the other

into the right. Large group sizes were selected for both socially exposed treatment groups to obtain sufficient numbers of subjects that mated during cohabitation.

*Cohabitation*

3 hours following aCSF/OTA infusion, subjects underwent a 30 minute, video-recorded cohabitation with a sexually experienced, ovariectomized, estrogen-primed, adult stimulus female. In the three days preceding cohabitation, stimulus females were brought into estrus with daily subcutaneous injections of 4.0 μg 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 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. Following the 30 minute session, stimulus females were removed and subjects remained in their home cage for 45 minutes before transcardial perfusion. 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.

*Perfusion, post-fixation, and sectioning*

45 minutes following the end of cohabitation (or control sessions), subjects were administered an overdose of isofluorane and were immediately perfused transcardially at a rate of approximately 4 mL/minute with 40 mL of 1x phosphate buffered saline (pH=7.4; diluted to 1x with distilled, deionized 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 after 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 µm sections using a Microm HM 440E freezing microtome and were stored in 1x PBS with 0.05% sodium azide until Cresyl violet or immunohistochemical staining.

*Behavioral scoring*

Six 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, huddling, and attack behavior. The operational definitions used for each behavior were as follows. Non-interaction included any male behavior not directed towards the female, including autogrooming, running, freezing, eating, drinking, sleeping, digging, and exploration. Investigation included social behavior directed immediately towards the female, including physical pursuit, olfactory investigation, and allogrooming. Mounting was defined as placement of the forepaws on the female from the rear without stereotypical intromission movement. 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. Attack behavior was defined as rapid, aggressive lunging or biting directed toward the female.

*Cresyl violet staining*

Sections containing cannula implant tracks were mounted onto Superfrost Plus slides (Fisher Scientific; 12-550-150) and allowed to dry overnight before cresyl violet staining. Immediately prior to staining, sections were submerged in 100% 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 $H_2O$ 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).

*Injection site validation*

Anatomical placement of cannula implants was determined on sections stained with cresyl violet. 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 NA. 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 NA. All remaining subjects had cannulation tracks that terminated within 0.5 mm of the dorsal NA shell and were included in subsequent analyses (n=13 aCSF-treated, exposed; n=15 OTA-treated, exposed; n=5 aCSF-treated, unexposed).

*Immunohistochemistry*

Sections underwent 3 washes in 1x PBS, 10-minute incubation in 1% sodium hydroxide in 1x PBS, and 3 washes in 1x PBS with 0.5% Triton-X (Sigma) (PBST) before treatment with 5% normal goat serum (Fitzgerald; Acton, MA) in PBST for 1 hour at room temperature. Sections were then incubated for 48 hours in primary rabbit polyclonal anti-Fos antibody (1:20,000; Calbiochem ABE457) on an orbital shaker at 4°C. Following primary incubation sections were washed 5 times in 1x PBS, once in 1x PBST, and were incubated in secondary biotinylated goat anti-rabbit IgG antibody (1:500; Vector Labs BA-1000) for 2 hours. After secondary incubation, sections were treated with an avidin-biotin peroxidase system (Vectastain Elite ABC System; Vector Labs: PK6100) for 1 hour 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 minutes in 70% EtOH, 10 minutes in fresh 95% EtOH twice, 10 minutes in fresh 100% EtOH twice), bathed in Xylenes (15 minutes in Xylenes twice), mounted onto Superfrost Plus slides (Fisher Scientific; 12-550-150) while still partially wet, and coverslipped using Krystalon (EMD Chemicals Inc., Gibbstown, NJ).

*Selection of PBN nuclei*

Fos expression was analyzed in 7 PBN nuclei: the nucleus accumbens shell (NAs), prefrontal cortex (PFC), anterior olfactory nucleus (AON), medial medial amygdala (MeA), basolateral amygdala (BLA), paraventricular hypothalamic nucleus (PVN), and ventral tegmental area (VTA). All of the analyzed regions are innervated by OTergic fibers, reciprocally interconnected with other PBN nuclei, and express OTRs in male prairie voles. The NA shell was analyzed (and selectively targeted with OTA) because, in addition to other neuromodulatory systems, both OTR density and activation within this nucleus have been associated with social learning and behavior in prairie voles and other rodents [85, 91, 98, 99, 105, 108, 111, 115, 116, 155, 165, 200, 202, 203]. The PFC was analyzed because OTRs in this nucleus modulate multiple forms of social learning and behavior [114, 204], including pair bonding in prairie voles [108]. The AON and MeA were analyzed because of their role in social olfactory odor processing [113] and social recognition [107, 113, 205]. The BLA was analyzed because it transmits social olfactory information to the mesolimbic reward system, including the NA [206]. The PVN was analyzed because it is a major site of OT synthesis and is the predominant source of OTergic fiber innervation to the NAs [115, 153] and other PBN nuclei [180]. Finally, the VTA was analyzed because it modulates pair bonding in male prairie voles [110]; projects to, releases dopamine (DA) within, and modulates IEG expression in multiple PBN nuclei [207]; and contains OT-sensitive neurons that modulate both social behavior and dopamine release into the NA [208, 209].

*Imaging and analysis*

As described previously [198], analysis of Fos expression in socially exposed subjects was restricted to males that mated (n=11 aCSF-treated; n=12 OTA-treated) to maximize divergence in presumed OTR signaling in the NA. Images were captured through a Nikon E800 microscope and a 10x 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 each brain region, Fos was quantified in both left and right hemispheres across 3 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 region using the MCID grain count function with a constant size, density, and shape thresholds applied across all subjects. For each subject, Fos expression counts within each region were averaged, yielding a single average Fos count per region per subject.

*Statistics*

*Data transformation*

Immunohistochemistry was performed in two batches. To standardize the data between batches, raw Fos count values were transformed into z-scores for each batch. These z-scores were used for subsequent statistical analyses of Fos expression.

*Sociosexual behaviors*

Behavioral data from the 30 minute cohabitation period were analyzed using a two-way repeated-measures ANOVA with treatment (OTA versus aCSF) as a between subjects factor and behavior as a within subjects factor. Planned Student's t-tests were used to compare behavioral measures between treatment groups when appropriate. Poisson regression was used for analysis of count

outcome variables (e.g. mating frequency). For all ANOVAs, effect size (Eta Squared) was calculated by dividing the individual effect sum of squares by the sum of the residual sum of squares and the effect sum of squares.

*PBN Fos expression*

Fos expression in the PBN was first analyzed using a two-way ANOVA with treatment (OTA exposed versus aCSF exposed versus aCSF unexposed) as a between subjects factors and region as a within subjects factor. *Post-hoc* one-way ANOVAs were used to compare PBN Fos expression in aCSF-mated versus OTA-mated subjects and in aCSF-unexposed versus all mated subjects (pooled across treatments). Effect size (Cohen's *d*) was calculated by dividing the mean difference by the pooled standard deviation.

*Fos expression in the NAs*

Fos expression in the NAs was analyzed across treatments using a one-way ANOVA with treatment (OTA-mated versus aCSF-mated versus aCSF-unexposed) as a between subjects factor. *Post-hoc* Student's t-tests were used to compare Fos expression in aCSF-unexposed control subjects versus OTA-mated subjects and versus aCSF-mated subjects. An additional *post-hoc* Student's t-test was used to compare Fos expression between OTA-mated subjects and aCSF-mated subjects.

*Fos expression covariance between the NAs and other PBN nuclei*

We hypothesized that blocking OTRs specifically in the NA would result in disruption of covariance between the NA and the other PBN nodes investigated, rather than the more global effect on correlated Fos activation we observed in our previous work (ref). Even though we anticipated no overall effect, we tested for effect specificity by investigating how NA injection of

OTA effected covariation across all PBN brain regions. These analyses were performed as previously described (ref) and combined principal component analyses (PCA) to get estimates of effect size (difference in variance explained by the first component) and permutation testing to assess the statistical significance of this effect.

To investigate the hypothesis that blocking OTRs in the NA specifically disrupts the correlation between the NA and the other PBN nodes, we used multiple linear regression (the "lm" function in R) with NA Fos expression as the dependent variable and the six other regions imaged as independent variables. We extracted $R^2$ values from the regression analyses and compared how much of the variance in NA Fos expression was explained by expression in the other PBN brain regions in animals given OTA vs. aCSF. This method assesses the relationship between the dependent variable and the independent variables while at the same time accounting for the correlation among the independent variables. 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 between these values. This procedure was repeated 10,000 times and the p-value for the difference in variance explained was calculated as the proportion of permuted differences exceeding the initial observed difference value.

*Fos expression covariance across the PBN*

The correlation patterns across brain regions for the different treatment/exposure groups were first examined descriptively by visualizing the data in heat maps. These maps, showing Pearson's correlation coefficients, were generated using the data visualization package ggplot2 in R. Principal component analyses (PCA) were performed using the "PCA" function from the

"FactoMineR" package to analyze differences in patterns of FOS expression between aCSF-mated and OTA-mated subjects. We generated a permuted null distribution through multiple, random assignment of individuals to one of the investigated treatment groups, without replacement. For each permutation we calculated the variance explained by the first principal component between groups and then the absolute value of the difference between these values. This procedure was repeated 10,000 times and the p-value for the difference in variance explained was calculated as the proportion of permuted differences exceeding the initial observed difference value.

## Results

*Effect of OTR activation in the NA on mating frequency*

In mammals, OT is released during sexual behavior in both males and females [152]. Central release during sexual behavior has been demonstrated in male rats [154], and increases in extracellular OT specifically within the NA during sexual behavior have been demonstrated in female prairie voles [153]. In male prairie voles, the NA is densely innervated by OTergic projections from the PVN [115], exhibits high levels of OTR expression, and exhibits Fos induction in response to sociosexual interaction and mating with a female [198]. We previously demonstrated that central OTR blockade reduces mating frequency in male prairie voles [198], consistent with findings in rats [181]. Here, we tested the hypothesis that this effect is mediated specifically by OTRs within the NA. aCSF-treated males (n=13) engaged in an average frequency of 4.11±1.05 intromission bouts per 30 minutes with the stimulus female compared to 5.56±1.19 bouts per 30 min in OTA-treated males. 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.

*Effect of OTR activation in the NA on social and sexual behaviors*

OTR density and signaling within the NA have been linked to social learning and behavior in a variety of rodent species through a variety of different experimental paradigms (see "Methods-Selection of brain nuclei" above). Here we tested whether site-specific OTR blockade within the NA was associated with differences in social behavior (non-interaction, investigation, mounting, intromission, or huddling) in this experimental paradigm. Among mated subjects (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; $p<4.06x10^{-13}$; Eta-squared=0.38), but not of treatment (aCSF versus OTA; $F_{1,109}$=0.00; p=1.00; Eta-squared=0.00) or the interaction between behavior and treatment ($F_{1,109}$=0.01; p=0.93; Eta-squared=0.00) on time engaged in the analyzed social behaviors during the 30 minute period of cohabitation (Figure 6C). 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 [198]. Importantly, these data suggest that any observed differences in Fos expression between OTA- and aCSF-treated subjects in these experiments are not due to differences in performance of the analyzed social behaviors.

*Effects of sociosexual interaction on PBN Fos expression*

We previously found that sociosexual interaction and mating with a female is associated with a robust increase in Fos expression across the PBN in male prairie voles, regardless of aCSF- versus OTA-treatment. We measured this effect as a positive control to validate that our paradigm induced Fos expression. A two-way repeated-measures ANOVA with treatment (aCSF-mated versus OTA-mated versus aCSF-unexposed subjects) as a between subjects factor and brain region as a within subjects factor revealed a significant main effect of treatment ($F_{1,190}$=52.055; $p=1.25x10^{-11}$; Eta-

squared=0.21), but not brain region ($F_{1,190}$=0.30; p=0.57; Eta-squared=0.00) or the interaction between treatment and brain region ($F_{1,190}$=1.86; p=0.15; Eta-squared=0.01), on Fos expression in the analyzed brain nuclei. These results suggest a strong effect of treatment on Fos expression that did not differ significantly across brain regions; therefore, region was excluded as a predictor for subsequent analyses. Excluding unexposed subjects, a post-hoc one-way repeated-measures ANOVA revealed that Fos expression across the PBN did not differ by treatment among mated animals ($F_{1,157}$=0.27; p=0.60; Eta-squared=0.00); therefore, OTA- and aCSF-treated subjects that mated were pooled for subsequent post-hoc analyses. A one-way repeated measures ANOVA revealed a significant effect of sociosexual interaction and mating on Fos expression across the PBN between mated animals (pooled) and unexposed controls ($F_{1,192}$=26.96; p=5.27x$10^{-7}$; Eta-squared=0.12), consistent with our previous results.

*Effect of accumbal OTRs on Fos expression in the NAs*

Sociosexual interaction and mating is associated with Fos induction in the NA in male prairie voles. Although it hasn't been tested specifically in males, sociosexual interaction increases extracellular concentrations of OT in the NA in female prairie voles [153]. For these reasons, we investigated whether NA OTR signaling during sociosexual interaction modulates local Fos expression in male prairie voles. A one-way ANOVA with treatment as a between subjects factor (aCSF-mated versus OTA-mated versus aCSF-unexposed) revealed a significant effect of treatment on Fos expression in the NA shell ($F_{1,26}$=12.89; p=1.35x$10^{-3}$; Eta-squared=0.33; Fig. 2A). *Post-hoc* Student's t-tests revealed significantly increased Fos expression between both mated subjects treated with OTA ($t_{1,15}$=-3.20; p=6.01x$10^{-3}$; Eta-squared=0.41) and mated subjects treated with aCSF ($t_{1,14}$=-2.48; p=0.029; Eta-squared=0.30) compared to unexposed controls. An additional *post-hoc* Student's t-test revealed a trend toward *increased* Fos expression in OTA-

versus aCSF treated subjects ($t_{1,21}$=-1.84; p=0.080; Eta-squared=0.14), similar to previous results following ICV infusion of OTA [198]. Taken together, these data provide modest evidence that NA OTRs, in aggregate, promote local inhibition of Fos expression during sociosexual interaction.

*Correlated Fos expression between the NA shell and other PBN nodes*

We previously found that central OTRs modulate correlated Fos expression across the PBN in male prairie voles during sociosexual interaction with a female [198]; however, ICV administration of OTA in these experiments (and therefore brain-wide OTR blockade) obscures the functional role of region-specific OTR populations. We hypothesized that if central OTR populations modulate functional coupling across PBN (and SDMN) nuclei, then site-specific infusion of a highly selective OTA into the NA would modulate correlated Fos expression between the NA shell and other PBN nodes. First, we looked at if site-specific blockade of OTRs resulted in PBN global effects on correlated Fos expression, by investigating if this treatment collectively modulate correlated Fos expression across the network as a whole. In line with our hypothesis we found no evidence suggesting this; the variance explained by the first principal component was virtually identical in animals treated with OTA and aCSF (difference in $R^2$ = 0.25%, p = 0.98). We did however find that correlated Fos expression between the NA shell and other PBN nodes is significantly decreased in OTA- versus aCSF-treated subjects, as seen in Figure 7. The multiple linear regression analyses revealed that the Fos expression across non-accumbal PBN nodes explains a very large amount of the variance in NA Fos expression in aCSF treated animals ($R^2$ = 91%) but not in animals treated with an OTA ($R^2$ = 20%). The result from the permutation test indicated that this difference in variance explained (71%) was statistically significant (p = 0.02). These results are consistent with the hypothesis that endogenous, region-specific OTR signaling

during sociosexual interaction modulates functional connectivity of individual nodes within conserved neural networks.

*Correlated Fos expression across the PBN*

We previously found that blockade of central OTRs during sociosexual interaction was associated with a global decrease in in correlated Fos expression across the PBN. We hypothesized that this effect was mediated by disruption of OTR signaling simultaneously across many interconnected forebrain nuclei during sociosexual interaction and mating. To test whether this effect could be driven solely by accumbal OTR signaling, we combined PCA and permutation testing to measure global patterns of covariance in Fos expression between aCSF- and OTA-treated males that mated with a stimulus female. In line with our hypothesis we found no evidence that accumbal OTRs modulate global covariance across the PBN; the variance explained by the first principal component was virtually identical in animals treated with OTA and aCSF (difference in $R^2$ = 0.25%, p = 0.98).

## Discussion

Central OTRs modulate mating frequency in male prairie voles [198], providing indirect evidence that OT is released centrally during sociosexual interaction, a phenomenon that has been measured directly in male rats [154]. To our knowledge, site-specific OT release into the NA during sexual behavior has only been investigated in female prairie voles, and these experiments found local increases of extracellular OT during sociosexual interaction [153]. Here, we tested the hypothesis that OTRs in the NA modulate sexual behavior in male prairie voles and found no difference between OTA- versus aCSF-treated males (Fig. 1). These data suggest that mating behavior in male prairie voles is modulated by OTRs in other nuclei, consistent with experiments in rats [101, 207].

Using the same behavioral paradigm, we previously showed that ICV administration of a highly selective OTA has no effect on multiple social behaviors in mated male prairie voles [198]; however, brain-wide OTR blockade may obscure behavioral effects that are modulated by region-specific OTR populations. In other experimental contexts, OTR density and activation in the NA have been linked with multiple social behaviors in prairie voles and other rodents [76, 85, 91, 98, 99, 105, 108, 111, 115, 116, 155, 165, 200, 202, 203]. Therefore, we investigated the hypothesis that endogenous activation of OTRs in the NA modulate sociosexual behaviors in male prairie voles. There were no differences between OTA- versus aCSF-treated subjects in any of the analyzed behaviors, regardless of whether all subjects or only subjects that mated (the subset investigated for Fos analyses) were analyzed, suggesting that activation of OTRs in the NA do not modulate these behaviors in this experimental paradigm. Most importantly, these results suggest that any significant differences in Fos expression between treatment groups are not due to differences in any of the analyzed social behavior.

The PBN is a simplified model of deeply interconnected social olfactory, mesolimbic reward, and neuropeptide-synthesizing brain nuclei (including multiple SDMN nuclei) and is thought to modulate perception and associative learning of social olfactory signatures. In prairie voles, it is hypothesized that transient neuropeptide signaling during repeated sociosexual interaction, combined with unique neuropeptide receptor distributions, biases the network toward encoding the mating partner's olfactory profile with positive valence (and subsequent, unfamiliar olfactory signatures with negative valence). Within this framework, the neuroplastic changes underlying this associative encoding of the partner's sensory signature facilitates plasticity in a suite of context-dependent, SDMN-regulated social behaviors characteristic of pair bond formation (e.g. sexual

behavior, social contact), expression (e.g. cooperative nesting, allogrooming, paternal care), and maintenance (consoling, selective aggression).

The NA is a PBN and SDMN nucleus in which OTRs (or homologous receptors) are expressed across distant vertebrate lineages [31]; OTRs directly modulate multiple social behaviors [108, 111, 116, 200]; OTR densities correlate with intra- and interspecific variation in multiple social behaviors [76, 85, 98, 203]; and OTR densities vary remarkably across species [76, 87, 203]. The NA receives projections from olfactory processing nuclei and OT neurons in the PVN, and is deeply interconnected with OT-sensitive mesolimbic reward nuclei (e.g. PFC and VTA) that also express OTRs and receive OTergic projections from the PVN [115, 180]. Signaling at kappa-opioid, corticotrophin releasing factor, D1 dopamine, D2 dopamine, and OT receptors in the NA all modulate social behavioral plasticity associated with pair bonding in prairie voles [87]; demonstrating that the NA is a structure in which transient neuromodulatory signaling during social contexts is critical for social behavioral plasticity. Here we use Fos expression to measure neural "activity" in the NAs and other PBN nuclei during sociosexual interaction. Briefly, Fos heterodimerizes with JUN to form AP-1 transcription factor complexes that modulate expression of AP-1 responsive genes involved in a variety of intracellular processes, including synaptic plasticity [189]. Thus, central Fos expression is thought to reflect rapid adaptation of neuronal physiology through robust transcriptional regulation in response to a stimulus, and has been linked with neuronal firing across many (but not all) neuronal phenotypes. We use site-specific administration of a highly selective OTA prior to sociosexual interaction to investigate how endogenous activation of OTRs in the NA modulates both local Fos expression in the NAs and correlated Fos expression across the PBN.

As described previously [198], analyses of Fos expression were restricted to subjects that mated to maximize divergence in presumed OT signaling within the NA between treatment groups. We previously demonstrated that sociosexual interaction and mating with a female induces Fos expression across the PBN as a whole. We tested for this effect as a positive control and found a significant increase in Fos expression across the PBN in mated versus unexposed subjects (regardless of OTA- versus aCSF-treatment), consistent with our previous results.

Next we tested whether OTR signaling in the NA during sociosexual interaction modulates local Fos expression in the NAs. We found a trend toward *increased* Fos expression in OTA- versus aCSF treated subjects, similar to previous results following ICV infusion of OTA [198]. Taken together, these data provide modest evidence that signaling at NA OTRs, in aggregate, during sociosexual interaction may promote inhibition of local Fos expression in male prairie voles. Obstacles in the development of selective OTR antibodies have hindered phenotypic characterization of OTR-expressing neurons, making cellular-level interpretations of these results difficult. OTRs may be expressed presynaptically on glutamatergic and/or GABAergic projections into the NA, as well as postsynaptically on local populations of interneurons and/or MSNs; and may serve a unique neuromodulatory role in each instance [210, 211]. Activation of presynaptic OTRs on serotonergic inputs to the NA (projecting from the dorsal raphe) depresses postsynaptic excitatory transmission onto MSNs in male mice [202], suggesting one potential mechanism by which NA OTRs may modulate inhibition of local Fos expression. In addition, it is hypothesized that OTRs expressed postsynaptically on NA MSNs heterodimerize with dopamine type-2 receptors to inhibit depolarization [206]. Future investigations are necessary to dissect the functional role(s) of OTRs in modulating electrophysiology of specific neuronal populations in the NA.

Although it has long been hypothesized that modulation of functional connectivity across neural networks by neuropeptides (and steroid hormones) is important in vertebrate social behavior, remarkably few experiments have directly tested this hypothesis [140]. Evidence that central OTRs modulate functional connectivity across neural networks during social contexts is derived almost entirely from experiments that have used indirect methods of investigation. For example, multiple brain imaging experiments in humans have found effects of intranasal OT on functional connectivity across limbic, striatal, cortical, and/or brainstem nuclei in a variety of social contexts [193, 212-215]; however, investigations using intranasal OT should be interpreted with caution [216, 217]. It is particularly important to note that intranasal OT administration does not necessarily mimic endogenous central release, and that OT can act via binding to vasopressin receptors [161]; thus, experiments using intranasal OT administration cannot draw specific conclusions about endogenous OTR signaling. Two studies have shown that polymorphisms in OXTR are associated with differences in functional connectivity between hypothalamic, limbic, and/or cortical brain areas during social tasks [131, 132], and one has shown that methylation of OXTR (the gene encoding OTR) in peripheral blood cells is associated with reduced functional connectivity among limbic and cortical areas while viewing fearful and angry faces [218]. Unfortunately, it is unclear whether human OXTR polymorphisms or OXTR methylation in blood cells are associated with differences in central OTR expression, and it is impossible to determine whether genetic/epigenetic associations are mediated directly by differences in central OTR signaling or indirectly through downstream effects of variable temporal/developmental OTR expression and/or differences in peripheral OTR signaling. Finally, hurdles in the development of specific ligands for primate OTRs (in contrast to many non-primate species) have hindered

mapping of central OTR distributions in the human brain [219], further limiting interpretations of these data.

Non-primate experiments have used correlated cytochrome oxidase and IEG expression to investigate functional connectivity across the SDMN in specific social contexts [141-144]. We recently combined this approach with ICV administration of a highly selective OTA to show that central endogenous OTR signaling associated with sociosexual interaction modulates correlated Fos expression across the PBN in male prairie voles [198], consistent with the hypothesis that central OTRs modulate patterns of functional network connectivity during social contexts. However, multiple important questions cannot be addressed with these data. For example, ICV administration cannot address whether OTR-mediated patterns of correlated activity are tied to region-specific OTR populations or instead represent an emergent property of simultaneous OTR signaling (or blockade) across the entire brain. Further, while central administration of OTA disrupts "global" correlated Fos expression across the PBN, it is unclear whether more specific patterns of connectivity are modulated by region-specific OTRs. Thus, although region-specific neuropeptide and steroid receptors have been linked to intra- and interspecific differences in a wide variety of social behaviors across a wide variety of species, currently there are no established links between these receptor populations and specific patterns of functional network connectivity during social contexts.

For these reasons, we investigated whether endogenous activation of OTRs in the NA during sociosexual interaction modulates correlated Fos expression between the NAs and other PBN nuclei, and found that local OTRs modulate "coupling" (i.e. correlated Fos expression) between the NAs and other PBN nuclei. We next used principal components analysis and permutation testing to investigate whether OTRs in the NA modulate global patterns of covariance in Fos

expression across the PBN, as described previously following ICV administration of OTA [198], and we found no differences between OTA- and aCSF-treated subjects. Taken together, these data indicate specificity in how endogenous activation of OTRs in the NA modulates patterns of functional coupling across the PBN. Because no behavioral differences were observed between treatment groups in these experiments, significant differences in correlated Fos expression do not likely reflect differences in behavior, and may reflect differences in perception or associative learning. The OT-mediated decrease in Fos expression (Fig. 7A) yet increase in functional coupling with other PBN nuclei (Fig. 7B) may indicate that OT increases signal-to-noise ratio in the NAs, suppressing noise while "tuning in" neurons in the NAs to inputs from other PBN nuclei that collectively represent the social olfactory profile, reward value, and other contextual information associated with the mating partner. This interpretation is consistent with hippocampal slice recordings from rats showing selective OTR agonists greatly improve signal-to-noise ratio and cortical information transfer by suppressing background firing (through inhibitory interneuronal networks) while increasing fidelity of spike transmission and spike timing onto CA1 pyramidal neurons [220]; and recent *in vivo* recordings in anesthetized rats showing administration of selective OTR agonists into the AON increases signal-to-noise ratio in main olfactory bulb mitral and tufted cells during odorant responses [221].

There are several important limitations to these data. Firstly, the neuronal phenotypes and connectivities of Fos-expressing neurons within each nucleus are unknown; identifying specific neuronal subpopulations that become functionally connected across PBN and SDMN nuclei during specific social contexts is a critical area for future investigation. Secondly, site-specific OTA administration targets all locally-expressed OTRs, potentially obscuring distinct functional roles that are dependent on neuronal phenotype and presynaptic versus postsynaptic expression; future

electrophysiological investigations are necessary to characterize the neuromodulatory function of OTRs in each context. Thirdly, Fos expression in this paradigm lacks temporal, behavioral, and electrophysiological specificity; we are currently using electrophysiological recordings and optogenetic stimulation in behaving prairie voles to investigate dynamic neural activity across PBN nuclei during sociosexual interaction. Fourthly, these experiments focused on OTRs in a single PBN nucleus in male prairie voles. Future investigations of other steroid and neuropeptide systems, additional brain areas, female subjects, and alternative species are imperative. Finally, site-specific OTR blockade does not likely reflect natural intra- and interspecific variation in local OTR signaling. Future studies should investigate how natural variation in central OTR expression modulates functional network connectivity within and across species.

Despite these limitations, these data represent a significant advance in our understanding of how neuropeptide receptors modulate neural network function. To our knowledge these are the first data to demonstrate that endogenous signaling at region-specific neuropeptide receptors in behaving animals modulates patterns of functional coupling across neural networks, establishing an important conceptual link between specific receptor populations known to be critical for social behavior and functional network principles that have long been thought to be critical for social behavior. Further, this is the second line of experiments from our laboratory demonstrating that central OTRs modulate functional coupling across the PBN, providing a necessary complement to brain imaging experiments in humans which use less direct methods, and electrophysiological studies in rats using peptide agonists in slice or in anesthetized subjects to show similar effects. Collectively, these experiments lend support to the hypothesis that central OTRs are a deeply conserved mechanism allowing for diverse patterns of functional connectivity across neural networks during social contexts.

# Chapter 4

## A single nucleotide polymorphism in OXTR is associated with differences in correlated immediate early gene expression across forebrain nuclei in male prairie voles

**Abstract**

The oxytocin (OT) system has been implicated in reproductive and social behaviors across distantly related bilaterian animals, including humans. In the brain, central distribution patterns and region-specific densities of oxytocin receptors (OTRs) are correlated with intra- and interspecific variation in social behavior in birds and mammals. Furthermore, blockade or knockdown of central and brain region-specific OTRs inhibits a variety of social behaviors across vertebrate lineages. We recently demonstrated that both central and brain region-specific blockade of OTRs robustly modulates correlated expression of activity-dependent IEGs across distributed OTR-expressing brain nuclei during sociosexual interaction in male prairie voles. However, individual prairie voles are known to vary substantially in region-specific OTR expression. It was recently demonstrated that a single nucleotide polymorphism (SNP) in *Oxtr*, the gene encoding the OTR, strongly predicts individual variation in both region-specific OTR expression as well as social behavior in prairie voles. Here, we investigate whether this SNP also predicts variation in distributed IEG expression across a hypothesized "pair bonding network" (PBN) during sociosexual interaction in male prairie voles. We find a significant association between genotype at this SNP and distributed covariance in Fos expression across the PBN during sociosexual interaction.

## Introduction

The oxytocin (OT) system has emerged as an evolutionarily ancient biological mechanism regulating social and reproductive behavior across distantly related species, spanning nematode worms and humans [146, 152]. In mammals, OT is synthesized in hypothalamic neurons that project peripherally to the posterior pituitary and centrally to a variety of hindbrain, midbrain, and forebrain nuclei [62]. OT release is triggered in a variety of reproductive contexts, including parturition, lactation, and mating [152]; and in response to more subtle social stimuli, such as social vocalizations, eye contact, and touch [151, 156, 157].

In contrast to the general conservation in anatomical "wiring" of mammalian OTergic neurons, distribution patterns and region-specific densities of oxytocin receptors (OTRs) in the brain are extraordinarily diverse within and across species. Region-specific OTR densities are correlated with variation in social behavior in multiple vertebrate lineages [79, 86, 87, 203]. For example, in microtine rodents, region-specific OTR densities are correlated with variation in social investigation, social contact, alloparental care, sociospatial behavior, and social monogamy [85, 98-100], and selective OTR antagonists (OTAs) and RNAi knockdown have been used to demonstrate that region-specific OTR populations modulate behavior in wide variety of social contexts [5, 108, 112, 116, 200]. These and many other experiments have revealed anatomical and functional principles of central neuropeptide systems that have been integrated into models of neural networks, such as the Social Decision Making Network (SDMN). The SDMN is a deeply conserved, reciprocally interconnected network of limbic and mesolimbic reward nuclei that is thought to regulate social communication, parental, sexual, and aggressive behaviors across all jawed vertebrates [31, 139, 140]. In addition to more traditionally analyzed levels of neural function (e.g. synapses, region-specific circuits, phenotype-specific neuronal populations,

anatomical subregions, individual brain nuclei, etc.), models of SDMN function emphasize the importance of distributed, interregional activity as an important organizational level of information processing that should be subjected to experimental analysis. Specifically, these models emphasize *patterns* of activity, including functional "connectivity" or "coupling" (i.e. correlated activity across spatially distinct neural loci), across the network as being strongly linked to social context and behavior [140]. In this framework, individual nuclei can serve dynamic roles depending on their relative weight or functional connectivity within the network; and intra- and interspecific variation in neuropeptide receptor (e.g. OTR) expression across the network is thought to reflect diverse network functions during social contexts that elicit transient release of neuromodulatory ligands, such as OT [140].

Previous investigations in non-mammalian species have used correlated expression of cytochrome oxidase (a metabolic marker) and immediate early genes (IEGs) to link patterns of functional coupling across the SDMN to distinct social contexts [53, 141-144]. We recently used ICV and site-specific administration of a highly selective OTA to investigate the role of central OTRs in modulating expression of the IEG product Fos across a hypothesized pair bonding network (PBN). Briefly, the PBN is a network of hypothalamic, social olfactory, and mesolimbic reward nuclei that is thought to modulate associative social olfactory learning. These experiments revealed that central and region-specific OTR signaling during sociosexual interaction modulate patterns of functional coupling across the PBN in male prairie voles [198], consistent with the hypothesis that neuropeptide systems modulate distributed, interregional network function during social contexts.

While these experiments provide important empirical support for hypothesized functional principles, OTR blockade using antagonists does not reflect differences in central OTR signaling due to natural variation in OTR expression. Thus, although it has been hypothesized for decades

that natural variation in central organization of neuropeptide, steroid hormone, and other neuromodulatory systems is the predominant neurobiological mechanism underlying intra- and interspecific diversity in SDMN function, this hypothesized functional principle severely lacks direct experimental support. Interestingly, two studies have reported that single nucleotide polymorphisms (SNPs) in human OXTR (the gene encoding OTR) are associated with differences in functional connectivity between hypothalamic, limbic, and/or cortical brain areas [131, 132]; and methylation status of OXTR in blood cells predicts functional connectivity between the amygdala and other limbic, striatal, and cortical regions during social contexts. However, obstacles in developing selective ligands for primate OTRs have hindered definitive mapping of central OTR expression in these regions, and the relationship between OXTR methylation in blood cells and OTR expression in the brain has not been investigated in any species. Thus, it is currently unclear whether genetic and epigenetic variation in human OXTR reflects variation in central OTR expression.

A recent study from our laboratory identified a naturally occurring SNP (NT213739) in the prairie vole OXTR that is associated with individual variation in both central OTR expression and social behavior [118]. Remarkably, NT213739 explains 74% of variance in OTR expression in specific PBN nuclei in prairie voles, and therefore represents an excellent opportunity to investigate whether natural intraspecific variation in central OTR expression is associated with differences in neural network function during social contexts. Here, we test whether individual genotype at this SNP in male prairie voles is associated with differences in sociosexual behavior, region-specific Fos expression, and patterns of functional coupling across the PBN.

## Methods

### Subjects

Male prairie voles were either toe clipped at post-natal day 7 (PND7) or ear clipped at PND21 to provide individual identification and collect tissue for genotyping. Males were housed in groups of two or three until behavioral testing during adulthood (60-180 days). Housing consisted of a ventilated 26x18x19 cm Plexiglas cage filled with Bed-o-cobbs Laboratory Animal Bedding under a 14:10 h light/dark cycle at 22°C with *ad libitum* access to food (rabbit LabDiet) and water. Subjects were drawn from our laboratory breeding colony originally derived from field captured voles in Illinois. Subjects were weaned at 21 days. Stimulus animals were sexually experienced, ovariectomized, estrogen-primed (see below), adult female prairie voles. All procedures were conducted in accordance with the National Institutes of Health Guide for the Care and Use of Laboratory Animals and were approved by the Emory University Institutional Animal Care and Use Committee.

Genotyping

As previously described [118], DNA was isolated from toe clips using a Qiagen DNeasy Kit. A 117 bp amplicon including the intronic SNP at NT213739 was amplified by PCR, using a Qiagen Taq PCR Master Mix Kit. The thermocycler program used included 1) initial denature at 94˚C for 5 min 30 s; 2) 35x cycles of denaturing at 94˚C for 30 s, annealing at 55˚C for 30 s, and elongation at 72˚C for 30 s; and 3) final elongation at 72˚C for 10 min. Amplicons were digested for 1.5 hours at 65˚C with the BsiHKAI restriction enzyme (New England Biolabs), which cuts the C-allele of NT213739 but not the T-allele. Thus, resulting banding patterns for each subject were used to identify genotype. The primers used for this reaction were, forward: 5'-GGGACGTTCACGTTACATGG -3', reverse: 5'- AGACGGGACAGAGTCTCCAG -3'.

Cohabitation

Male subjects underwent a 30 minute, video-recorded cohabitation with a sexually experienced, ovariectomized, estrogen-primed, adult stimulus female. In the three days preceding cohabitation, stimulus females were brought into estrus with daily subcutaneous injections of 4.0 μg 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 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. Following the 30 minute session, stimulus females were removed and subjects remained in their home cage for 45 minutes before transcardial perfusion. For unexposed control subjects (n=8), 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.

Perfusion, post-fixation, and sectioning

45 minutes following the end of cohabitation (or control sessions), subjects were administered an overdose of isofluorane and were immediately perfused transcardially at a rate of approximately 4 mL/minute with 40 mL of 1x phosphate buffered saline (pH=7.4; diluted to 1x with distilled, deionized 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 after 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 μm sections using a Microm HM 440E freezing microtome and were stored in 1x PBS with 0.05% sodium azide until immunohistochemical staining.

Behavioral Scoring

Six 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, huddling, and attack behavior. The operational definitions used for each behavior were as follows. Non-interaction included any male behavior not directed towards the female, including autogrooming, running, freezing, eating, drinking, sleeping, digging, and exploration. Investigation included social behavior directed immediately towards the female, including physical pursuit, olfactory investigation, and allogrooming. Mounting was defined as placement of the forepaws on the female from the rear without stereotypical intromission movement. 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. Attack behavior was defined as rapid, aggressive lunging or biting directed toward the female. All was conducted by experimenters blind to treatment groups.

Immunohistochemistry

Sections underwent 3 washes in 1x PBS, 10-minute incubation in 1% sodium hydroxide in 1x PBS, and 3 washes in 1x PBS with 0.5% Triton-X (Sigma) (PBST) before treatment with 5% normal goat serum (Fitzgerald; Acton, MA) in PBST for 1 hour at room temperature. Sections were then incubated for 48 hours in primary rabbit polyclonal anti-Fos antibody (1:20,000; Calbiochem ABE457) on an orbital shaker at 4°C. Following primary incubation sections were washed 5 times in 1x PBS, once in 1x PBST, and were incubated in secondary biotinylated goat anti-rabbit IgG antibody (1:500; Vector Labs BA-1000) for 2 hours. After secondary incubation, sections were treated with an avidin-biotin peroxidase system (Vectastain Elite ABC System; Vector Labs: PK6100) for 1 hour 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 minutes in 70% EtOH, 10 minutes in fresh 95% EtOH twice, 10 minutes in fresh 100% EtOH twice), bathed in Xylenes (15 minutes in Xylenes twice), mounted onto Superfrost Plus slides (Fisher Scientific; 12-550-150) while still partially wet, and coverslipped using Krystalon (EMD Chemicals Inc., Gibbstown, NJ).

Selection of brain nuclei

Fos expression was analyzed in 7 PBN nuclei: the nucleus accumbens shell (NAs), prefrontal cortex (PFC), anterior olfactory nucleus (AON), medial medial amygdala (MeA), basolateral amygdala (BLA), paraventricular hypothalamic nucleus (PVN), and ventral tegmental area (VTA). All of the analyzed regions are innervated by OTergic fibers, reciprocally interconnected with other PBN nuclei, and express OTRs in male prairie voles. A brief description of the role of OTR signaling in these nuclei has been described previously (see Chapter 3: Methods: "Selection of PBN nuclei").

Imaging and analysis

Images were captured through a Nikon E800 microscope and a 10x 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 each brain region, Fos was quantified in both left and right hemispheres across 3 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 region using the MCID grain count function with a constant size, density, and shape thresholds applied across all subjects. For each subject, Fos expression counts within each region were averaged, yielding a single average Fos count per region per subject.

## Statistics

All statistical analyses were performed in R 3.1.1.

### *Data transformation*

Immunohistochemistry was performed in two batches. To standardize the data between batches, raw Fos count values were transformed into z-scores for each batch. These z-scores were used for subsequent statistical analyses of Fos expression.

### *Sociosexual behaviors*

Behavioral data from the 30 minute cohabitation period were analyzed using a two-way repeated-measures ANOVA with genotype (T/T versus C/T versus C/C at NT213739) as a between subjects factor and behavior as a within subjects factor. Planned Student's t-tests were used to compare behavioral measures between genotypes when appropriate. Poisson regression was used for analysis of mating frequency. For all ANOVAs, effect size (Eta Squared) was calculated by dividing the individual effect sum of squares by the sum of the residual sum of squares and the effect sum of squares.

### *PBN Fos expression*

Fos expression in the PBN was first analyzed using a two-way repeated measures ANOVA with genotype (T/T versus C/T versus C/C at NT213739) as a between subjects factors and brain region as a within subjects factor. *Post-hoc* one-way ANOVAs were used to compare PBN Fos expression between T/T-mated, C/T-mated, and C/C-mated subjects; as well as between the "mixed" genotype (T/T, n=3; C/T, n=3; C/C, n=3) unexposed subjects and all mated subjects (pooled across genotypes).

### *Fos expression in the NAs*

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

*Fos expression covariance between the NAs and other PBN nuclei*

To investigate the hypothesis that genotype modulates coupling between the NA and the other PBN nodes, we used multiple linear regression (the "lm" function in R) with NA Fos expression as the dependent variable and the six other regions imaged as independent variables. We extracted $R^2$ values from the regression analyses and compared how much of the variance in NA Fos expression was explained by expression in the other PBN brain regions in. This method assesses the relationship between the dependent variable and the independent variables while at the same time accounting for the correlation among the independent variables. Because these samples were too small to establish an additive effect of genotype, we restricted our analysis to only "high" and "low" OTR expression genotypes. To test for statistical significance, we generated a permuted null distribution through multiple, random assignment of individuals to either of the genotype groups, without replacement. For each permutation we calculated the multiple $R^2$ for both groups and the absolute value of the difference between these values. This procedure was repeated 10,000 times and the p-value for the difference in variance explained was calculated as the proportion of permuted differences exceeding the initial observed difference value.

*Fos expression covariance*

Principal component analyses (PCA) were performed using the "PCA" function from the "FactoMineR" package in R to analyze differences in patterns of correlated Fos expression between T/T, C/T, and C/C genotypes. We generated a permuted null distribution through multiple, random assignment of individuals to one of the investigated treatment groups, without replacement. For each permutation we calculated the variance explained by the first principal component between groups and then the absolute value of the difference between these values. This procedure was repeated 10,000 times and the p-value for the difference in variance explained was calculated as the proportion of permuted differences exceeding the initial observed difference value. This approach was used to analyze covariance in Fos expression across the mesolimbic reward network and across the PBN.

## Results

*Association between* NT213739 *and sociosexual behaviors*

In the NA, region-specific variation in OTR expression and endogenous activation of OTRs have been linked to social learning and behavior in prairie voles and other rodents [85, 91, 98, 99, 105, 108, 111, 115, 116, 155, 165, 200, 202, 203]. In this paradigm, male prairie voles exhibit variation in multiple sociosexual behaviors (non-interaction, investigation, mounting, intromission, and huddling) during interaction with the stimulus female. We investigated whether variation in these behaviors is associated with individual genotype at NT213739, a SNP explaining 74% of the variance in accumbal OTR density that has previously been associated with partner preference formation in male prairie voles [118]. A "T" (thymine) at NT213739 is associated with low accumbal expression of OTR relative to a "C" (cytosine). As seen in Fig. 8, among mated subjects (T/T "low," n=10; C/T "mid," n=10; C/C "high," n=11), a two-way repeated-measures ANOVA revealed a significant main effect of behavior (non-interaction, investigation, mounting, or

intromission; $F_{1,114}$=229.0; $p<2x10^{-16}$; Eta-squared=0.67), but not of genotype (T/T versus C/T versus C/C; $F_{1,114}$=0.10; p=0.76; Eta-squared=0.00) or the interaction between behavior and genotype ($F_{1,114}$=0.05; p=0.82; Eta-squared=0.00) on overall time engaged in any of these behaviors during the 30 minute test. Thus, among mated animals, genotype had no detectable effect on any of the analyzed sociosexual behaviors, consistent with previous results following ICV and site-specific administration [198]. Importantly, these data suggest that any associations between genotype and Fos expression in these experiments are not likely due to differences in any of the analyzed behaviors.

*Association between* NT213739 *and PBN Fos expression*

We have previously found that sociosexual interaction and mating with a female is associated with a robust increase in Fos expression across the PBN in male prairie voles, regardless of ICV and region-specific administration of OTAs. We measured this effect as a positive control to validate that Fos expression was induced across the PBN in these experiments. A two-way repeated-measures ANOVA with treatment (interaction and mating versus unexposed) as a between subjects factor and brain region as a within subjects factor revealed a significant main effect of treatment ($F_{1,274}$=71.960; $p=1.39x10^{-15}$; Eta-squared=0.21), but not brain region ($F_{1,274}$=0.92; p=0.34; Eta-squared=0.00) or the interaction between treatment and brain region ($F_{1,274}$=1.86; p=0.97; Eta-squared=0.00), on Fos expression in PBN nuclei (Figure 9). These results suggest a strong effect of sociosexual interaction and mating on Fos expression that did not differ significantly across brain regions; therefore, region was excluded as a predictor for subsequent analyses. Excluding unexposed subjects, a post-hoc one-way repeated-measures ANOVA revealed that Fos expression across the PBN did not differ by genotype among mated animals ($F_{1,213}$=0.29; p=0.59; Eta-squared=0.00); therefore, subjects that mated were pooled by genotype for subsequent post-hoc

analyses. A one-way repeated measures ANOVA revealed a significant effect of sociosexual interaction and mating on Fos expression across the PBN between mated animals (pooled genotype) and unexposed controls ($F_{1,276}$=27.91; p=2.58x$10^{-7}$; Eta-squared=0.09), consistent with previous experiments.

*Association between* NT213739 *and Fos expression in the NAs*

Although not yet tested in males, sociosexual interaction increases extracellular concentrations of OT in the NA in female prairie voles [153]. In male prairie voles, sociosexual interaction and mating with a female is associated with increased Fos expression in the NAs relative to unexposed controls [198]. In previous experiments, both ICV and NA-specific selective blockade of OTRs was associated with a trend toward increased Fos expression in the NAs (Fig. 4A and Fig. 7, respectively), although though these effects were not statistically significant. Together, these data suggest that differential OTR activation in the NA during sociosexual interaction may modulate Fos expression in male prairie voles. For these reasons, we investigated whether genotype at NT213739, which strongly predicts OTR densities in the NA, is associated with differences in Fos expression in the NAs. A two-way ANOVA with treatment (interaction and mating versus unexposed) and genotype (T/T versus C/T versus C/C) as between subjects factors revealed a significant effect of treatment ($F_{1,36}$=11.7; p=1.55x$10^{-3}$; Eta-squared=0.24) but not genotype ($F_{1,36}$=1.45; p=0.20; Eta-squared=0.04) on Fos expression in the NAs. A *post-hoc* one-way ANOVA confirmed that genotype at NT213739 is not associated with differential levels of Fos expression in the NAs among males that interacted and mated with a female ($t_{1,29}$=0.94; p=0.34; Eta-squared=0.03).

*Effect of* NT213739 *on covariance in Fos expression between the NAs and other PBN nuclei*

Genotype at NT213739 strongly predicts levels of OTR expression in the NA, and we previously showed (Chapter 3) that region-specific OTRs in the NA modulate correlated Fos expression between the NAs and other PBN nuclei during sociosexual interaction with a female [118]. Based on this, we hypothesized that genotype at NT213739 would be associated with differences in correlated Fos expression between the NAs and other PBN nuclei. As seen in Figure 7, in most cases there is an additive genotype effect on how correlated PBN nodes are with the NAs. Although this trend was verified using multiple linear regression, showing that the variance in NAs Fos expression explained by the other 6 PBN regions differ between genotypes ($R^2$ for T/T=68%, C/T=43%, and C/C=10%), in this relatively small sample we are not sufficiently powered to establish that these differences are statistically significant. For example, using the same permutation test approach described above, the difference in variance explained in T/T compared to C/C animals (58% difference in $R^2$) is only trending towards significance (p-value = 0.08) in this sample.

*Association between* NT213739 *and FOS expression covariance across the mesolimbic reward network*

OTR signaling in the NAs and PFC are necessary for pair bonding in female prairie voles, and NT213739 genotype significantly predicts OTR expression in both of these nuclei [108]. The VTA is reciprocally connected with the NAs and PFC and is necessary for pair bond formation in male prairie voles [110]. For these reasons, we investigated how the NT213739 alleles specifically associate with connectivity across NAs, PFC, and VTA. We assessed the strength of the covariance across these nodes as described above by running principal components analyses and extracting the variance explained by the first component. These analyses show that among individuals carrying two copies of the T allele the first principal component explains 78.8% of

the variance. In contrast, in the two other genotype groups the first component explains less of the variance (C/T = 42.7%, C/C = 45.0%). Since no apparent difference was found between C/T and C/C animals, these two groups were combined and in the pooled group the first principal component explains 42.3% of the variance. To test if the difference in explained variance between T/T animals and the combination of the other two groups (36.6%) is statistically significant we used permutation testing. Animals were randomly assigned to either of the two groups and the difference in variance explained was calculated. This procedure was repeated 10,000 times and statistical significance was determined by the proportion of the generated null distribution exceeding the observed difference. This resulted in a two-sided p-value of 0.003.

*Association between* NT213739 *and FOS expression covariance across the PBN*

As seen in figure 10A, homozygotes for the allele associated with low expression of OTR show a stronger and more homogenous pattern of correlation across PBN nodes than heterozygotes and homozygotes for the high expressing allele (Figure 10B and 10C). We assessed the strength of overall correlation in the different genotype groups by running principal components analyses and extracting the variance explained by the first component. These analyses show that among individuals carrying two copies of the T allele, the first principal component explains 61.2% of the variance. In contrast, in the two other genotype groups the first component explains less of the variance (C/T = 36.4%, C/C = 35.4%). Since no apparent difference was found between C/T and C/C animals, these two groups were combined and in the pooled group the first principal component explains 33.4% of the variance. To test if the difference in explained variance between T/T animals and the combination of the other two groups (27.8%) is statistically significant we used permutation testing. Animals were randomly assigned to either of the two groups and the difference in variance explained was calculated. This procedure was repeated

10,000 times and statistical significance was determined by the proportion of the generated null distribution exceeding the observed difference. This resulted in a two-sided p-value of 0.02.

**Discussion**

Among mated males, there was no association between genotype at NT213739 and any of the measured sociosexual behaviors, consistent with previous experiments in which ICV and NA-specific administration of a highly selective OTA had no effects on these behaviors in males that mated with the stimulus female. This result did not change when all subjects (including males that did not mate) were analyzed. Interestingly, in a previous experiment, fewer males mated following ICV administration of OTA and therefore a significant decrease in mating behavior was observed when all subjects (including males that did not mate) were analyzed. It may be that male sexual behavior can be inhibited by selective antagonism of central OTRs but not by differences in OTR signaling due to natural variation in central OTR expression. Alternatively, male sexual behavior in prairie voles may be modulated by natural variation in region-specific OTR signaling in brain regions where OTR expression varies independently of genotype at NT213739. Importantly, these data suggest that any associations between genotype and Fos expression observed in these experiments do not reflect differences in the analyzed behaviors.

Briefly, neuronal expression of Fos (and other IEG product) is thought to be induced by synaptic inputs and depolarization, after which Fos heterodimerizes with Jun proteins to form transcription factor complexes that regulate genes involved in synaptic plasticity and other neurophysiological processes. Therefore, Fos expression is thought to reflect integration of synaptic activity into long-term neurophysiological plasticity and has been used to map neural networks involved in learning and memory formation in a wide variety of species and experimental contexts. Importantly, Fos is not expressed in all neuronal phenotypes, and therefore its region-specific expression likely

reflects activity in only a subset of neuronal phenotypes. Here, increases in Fos expression within PBN nuclei are thought to reflect increases in synaptic plasticity in region-specific subsets of neuronal phenotypes (those capable of expressing Fos protein in this experimental context) embedded within PBN nuclei. Similarly, correlated Fos expression across the PBN is thought to reflect correlated synaptic plasticity across these region-specific neuronal subsets, suggesting they are embedded within common functional networks modulated by sociosexual interaction.

Site-specific administration of a selective OTA into the NA decreases correlated Fos expression between the NAs and other PBN nuclei (Fig. 7), and ICV administration of OTA decreases correlated Fos expression across the PBN as a whole, suggesting that central and region-specific OTRs modulate functional coupling of neuronal populations across PBN nuclei during sociosexual interaction in male prairie voles. Based on these observations, we hypothesized that genotypes associated with increasingly high levels of OTR expression in the NAs (and potentially other PBN nuclei) would also predict increasingly strong patterns of covariance in Fos expression between the NAs and other PBN nuclei, across mesolimbic reward nuclei, and across the PBN as a whole. Remarkably, a trend was observed between genotype at NT213739 and covariance in Fos expression between the NAs and other PBN nuclei (but not between the AON and other PBN nuclei); and a strong and significant relationship was observed between NT213739 and correlated Fos expression across mesolimbic reward nuclei (i.e. VTA, NAs, and PFC), and across the PBN as a whole, supporting hypotheses that natural variation in neuropeptide receptor expression across conserved neural networks reflects functional diversity of these networks during social contexts [140]. Surprisingly, however, these effects were in the *opposite* direction of our original hypothesis, with the “high” OTR-expressing genotype predicting *decreased* covariance in Fos expression in all three cases.

Though unexpected, these data may provide important clues regarding the organization and function central OTRs, and multiple important factors should guide their interpretation. For example, King et al. previously calculated the respective frequencies of "C" (high) and "T" (low) polymorphisms in our outbred laboratory colony of prairie voles as 0.32 and 0.68, respectively, suggesting C/C homozygotes ("high" accumbal OTR expression) likely represent approximately 10% of subjects tested in previous experiments. In contrast, "low" (T/T; ~46% of colony) and "moderate" (C/T; ~44% of colony) levels of accumbal OTR expression likely represent the vast majority of subjects tested in previous experiments, including experiments demonstrating strongly correlated Fos expression between across the PBN nuclei during sociosexual interaction in aCSF-treated males. Because T/T males likely represented a plurality of test subjects in these experiments, it is not surprising that strongly correlated Fos expression across the PBN is observed among T/T males; instead, it is surprising that C/T and C/C genotypes—associated with *higher* levels of region-specific OTR expression—exhibit more *weakly* correlated Fos expression across the PBN in comparison.

It is also important to consider that OTRs can be expressed presynaptically and/or postsynaptically on different neuronal phenotypes and can have different functional, region-specific roles in each of these contexts [211]. In the NA, OTRs may be expressed presynaptically at glutamatergic and/or GABAergic terminals or postsynaptically on interneurons and/or medium spiny neurons (MSNs); however, the relationship between genotype at NT213739 and OTR expression in each of these contexts has not yet been directly investigated. NT213739 is strongly associated (explaining 69% of the variance) with robust differences in accumbal OXTR mRNA expression, and accumbal OXTR mRNA expression is strongly correlated with accumbal OTR protein binding density, suggesting that differential accumbal OTR expression associated with genotype at NT213739 is

likely explained by differential OTR expression on accumbal neurons as opposed to presynaptic terminals [118]. In this framework, one possibility is that the NT213739 SNP predicts varying levels of OTR expression in the same subpopulations of accumbal neurons across genotypes; alternatively, the "C" allele may be associated with OTR expression in distinct subpopulations of accumbal neurons that do not express OTRs in "low" T/T animals. In either scenario, natural variation in OTR expression on specific subpopulations of accumbal neurons would almost certainly reflect shifts in a different set of functional parameters compared to manipulations of all presynaptic and postsynaptic OTR signaling using OTAs. Conceptually these principles may extend to other regions, and thus the effects of region-specific and ICV manipulations with OTAs should not be expected to reflect functional differences associated with natural variation in region-specific OTR expression, or vice versa.

Finally, it is also important to highlight that genotype at NT213739 may predict levels of OTR expression in other PBN and SDMN nuclei. Consistent with this possibility, King et al. investigated 12 brain regions and reported that genotype at a second OXTR SNP (NT204321)—which is in strong linkage with genotype at NT213739—significantly predicts OTR expression in the NA, caudate putamen (CP), olfactory bulb (OB), and AON; and factor analyses further revealed that OTR expression in the NA strongly covaries with OTR expression in multiple additional forebrain nuclei including the olfactory tubercle (Tu), lateral septum (LS), central amygdala (CeA), and PFC [118]. Although the NT213739 SNP strongly predicts levels of accumbal OTR expression, its relationship with OTR expression in these regions and other PBN nuclei has not yet been investigated. Therefore, genotypic differences in Fos expression across the PBN in these experiments should not be interpreted as the consequence of natural variation in

accumbal OTR signaling, as these differences may reflect variation in OTR signaling across many forebrain nuclei.

In light of these considerations, our data reveal that "moderate" (C/T) and "high" (C/C) NT213739 genotypes are associated with decreased covariance in Fos expression between the NAs and other PBN nuclei, across mesolimbic reward nuclei, and across the PBN as a whole, relative to "low" OTR genotypes. These data show that natural genetic variation at a single nucleotide in OXTR predicts significant shifts in PBN function during sociosexual contexts, presumably due to variation in region-specific OTR expression. It is particularly interesting that this effect is observed specifically across mesolimbic reward nuclei; OT and dopamine (DA) systems in these regions modulate social and sexual behaviors across species, and have been specifically implicated in pair bonding in both birds and mammals [44, 108, 110].

There are multiple interpretations of more weakly correlated Fos expression across the PBN in "moderate" (C/T) and "high" (C/C) OTR-expressing males. For example, strong positively correlated Fos expression across PBN nuclei may indicate neuronal populations that are functionally coupled by co-activation or co-(dis)inhibition (or both) during the experimental manipulation. In contrast, strong negatively correlated Fos expression across PBN nuclei may indicate neuronal populations whose respective activation potentials are co-modulated in opposing directions (e.g. excitatory inputs onto one predict inhibitory inputs onto the other). In this simplified framework, decreases in correlated Fos expression associated with genotype may represent functional de-coupling of neuronal populations distributed across PBN nuclei, or may represent more complex shifts in the balance of excitatory and inhibitory modulation of region-specific subpopulations; interestingly, the latter interpretation is consistent with previous investigations of OTR function in the left auditory cortex of maternal mice in response to pup calls

[222, 223]. Due to the limitations of Fos analyses, detailed interpretation of these data at the level of specific neural circuits and neuronal phenotypes will require complementary electrophysiological, neuroanatomical tract tracing, and cell-type specific investigations.

There are additional limitations to these experiments, some of which we have described previously [198]. For example, region-specific OTRs may modulate activity of neuronal phenotypes that are critical for social behavior but that do not express Fos; alternative activity-dependent markers may be useful for investigating such populations [224]. In these experiments neural function was investigated across genotypes, and therefore the effects of variable OTR expression through development cannot be separated from immediate differences in central OTR signaling during sociosexual interaction; future investigations using viral and/or transgenic approaches allowing temporally-controlled central and region-specific gene editing (e.g. CRISPR, transiently regulated Cre-recombination, etc.) can resolve these factors. Sample sizes in these experiments were relatively small; investigations using larger samples are important for replication, and increased statistical power may identify additional behavioral and neural effects associated with natural variation in central and region-specific OTR expression that were not detected in this study. Finally, anatomical resolution of OTR (and V1aR) expression has hardly advanced beyond the level of whole brain regions due to limitations in development of specific antibodies, greatly hindering interpretations of these and other data at the cellular level. More finely resolved clues will continue to emerge from electrophysiological slice recording experiments and double in-situ hybridization experiments; additionally, advances in gene editing techniques such as CRISPR will likely provide powerful and high-throughput tools for characterizing a more comprehensive and anatomically precise understanding of OTR function.

Despite these limitations, there are enormous advantages to these experimental approaches. Genotyping combined with analysis of endogenous Fos expression in behaving prairie voles represents a non-invasive and high-throughput approach toward linking intraspecific genetic variation in OXTR, patterns of region-specific OTR expression in the brain, and properties of distributed neural network function during naturalistic social contexts. Here we demonstrate for the first time that naturally occurring genetic variation in OXTR which predicts region-specific patterns of central OTR expression also predicts patterns of endogenous and correlated gene expression across sensory, limbic, and mesolimbic reward nuclei during social contexts in a vertebrate. These data provide a necessary complement to functional brain imaging studies in humans demonstrating that genetic variation in OXTR predicts patterns of correlated BOLD signals across cortical, limbic, and mesolimbic reward nuclei during social tasks [131, 132], and suggest that evolutionary plasticity in OXTR may represent a conserved mechanism underlying diverse neural network function during social contexts in mammals. Finally, these data represent the third line of evidence from our laboratory linking central neuropeptide receptors with patterns of distributed activity across neural networks during social contexts, and provide critical experimental support for previously hypotheses that natural diversity in neuropeptide receptor distribution across evolutionarily conserved neural networks (e.g. the vertebrate SDMN) reflects diversity in distributed network function during social contexts.

# Chapter 5

## Discussion

**The OT system: evolutionary origins and deep homology**

Paracrine secretion of hormone-like peptides that bind to target receptors and trigger intracellular signaling cascades—as well as G proteins, $Ca^{2+}$ and cAMP second messenger pathways, enzymes with cyclase activity, and protein kinases—are involved in quorum sensing in colonially living bacteria [225-228]; similarly, autocrine secretion of hormone and pheromone peptides that bind to G protein coupled receptors (GPCRs; a diverse group of receptors found in some eukaryotes that contain seven putative transmembrane domains as well as three extracellular and three intracellular loops) in diverse signaling pathways have been discovered in yeast, plants, and other eukaryotic organisms lacking nerve cells; demonstrating that many of the biological building blocks and evolutionary precursors of neuropeptide systems were present shortly after the rise cellular life [229, 230]. In eumetazoans, neuropeptides are signaling molecules that are cleaved from proneuropeptides (PNPs) in neurons and typically activate GCPRs. Interestingly, recent comparative genomic analyses have identified PNPs and GPCRs in placozoans, suggesting the intriguing possibility that "neuropeptide" signaling may have predated the evolution of neurons [231]. Here we briefly highlight a sample of deeply homologous anatomical and functional properties between OT-like neuropeptide systems that are separated by more than a billion collective years of divergent evolution.

OT-like peptide receptors are GPCRs that are coupled to both $Ca^{2+}$ and cAMP second messenger pathways and are thought to have evolved following duplication events of pre-existing neuropeptide ligands and/or their respective receptors [232-234]. OT-like peptides and their respective GPCRs have been identified in both neurons and reproductive tissue across a wide variety of protostimian and deuterostimian lineages, suggesting strong selective pressures for the

neural and reproductive functions of these systems for more than 700 million years of evolution [235].

While a single OT/AVP-like peptide homologue is typically present in invertebrates, a duplication event is thought to have occurred in jawless fish approximately 500 million years ago, giving rise to the two AVP-like and OT-like neuropeptide lineages that are observed across virtually all modern vertebrates [236, 237]. Vertebrate OT-like peptides contain a strongly conserved nine amino acid sequence and—like all neurohypophyseal hormones—contain a disulfide bridge between Cys residues 1 and 6 that creates a cyclic structure. The mature nonapeptide is cleaved from a neurohypophyseal propeptide that also exhibits a cyclic structure and contains four main components: a translocator signal, the nonapeptide hormone, a cleavage site, and a neurophysin carrier molecule that facilitates axonal transport of the mature OT-like peptide to terminals [233]. Many OT-like homologues in invertebrates are also nonapeptides, but longer OT-like homologues have been discovered in nematodes and tunicates [235]. The structural characteristics of these invertebrate homologues are remarkably similar: the OT-like peptide is cleaved from a cyclic prepropeptide containing a translocator signal, the OT-like peptide, a cleavage site, and a neurophysin. The mature OT-like homologues are also cyclic, suggesting strong selection for these structural features, perhaps due to coevolution with their respective receptors or processing enzymes [233, 238].

Investigations in nematodes, leeches, and annelid worms have demonstrated that OT-like systems share a deeply conserved role in neurotransmission, reproductive behavior, and learning [101, 146, 152, 232, 235, 239-243]. In both sessile tunicates and mammals, OT-like peptides are synthesized in neurons sensitive to salt concentration, and OT-like receptors are expressed in neural, heart, and reproductive tissue, revealing deeply conserved patterns of tissue-specific expression [233, 244].

Tunicate OT-like receptors expressed in the osmoregulatory neural gland have been shown to facilitate siphon contraction in response to hypotonic seawater, preventing its flow into the body demonstrating an ancient role of OT-like peptides in modulating fluid homeostasis [245].

Invertebrate OT-like peptides are synthesized in neurosecretory cells that can project centrally and peripherally, in some cases directly contacting peripheral circulatory systems [235]. Although important specializations of OT-like systems have evolved in vertebrate lineages, this basic anatomical structure has been conserved; OT-like peptides are synthesized in neurosecretory cells of the hypothalamus and can be released into the brain or into the peripheral circulation via the posterior pituitary and median eminence [62]. Interestingly, neurosecretory cells have been shown to express both OT-like peptides as well as their target receptors in both invertebrates and mammals, suggesting deeply conserved autoregulatory mechanisms of these systems [246]. In mammals, OT signaling at these autoreceptors mobilizes intracellular $Ca^{2+}$ stores and "primes" the neuron to dendritically secrete OT in response to depolarization [247-249].

One study found that OT -like neurosecretory cells in annelid worms and zebrafishes express common sets of cell-type specific transcription factors, microRNAs, and opsins characteristic of light-sensitive cells, suggesting common evolutionary origins and an ancient coupling of OT -like neurosecretion with the light cycle [250]. Interestingly, OT (and AVP) neurons in rodents express clock genes, and OT (and AVP) has been repeatedly linked to circadian rhythm in both rodents and primates [251-257]. In nematodes, OT-like neurons respond to mechanical and thermal stimuli and receive direct sensory inputs, suggesting an ancient coupling of OT-like neurosecretion to multiple sensory modalities [250]. In mammals, OT neurons have been shown to receive monosynaptic inputs from the olfactory bulbs, and OT release can be triggered by visual, auditory, and mechanical social stimuli [151, 156, 157, 258]. It may be that top-down limbic and cortical

modulation of sensory inputs to the hypothalamus in vertebrates facilitates coupling of OT release to social stimuli across sensory modalities. In rodents, OTRs are expressed densely throughout olfactory pathways and are also present in auditory cortex; and OTR signaling directly modulates processing of olfactory and auditory stimuli, suggesting the capacity for reciprocal modulation between primary sensory and OT systems [221, 223]. In primates, vision and audition are more dominant forms of sensory processing, and OTRs are expressed in visual and auditory processing regions [13, 14, 259].

In nematodes, the OT-like peptide and its receptor are critical for associative gustatory learning—or behavioral plasticity in response to conditioned stimuli—specifically by modulating neuronal circuits that are essential for approach/avoidance behavior in response to taste cues [235]. In vertebrates, OTRs modulate many forms of associative learning and approach/avoidance behavior during social contexts; and in rodents, exogenous manipulations of central OT signaling modulate conditioned fear learning, drug reinforcement, and auditory processing of non-social cues; suggesting that OT systems are embedded within generalized sensory, learning, and reward circuits [87, 152, 223, 239, 260-264]. In both nematodes and rodents, OT-like systems interact with dopaminergic and serotinergic systems to modulate associative learning, suggesting a deeply conserved coupling between OT-like neurosecretion and transient modulation of these ancient neurotransmitter systems to facilitate adaptive behavioral plasticity [34, 202, 232]. Interestingly, many electrophysiological investigations have shown depolarizing effects of OT-like signaling in a wide variety of cellular contexts in both invertebrates and vertebrates, although there are exceptions to this generalization [211, 246, 265-268]. Thus, in nervous tissue, OT-like systems may share a deeply conserved role in transiently “priming” sensory, learning/memory, and

approach/avoidance circuits in response to stimuli that trigger release of their respective OT-like peptides.

**Mammalian OT release and signaling**

In mammals, OT is synthesized in magnocellular and parvocellular neurons residing in the paraventricular, accessory, and supraoptic nuclei of the hypothalamus (PVN, AN, and SON, respectively). OT can be released into the periphery via magnocellular projections to the posterior pituitary or centrally through magnocellular dendrites or magnocellular and parvocellular projections to extrahypothalamic sites in the brain. A wide variety of stimuli have been shown to trigger OT release, including mating, parturition, nursing, hypertonic solutions, a variety of pharmacological agents, and more subtle social stimuli [233]. Interestingly, it was recently shown that OT neurons in the SON receive synaptic input from glomeruli in the ventral portion of the olfactory bulb (OB), a division that is thought to be involved in processing social cues [269-271]. These glomeruli have been shown to receive unusually organized (i.e. monoglomerular) projections from olfactory sensory neurons that express the OR37 receptor, which has been shown to bind ligands derived from mammalian anal glands [272, 273]. These independent studies reveal a direct pathway by which social olfactory cues can make direct contact with the OT system.

Like other neuropeptides, OT is stored in and released from large dense core vesicles (LDCVs) that are distributed throughout the dendrites, soma, and axons of OT neurons and are released in response to increased concentrations of intracellular $Ca^{2+}$ [265]. For example, increased concentrations of intracellular $Ca^{2+}$ can trigger exocytosis of LDCVs from the dendrites into the extracellular space, allowing OT to diffuse to target receptors in the hypothalamus and potentially in extrahypothalamic sites [247]; however, anatomical evidence from rats suggests that, similar to fishes and amphibians, dendritic innervation of the third ventricle may also allow OT release

directly into the CSF in mammals [248]. Interestingly, dendritic release can be uncoupled from depolarization by signaling molecules (e.g. α-MSH) that mobilize intracellular $Ca^{2+}$ without depolarizing the neuron, as has been demonstrated on OT neurons in the SON [248].

OT can also be released focally through axons that innervate a wide variety of forebrain regions, allowing for rapid modulation of behavior [180, 268]. It was long thought that these projections originate from parvocellular OT neurons in the PVN; however, recent evidence in fishes and rodents has demonstrated that many of these fibers project from magnocellular OT neurons in the PVN, AN, and SON, suggesting a mechanism by which peripheral and central release may be coupled [153, 180, 274, 275]. Interestingly, electrophysiological and behavioral effects following optogenetically evoked axonal OT release occur relatively slowly (on the order of 2-20 seconds); this, along with anatomical evidence, has led Knobloch et al. to propose that axonal OT release occurs along the axon in a non-synaptic fashion, an event that can be triggered without depolarization [62, 276]. Importantly, in the PVN and SON, OT neurons have been found to co-express VGLUT2 but not GAD67, suggesting that depolarization of these neurons is coupled with synaptic glutamate release [277]. It has been hypothesized that these multiple release mechanisms—slow volume diffusion through the extracellular space, distribution through the CSF, and rapid and focal axonal release into target structures—can occur simultaneously to modulate behavior in a multimodal fashion [148, 278].

Upon release, OT has an extremely high affinity for OTR and can exert its effects at low nanomolar concentrations, suggesting high signaling efficacy following the release of a small number of LDCVs [248]. Because OT neurons express OTRs, dendritic OT release has been shown to have an autoregulatory effect whereby OT binds to autoexpressed OTRs and “primes” the neuron into a more excitable state for as long as an hour, an effect that is described in more detail below (see

"Paraventricular nucleus of the hypothalamus" in the follow section) [248, 279]. Intrahypothalamic glutamatergic synapses have been shown to link magnocellular neurons across the PVN and SON as well within the PVN, fitting with observations of synchronization in recordings from pairs of OT neurons in fishes and rodents, and suggesting a mechanism by which this priming effect could translate to heightened excitability across a broader hypothalamic magnocellular OT network, priming it for multimodal OT release in response to sociosensory stimuli [280-283].

OTR signaling is traditionally thought to be coupled to a Gq/11 protein which triggers phospholipase Cβ pathways and mobilization of intracellular $Ca^{2+}$ stores, a sequence that is generally thought to increase neuronal excitability; however, it was recently demonstrated that higher concentrations of OT (5-50 nmol/L) can activate Gi or Go proteins, which is generally thought to have inhibitory effects on neuronal excitability [65, 284, 285]. This may add an important qualification to the traditionally described positive feedback loop that OT has on its own release, suggesting that it may have an inhibitory autoregulatory effect at high concentrations. Thus, the effects of OTR signaling on neuronal excitability and intracellular neuronal signaling may be more complex than previously thought.

## The pair bonding network: behavioral, anatomical, and electrophysiological perspectives

The pair bonding network (PBN) represents an OT-sensitive network of interconnected olfactory, limbic, and mesolimbic reward nuclei. The PBN is not intended to be a comprehensive set of OT-sensitive forebrain nuclei that are involved in social behavior and should be subject to future revision based on new data; instead, it is intended to be a simplified but useful model of a core neural network that 1) is integral for associative social olfactory learning and 2) exhibits a unique organization of OTR expression in the prairie vole brain that is thought to facilitate pair bond

formation by modulating distributed encoding of the partner's olfactory profile. All PBN nuclei are innervated by hypothalamic OT projections; OTR expression in each nucleus has been demonstrated in multiple representative rodent species (but region-specific OTR densities can exhibit remarkable intra- and interspecific variation); and OTR signaling has been shown to modulate rodent behavior in every PBN nucleus. Electrophysiological studies in mice and rats have demonstrated that OT signaling can mediate a wide variety of effects across PBN nuclei depending on brain region and pre- versus postsynaptic localization of OTRs. The basic neuroanatomical interconnectivity (predominantly monosynaptic inputs to/from other PBN nuclei), OT-modulated behavioral effects, and OT-modulated electrophysiological effects will be briefly reviewed for each PBN nucleus.

*Anterior olfactory nucleus*

The AON projects to the olfactory tubercle and is reciprocally interconnected with the OB, piriform cortex, and the PFC; thus, the AON is positioned at the core of an olfactory network that feeds directly into the PVN, prefrontal cortex (PFC), nucleus accumbens (NA), and medial and basolateral amygdala (MeA and BLA, respectively) [286, 287]. In addition to the PFC, the AON also receives direct projections from the MeA [286]. OTR signaling in the OB has been shown to modulate maternal behavior, and endogenous OTR signaling specifically within the AON is critical for social recognition in rodents [205, 288]. In cultured olfactory bulb (OB) neurons, activation of OTRs acts both presynaptically (mitral/tufted cell axons) and postsynaptically (granule cells) to enhance glutamatergic transmission from mitral/tufted cells to granule cells [289]. In the rat anterior olfactory nucleus (AON), OTR signaling increases glutamatergic synaptic drive onto OB granule cells to increase signal-to-noise ratio during odor processing [205]. OTR signaling mediates this effect by regulating $Na^+$ channels and increasing excitability of putative

fast-spiking interneurons in the AON as well as glutamatergic pyramidal neurons projecting to the OB. This drives excitation of inhibitory granule cells which project onto and lower baseline firing of M/TCs while simultaneously increasing their peak odor responses, presumably enhancing information extraction [205]. Similarly, OTergic inputs from the PVN have an excitatory effect on granule cell firing and an inhibitory effect on mitral/tufted cell firing in the accessory olfactory bulb [290]. OT paired with 100 Hz stimulation has also been shown to potentiate NMDA-mediated long term potentiation (LTP) of mitral/tufted cell to granule cell synaptic input [291].

*Prefrontal cortex*

The PFC (specifically the prelimbic subdivision, which was analyzed in chapters 2, 3, and 4) receives afferent projections from the AON (and other olfactory nuclei), BLA, and VTA [287]. In turn, the PFC projects to the AON, NAs, PVN (sparsely), MeA, BLA, and VTA [292]. OTR signaling in the PFC has been shown to modulate partner preference formation in prairie voles; sociosexual behavior in mice; and anxiety-like behavior, maternal care and aggression, and socially-buffered fear extinction in rats [108, 114, 204, 293, 294]. OTRs are expressed on somatostatin-positive GABAergic interneurons in the PFC in mice, and OTRs are expressed at significantly higher levels in prairie voles compares to non-monogamous montane voles [76, 114]. OT has been found to modulate plasticity at glutamatergic synapses onto pyramidal neurons in layer V (the primary source of PFC input to the NA); however, these effects are complex. Similar to theta stimulation of layer II/III, high concentrations (100 nmol/L) of OT—but not low concentrations of OT (1nmol/L)—suppress basal levels of glutamatergic input onto layer V pyramidal neurons, an effect that is mediated by presynaptic activation of CB1 receptors. Interestingly, pretreatment with OT (1 hour) causes theta stimulation to induce LTP of

glutamatergic input onto these neurons, an effect that is mediated by postsynaptic insertion of $Ca^{2+}$ AMPA receptors [295].

*Nucleus accumbens shell*

The nucleus accumbens shell (NAs) receives dense olfactory input from the olfactory tubercle and receives afferent projections from the PFC, MeA, BLA, and ventral tegmental area (VTA) [296, 297]. In turn, the NAs sends projections to the PVN and VTA [298-300]. It is also important to note that the NAs also projects heavily to the ventral pallidum (VP), a nucleus that in turn projects to PFC, PVN, MeA, BLA, and VTA [301]. Endogenous OTR signaling and/or OTR binding density in the NA (in some instances specifically within the shell) is associated with social approach, social reward learning, space use, mating tactics, alloparental behavior, maternal behavior, partner preference formation, passive stress coping-like behavior during social separation, and other social behaviors in prairie voles and other rodents [85, 91, 98, 99, 105, 108, 111, 115, 116, 155, 165, 200, 202, 203, 302]. In mice, activation of presynaptic OTRs expressed on serotonergic projections from the dorsal raphe to the NA mediates local serotonin release; and this causes serotonin-dependent LTD through depression of postsynaptic excitatory transmission onto medium spiny neurons (MSNs) [202]. ICV administration of OT increases mean firing rate of slow firing neurons specifically in the NA shell, and this effect is inhibited by repeated pre-treatment with morphine [303].

*Paraventricular nucleus of the hypothalamus*

The PVN receives olfactory input from the OB and also receives projections from the PFC, NAs, MeA [243, 298, 304]. OT neurons in the PVN project to every other PBN nucleus [180]. Endogenous OTR signaling in the PVN modulates social buffering in prairie voles and maternal behavior in rats [57, 305]. Interestingly, social buffering can also be induced by exposure to

agonists for the OR37 receptor [306]. RNAi knockdown of OT specifically within the PVN modulates a variety of social behaviors in zebra finches, and optogenetically evoked axonal OT release from PVN projections to the AON modulates social odor processing in rats [21, 205]. OT neurons can exhibit small increases in spontaneous firing in response to some stimuli (e.g. hyperosmotic treatment) or robust increases in synchronized burst firing (10-30Hz) during parturition and nursing, which is associated with robust and pulsatile OT release into the periphery [307]. A wide variety of pharmacological agents have also been shown to modulate OT neuronal electrophysiology, including estrogen and CRFR2 agonists [115, 308]. Excitation of OT neurons is potentiated by OT, similar to effects that have been demonstrating following dendritic OT release [309]. This effect depends on tonic OT release from the "postsynaptic" OT neuron. Through binding to postsynaptic OTRs, OT triggers mobilization of intracellular $Ca^{2+}$ concentrations and release of endocannabinoids, which bind to presynaptic CB1 receptors and inhibit GABAergic input [309]. Interestingly, electrical stimulation of GABAergic inputs to the PVN has been shown to have a general excitatory effect on OT neurons and an inhibitory effect on AVP neurons, consistent with findings in nursing rats [310, 311].

*Medial amygdala*

In addition to olfactory input from the pirifirm cortex and OB (including sensory cells that express the OR37 receptor, which has implicated in social olfactory processing), the MeA (particularly the posterodorsal subdivision which is the focus of chapters 2, 3, and 4) receives projections from the PFC, sparse projections from the BLA, and is reciprocally (though sparsely) interconnected with the VTA [273, 292, 312-314]. In turn, the MeA projects to the AON, PVN, and NAs [286, 304, 314-316]. OTR signaling in the MeA modulates social recognition as well as social approach behavior toward immune-compromised conspecifics [107, 317-319]. Theta stimulation of the

accessory OB induces LTD in the MeA and is thought to reflect a critical component of social memory formation; during theta stimulation of the accessory OB, OT potentiates LTD in the MeA and OTR antagonists inhibit LTD formation [318] .

*Basolateral amygdala*

The BLA receives dense olfactory input from a variety of olfactory nuclei, including the piriform cortex, which is densely interconnected with the AON; in turn, the BLA projects to the NAs, weakly to the MeA, and is reciprocally interconnected with the PFC [296, 312]. Manipulations of OT signaling have been shown to modulate contextual fear learning and social decision making in rodents and primates, respectively [262, 320, 321]. To our knowledge, the electrophysiological effects of OTR signaling within the BLA have not been investigated; however, BLA neurons have been shown to respond to social stimuli, and OTR signaling modulates BLA input to the medial central amygdala (CeM) [322-324]. Endogenous OTR signaling in the CeM is critical for contextual fear learning and maternal behavior, and the BLA is hypothesized to play a critical role in these processes [262, 325, 326].

*Ventral tegmental area*

The VTA is reciprocally interconnected with the NAs, PFC, and MeA [287, 297, 300, 327, 328]. In rats, endogenous OTR signaling in the VTA has been shown to modulate the onset of maternal behavior (OTA inhibits maternal behavior) and sucrose intake (OTA increases sucrose but not water intake); and exogenous activation of OTRs in the VTA in females induces progesterone-mediated lordosis; in males, exogenous activation of OTRs in the VTA induces penile erection and increases extracellular DA concentrations in the NAs and PVN; and antagonism of GABA or AMPA receptors in the VTA during cohabitation accelerates pair bond formation in male prairie voles [110, 209, 329-332]. Electrophysiological recordings have shown that high concentrations

of OT (1μm) strongly depolarize small-sized but not large-sized neurons in the VTA [333]. Interestingly, I.C.V. administration of OT potentiates excitation of magnocellular OT neurons in the supraoptic nucleus (SON) during suckling in response to VTA stimulation [334]; dendritic release of OT in the SON has been implicated in social recognition [335]; and both OT and 10Hz stimulation of the SON (and contralateral PVN) has been shown to excite OT neurons in the PVN (and inhibit AVP neurons), perhaps through internuclear glutamatergic circuits as has been previously proposed [280, 336, 337]; together these data suggest multiple mechanisms by which the VTA may interact with the OT system to modulate social cognition and behavior.

**Experimental results**

Like virtually all biological systems, the brain is a deeply interconnected web of functional networks that can be studied at many levels. These neural networks vary along a spectrum of hierarchical versus distributed information processing and have been subjected to varying degrees of rigorous investigation. Decades of advance in neuroscience have caused increasing emphasis to be placed on the role of distributed network function in cognition and behavior [338]. For example, in the wake of the pioneering work of Ann Kelley, Sarah Newman, and others, the limbic and mesolimbic reward systems (and other brain networks) have come to be understood as possessing fundamentally distributed anatomical and functional properties. This view emphasizes that just as information can be processed and encoded in well-defined multi-synaptic microcircuits within brain regions (e.g. CA3/Schaeffer collateral/CA1 pathway), it can also be processed and encoded across poorly understood and interregional networks of distributed and reciprocally interconnected neuronal populations. Studying brain function at this network-level of analysis has grown increasingly important in advancing our understanding of behavior, cognition, and psychiatric phenotypes.

One of the most fascinating characteristics of the OT (and AVP) system is the juxtaposition of 700 million years of deeply conserved tissue-specific expression and function (i.e. fluid balance, reproductive physiology and behavior, neurotransmission) with its extraordinary organizational diversity in the vertebrate forebrain. Diverse patterns of OTR expression across limbic and mesolimbic reward systems have been correlatively and causatively linked with numerous dimensions of vertebrate sociality, revealing evolutionary plasticity in central receptor distribution as a major mechanism by which the central OT system has been exapted to modulate social behavior while maintaining its ancient physiological roles. The primary goal of these experiments is to inch forward our understanding of how plasticity in the central OT system may be linked to brain function, and to push into new territory so that new hypotheses can be generated.

One of the hallmark observations that led to distributed network models of social behavior was the observation of "redundancy" or distribution of apparently similar behavioral functions across multiple limbic nuclei. For example, it was shown that male sexual behavior could be facilitated in castrated hamsters with similar estrogen manipulations in the medial preoptic area or the medial amygdala. While this may not be surprising, it challenged the predominant hierarchical models of sexual behavior that were largely accepted at the time. These and other advances in the study of reward learning simultaneously led to network models of reciprocally modulatory limbic and mesolimbic networks that integrate sensory, hormonal, and reinforcement cues in a distributed manner to guide adaptive behavior. Major hypotheses that emerged from these lines of work were: 1) social context and behavior are more strongly linked to patterned activity (i.e. relative "weighting" of nodes, or "functional connectivity" of nodes) across the whole network than to activity at more traditional levels of analysis, and 2) variation in steroid hormone and neuropeptide receptor organization in the forebrain reflects variation in distributed function of these networks

during social contexts. The first of these hypotheses has been explicitly tested [143]; but to our knowledge they have not been directly tied to endogenous central steroid hormone or neuropeptide signaling.

In these experiments we first demonstrated that endogenous central OTR signaling is critical for pair bond formation in socially monogamous male prairie voles, reversing a notion of sex-specific function that has pervaded the literature for decades. Briefly, prairie voles exhibit a unique central OTR organization compared to closely related species as well as high intraspecific variation in region-specific OTR expression. We then used male prairie voles to investigate how central OTR signaling during naturalistic social interactions modulates correlated IEG expression across an OT-sensitive neural network that is thought to modulate associative social olfactory learning and pair bond formation. We used a relatively simple but high-throughput Fos protocol combined with pharmacological and genetic techniques to generate three independent lines of converging evidence suggesting that the OT system modulates correlated expression of Fos across nodes of this network.

There are limitations to these experiments that are outlined in the "Discussion" sections of chapters 2, 3, and 4. Among the most significant are limitations in interpreting expression of Fos protein itself. For example, Fos protein is expressed over a time course of 60-120 minutes following a stimulus, which forces experimenters to use a relatively acute stimulus or to forfeit behavioral specificity. For multiple reasons we chose to forfeit behavioral specificity in these experiments, favoring an extended period of social interaction, mating, and presumably sustained OT release, diffusion, and signaling. Another major limitation is that many neuronal phenotypes have been characterized that do not typically express Fos protein but express other IEG protein products (e.g. accumbal MSNs) neurons [224]. Combined with lack of cell-type specific labeling, these factors

prevent delineation of specific neuronal phenotypes that are modulated by OT signaling. Finally, links between expression of Fos protein, electrophysiological activity, and neural plasticity have not been fully characterized. While expression of Fos protein may be a valuable marker for mapping neural plasticity in some neuronal populations, it is important to consider that post-transcriptional regulation may inhibit translation of the protein in other subsets of activated neurons. Thus, expression of Fos mRNA may be more precise for mapping neuronal activity. Alternative methods (e.g. alternative IEGs, measuring IEG mRNA expression, and electrophysiological recordings) are necessary to address these limitations.

Despite these limitations, these experiments have shown conclusively that both endogenous OTR signaling and natural genetic variation in OXTR as associated with significant differences in distributed patterns of Fos expression across brain nuclei that are important for social olfactory processing and reward learning. We observe dramatic and highly significant shifts in the distributed response of "Fos-capable" neuronal populations embedded within these nuclei that are associated with variation in multiple dimensions of the OT system (i.e. global OTR signaling, region-specific OTR signaling, and natural genetic variation associated with robust differences in region-specific OTR expression). Of particular note is the robust effect of genotype on patterned Fos expression across mesolimbic reward nuclei (i.e. NAs, PFC, and VTA). Mesolimbic reward networks have been implicated in not only pair bond formation but more general forms of reward learning and reinforcement. We have transformed data from all three experiments into z-scores to highlight one particular relationship, the PFC-NAs, in Fig. 11. In an interesting complement to these Fos results, the PFC-NAs nuclei exhibit electrophysiological coherence during mating in prairie voles. We have used optogenetics to investigate this circuit and have shown that synchronized activity between these regions is important for the formation of selective social

preferences. Interestingly, altered functional connectivity of this circuit has been implicated in a variety of human psychiatric phenotypes [339-342].

Also striking was the finding that the genotype associated with *high* OTR expression, which has been associated with accelerated pair bond formation in the laboratory and socially monogamous behavior in the field, is associated with more weakly correlated Fos expression in this paradigm. Interpreting strongly positive or negative Fos expression is less challenging than interpreting elevated but uncorrelated Fos expression, which could reflect complex shifts in how inputs translate to local excitation and inhibition of Fos-capable neurons. This is discussed in more detail in the "Discussion" section of Chapter 4, but one potential interpretation is that this reflects a shift in the relationship between afferent input and local excitatory:inhibitory balance. In other words, under the assumption Fos expression reflects a response to excitatory input, strongly positively correlated Fos expression in presumed "low" genotypes may reflect a network that is largely connected by excitatory or disinhibitory inputs (manifested by greater levels of Fos expression in one region predicting greater levels of Fos expression in another). High OTR expression may tip this scale by augmenting the influence of inhibitory transmission, reducing positively correlated Fos expression across the network.

There is a modest level of support for this idea derived from the electrophysiological literature. Namely, a sizeable body of work has demonstrated potentiation of GABAergic neurons (predominantly interneurons) by OTR activation, which shifts the excitatory to inhibitory balance to increase signal-to-noise ratio and enhance synaptic transmission. This model of OTR signaling is undoubtedly simplified and has been outlined in more detail by Froemke et al. [222, 223]; however, it may provide a useful framework for interpreting these data, guiding future hypotheses, and synthesizing common neuromodulatory themes of OTR signaling.

More broadly, shifts in correlated IEG expression across distributed brain regions during social contexts may reflect shifts in network "states" that shape how sociosensory information is transmitted and encoded across distributed brain regions during social contexts. In this view, a measurable range of network states during a given social context (e.g. sociosexual interaction and mating) may be necessary for specific forms of behavioral plasticity (e.g. pair bond formation). Furthermore, there may be a variety of evolutionary pathways toward achieving these ranges of network states that involve independent organizational parameters (e.g. OTR densities in different regions, or different within region neuronal populations) and neural systems (e.g. OTR versus V1aR). In this framework, the relative balance of multiple neuromodulators (e.g. neuropeptides, steroid hormones, etc.) released during a specific social context would be integral in determining the transient network state, and diverse network states could evolve through variation in any of the aforementioned parameters, as well as others (e.g. AVP soma distribution).

These interpretations are intentionally speculative and should not detract from the principal findings of these experiments, which definitively show that central OTR signaling during naturalistic social interaction modulates patterned expression of an activity-dependent IEG product across behaviorally important neural networks. These results suggest shifts in how distributed Fos-capable neuronal populations are modulated across these nuclei that are associated with multiple dimensions of the endogenous OT system, providing critical support for previously outlined models.

## Future Directions

Future studies should build upon these results using a variety of complementary techniques and experimental approaches. For example, similar high-throughput approaches can investigate expression of alternative IEG mRNA or protein products; different social contexts; different

neuropeptide or steroid hormone systems; females; alternative species; multiple developmental time points; and alternative neural networks. Neuroanatomical tracers and cell-type specific markers can be used in tandem with IEGs to investigate specific circuits, projections, and neuronal phenotypes. Multiregional electrophysiological recordings coupled with manipulations of specific steroid hormone or neuropeptide systems will provide critical mechanistic insights into how endogenous steroid hormone and neuropeptide systems modulate functional connectivity across brain regions and network states. Optogenetics can be used to test the role of interregional projections in modulating social behavior. CRISPR can be used to genetically delete or manipulate specific neuronal populations or region-specific neuropeptide systems (e.g. altering region-specific OTR densities) to determine their role in driving patterns of functional connectivity, specific network states, and specific forms of social behavior.

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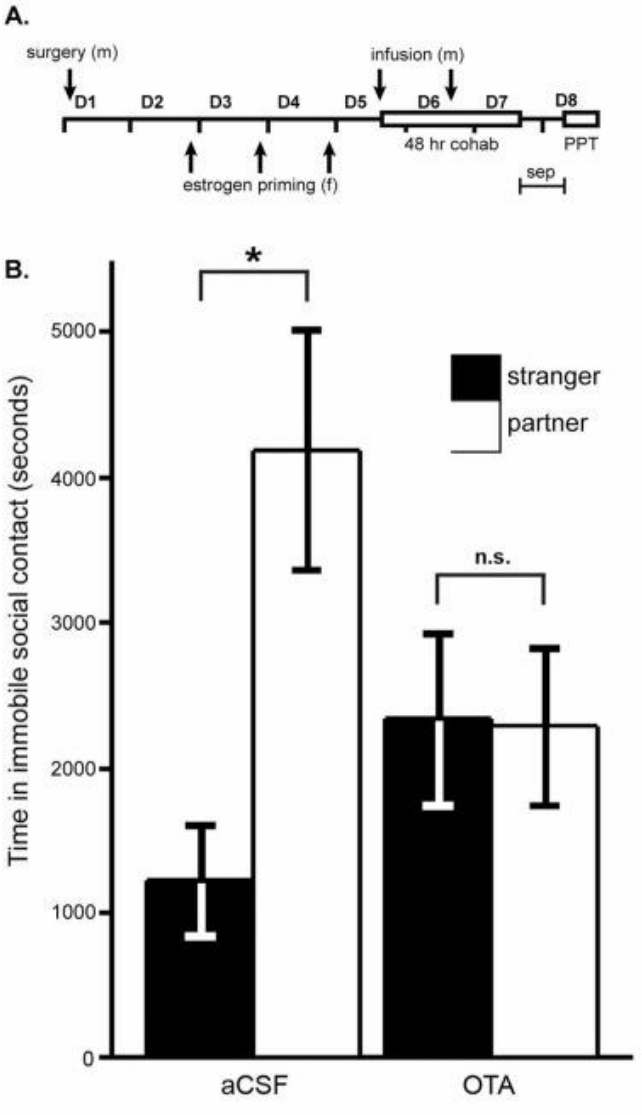



**Figure 1. Central OTR signaling modulates pair bond formation in male prairie voles.** Figure 1A represents a schematic of the experimental timeline and design (D=day; m=male; f=female; sep=overnight separation; PPT=partner preference test). Prior to testing, adult males received either aCSF or OTA (administered ICV) throughout a 48-hour cohabitation period with an ovariectomized, estrogen-primed, sexually experienced adult female. Figure 1B depicts time spent in immobile social contact (e.g. huddling) with the partner versus the stranger in a 3-hour partner preference test in aCSF- and OTA-treated animals. There was a significant interaction effect of treatment (OTA versus aCSF) by stimulus (partner versus stranger) on time spent in immobile

social contact ($F_{1,60}$=6.208, p=0.016; two-way repeated measures ANOVA). aCSF-treated males (n=16) spent significantly more time in immobile social contact with the partner (4193±826 seconds) compared to the stranger (1226±387 seconds; p=0.0028; Student's t-test). In contrast, time spent in immobile social contact with the partner (2285±541 seconds) versus the stranger (2339±588 seconds) did not significantly differ within OTA-treated subjects (p=0.95; Student's t-test). Asterisk indicates a p-value below 0.05.

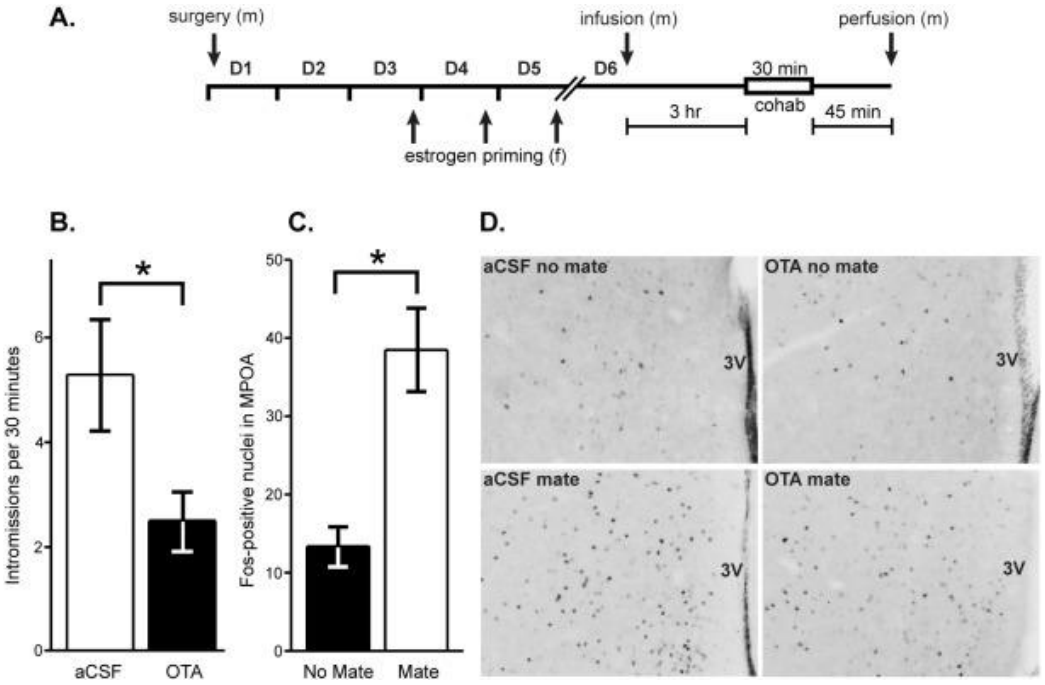



**Figure 2. Central administration of OTA reduces frequency of intromission bouts and mating is associated with elevated MPOA Fos expression.** Figure 2A shows a schematic of the experimental timeline and design for Fos experiments (D=day; m=male; f=female). Prior to testing, adult males received either aCSF or OTA (administered ICV). Figure 2B shows the number of intromission bouts exhibited during a 30-minute period of sociosexual interaction with an ovariectomized, estrogen-primed, sexually experienced adult female. aCSF-treated subjects (n=14) engaged in an average frequency of 5.30±1.14 intromission bouts with the stimulus female per 30 minutes, which differed significantly relative to OTA-treated males (2.49±0.62 bouts per 30 min; z=-4.30; $p=2.35x10^{-5}$; Poisson regression). Figure 2C shows the number of Fos positive nuclei within the medial preoptic area between subjects that did not mate (n=11) compared to those that mated (n=20; pooled across OTA and aCSF treatment) during the 30-minute period of sociosexual interaction. Subjects that did not mate exhibited significantly fewer Fos-positive nuclei (13.49±2.51) compared to subjects that mated (38.54±5.34; $t_{28}$=-3.34, p=0.0024; Student's

t-test). Asterisks indicate p-values below 0.05. Figure 2D shows representative images of Fos expression within the MPOA across all treatment groups.

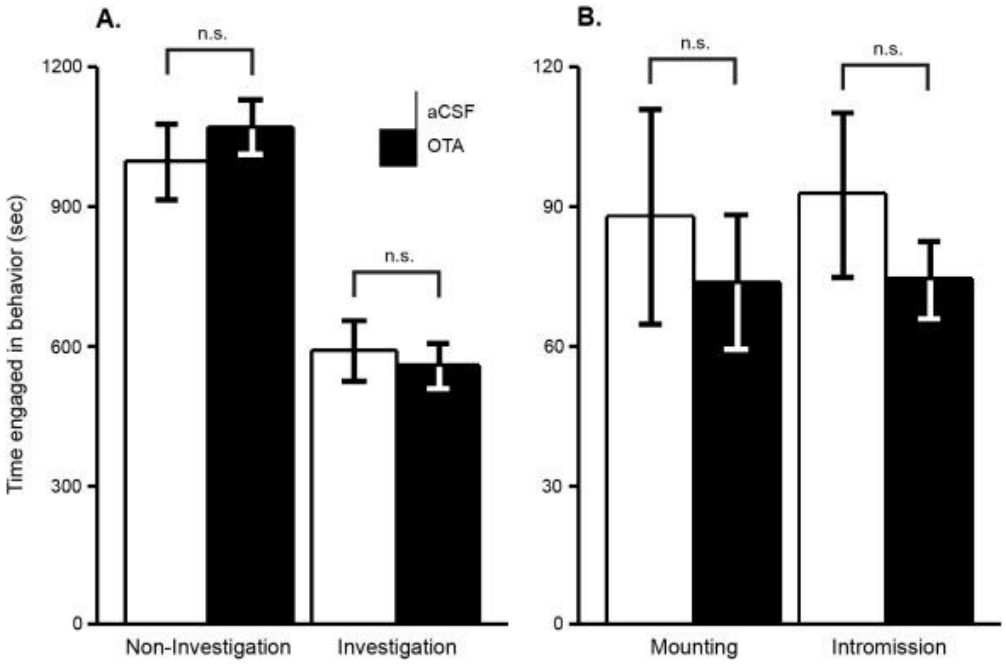
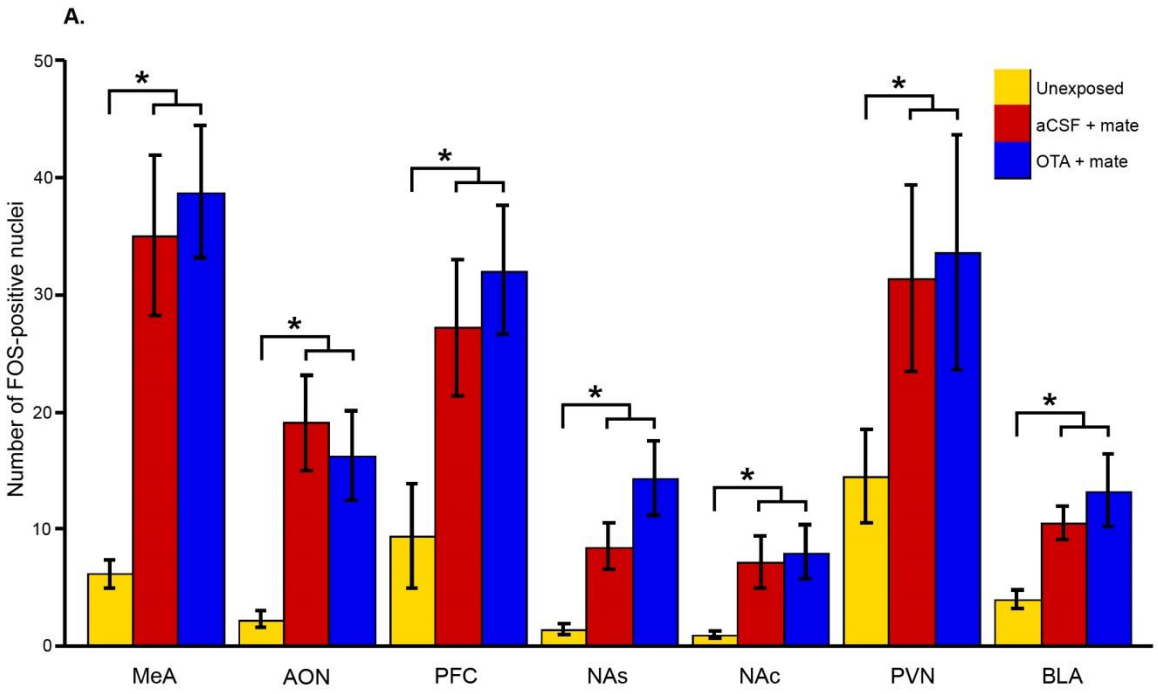
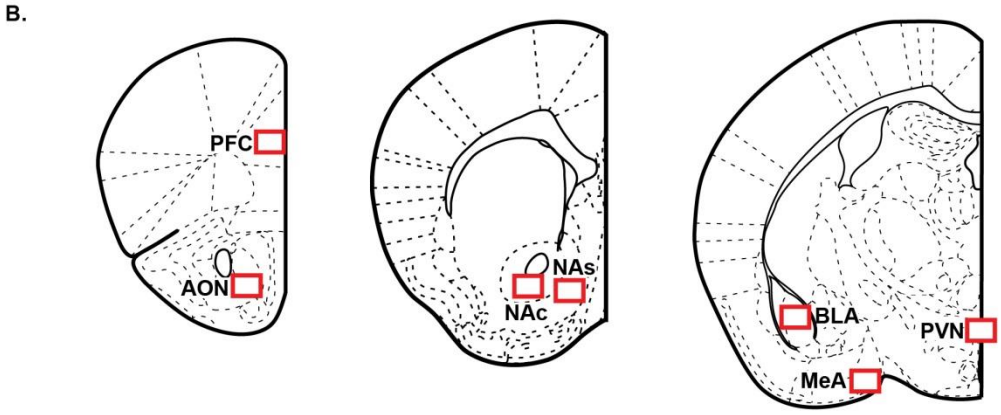



**Figure 3. OTA- and aCSF-mated subjects do not differ in time engaged in non-interaction, social investigation, mounting, or intromission.** Figure 3 shows the time engaged in non-interaction, social investigation, mounting, and intromission between aCSF- (n=11) and OTA-mated (n=9) males during a 30-minute period of sociosexual interaction with a female. There was no main effect of treatment on time engaged in any of these behaviors ($F_{1,138}$=0.064, p=0.801, two-way repeated measures ANOVA). "n.s." indicates that the difference is not statistically significant.


A.
Number of FOS-positive nuclei
50
40
30
20
10
0
Unexposed
aCSF + mate
OTA + mate
*
MeA
AON
PFC
NAs
NAc
PVN
BLA


B.
PFC
AON
NAs
NAc
BLA
PVN
MeA

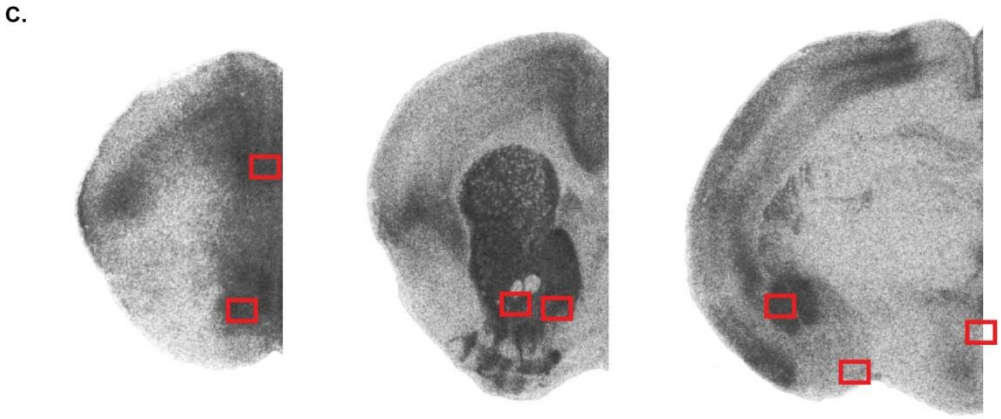

C.

**Figure 4. Sociosexual interaction is associated with elevated FOS expression across OTR-expressing PBN nuclei.** Figure 4A shows measures of FOS-positive nuclei across seven PBN nodes in aCSF-unexposed, aCSF-mated, and OTA-mated male prairie voles. There was a significant main effect of treatment on FOS expression across these areas ($F_{1,189}$=22.06, $p=5.05x10^{-6}$; two-way repeated measures ANOVA). Within mated subjects, there was no main effect of treatment (OTA versus aCSF) on FOS expression across these areas ($F_{1,129}$=1.01; p=0.32). Pooled by treatment, mated subjects exhibited elevated FOS expression in all of the analyzed brain areas relative to unexposed controls. Asterisks indicate p-values below 0.05. Figure 4B shows the neuroanatomical positions (rectangular frames) at which images were captured for FOS expression analysis in each of these regions using illustrations from The Mouse Brain atlas (Franklin and Paxinos Third Edition). Figure 4C highlights these regions (rectangular frames) on representative autoradiogram films depicting OTR densities (black) in a male prairie vole. Abbreviations: MeA=medial amygdala; AON=anterior olfactory nucleus; PFC=prefrontal cortex; NAs=nucleus accumbens shell; NAc=nucleus accumbens core; PVN=paraventricular hypothalamic nucleus; BLA=basolateral amygdala.

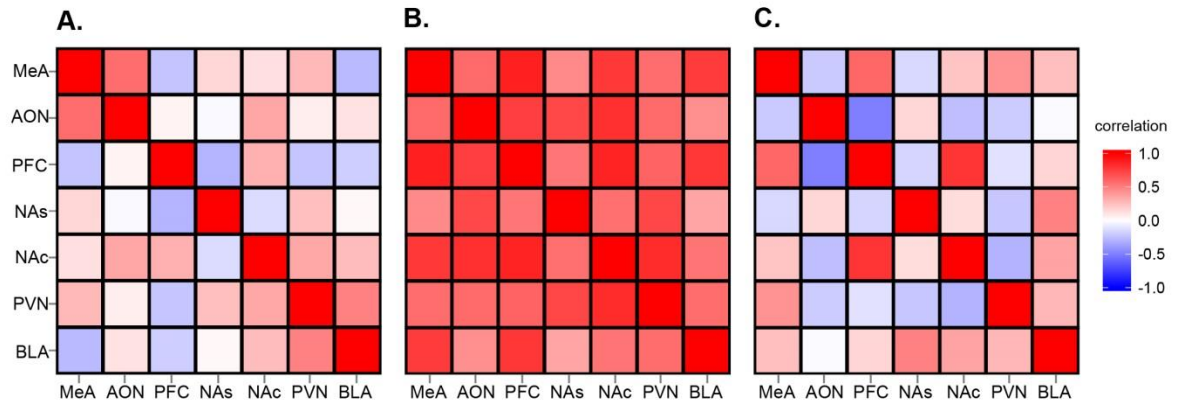



**Figure 5. Patterns of covariation in Fos expression between brain regions within the pair-bonding network.** Figure 5 shows pair-wise comparisons (Pearson's correlation coefficients) of Fos expression across brain regions involved in pair bonding. The heatmaps are symmetrical, meaning that the upper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

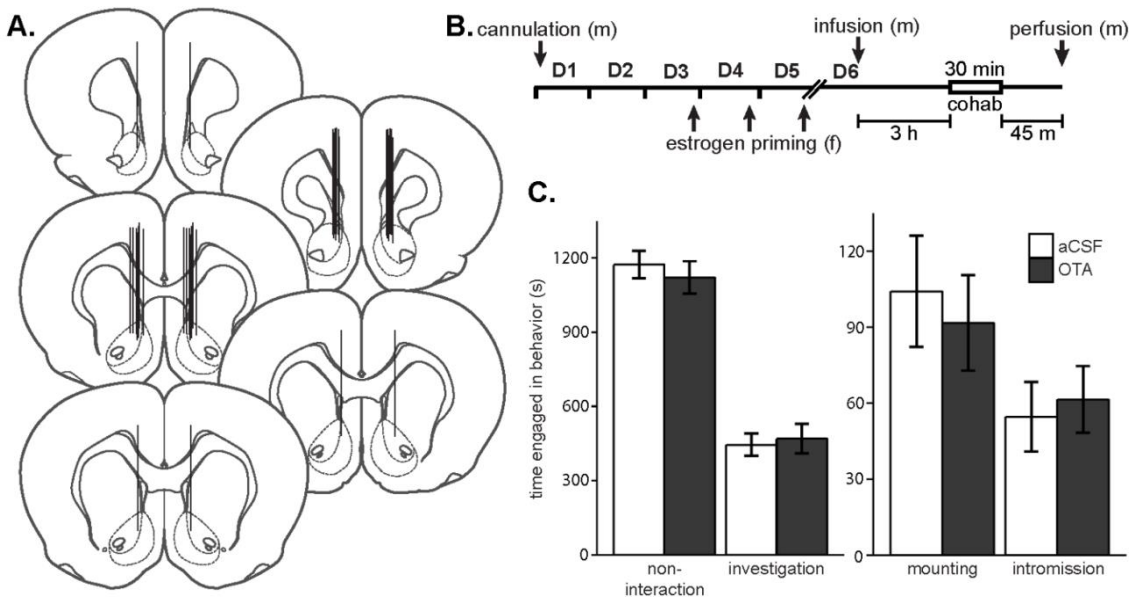



**Figure 6. Site-specific administration of OTA into the NA has no effect on multiple sexual and social behaviors.** Figure 6A depicts cannula placement across all subjects, with cannula tracks represented by solid black vertical lines and the NA represented by dotted boundaries. Figure 6B shows a schematic 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 NA. Figure 6C shows the time engaged in non-interaction, social investigation, mounting, and intromission among mated males (n=11 aCSF; n=12 OTA) during 30 minutes of sociosexual interaction with a female. There was no main effect of treatment on time engaged in any of these behaviors (aCSF versus OTA; $F_{1,109}$=0.00; p=1.00; Eta-squared=0.00; two-way repeated measures ANOVA). "n.s." indicates that the difference is not statistically significant.

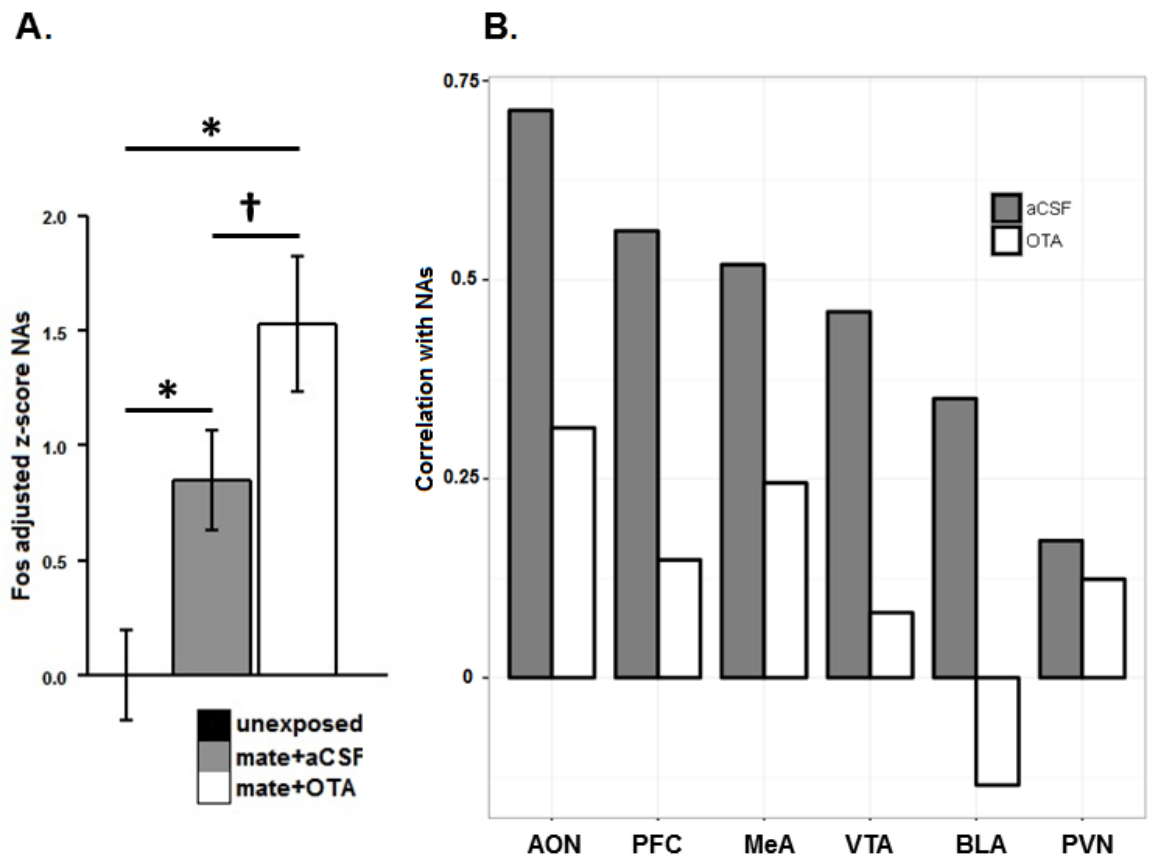



**Figure 7. Effects of sociosexual interaction and local OTR signaling on Fos expression in the NAs.** Figure 7A shows the adjusted z-score for Fos expression in the NAs across aCSF-unexposed (n=5), aCSF-mated (n=11), and OTA-mated (n=12) males. Unexposed males exhibited significantly lower Fos expression in the NAs compared to aCSF-mated ($t_{1,14}$=-2.48; p=0.029; Eta-squared=0.30; Student's t-test) and OTA-mated ($t_{1,15}$=-3.20; p=6.01x$10^{-3}$; Eta-squared=0.41; Student's t-test) males. Among mated subjects, aCSF-treated males exhibited a trend toward reduced Fos expression in the NAs relative to OTA-treated males ($t_{1,21}$=-1.84; p=0.08; Eta-squared=0.14; Student's t-test). Figure 7B shows Pearson's R correlation values for Fos expression between the NAs and every other other PBN nucleus between aCSF-mated and OTA-mated males. A multiple linear regression analysis revealed that Fos expression across non-accumbal PBN nuclei explains a large amount of the variance in NAs Fos expression in aCSF treated animals ($R^2$

= 91%) but not in animals treated with an OTA ($R^2$ = 20%). A permutation test indicated that this difference in variance explained (71%) was statistically significant (p = 0.02). Asterisks indicate p < 0.05, daggers indicate p < 0.1.

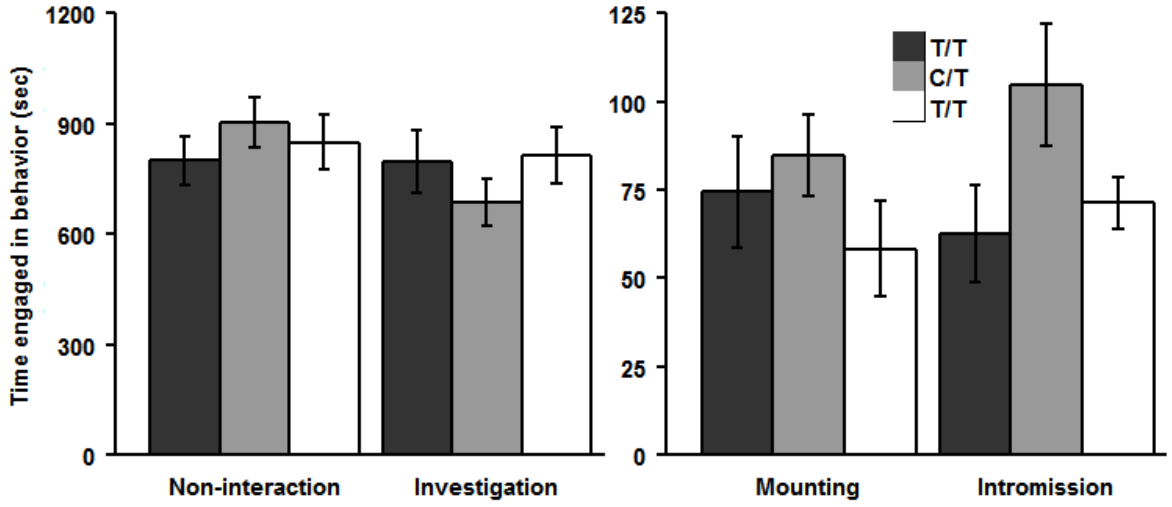



**Figure 8. Genotype at NT213739 has no effect on male sociosexual behaviors.** Figure 8 shows the time engaged in non-interaction, social investigation, mounting, and intromission between T/T, C/T, and C/C males during a 30-minute period of sociosexual interaction and mating with an estrogen-primed stimulus female. There was no main effect of genotype on time engaged in any of these behaviors ($F_{1,114}$=0.10; p=0.76; Eta-squared=0.00, two-way repeated measures ANOVA). "n.s." indicates that the difference is not statistically significant.

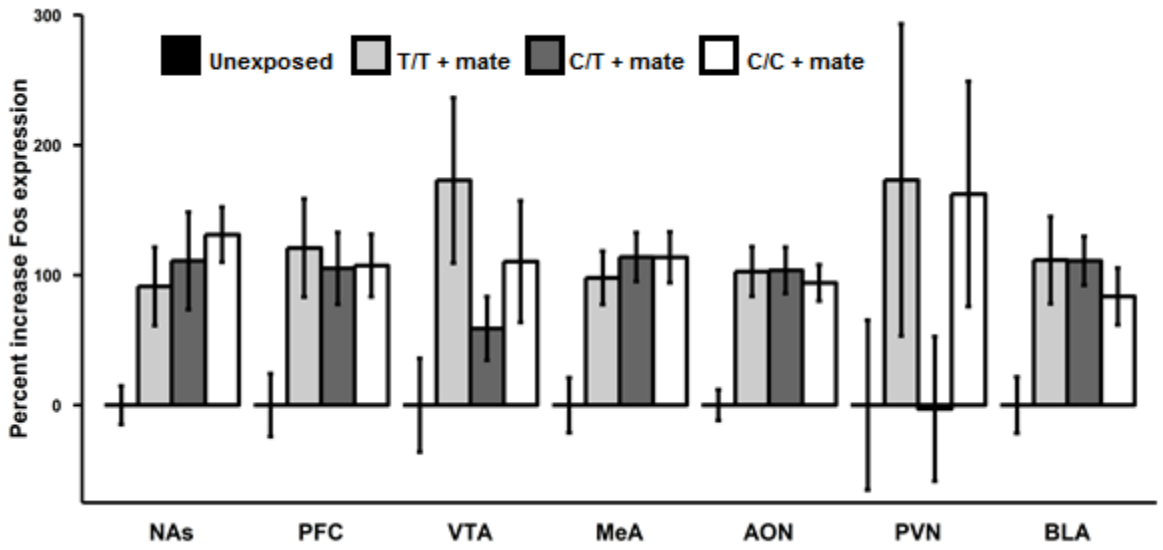



**Figure 9. Effect of genotype at NT213739 on Fos mean Fos expression across PBN nodes.** Figure 9 shows measures of FOS-positive nuclei across seven PBN nodes in unexposed (mixed genotype), T/T-mated, C/T-mated, and C/C-mated male prairie voles. There was a significant main effect of treatment on Fos expression across these areas ($F_{1,274}$=71.960; p=1.39x$10^{-15}$; Eta-squared=0.21). Within mated subjects, Fos expression across the PBN did not differ by genotype among mated animals ($F_{1,213}$=0.29; p=0.59; Eta-squared=0.00). Pooled by genotype, mated subjects exhibited elevated Fos expression across the PBN relative to unexposed controls ($F_{1,276}$=27.91; p=2.58x$10^{-7}$; Eta-squared=0.09). Asterisks indicate p-values below 0.05. Abbreviations: NAs=nucleus accumbens shell; PFC=prefrontal cortex; VTA=ventral tegmental area; MeA=medial amygdala; AON=anterior olfactory nucleus; PVN=paraventricular hypothalamic nucleus; BLA=basolateral amygdala.

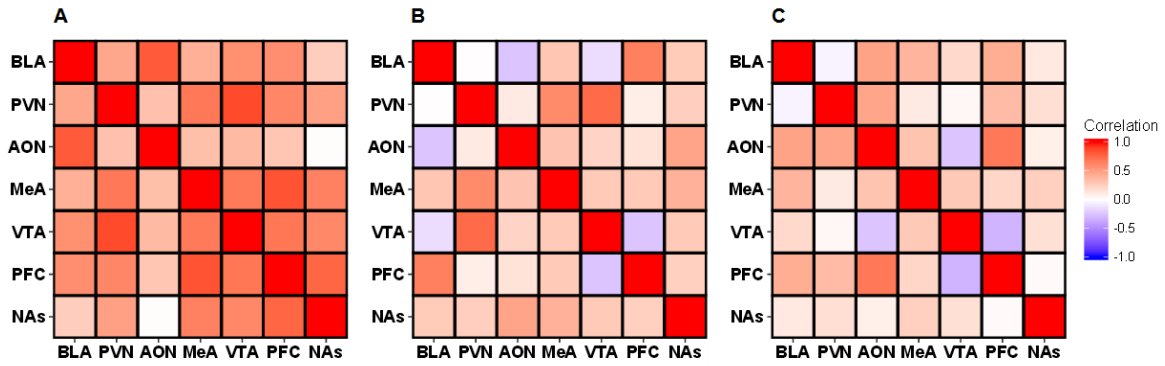



**Figure 10. Genotype at NT213739 is associated with differences in covariance in Fos expression across the pair bonding network.** Figure 10 shows pair-wise comparisons (Pearson's correlation coefficients) of Fos expression across OTR-expressing forebrain regions involved in social learning. The heatmaps are symmetrical, meaning that the upper and lower triangle in each map represents the same data. Figure 10A represents Fos expression covariance in mated homozygous T/T males with the "low" accumbal OTR expression phenotype. Figure 10B and 10C represent Fos expression covariance in mated heterozygous C/T and C/C males, exhibiting the "moderate" and "high" accumbal OTR expression phenotypes, respectively. Abbreviations: BLA=basolateral amygdala; PVN= paraventricular hypothalamic nucleus; AON= anterior olfactory nucleus; MeA=medial amygdala; VTA=ventral tegmental area; PFC=prefrontal cortex; NAs=nucleus accumbens shell.

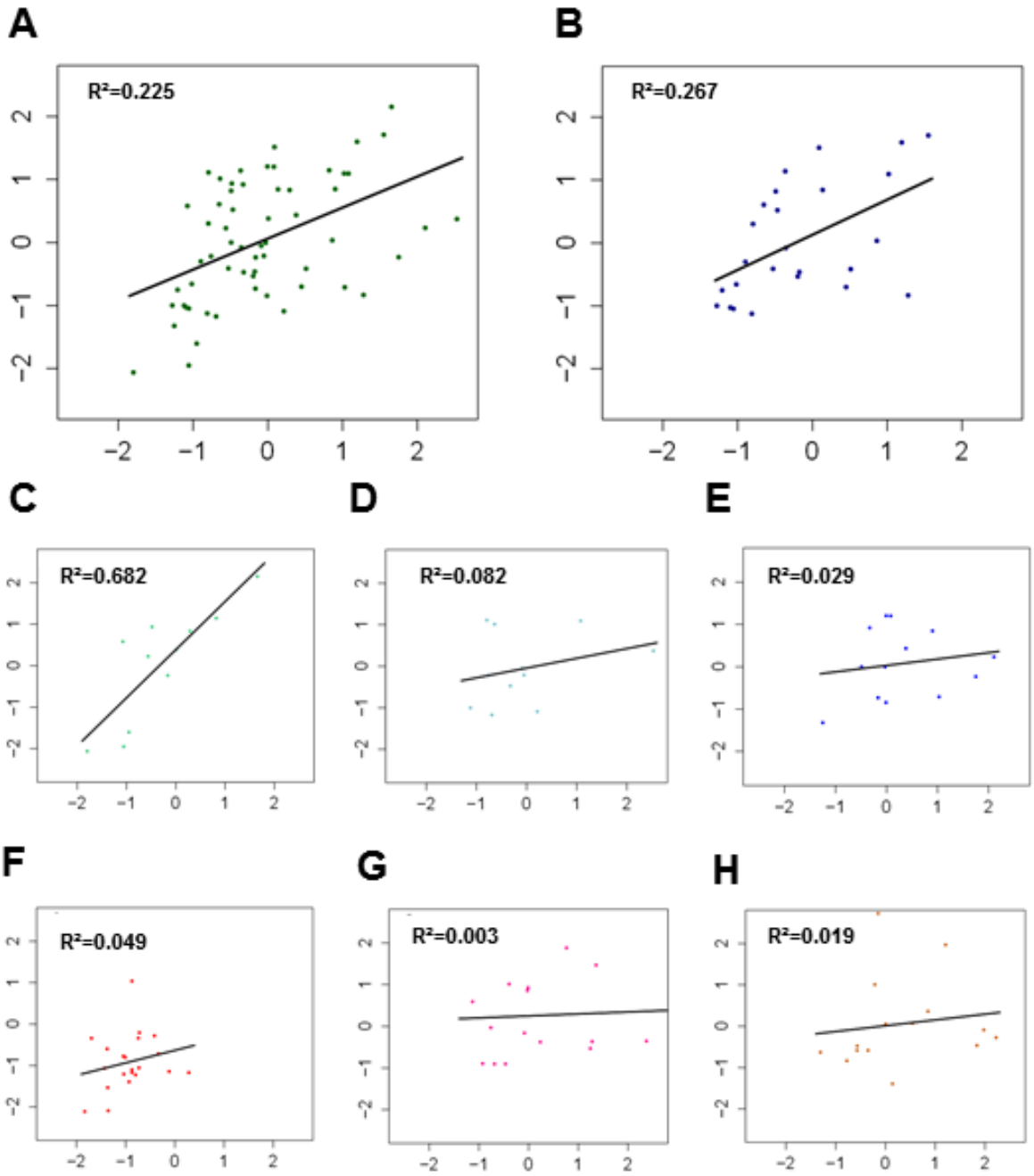



**Figure 11. NAs to PFC as a candidate OT-regulated pathway.** Pairwise comparisons for all experiments (Chapters 2, 3, and 4) of transformed z-scores for Fos expression in the NAs (x-axis) and PFC (y-axis).

A. Pooled male subjects that interacted with a female (Ch. 2, Ch. 3, and Ch. 4)

B. Pooled male aCSF-treated subjects that interacted with a female (Chapter 2 and Chapter 3)

C. T/T ("low OTR") subjects that interacted with a female

D. C/T ("moderate OTR") subjects that interacted with a female

E. C/C ("high OTR") subjects that interacted with a female

F. All unexposed subjects pooled (Ch. 2, Ch. 3, Ch. 4)

G. All subjects ICV-treated with OTA (Ch. 2)

H. All subjects NA-treated with OTA (Ch. 3)