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Genetic Variation of the Oxytocin System and Social Behavior

of Adult Female Rhesus Macaques

By

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Doctor of Philosophy

Graduate Division of Biological and Biomedical Sciences

Neuroscience

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B.S., B.A., Indiana University, 2011

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Abstract
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Affiliative behavior, which underlies the formation and maintenance of social bonds, is a vital component of human mental health. This behavior varies on a spectrum across typical individuals and deficits are observed in those with autism spectrum disorder (ASD), schizophrenia, and adverse early life experiences. Decades of human genetic research have established that most complex traits are highly heritable. Thus, investigating the genetic contributions to individual variation in these socio-behavioral traits and the neural mechanisms that precede them is important for understanding how affiliative bonds are either maintained, supported, or disrupted. The neurohormone oxytocin (OXT) and its receptor OXTR as well as the genes that encode them have established history of shaping social behavior in a variety of mammals. As such, in this dissertation I examine the genetic and epigenetic variation of these oxytocin-related genes and measure their effect on various social traits of adult female rhesus macaques. I quantify the predictive value of 13 genetic variants in *OXTR* as well as the methylation patterns at CPG sites in both *OXT* and *OXTR.* I do not find evidence of robust influence of any of these markers on behavioral outcomes or OXT in cerebrospinal fluid. These findings support the conclusion that has emerged in human genetic studies of complex traits, which is that individual markers have negligibly small effects. To reliably detect effects of small sizes, genetic association studies would need to be carried out in much larger sample sizes. While the rhesus macaque model has a social and neuroanatomical similarity to humans with high translational value, to maximize the validity of genetic association findings from non-human primate (NHP) research, funding agencies and NHP centers should optimize experimental designs for maximum statistical power.

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Chapter 1. Introduction

Primates, including humans, are among the most social animals. For humans, the ability to engage in appropriate social behaviors that form and maintain bonds with others are an essential component of mental health. The factors that give rise to the complex social repertoires that make social bonds possible are as diverse and varied as the social behaviors themselves.

1.1 The importance of studying social behavior

It is inherently interesting to understand the biological basis for a component of the human experience that is so highly developed in comparison to other species. Because the complexity of human social behavior tops that of all other species, by identifying its biological origins, we can gain insights into the evolution of the behavior and important evolutionary questions.

A more pressing need for studying social behavior also arises when we consider psychiatric disorders marked by social deficits. Once we begin to understand the psychological, neural, hormonal, and genetic mechanisms that support the rich human social behaviors and structure, we also identify the neurobiological correlates that generate individual variability in social behavior and its pathology. This is not only useful for understanding the rudiments of natural variation in the normative population, but is also a critical first step in revealing the common pathways that may be altered or impaired in psychiatric conditions in which the ability to engage in appropriate social behavior is disrupted (e.g. autism spectrum disorder, ASD; schizophrenia; social anxiety).

Social behavior is regulated by many biological factors, including a mélange of neurochemical signals and the sensitivity of their receptors in brain tissue. The genes that encode both the neurochemicals and their receptors can also be sources of variation for the diverse behavioral

phenotypes that are ultimately observed. On top of this, early life experience and environmental factors interact with the existing neurochemical and genetic architecture, leaving an experiential "fingerprint" that is encoded through epigenetic mechanisms.

In this chapter, I will provide an overview of what is known about individual variation of adult social behavior with respect to the neurohormone oxytocin (OXT). Then, I will focus on non-human primates (NHPs) as a highly translational model for human social neurobiology. But, in order to provide a historical context for how this work fits into the broader scope of OXT research, I will first summarize the insights from human and rodent literature. After reviewing what is known about OXT and NHPs, I will then briefly cover how these findings have translational relevance in disorders with social behavior deficits, specifically ASDs.

1.2 Environmental and genetic influences on complex social behavior

Social behavior that is observed in adulthood is often influenced by learning and experience. Early life experience, in particular, can have profound effects. This has been well-demonstrated in circumstances of adverse life events (Nemeroff 2016; McCrory et al. 2010). For example, in humans early life stress such as neglect or childhood maltreatment has been well-documented to lead to increased risk of numerous psychiatric disorders later in life such as depression or anxiety (Tollenaar et al. 2017; Heim et al. 2010), anti-social behavior (Widom and Brzustowicz 2006), and schizophrenia (Morgan and Fisher 2007). In monkeys, atypical rearing environments subjected either to social isolation or raised under conditions of maternal deprivation, or the experience of maltreatment by the mother lead to inadequate development of social skills, often displaying excessive aggression and emotional reactivity (Harlow et al. 1965; Suomi 1991; Howell and Sanchez 2011; Sánchez et al. 2001). In rodents, early postnatal stressors in mice

such as maternal separation or handling also leads to changes in social interaction among conspecifics and anxiety-like behavior as adults (Bondar et al. 2018; Sánchez et al. 2001). Neonatal isolation in the prairie vole model also leads to impaired ability to form selective bonds with a partner in adulthood (Barrett et al. 2015).

These environmental perturbations undoubtedly play key roles in shaping social behavior later in life. Nevertheless, decades of human genetics research, from twin and adoption studies and from multiple longitudinal studies of cohorts from the 1920s and 1930s have provided evidence that in typical populations, most complex traits are highly heritable (Polderman et al. 2015; Davies et al. 2011). This includes traits in socio-behavioral domains and underscores a strong genetic influence. A recent large study compiling a sample of more than 14.5 million twin pairs, 20 thousand traits, and more than 3000 individual studies from the past half-century found that on average, complex traits are approximately 50% heritable (Polderman et al. 2015). For example, anxiety in adult females was roughly found to be 31% heritable. In the past decade, genome wide association studies (GWAS) have also ushered in an era of attempts to identify specific genetic factors that explain the substantial heritability that behavioral genetic studies identified. While GWAS have produced heritability estimates for traits that are lower than what twin studies have produced (a discrepancy in these estimates is referred to as “missing heritability”) (Manolio et al. 2009), there is still consensus that genetic factors play key roles in shaping behavior. Indeed, even for traits which are primarily environmentally-determined, such as educational attainment, genetic factors can account for 20% of individual variation (Okbay et al. 2016), reflecting the biological contributions of related phenotypes such as cognition. Because genetic factors will have substantial influence on how diverse phenotypes manifest, it is important then to identify the genes and encoded products that give rise to the behaviors of interest.

1.3 A brief history of oxytocin

Genetics and experience can affect the neurochemical milieu which influences social cognition and sociosexual behaviors. Of the many neurochemical influences, OXT in particular has been intensively studied for its role in various aspects of social affiliation and caregiving, as well as broader social function.

OXT, a neurohormone produced in the hypothalamic nuclei, was initially known for its role in parturition and nursing— the Greek origins of the word itself mean “sudden birth”. The nonapeptide is synthesized in the supraoptic and paraventricular nuclei of the hypothalamus, and axons of the cells in these regions extend down into the posterior pituitary where they release OXT directly into the bloodstream for peripheral circulation (Burbach et al. 2006). The most well-characterized action of OXT in the periphery involves triggering uterine smooth muscle contractions during childbirth and facilitating milk ejection during breastfeeding by triggering the contraction of mammary myoepithelial cells (Jurek and Neumann 2018).

However, axon collaterals extending from these neurons also project to other regions of the brain, allowing OXT to have central effects related to complex social interactions, including parental nurturing and pair bonding. The effects of OXT are instantiated through its only receptor, OXTR, though it can also act as a ligand on the vasopressin receptors, (AVPR1a, AVPR1b, and AVPR2), albeit with lower affinity (more details of its binding to AVPR1a receptors in section 1.7)(Chini and Manning 2007; Freeman, Inoue, et al. 2014).

Though OXT was first characterized for its involvement in the mechanics of childbirth and nursing, its simultaneous release in the brain was later understood to promote maternal

nurturing behavior in mammals and to facilitate bonding between mother and child (Rilling and Young 2014). Indeed, OXT is important for maternal care in rats as well as sheep and reflects the elegantly-orchestrated suite of changes that must occur in the brain to motivate the mother to engage in maternal nurturing behaviors (Ross and Young 2009).

Eventually the understanding of OXT's role was further extended to bonding in the context of monogamous pair bonds. The pair bond is defined as being a strong social relationship between two partners of a socially-monogamous species that persists throughout breeding and non-breeding periods (Walum and Young 2018; Carter et al. 1995). This phenomenon is observed in several mammalian species, including prairie voles (Carter and Getz 1993), titi monkeys (Fernandez-Duque et al. 1997; Fuentes 1998), marmosets (French et al. 2018), and California mice (Jašarević et al. 2013). It is hypothesized that in many mammalian species, OXT serves to make the identity of the pair-bonded partner more salient and rewarding. In humans, OXT has been shown to enhance the response of reward-related regions in the brain when men view the face of their female partner (Scheele et al. 2013) and also stimulates men in monogamous relationships to keep a greater distance between themselves and women who are not their partner during a close-approach paradigm (Scheele et al. 2012). There have also been some reports (albeit, inconsistent ones) in humans that endogenous OXT levels in plasma are related to partner support (Grewen et al. 2005; Taylor et al. 2010). In pair-bonded tamarins, partners show correlated levels of urinary OXT, and this is also related to frequency of sexual behavior or affiliative interaction (Snowdon et al. 2010).

In addition to this, the role of OXT has since been described in broader social contexts, particularly in pro-social behavior and social cognition generally. The experience of gentle, tactile stimulation in rodents, mimicking affiliative behavior such as licking and grooming,

induces immediate early genes in OXT neurons and stimulates OXT release (Barrett et al. 2015). In dogs, the sight of a familiar person increases plasma OXT levels and induces contact-seeking behavior (Rehn et al. 2014), and mutual eye gaze between dogs and their owners initiates a positive feedback loop of increased urinary concentrations of OXT and affiliative behavior in both the dog and owner (Nagasawa et al. 2015). In humans, young girls speaking to their mothers on the phone induces social buffering and increases peripheral measures of OXT after experiencing a psychosocial stressor (Seltzer et al. 2010). OXT has also been shown to improve one's ability to infer emotional states by viewing images of socially-salient facial features, such as the eyes (Domes et al. 2007). OXT is involved in many facets of social interactions, implicating the oxytocinergic system as an important hub for coordinating social roles. As a field, uncovering how OXT shapes and defines these various relationships among mammals is still relatively in its infancy.

1.4 Manipulations of the OXT system

Our ability to understand the mechanisms that underlie these social behaviors and OXT's action come from studies of animal models, which not only allow for strict environmental control but also provide the possibility for experimental manipulation and the harvesting of brain tissue. Most of the literature covering the mechanisms of social behavior have been based primarily on rodent studies.

The classic example of this comes from the comparison of two closely-related species, the prairie and meadow voles, with very different socio-behavioral profiles: the prairie vole is highly-social, monogamous, and exhibits biparental behavior, while the meadow vole is generally solitary, exhibits a promiscuous mating strategy, and care of young is uniparental (Insel and Shapiro 1992).

These social and behavioral differences arise in part from distribution differences of the OXTR receptor, particularly in regions that are critical for social reward (Insel and Shapiro 1992; Young et al. 2001; Olazábal and Young 2006b). A variety of infusion, antagonist, and knockout studies have allowed the mechanistic exploration of OXT and its receptor. Signaling differences in the nucleus accumbens (NAcc) are especially important. Indeed, increased levels of OXTR (induced through viral vectors) in the NAcc accelerate the formation of a pair bond in female prairie voles (Ross et al. 2009). Blocking OXTR signaling in the NAcc, either via antagonists or with viral-vector-mediated RNA interference (RNAi) knockdown of the receptor, prevents pair bond formation among prairie voles (Young et al. 2001; Liu and Wang 2003; Olazábal and Young 2006a). The pair bond formation is contingent on the ability to discriminate between the identity of a familiar partner and a novel conspecific. In rodents, the infusion of OXT into the brain facilitates this social recognition (Dluzen et al. 1998; Popik and van Ree 1998). OXT's role in social cognition has been further supported with the use of knockout mice. For example, mice that are knockouts for the murine *Oxt* gene demonstrate social amnesia (Ferguson et al. 2000), and reinstatement of exogenous OXT rescues this deficit. Additionally, a null mutation in the murine gene for OXTR (*Oxtr*) results in social impairments in mince as well as deficits in maternal care toward their pups (Takayanagi et al. 2005). Taken together, these findings demonstrate the various ways that experimental manipulation in animal models has allowed the field to home in on the contributions of the OXT system in pro-social and bonding behavior.

1.5 Variation in the OXT system

While the pharmacological and genetic manipulations of the oxytocinergic system in rodents make clear that OXT plays a critical role in shaping social behavior, a great deal of intraspecies variation in social behavior still exists. In order to understand how this natural variation in social

behavior within a species emerges, it is prudent to investigate how diversity of the OXT system itself may specifically contribute.

1.5.1 Genetic variation of the OXT system

One way in which this diversity is encoded is via genetic variants in the gene sequences that code for OXT and OXTR (*OXT* and *OXTR,* respectively, in primates). This could include variation encompassing long stretches of nucleotides, such as copy number variants (CNVs) or focal changes such as single nucleotide polymorphisms (SNPs). With respect to the *OXT* and *OXTR* genes, the vast majority of variation identified has been due to SNPs (Tops et al. 2018), though a rare 0.7 Mb genomic deletion which included *OXTR* has also been identified in at least one family with ASD with multiple affected individuals (Gregory et al. 2009).

Genetic association studies suggest that variation in *OXT* and *OXTR* may explain part of the variance in social phenotypes in both healthy and patient groups. In humans, *OXT* variants have been associated with individual variability in attachment-related anxiety and dopaminergic responses to stress (Love et al. 2012), as well in maternal behaviors (Mileva-Seitz et al. 2013). However, considerably more attention has been given to *OXTR* variants compared to *OXT* SNPs (see Feldman et al. 2016 for review). Reported associations between *OXTR* and social behavior in humans include differences in pair-bonding (Walum et al. 2012), social recognition (Skuse et al. 2014), prosocial temperament, and underlying limbic structures in the brain (Tost et al. 2010), susceptibility for displaying conduct problems (Notzon et al. 2016), as well as brain responses to social cues, stressors, or the salience of social rewards (Loth et al. 2014; Feng et al. 2015). Multiple *OXTR* variants have also been reported with associations to social impairments in individuals with (Wu et al. 2005; Wermter et al. 2010) and without ASD (Parker et al. 2014), although *OXTR* genetic associations with ASD are discussed in greater detail in a

later section. The *OXTR* SNPs that have received the most attention for associations with behavior or psychiatric disorders have primarily occurred in non-coding regions (see Tops et al. 2018 for review).

The magnitude and direction of each of the reported effect sizes for these SNPs have been marked by inconsistencies in the literature, and at least two meta-analyses reflect this (Bakermans-Kranenburg and van Ijzendoorn 2014; Li et al. 2015). The reasons vary, but are partly explained by the fact that most genetic association studies are underpowered (Ioannidis et al. 2014), and this has been specifically demonstrated in neuroscience candidate gene studies (Nord et al. 2017; Button et al. 2013). Other reasons include that many studies of *OXTR* SNPs have been carried out as haplotype studies, which test whether particular combinations of SNPs explain a trait. A challenge with synthesizing results of *OXTR* haplotype studies is that haplotype structure has varied across samples and no two studies have included the same loci nor have they yielded the same results (Lerer et al. 2008; Wermter et al. 2010; Wu et al. 2005; Liu et al. 2010). While the reported associations of OXT related genes do not imply a causal effect on behavior, the associations between genotype and particular social phenotypes create a starting point for identifying the most likely of the candidate variants that may contribute to differences in gene expression and subsequent changes in behavior. In fact, brain banking efforts have begun to collect and quantify expression differences in various human brain samples, which will undoubtedly be critical for the evaluation of potentially casual SNPs (Carithers et al. 2015).

The contributions of individual SNPs are small when associated with behavior (Visscher et al. 2017), but brain endophenotypes such as expression levels in brain tissue may have larger effects and be more easily studied and quantified. This has been demonstrated most

prominently in the prairie vole. Within *Oxtr*, a single SNP served as a highly predictive locus for expression in the NAcc specifically (King et al. 2016), explaining more than 70% of *OXTR* variance and supporting the hypothesis that individual genetic markers can be an important source of individual differences in brain signaling in regions important for regulating social behavior.

1.5.2 Epigenetic variation of the OXT system

While genetic variants such as SNPs provide one possible source of encoding variation in gene expression within the OXT system, epigenetic regulatory mechanisms are additional means by which a gene's function and product may be affected. Epigenetic marks are shaped by environmental influences and are one way in which life experience, especially early-life experience, can be embedded biologically (Kundakovic and Champagne 2015). While epigenetic modifications do not alter the DNA sequence, they can influence transcriptional activity (Tammen et al. 2013; Powledge 2011), which can ostensibly lead to downstream variability in behavioral phenotypes. Examples of epigenetic modification include DNA methylation, chromatin modification (including modifications to histones such as methylation, phosphorylation, acetylation, or ubiquitylation), and regulation of mRNA expression by microRNA (Jaenisch and Bird 2003). The most commonly investigated epigenetic mechanisms is DNA methylation. In mammals, this typically occurs when a methyl group is covalently attached to the DNA strand on a cytosine in a cytosine-guanine dinucleotide sequence (i.e. CpG site) (Gardiner-Garden and Frommer 1987). DNA methylation can alter gene expression by influencing how transcription factors are recruited or bind to the DNA sequence (Bonasio et al. 2010). Though not universally true across tissues types, it is generally held that increased methylation of the promotor region of a gene signals the repression of that gene (Razin 1998; Kader et al. 2018). Stretches of DNA with G + C content greater than 50% and many CpG sites

are called CpG islands (Gardiner-Garden and Frommer 1987). They are often located within or near promotor regions of genes (Janitz and Janitz 2011; Saxonov et al. 2006), and thus CpG islands are particularly equipped to regulate gene activity via methylation.

Decreased methylation of the *OXT* gene is associated with increased measures of human sociability (Haas et al. 2016). It has also been suggested that methylation of the *OXTR* gene is related to a range of human psychosocial traits and behavior from callous-unemotional traits related to psychopathy (Dadds et al. 2014), anxiety and depression (Chagnon et al. 2015; Ziegler et al. 2015; Ebner et al. 2018; Reiner et al. 2015), perceptions of fear and anger (Puglia et al. 2015), and neural responses to social stimuli (Jack et al. 2012). There is also some evidence that particular *OXTR* CpG sites may have elevated levels of methylation among individuals with ASD (Gregory et al. 2009). Most of these studies have investigated peripheral methylation levels in blood samples, though a few have used saliva samples (Haas et al. 2016; Chagnon et al. 2015), and one has used brain tissue (Gregory et al. 2009).

A limited number of methylation studies of rodent *Oxtr* have been conducted. These have mostly been carried out in the context of varying maternal care experiences. For example, rats which receive low levels of licking and grooming from dams showed reduced *Oxtr* methylation in peripheral blood samples but no changes in brain tissue (Beery et al. 2016). A more recent study in prairie voles found the opposite result: low levels of parenting resulted in higher *Oxtr* methylation, and this was evident in both peripheral and central tissue (Perkeybile et al. 2019).

In order to understand how both genetic and epigenetic diversity of the OXT system may lead to varying social outcomes, more work must be continued in animal models so that the strongest candidate markers can be further tested with manipulation under controlled environments. In

particular, the use of NHP models provide an ideal opportunity for doing so. However, compared to more commonly used rodent models, we know relatively little about the OXT system in primates.

1.6 Why use NHPs for studying social behavior?

The use of NHPs confers several advantages for studying social behavior in comparison to rodents. While rodent studies have laid a foundation for precise OXT mechanisms that influence behavior, the ceiling for rodent social complexity will never reach that of primates. In particular, the primate brain evolved to deal with environments with rich social complexity, which has shaped a "social brain" with a much higher capacity for social memory and sophisticated social responses (French et al. 2018). Thus, NHP models more closely preserve the rich behavioral, cognitive, and social complexity that is observed in humans, while still allowing for experimental manipulation, invasive tissue sample collection, and environmental control that is either not feasible or unethical to carry out in human subjects (Capitanio 2017). Behaviorally, rhesus monkeys, for example, form reciprocal social alliances within elaborate social hierarchies (Altmann 1962; Bernstein et al. 1974), and use policing behavior to limit the occurrence of social conflicts (Beisner et al. 2016). In social groups cercopithecine monkeys, including rhesus, with a high social rank will use aggressive behavior at random to reinforce existing dominance relationships (Silk 2003). Most NHPs also form strong filial bonds with kin. For example, rhesus macaques maintain matriarchal, matrilineal societies, in which they form lifelong bonds with all female kin (Suomi 2005), and studies of the influence of maternal care of this NHP species have shaped maternal nurturing approaches in our own species (Ruppenthal et al. 1976).

The rich behavioral repertoire that NHPs display can still be captured in controlled lab and semi-naturalistic environments (Graves et al. 2002; Michopoulos et al. 2016; Wilson et al. 2013;

Kovacs-Balint et al. 2018), which highlights an additional benefit of using this model. The use of NHP models provides a potential statistical advantage (in comparison to humans), especially for observational studies. Studies with human subjects will often require much larger samples in order to account for the sample heterogeneity in life experiences such as upbringings, diets, drug/alcohol exposure and culture. In contrast, NHP studies afford the possibility of controlling many aspects of the subjects' lives, leading to reduced noise from non-genetic factors. This means it is possible that smaller samples of NHPs may allow for the detection of stronger associations that would not be detectable in a human study of the same size.

Finally, NHPs peripheral and central nervous systems are highly homologous to humans. In contrast to rodents, the cortical development and organization of NHP brains (e.g. cytoarchitectonics, connectivity patterns, amount of myelination, and number and density of cortical neurons) is more comparable to that of humans (Roth and Dicke 2005; Ventura-Antunes et al. 2013; Phillips et al. 2014; Preuss 1995). For example, the NHP visual cortex and prefrontal cortex can be subdivided into functional subsections similar to those in the human brain (Preuss 1995; Uylings et al. 2003). Well-developed frontal and temporal lobes in NHPs also allow for their participation in complex cognitive tasks (e.g. facial recognition tasks (Parr et al. 2000) and modified versions of tasks which tap into executive function processes such as the Wisconsin Card Sort Test, similar to those used with human subjects (Moore et al. 2005). Additionally, NHPs demonstrate a cognitive awareness that affords them the ability to be sensitive to behavioral states of others (Drayton and Santos 2016). All of these features make NHP models particularly well suited for social-behavioral studies.

1.7 NHPs and OXTR distributions

Mapping OXTR distribution in the brain of NHPs is important to understand the mechanisms underlying OXT action. In rodents, the precise characterization of the distributions allowed for elegant site-specific pharmacological manipulations. Such studies revealed whether OXT in that particular brain region was necessary for behavioral phenomenon such as pair bonding or maternal nurturing. In primates, this objective has been more challenging. I describe why and summarize the interspecies variation among select groups of NHPs below.

In rodents, OXTRs have typically been visualized using receptor binding autoradiography, using a specific OXTR-radioligand (Elands et al. 1988). In primates, however, this approach has been problematic because of the high degree of structural similarity between primate OXTR and AVPR1a receptors and the resulting cross-reactivity of the ligands (Manning et al. 2012). The commercially-available radioligands for OXTR and AVPR1a happen to be highly selective in rodents; but in primate tissue, both radioligands are less selective and unfortunately exhibit a high affinity for both receptors (Manning et al. 2012). Initial efforts to map OXTR in human brain tissue were carried out nearly 30 years ago, before the pharmacological cross-reactivity between OXT and AVP systems was fully recognized (Loup et al. 1991; Loup et al. 1989), so findings from those early studies almost certainly represent both OXTR and AVPR1a binding. Not until very recently has OXTR expression in human tissue been re-characterized in specific regions of interest using more elegant techniques, described further below (Freeman et al. 2017; Freeman et al. 2018).

Attempts to employ immunohistochemistry have also not been fruitful because, at present, no antibodies with sufficient sensitivity and selectivity exist for OXTR (Yoshida et al. 2009). In mice, various OXTR antibodies have been tried, but all yield moderate staining in brain tissue where no OXTR is present (i.e. of OXTR knockout models), rendering them ineffectual (Yoshida et al.

2009). Boccia et al. (2013) implemented a custom OXTR antibody in human tissue, but the specificity of the antibody was never validated in control tissue. Thus, the results from the Boccia et al. (2013) study remain to be confirmed. *In situ* hybridization has also been used to localize OXTR mRNA and confirm its overlap with OXTR radioligand binding (Freeman, Inoue, et al. 2014). Identifying OXTR-synthesizing neurons with *in situ* hybridization alone, however, is insufficient because this technique highlights only the cell body and does not allow visualization of OXTRs that may be present in other brain regions where axons of these cell bodies may project. As such, it is possible that OXTR in primates is expressed more widely in regions other than those highlighted below, but commonly-available techniques are not yet sufficiently fine-grained to visualize them (Putnam et al. 2018).

The solution for mapping OXTR localization in primate brain has been to use competitive binding receptor autoradiography. In this process, selective unlabeled competitors for the AVPR1a receptor are co-incubated with the radioligand for OXTR (Freeman, Inoue, et al. 2014; Freeman et al. 2017; Freeman, Walum, et al. 2014). Using this method, OXTR has been mapped onto regions involved in auditory and visual processing and attention including the superior colliculus, pulvinar, and primary visual cortex (Freeman and Young 2016), which is not surprising given that the most salient social information for primates comes from auditory and visual sensory inputs (Ghazanfar and Santos 2004). This is in contrast to rodents, for whom social information is primarily olfactory (for review see Brennan 2004) and whose OXTR distributions are located primarily in olfactory regions (e.g. olfactory bulb and the accessory olfactory nucleus and their downstream projections, including the medial and central amygdala, the bed nucleus of the stria terminalis, and piriform cortex) and regions that play roles in processing social information (e.g. NAcc, hippocampus, lateral septum, and prefrontal cortex) (Freeman and Young 2016; Insel et al. 1993).

Across common marmosets, rhesus macaques, titi monkeys, and human species, conserved OXTR expression is observed in the nucleus basalis of Meynert (NBM) (Freeman and Young 2016; Freeman, Walum, et al. 2014; Loup et al. 1991; Freeman et al. 2018). This area is responsible for cholinergic input to the entire neocortex and is also a main source of cholinergic inputs to the amygdala (Mesulam et al. 1983). The NBM is involved in mediating visual attention and arousal, and its inputs to the amygdala play a role in memory consolidation (Richardson and DeLong 1988; Mesulam et al. 1983). The region has been hypothesized to modulate an overall behavioral state of “readiness”, during which the ability to process and encode salient stimuli is enhanced (Gratwicke et al. 2013). The pedunculopontine tegmental nucleus (PTT) is another primarily cholinergic region which expresses OXTR in both marmosets and rhesus (Freeman and Young 2016). The PPT’s function is tied to regulation of arousal states and motivation, and it has been recently proposed to be involved in updating behavioral states in response to changing environmental contexts (Mena-Segovia and Bolam 2017). Thus, the NBM and PPT may play important roles in attending to and mediating behavioral responses to salient social information. OXT radioligand binding has also been observed in the superior colliculus of all primate species examined (Freeman and Young 2016; Freeman et al. 2018). Given the importance of even subtle shifts in eye gaze as well as reciprocal gaze between two individuals in social communication (Emery 2000), it is unsurprising that OXTR would be expressed in this region responsible for saccadic eye movements and orienting towards salient stimuli (Sparks 1986; Gandhi and Katnani 2011).

Selective OXTR binding has also been reported in human tissue in the spinal trigeminal nucleus (Freeman et al. 2017), a brain region responsible for relaying sensory input (pain and tactile) from the face (Patel and M Das 2019). OXTR binding in this nucleus is also present in

marmosets (Schorscher-Petcu et al. 2009), titi monkeys (Freeman, Walum, et al. 2014), and rhesus macaques (undiscussed findings from Freeman, Inoue, et al. 2014; explained in Freeman et al. 2017). OXTR's role in the spinal trigeminal nucleus is not well understood, though it may be related to somatosensory functions of OXT, such as analgesic effects and decreasing responses to noxious stimuli (Grinevich and Charlet 2017; Grinevich and Stoop 2018; Tzabazis et al. 2016).

Distribution differences between primate species have also been detected. It is not yet well understood how these expression differences may explain the behavioral differences in these species, though future pharmacological and electrophysiological experiments targeting areas sensitive to OXT may help elucidate this. Some of the differences include dense OXTR expression in the NAcc that is only observed in marmoset, which has a selective, but flexible breeding system (i.e. they are not strictly monogamous) (Schorscher-Petcu et al. 2009; Goldizen 1988). Binding in another region relevant to reward, the ventral pallidum, has been observed in human tissue (Freeman et al. 2018). The non-monogamous rhesus macaque shows no OXTR binding in either of these areas (Freeman, Inoue, et al. 2014). Unexpectedly, the monogamous titi monkey does not exhibit dense binding in the NAcc either (as might be otherwise be expected from comparisons to the monogamous prairie vole which does) (Freeman, Walum, et al. 2014). Instead, the titi monkey expresses dense OXTR in the dentate gyrus of the hippocampus (Freeman, Walum, et al. 2014). Whether OXT may act in this region to modulate social spatial memory is question for future investigations.

In general, binding of OXT tends to be more diffuse and less abundant than AVP binding across primate tissue (Freeman, Walum, et al. 2014; Freeman and Young 2016; Schorscher-Petcu et al. 2009). Some exceptions to this include the dense OXTR binding in the NAcc of the

marmoset, and findings from the most recent human expression studies which did not detect AVPR1a binding in any of their regions of interest (NBM, ventral pallidum, globus pallidus, periaqueductal gray, and superior colliculus) (Freeman et al. 2018). Of the investigated primate species, OXTR expression has been the most restricted in rhesus macaques (Freeman and Young 2016). The strongest OXTR expression in rhesus is in the NBM and the superficial gray layer of the superior colliculus. Rhesus OXTR expression is also observed in the PPT (previously described), the trapezoid body (involved in sound localization and auditory processing), and the ventromedial hypothalamus (involved in sexual behavior and satiety) (Freeman, Inoue, et al. 2014). Expression in these visual, and auditory regions still supports OXTR's role in regulating attention to social cues in this species. It is also important to note that receptor autoradiography is not sensitive enough to detect OXTRs that may be expressed in small quantities on axon terminals, for example, in brain regions that may still be sensitive to OXT. As a result, these studies should not be considered comprehensive mappings of all regions where OXT may be acting (Putnam et al. 2018). As more studies begin to use NHP models to test the clinical applications of OXT-based therapies for neuropsychiatric disorders continued OXTR localization studies become particularly useful (Chang and Platt 2014; Modi et al. 2014; Dal Monte et al. 2014; Freeman et al. 2016).

1.8 Rhesus macaques and OXT

The rhesus macaque is the most widely used model in biomedical research and by nature of rhesus being Old World monkeys, they share many biobehavioral characteristics with humans, more so than other primate groups (Thierry et al. 2004). This makes rhesus particularly well-suited and translationally-valuable for electrophysiological and pharmacological investigations into the complex cognitive and biological processes that govern social behavior. For these reasons, the discussion henceforth focuses on the OXT system in this particular species.

1.8.1 Experience and OXT

The rhesus model offers a unique opportunity to investigate the relationship between experience and OXT. Some of the earliest work related to the OXT system, social behavior, and the rhesus monkey was done in the mid-1980s, showing that concentrations of OXT decrease in plasma in the hour following exposure to acute behavioral stressors (Kalin et al. 1985). Years later, OXT in CSF was shown to be significantly reduced in rhesus macaques with early adverse rearing (i.e. nursery-rearing) and correlated with decreased social behavior through adulthood (Winslow et al. 2003). Interestingly, parallel findings of decreased OXT have since been observed in studies of cerebrospinal fluid of adults exposed to maltreatment or emotional abuse early in life (Heim et al. 2009) and studies of peripheral OXT from children in orphanages (Wismer Fries et al. 2005), suggesting that OXT may contribute to the mechanisms underlying failure to form filial attachments in these populations (Wismer Fries et al. 2005; Rutter et al. 1999). More current work has shown that peer-reared rhesus also show epigenetic alterations in *OXTR* and less *OXTR* mRNA in hippocampal tissue (Baker et al. 2017). Additionally, an increased number of reciprocal play relationships in female juvenile rhesus (but not males) positively predicts plasma OXT at time points later in development (Weinstein et al. 2014).

1.8.2 Exogenous OXT and rhesus

In recent years, most studies of rhesus macaques and OXT that are relevant to social behavior have been related to intranasal or aerosolized administration of OXT, and its effects on social cognition. The use in intranasal OXT (IN-OXT) in humans as a potential therapeutic for social deficit disorders has drawn both enthusiasm and skepticism (Evans et al. 2014; Leng and Ludwig 2016), as discussed further below. Thus, its application in NHPs has been an important

attempt to elucidate IN-OXT's potential efficacy in matters such as how it reaches the central nervous system, as well as in a variety of social cognition tasks.

Studies in rhesus monkeys in which OXT has been manipulated in some way have generally shown OXT to have a prosocial or anxiolytic effect. Administration of aerosolized OXT decreases attention to images with negative facial expressions in dot probe tasks (Parr et al. 2013), and also blunts vigilance towards social threats in a task related to social attention (Ebitz et al. 2013). While it may seem contradictory that OXT would decrease attention towards social cues, it is possible that in the case of social stimuli that are perceived as threatening, reduced attentiveness may serve as a pro-social lubricant, ultimately promoting social exploration, and reducing social stress. This logic is further supported by a recent study in a small sample of male rhesus, which found that administration of OXT and the closely-related neuropeptide vasopressin (AVP) resulted "relaxed" social interactions, which the authors operationalize as reduced starting between pairs of rhesus of varying social hierarchy positions (Jiang and Platt 2018). These pharmacological manipulations were initially carried out through a nebulizer mask and then were recapitulated with focal injections into the anterior cingulate gyrus. Though the effects of AVP in this study were more robust than those of OXT, this may be reflective of the numerous receptors for AVP located in the rhesus brain (to which OXT can bind), as discussed previously (Freeman and Young 2016).

Other studies have used IN-OXT to modulate attention to the face as well as gaze-following (i.e. when one perceives a social partner's gaze, then also directs their own attention to the event or object being attended to). For example, under normal conditions, the repeated presentation of a video of a monkey engaging in natural behavior would typically result in habituation and reduced gaze-following by the viewer. However, when Putnam et al. (2016) administered IN-OXT the

monkey viewing the videos in a sample of four adult male rhesus, habitation is blunted and the frequency of gaze-following saccades is not as reduced. A parallel finding has been reported in a group of infant macaques (N=24), in which exogenous OXT increases gaze-following behavior in females (but not males) (Simpson et al. 2017).

IN-OXT in rhesus has also been used to investigate social decision making. In a study that used a social-choice paradigm, the subject could choose whether or not to deliver a juice reward to another rhesus (a pro-social choice), itself (a selfish choice), no one, or a combination of these choices (Chang et al. 2012). Notably, when the subject received IN-OXT, the frequency of pro-social choices increased, and this was also accompanied by an increase in the subject gaze-shifting to the recipient's face after delivery of the reward. This study involved two decision-making monkeys, and one recipient monkey (Chang et al. 2012). Notably, when this paradigm was repeated with a direct unilateral injection of OXT into the basolateral amygdala, but not the dorsolateral prefrontal cortex, it also led to a preference for making pro-social decisions (Chang et al. 2015). For both brain regions, focal injections were completed in a sample of N=2.

The effects observed in the amygdala suggest that this region may be an important site for OXT modulation of social behavior. Indeed, in additional to its well-known role in emotional and stress responses, the amygdala also plays an important role in social cognition in humans and NHPs (Kirsch et al. 2005) and in recognition of salient social features such as facial expressions and eye contact (Gothard et al. 2007; Mosher et al. 2014). In rhesus macaques, when intranasal OXT is administered, fMRI responses to fearful and aggressive faces are reduced, and this is coupled with reduced functional connectivity between the amygdala and the occipital and inferior temporal cortex (Liu et al. 2015). Furthermore, in the aforementioned study of pro-social decision-making, firing rates from a subset of neurons in the basolateral amygdala were

associated with the value of a juice reward when given to either oneself or to the other monkey, and additionally neuronal activity was associated with pro-social decisions (Chang et al. 2015). It is possible that these effects may be mediated by OXT action on OXTR receptors located on axon terminals in the amygdala that cannot be visualized with current methods using receptor autoradiography or in situ hybridization (Freeman and Young 2016).

Finally, rhesus have also been used to explore the question of whether or not IN-OXT reaches the brain, as measured by increased CSF concentrations. In a recent study by Lee et al. (2018), doses of 80 IU of d5-deuterated OXT were administered both intranasally and intravenously. Both routes resulted in increased OXT CSF levels 60 minutes later. Intravenous administration was just as effective as intranasal administration. However, this dosage of OXT is much higher than the doses typically used in other rhesus studies (typically 20-50 IU) (Bauman et al. 2018). In contrast, an earlier study which administered smaller doses of OXT through either intranasal (24 IU), aerosolized (24 IU) or intravenous (48 IU) routes, measured plasma and CSF concentrations of OXT at baseline and post- 5, 15, 60, and 120 minute timepoints (Modi et al. 2014). This study found that only aerosolized OXT elevated OXT CSF levels. All of these studies have been completed in relatively small samples, and additional work needs to be done in larger samples of rhesus to clarify which routes provide the most efficacious entry of exogenous OXT into the central nervous system. One additional caveat is that assays for detecting OXT have been notoriously unreliable and inconsistent (Leng and Sabatier 2016), pointing to the need for a more robust assay to assess the effects of these exogenous treatments.

1.8.3. Rhesus OXTR behavioral genetics

Despite the invaluable translational worth of the rhesus model, very few studies have directly investigated genetic variation in *OXTR* with respect to behavior in this species. Madlon-Kay et al. (2018) tested 3 *OXTR* variants, in addition to variants in the vasopressin family of receptors, but found negligible effects of all SNPs on social behavior. As a secondary outcome, Baker et al. (2017) reported that a nonsynonymous Ala6Ser variant of *OXTR* ameliorated the social-separation response in rhesus. The authors show that peer-reared animals are likely to demonstrate epigenetic downregulation of *OXTR* as a result of being peer-reared, and Ala6Ser variant may possibly confer resilience for peer-reared animals (Baker et al. 2017). Aside from these, only two other studies have reported *OXTR* variation in any NHP model. Staes et al. (2015) investigated an intronic variant but found no effects on individual variation in personality in a small group (N=62) of captive chimpanzees. Continued genetic work in large samples of rhesus is necessary to take full advantage of the research benefits the rhesus model can provide. A thorough understanding of the genetic diversity in the OXT system in rhesus provides the opportunity to build upon what has been learned from rodent studies and contribute to the understanding of what mechanisms govern social behavior in primates, including humans.

1.9 Translational relevance

The work described thus far has many clinical implications for disorders that are marked by social dysfunction, including several neurodevelopmental disorders, of which ASD has received the most attention with respect to OXT. First characterized in 1943 by Leo Kanner, the seminal report of what is now known today as ASD, characterized a group of children with traits including perseverative behaviors, language and communication challenges, and--most relevant to the present discussion—deficits in reciprocal social interaction (Kanner 1943; Lai et al. 2014). The prevalence of ASD is estimated to be as high as 3% for children in the United States

(Christensen et al. 2019), and the parallel impairments in affiliative bonding can be debilitating and lead to poor quality of life and social outcomes (Hofvander et al. 2009; Maïano et al. 2016).

Though ASD is a heterogeneous disorder, both in the source and manifestation of its phenotypic traits, estimates from twin studies have placed the heritability of ASD at more than 80% (Geschwind 2011; Lai et al. 2014), reflecting a strong genetic role in its etiology. However, the concordance of ASD in identical twins is seldom 100%, demonstrating that environmental factors also play a role, likely occurring early-on in development. However, aside from fetal exposure to valproate (Christensen et al. 2013), environmental risk factors have not been clearly elucidated.

Rare genetic mutations are estimated to be causal in up to third of individuals with ASD, and a few of these rare variants have been identified to have large effects sizes (Vorstman et al. 2017). Importantly, however, even for well-known variants, the complex genotype-phenotype architecture of ASD make it challenging to study, for example, the penetrance and expressivity vary widely for carriers of risk alleles (Vorstman et al. 2017).

Common variants (with a minor allele frequency greater than 5% in the general population), each with much smaller effects (on the order of a relative risk of approximately 1.1) are likely to contribute to most cases of ASD (Vorstman et al. 2017; Ramaswami and Geschwind 2018), such that the cumulative contribution of common variants across a number of genes is estimated to be between 15-50% (Gaugler et al. 2014; Klei et al. 2012). Though none of these common variants are causal on their own, the additive effect of a combination of them is thought to contribute to the continuum of variation of social functioning within the general population, as

well as the increased susceptibility for autism in the offspring of parents with sub-threshold autistic traits (Geschwind 2011; Lyall et al. 2014; Hovey et al. 2014).

One challenge of studying these small genetic contributions is that large cohorts of individuals with ASD need to be recruited to conclusively identify single variants at genome wide significance (Vorstman et al. 2017). Only one study thus far has identified common risk variants significantly associated with ASD, after having tripled the previous largest discovery sample, in conjunction with the use of a recently-introduced analytic technique, multi-trait analysis of genome-wide association (MTAG) (Turley et al. 2018) to increase statistical power for GWAS (Grove et al. 2019). As a result, to date it has mostly been the identification of rare variants with whole-genome sequencing and whole-exome sequencing methods that have been responsible for the discovery of ASD risk genes (Ruzzo et al. 2019). When considering ASD risk factors at the level of the gene (and not of individual variants), efforts such as the curation of the Simons Foundation Autism Research Initiative (SFARI) gene database are making considerable headway in identifying the most important genetic influences by comprehensively compiling and categorizing all ASD risk genes reported in the literature. Presently, 958 genes have been scored for how strongly the evidence links the particular gene to ASD (SFARI 2019). Scores range from category 1 “high confidence”, which meet genome wide statistical significance and have withstood an independent replication, to category 6 “Evidence does not support a role” (Abrahams et al. 2013). Less than 3% of currently assessed genes meet category 1 criteria (SFARI 2019), but this may change as investigations continue to accumulate stronger evidence with larger sample sizes, deep resequencing efforts, and the decreasing cost of genotyping. The most cited of the “high confidence” genes (reported 40 times or more) include *SHANK3*, *SCN2A*, *PTEN*, *RELN*, *SYNGAP1, GRIN2B,* and *CHD8*. These are involved in a range of neural processes, including those broadly related to synaptic formation and function, neuronal

excitability, or early developmental and structural processes in brain growth (SFARI 2019). The largest category of SFARI genes is category 4, “Minimal evidence”. Nearly 50% of reported ASD genes fall into this category and contain genes with unreplicated associations or those with no additional independent evidence for ASD association. More than 1000 genes are likely contribute to ASD risk (Lai et al. 2014), and pointing the specific gene and their variants to variations in neural circuitry and downstream cognitive or behavioral changes using animal models is critical for a mechanistic understanding of ASD emergence.

1.9.1 ASD and OXT genetics

Though neither *OXTR* nor *OXT* have been consistently ranked among the main risk genes for ASD in recent GWAS scans (their SFARI risk categories are 3.1 and 4.1, respectively) (Autism Spectrum Disorders Working Group of The Psychiatric Genomics Consortium 2017; Grove et al. 2019; SFARI 2019), many studies have reported *OXTR* variants to be associated with a variety of brain activity signatures and behavioral phenotypes relevant to ASD. However, the implication of the findings should be appropriately tempered by the limited sample sizes in each study.

OXTR gene polymorphisms could putatively contribute to traits affecting the social motivation theory of ASD, in which the rewarding aspects of social stimuli are not properly represented in affected individuals, leading to reduced motivation to interact socially, and subsequently fewer opportunities to engage in social learning. In support of this idea, at least two studies have reported altered circuitry in individuals with ASD that is tied to specific *OXTR* variants (Hernandez et al. 2017; Uzefovsky et al. 2019). For example, the study by Hernandez et al. (2017) found that functional connectivity between the NAcc and other reward circuit regions

such as the anterior cingulate gyrus was decreased in ASD youth (N=41) and exacerbated in individuals that carried additional *OXTR* risk alleles.

Additional studies have identified aberrant responses to reward- or socially-related stimuli in healthy adults who carry risk alleles (Damiano et al. 2014; Tost et al. 2010; Scott-Van Zeeland et al. 2010) (N=31, N=212, respectively), while others find associations with *OXTR* variants and ASD status in general (Campbell et al. 2011; Yrigollen et al. 2008; de Oliveira Pereira Ribeiro et al. 2018) (N=527, N=2333, N=209, respectively). However, the authors of these studies acknowledge that the associations they report do not survive correction for multiple comparisons. To date, two meta-analyses have been conducted with respect to OXTR-related polymorphisms and ASD phenotypes. The larger of the two (N=1700) (Bakermans-Kranenburg and van Ijzendoorn 2014) focused on two commonly studied *OXTR* SNPs but did not find any significant effects of these SNPs, while the second meta-analyses did (LoParo and Waldman 2015). The inconsistency of the reported associations are likely a result of smaller, early studies being underpowered and overestimating the true effect size (Kraft 2008).

1.9.2 Intranasal oxytocin

As previously mentioned, the use of IN-OXT as a potential therapeutic for ASD patients to alleviate impairments in social function has garnered great interest. Individual studies in humans have shown that a single dose of intranasal OXT increases the time spent looking at the eye-region (Guastella, Mitchell and Dadds 2008), social memory (Guastella, Mitchell and Mathews 2008), and theory of mind (Domes et al. 2007). In small samples, IN-OXT has been shown to increase socially-cooperative behavior among individuals with ASD (Andari et al. 2010).

While these results appear to be promising individually, mechanistic explanations for how IN-OXT may be having central effects are lacking, as many reports using IN-OT to probe central effects administer concentrations of OXT that also result in supraphysiological levels peripherally (Leng and Ludwig 2016). Additionally, both meta-analyses and results of randomized control trials investigating the effects of IN-OXT on social outcomes have led to mixed results (Walum et al. 2016; Keech et al. 2018; Ooi et al. 2017). Ultimately, the effectiveness of IN-OXT as a therapeutic for individuals with ASD or other social deficits should be considered speculative for the time being. A better understanding of mechanism, dosage, and the importance of social context is needed to elucidate the efficacy in the IN-OXT approach as a therapy or whether other factors need to be considered to explain what makes particular individuals more or less responsive to the proposed OXT-based therapeutics.

1.10 Overall goals and hypotheses

Based on the literature reviewed above, the overall **goal** of this dissertation is to gain insight into how the OXT system may contribute to natural variation in social behavior using an NHP model, the rhesus macaque. The body of OXT-related work in the rhesus model is budding, and an understanding of what contributes to individual variability in the species remains to be mapped. Using a rich data set from a large number of socially-housed adult rhesus that includes OXT in CSF, biological markers from blood, and extensive data on behavioral phenotypes and covariates, I carried out an exploratory analysis of genetic variants (SNPs) and epigenetic predictors of social behavior in the oxytocinergic system. While candidate genes studies have been notoriously challenging in humans due to small effect sizes and heterogeneous living environments, because of the controlled environment that NHPs can be studied in, the case could be made that larger effect sizes could be observed in candidate gene studies in moderately large groups of NHPs with rich behavioral data.

By quantifying the extent to which these gene variants, epigenetic markers, and levels of neuropeptide in the CSF contribute to variation in social behaviors, we begin to establish an inroad to learn more about the regulatory biology of complex social repertoires in primates. In ***chapter 2***, I estimate the heritability of prosocial, anxiety-like, and aggressive behaviors in a fairly large group of adult female rhesus macaques. I also examined whether 13 genetic variants in the promoter region of *OXTR* significantly predict variation in prosocial behavior of adult female rhesus. Because of OXT's implication in other behavioral domains, I also test whether there is a significant association to measures of anxiety and aggression. As a secondary outcome, I examined whether genotype affects concentrations of OXT in CSF in a subset of animals. Lastly, I test whether OXT CSF concentrations themselves predict any of the observed behaviors. In ***chapter 3***, I extend my exploration into variation of epigenetic marks of *OXT* and *OXTR* in the same sample of animals to quantify their association with prosocial, anxiety-like, and aggressive behaviors as well as OXT concentrations in CSF. Finally, in ***chapter 4***, I discuss the overall conclusions and interpretation of these studies and address some statistical limitations in the current approaches and practices that will need to be addressed in future studies to best move the research in OXT NHP models forward.

Chapter 2. Heritability and relationship of oxytocin receptor gene variants with social behavior and central oxytocin in colony-reared adult female rhesus macaques

Acknowledgment of Reproduction

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2.1 Abstract

The genetic contributions to sociality are an important research focus for understanding individual variation in social function and risk of social deficits in neurodevelopmental disorders (e.g. autism). The neuropeptide oxytocin (OXT) and its receptor, OXTR, influence social behavior across species. In humans and animals, common variants within the OXTR gene (*OXTR*) have been associated with varying socio-behavioral traits. However, the reported magnitude of influence of individual variants on complex behavior has been inconsistent. Compared to human studies, non-human primate (NHP) studies in controlled environments have the potential to result in robust effects detectable in relatively small samples. Here we estimate heritability of social behavior and central OXT concentrations in 214 socially-housed adult female rhesus macaques, a species sharing high similarity with humans in genetics, physiology, brain and social complexity. We present a bioinformatically-informed approach for identifying single nucleotide polymorphisms (SNPs) with likely biological relevance. We tested 13 common SNPs in regulatory and coding regions of *OXTR* for associations with behavior (pro-social, anxiety-like, and aggressive) and OXT concentration in cerebrospinal fluid (CSF). We found moderate rates of heritability for both social behavior and CSF OXT concentrations. No tested SNPs showed significant associations with behaviors or CSF in this sample. Associations between OXT CSF and social behavior were not significant either, though OXT CSF had a high

posterior probability of having a negative relationship to anxiety-like behavior ($p_{<0}$ = 0.97). SNP effect sizes were generally comparable to those reported in human studies of complex traits. While environmental control and a socio-biological similarity with humans is an advantage of rhesus models for detecting smaller genetic effects, it is insufficient to obviate large sample sizes necessary for appropriate statistical power.

2.2 Introduction

Oxytocin is an evolutionarily conserved neuropeptide involved in orchestrating reproductive, maternal, and social behaviors across species (Feldman et al. 2016). Individual variability in these traits is attributable to genetic variation, apart from the role of experience. As such, variants such as single nucleotide polymorphisms (SNPs) within the OXT receptor gene (*OXTR*) may contribute to the diversity of complex social repertoires. Genetic association studies suggest that variation in *OXTR* may explain part of the variance in social phenotypes including pair-bonding behavior, social recognition, prosocial temperament, and sensitivity to social stressors (Feldman et al. 2016; Walum et al. 2012; Skuse et al. 2014). Multiple *OXTR* SNPs have also been linked to social impairments in individuals with autism (LoParo and Waldman 2015).

Despite reports linking human *OXTR* variants to social phenotypes, other studies, including a meta-analysis (Bakermans-Kranenburg and van Ijzendoorn 2014), have failed to find effects of consistent magnitude and direction of these variants on social domains (see Tops et al. 2018 for review). Inconsistent results may be related to biases which disproportionately suppress non-significant results from publication (e.g. publication bias, selective reporting), and inflated effect sizes due to low statistical power (Ioannidis et al. 2014).

The use of animal models, however, has allowed the underlying mechanisms for SNP-behavior associations to be probed in the brain, including assessing the functional effects of differential *OXTR* gene expression caused by genetic variants in brain tissue. For example, in the prairie vole rodent model, a single *Oxtr* SNP predicted more than 70% of the variability in expression in the nucleus accumbens, a region critical for social reward (King et al. 2016). This suggests that individual variants have the potential to profoundly impact brain phenotypes which can mediate downstream behavior differences.

Non-human primates (NHPs), such as the rhesus macaque, have been widely used to study social behavior. Both rhesus and humans display rich behavioral repertoires, complex social hierarchies, and are very similar in their physiology and neuroanatomy. Additionally, rhesus have frequently been used to investigate the role of OXT on sociality (Putnam et al. 2018), and as such, an understanding of the genetic contributions to the oxytocinergic system in this NHP model is valuable for translation to humans.

While human genetic association studies necessitate extremely large samples to detect small effects reliably among the multitude of factors influencing complex traits, it is possible that NHP studies conducted in experimentally controlled, semi-naturalistic settings with known pedigrees can detect larger effects due to the reduction in environmental noise (e.g. standard diets and perinatal environments). The goal of the present study was to use an informed approach to examine the associations of novel rhesus *OXTR* (*rhOXTR*) SNPs with social behavior (pro-social, anxiety-like, and aggressive) and OXT in cerebrospinal fluid (CSF) in 214 adult female rhesus macaques housed in large social groups. We also report estimates of heritability and examine the effects of OXT CSF on each behavior.

2.3 Materials and Methods

2.3.1 Subjects and Housing

Subjects were 214 adult female rhesus monkeys (4-24 years in age; median: 7, interquartile range: 6-11) of known pedigrees living in complex social groups at the Yerkes National Primate Research Center (YNPRC) Field Station (Lawrenceville, GA) as described in Wilson et al. (2013). Subjects came from five social groups consisting of 28-94 adult females, their kin (sub-adult, juvenile, and infant offspring), and 2-10 adult males (see kin relationships in Table S2). Animals were selected from matrilines across all social status ranks (high, medium and low) and with varying degrees of relatedness for heritability analyses. All procedures complied with the Animal Welfare Act and U.S. DHHS “Guide for the Care and Use of Laboratory Animals” and were approved by the Emory IACUC.

2.3.2 Behavioral Data Collection

Focal observations were collected in real-time following published protocols (Wilson et al. 2013; McCormack et al. 2009) based on established ethograms (Altmann, 1962). Four 20 minute scoring sessions (mean: 80.06 min/animal, SD: 2.94) were collected across four weeks between 7:00-11:00AM (when animals are most active) during the mating season to reduce seasonal variability in behavior. We focused on representative behaviors: pro-social (percent of time spent in proximity to other adult females), anxiety-like (frequency of self-scratches) (Schino et al. 1991), and aggressive (non-contact, subject-initiated) behaviors (Table 2.1).

2.3.3 CSF and Blood Samples

Animals were habituated to experimental procedures to facilitate blood and CSF collection using methods minimizing arousal (Wilson et al. 2013). Each subject was accessed once, shortly after sunrise and during mating season, but never on the same day as the behavioral data collection.

Animals were anesthetized with Telazol™ (3 mg/kg, IM). CSF samples were collected in a subset of the subjects (n=166) to examine central OXT concentrations. CSF samples were collected (2 ml/subject) from the *cisterna magna* passively by gravity through a 22 G needle and placed immediately on dry ice. Latency to CSF sample collection from time of disturbance was recorded.Two 3 ml blood samples were collected in EDTA tubes for DNA extraction and immediately placed on ice. Samples were stored at -80ºC until time of processing. CSF samples were processed by the YNPRC Biomarkers Core Laboratory using commercially available ELISA kits produced by Assay Designs (Ann Arbor, MI), following manufacturer's recommendations. Sensitivity of the OXT assay was 15.6 pg/ml and the inter- and intra- assay CVs were 7.48% and 10.2%, respectively.

2.3.4 Covariates

All models included covariates for age and social rank (because rank can causally influence pro-social behavioral rates (Snyder-Mackler et al. 2016)). OXT concentrations were assayed in three batches; observations were mean-centered per batch to control for inter-batch variation. Latency to CSF collection did not have a significant effect on OXT CSF concentrations in this sample.

2.3.5 SNP Selection

SNP discovery efforts were completed in an independent group of rhesus and focused on 5' regulatory and coding regions of *rhOXTR,* approximately spanning the promotor region and first three exons. Within this region, candidate SNPs were identified in positions analogous to those in humans that have been cited as allele specific effects on *OXTR* expression (Chen et al. 2016) and suggested to predict individual differences in behavior and disease vulnerability (Wu et al. 2005; Lucht et al. 2009; Lucht et al. 2013; Kuessel et al. 2013; Loth et al. 2014). While SNPs are not conserved across species, comparably located SNPs in macaques may

confer functionally similar effects and therefore provide translational potential. Candidate SNPs were narrowed down based on their presence with sufficient minor allele frequency in multiple genetically-distinct populations of rhesus. This resulted in 13 loci to be genotyped for this study's sample (Fig 1), including one non-synonymous SNP (SNP 8), conferring an Ala6Ser amino acid change in rhesus, and which has been reported to rescue behavioral deficits in peer-reared animals (Baker et al. 2017).

2.3.6 Genotyping

Three different genotyping techniques were used. Archived next-generation sequencing data targeting *OXTR* exons was available for loci 4-13. Libraries were generated using the Illumina NexteraXT DNA kit and sequenced on an Illumina HiSeq1000. The remaining markers were genotyped using TaqMan or Sanger Sequencing. Cycle sequencing was performed using the Big Dye Terminator, version 3.1, reaction in 96-well optical plates (Applied Biosystems, Foster City, California). Variants were detected by visualization of electropherograms generated by ABI Sequencing Analysis software. Relevant assay and primer information are in Table 2.2.

2.3.7 Statistical Analysis

All statistical analyses were performed in R version 3.4.1 (R Project for Statistical Computing, Vienna, Austria). Each SNP's association with behavioral and CSF measures was tested independently. Mixed effect models known as "animal models" within the "MCMCglmm" package were used for all analyses (Hadfield 2010). This Bayesian framework allows for the inclusion of breeding values from the pedigree as a random effect to (1) estimate trait heritability and (2) to account for relatedness when testing SNP associations with behavior and CSF (Wilson et al. 2010). Narrow-sense heritability was calculated as the proportion of variance explained by additive genetic factors (via the pedigree) out of all phenotypic variance. SNPs and

covariates were included as fixed effects. To account for differences in total time observed, observation length was included as an offset for models including frequency behaviors (i.e. raw counts). The prior distribution for additive genetic variance (σ^2_A) was defined by commonly used non-informative parameters for the inverse-gamma distribution, IG(0.001, 0.001). Models were run with a minimum of 5 million iterations, a burn-in of 5,000, and thinning interval of 1,000 until adequate mixing was ensured.

A Gaussian distribution was specified for pro-social behavior and log-transformed OXT CSF measures. For anxiety-like behavior, a Poisson specification was used. Heritability was not estimable for aggression, even after trying multiple distributions and stronger priors on σ^2_A. This was most likely due to a combination of low occurrence of this behavior (i.e. low information) and a small sample size (Chi et al. 2014). Because controlling for relatedness was essential for all models, this outcome was excluded from subsequent analyses.

In post-hoc analyses, a likelihood-ratio test was used to compare whether inclusion of all SNPs (versus none) significantly improved model fit. To do so, frequentist versions of each model were run using R packages “pedigreemm” and “regress” to extract maximum log-likelihood values.

Finally, the effects of OXT CSF on behavior were also assessed. For these models, a probabilistic inference was also made by reporting the posterior probability of observing a positive ($p_{>0}$) or negative ($p_{<0}$) effect between OXT CSF and the behavior. This was quantified by reporting the proportion of the posterior distribution on the same side of zero as the mean effect.

2.4 Results

2.4.1 Heritability Estimates

We report moderate heritability for pro-social behavior ($h^2 = 0.312$), anxiety-like behavior ($h^2 = 0.283$), and OXT CSF ($h^2 = 0.183$), though all 95% credible intervals were wide (Table 2.3).

2.4.2. SNP Associations

All 13 markers investigated (Figure 2.1) conformed to Hardy-Weinberg equilibrium, except two (SNPs 1 and 9, Table 2.4). No significant associations were detected between individual markers and behavioral outcomes or OXT CSF in our sample, as all uncorrected 95% credible intervals overlapped zero (Table 2.3). When all markers were included in the model, no significant improvement in model fit was observed, indicating that the cumulative effect of all SNPs still results in negligible effects on the investigated traits.

2.4.3 Associations between OXT CSF and Behavior

Finally, the relationship between OXT CSF and pro-social and anxiety-like behavior yielded no significant effects (Table 2.3), as determined by 95% CI including zero. However, the effect of OXT CSF on anxiety-like behavior had a high probability of being in the negative direction ($p_{<0} = 0.97$). The posterior probability of observing a negative relationship between OXT CSF and pro-social behavior was also relatively high ($p_{<0} = 0.80$).

2.5 Discussion

Rhesus are a species with great translational value due to their behavioral and biological parallels to humans. Animals in this particular study also experienced complex social housing, and experimentally controlled environments (similar living, housing, and dietary conditions),

attenuating the variability introduced by environmental factors that impact human studies. These benefits might suggest that rhesus behavioral genetic studies, which aim to detect subtle genetic signals among various sources of environmental noise, are exempt from the large samples sizes necessary in analogous studies conducted in humans. However, if there is a true underlying effect of *OXTR* variants on the outcomes measured here, the use of the rhesus model did not produce effects large enough to be detected in this sample. Specifically, we report no significant associations between the 13 *rhOXTR* SNPs on pro-social or anxiety-like behavior, or on OXT concentrations in CSF. This included one non-synonymous variant (SNP8), which had previously been reported to rescue behavioral deficits in rhesus that were peer-reared (Baker et al. 2017). Further, OXT CSF did not significantly predict any behavioral outcomes. Interestingly, the posterior estimates indicated that a negative relationship between OXT CSF and anxiety-like behavior was most probable. This is consistent with the well-documented history of endogenous and exogenous OXT acting as an anxiolytic in both animals (Yoshida et al. 2009; Janezic et al. 2016) and humans (Neumann and Slattery 2016).

Although we did not find significant effects of individual markers, our heritability analysis showed moderate additive genetic effects on all investigated traits, underscoring the existence of genetic influence on these outcomes. This is unsurprising given extensive research in humans demonstrating that most complex traits are on average 50% heritable (Polderman et al. 2015).

Regarding SNP associations, explanations for our null results include: (1) Despite our informed strategy for selecting markers, it is possible we identified *rhOXTR* variants that do not affect expression (nor subsequent downstream normative behavior). This is a limitation of candidate-gene approach, which narrows the breadth of testable markers. Whole-genome association methods would be needed to comprehensively identify any and all robust markers (while

acknowledging the non-trivial challenges to statistical power this introduces). Alternatively (2), the contributions of individual SNPs across the genome, including these 13, may have small, incremental effects on polygenic, complex traits that studies like this one are too underpowered to detect. Decades of research in humans indicates the latter is more plausible and that the effect of an individual locus on a complex trait is likely negligible (Flint and Munafò 2013). As such, the experimental control afforded by NHP studies such as this one is not sufficient to generate effects robust enough to override the necessity for large samples required in human studies.

The estimation of the true effect sizes of SNPs in rhesus is difficult to assess because there are not enough studies to conduct a meta-analysis. However, our results are consistent with Madlon-Kay et al. (2018), who also investigated *rhOXTR* markers and social behavior in a similarly-sized sample of rhesus and likewise found no significant effects. We agree with their conclusions that behavioral genetic studies in NHPs likely face the same challenges as in humans: First, samples upwards of tens of thousands would be required to be adequately powered if effect sizes of SNPs on complex traits are as comparably small in NHPs as reported in humans. Second, reported associations with complex traits resulting from small genetic studies are more likely to be false-positives and fail to replicate, which has been well-documented in human literature.

While our approach to select influential *rhOXTR* markers was not effective for our specific behavioral outcomes in the context of this sample size, it could be suitable for identifying endophenotypes such as brain expression and neural activity, which bear a closer biological relationship to the proximate consequences of genetic variation. Future genetic associations studies of social behavior in NHPs should parallel the human genetics field in shifting to

extensive collaborative efforts, resulting in the possibility of acquiring large sample sizes, surpassing what would be feasible for individual research groups. Importantly, our results do not negate the wealth of OXT research demonstrating OXT's role in regulating social behavior, nor do they refute the possibility that *OXTR* SNPs may have measurable effects in larger samples, or under different experimental conditions (e.g. stress challenges). Whole-genome approaches, when appropriate sample sizes can be attained, can address the relative influence of *OXTR* variants on shaping primate social behavior.

Tables and Figures

Table 2.1. Specific behaviors analyzed for this study

Variable	Definition	Data Collected
Pro-social Behavior		
Proximity to other adult females[a]	Subject is within 1 foot of one or more other adult females (including physical contact)	Duration
Aggressive Behavior		
Non-contact aggression	Counts of agonistic chases, open-mouth threats, barks, and lunges of/by another animal	Frequency
Anxiety-like / Solitary Behavior		
Anxiety/self-directed	Counts of self-scratches[b]	Frequency

[a] Given the matrilineal structure of rhesus troops (Altmann 1962), proximity to adult females (as opposed to other members in the group) comprise the most frequent and relevant social interaction observed .

[b] In order for the same frequency behavior to be scored a second time, at least 3 seconds had to have passed from the first instance of the behavior. Bouts persisting an additional 3 seconds were counted as a second event.

Table 2.2. Assay and Primer information

PCR Program Parameters	**Hold**	**Denature**	**Annealing**	**Extension**	**Hold**	**Hold**
Temperature	95	96	see below*	68	72	4
Time	10 min	3 sec	3 sec	5 sec	10 sec	∞

Sanger Sequencing OXTR amplicon	**Forward Primer**	**Reverse Primer**	**Target length**	**Annealing Temperature***
1	GCTTCCAAACCAAGAAAGGA	CCTGCTTAGCACCCAGCTAC	495bp	55 °C
2	GGTGCTAAGCAGGGATGGAC	GGGACTGGGACTCTAAAACCA	538bp	61.6 °C
3	GGGGAGGTGGTTTGGTTTTA	GAGTTGACTGCCTCGGTGCT	521bp	59.4 °C
4	GCCTCTACACCCTCCGACAC	CAGGGACATGAGTAGCAGCA	496bp	61.4 °C
5	CAGTTGCTGTGGGACATCAC	GCCTTGGAGATGAGCTTGAC	521bp	61.4 °C
6	CAGAATTTGCGGCTCAAGAC	TGTGGGATTTCAAACCCTCTT	364bp	57.7 °C

TaqMan Assay	**Forward Primer**	**Reverse Primer**
SNP 1	GGGAGAGAAAAGGCTGAAAATTAACA	CCCGGGTAGATAGTGATGAAGTTAC
SNP 2	CCACTGCAAAATAAACCCGTTTGTT	CAGCCGCGCAGACC
SNP 3	GCGACCAGCCAGGCT	ATGCTAGGCTGAGGTCTCTGA
	Reporter 1 Sequence	**Reporter 2 Sequence**
SNP 1	CATTTTTGGAAAGTAATCAG	CATTTTTGGAAAATAATCAG
SNP 2	TTGGTCCCCGAGACTT	TGGTCCCGGAGACTT
SNP 3	CTGGAGCCTCGGTTGTA	TCTGGAGCCTCTGTTGTA

Table 2.3 Heritability and SNP Estimates

	Proximity to Adult Females (Pro-social)			Self-Scratches (Anxiety-like)			OXT in CSF		
Narrow sense heritability (h^2)	**Point Estimate**	**Lower 95% CI**	**Upper 95% CI**	**Point Estimate**	**Lower 95% CI**	**Upper 95% CI**	**Point Estimate**	**Lower 95% CI**	**Upper 95% CI**
Without fixed effects	0.312	0.046	0.591	0.213	4.98×10^{-4}	0.670	0.183	0.002	0.478
With fixed effects	0.306	0.038	0.587	0.297	3.95×10^{-4}	0.679	0.193	7.93×10^{-4}	0.500
SNPs	**β**	**Lower 95% CI**	**Upper 95% CI**	**β**	**Lower 95% CI**	**Upper 95% CI**	**β**	**Lower 95% CI**	**Upper 95% CI**
1) 140481032 G/A	-0.011	-0.043	0.023	0.003	-0.141	0.148	0.039	-0.056	0.136
2) 140497572 G/C	0.003	-0.028	0.037	0.058	-0.089	0.224	0.023	-0.072	0.123
3) 140497358 G/A	0.003	-0.028	0.038	0.079	-0.076	0.238	0.025	-0.074	0.122
4) 140497003 G/T	-0.037	-0.132	0.063	-0.481	-0.912	0.015	0.123	-0.177	0.431
5) 140496605 T/C	-0.006	-0.038	0.027	0.043	-0.126	0.19	0.015	-0.073	0.108
6) 140496348 A/G	-0.004	-0.038	0.027	0.031	-0.111	0.188	0.019	-0.073	0.108
7) 140496344 A/C	-0.005	-0.038	0.028	0.032	-0.126	0.181	0.018	-0.076	0.115
8) 140496196 G/T	0.014	-0.026	0.049	0.043	-0.128	0.22	-0.032	-0.131	0.072
9) 140495645 C/G	-0.007	-0.036	0.021	0.107	-0.037	0.242	0.038	-0.048	0.131
10) 140495542 A/T	-0.005	-0.096	0.087	0.056	-0.386	0.526	0.126	-0.117	0.387
11) 140495303 C/T	0.004	-0.028	0.034	-0.064	-0.21	0.084	0.06	-0.035	0.157
12) 140495244 G/A	-0.005	-0.052	0.037	-0.176	-0.381	0.04	0.044	-0.104	0.17
13) 140495203 T/G	-0.001	-0.033	0.03	-0.076	-0.229	0.072	0.068	-0.027	0.154
	β	**Lower 95% CI**	**Upper 95% CI**	**β**	**Lower 95% CI**	**Upper 95% CI**			
OXT CSF vs Behavior	-0.024	-0.080	0.032	-0.273	-0.543	0.006	-	-	-

Table 2.4. *OXTR* SNP Characteristics

	SNP	Hardy-Weinberg ($\chi 2$)	p	Minor Allele Frequency
1	chr3:140481032 G/A	80.365	<0.001 ***	0.204
2	chr3:140497572 G/C	0.001	0.981	0.315
3	chr3:140497358 G/A	0.132	0.716	0.314
4	chr3:140497003 G/T	0.150	0.698	0.026
5	chr3:140496605 T/C	0.494	0.482	0.462
6	chr3:140496348 A/G	0.054	0.817	0.479
7	chr3:140496344 A/C	0.054	0.817	0.479
8	chr3:140496196 G/T	1.103	0.294	0.265
9	chr3:140495645 C/G	4.628	0.031 *	0.430
10	chr3:140495542 A/T	0.246	0.620	0.033
11	chr3:140495303 C/T	0.494	0.482	0.462
12	chr3:140495244 G/A	0.042	0.837	0.143
13	chr3:140495203 T/G	0.051	0.821	0.448

*indicates significant deviation from Hardy-Weinberg equilibrium, as tested with a chi-square test

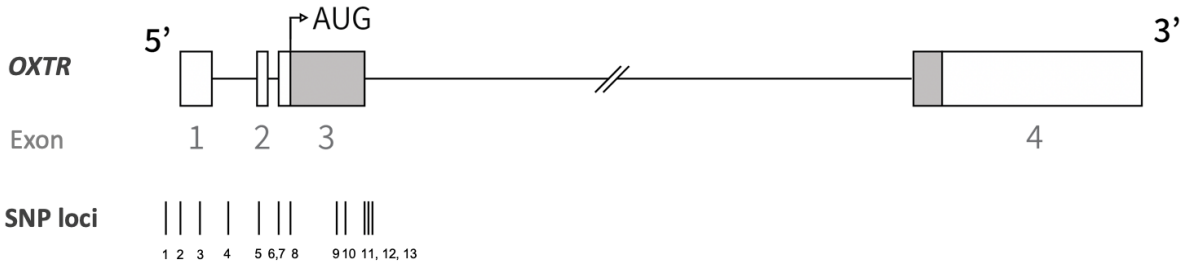

Figure 2.1. Schematic of OXTR SNPs probed in this study. Untranslated portions of the exons are indicated by the white bars. Genomic coordinates correspond to chromosome 3 of the MacaM reference genome (SNP 1) 140481032, (SNP 2) 140497572, (SNP 3) 140497358, (SNP 4) 140497003, (SNP 5) 140496605, (SNP 6) 140496348, (SNP 7) 140496344, (SNP 8) 140496196, (SNP 9) 140495645, (SNP 10) 140495542, (SNP 11) 140495303, (SNP 12) 140495244 G/A, (SNP 13) 140495203.

Supplementary Materials

Table S2.1. Kin information in our sample

	Unrelated		1st cousins		Half siblings			Full Siblings	Inbred
Coefficient of Relatedness	0	0.0625	0.125	0.1875	0.25	0.3125	0.375	0.5	0.75
Number of relationships	21504	31	261	6	859	15	12	102	1

Chapter 3. Methylation of *OXT* and *OXTR* genes, central oxytocin levels, and social behavior in female macaques

Acknowledgment of Reproduction

The following chapter has been modified slightly from a manuscript in preparation for publication*:* De Leon, D., Nishitani, S., Walum, H., McCormack, K.M., Wilson, M.E., Smith, A.K., Young, L.J., Sanchez. M.M. Methylation of *OXT* and *OXTR* genes, central oxytocin levels, and social behavior in female macaques.

3.1 Abstract

Oxytocin (OXT) and its receptor (OXTR) are encoded by *OXT* and *OXTR*, respectively. Variable methylation of these genes has been linked to variability in sociability and neuroendophenotypes. Here we examine whether *OXTR* or *OXT* methylation in blood predicts concentrations of OXT in cerebrospinal fluid (CSF) and social behavior in 207 socially-housed female rhesus macaques. We report a similarity between human and rhesus CpG sites for *OXT* and *OXTR* and a putative negative association between methylation of two *OXTR* CpG units with aggressive behavior (both P=0.003), though this finding does not survive the most stringent correction for multiple comparison testing. We did not detect a statistically significant association between methylation of any CpG sites and CSF OXT concentrations. Because none of the tested associations survived statistical corrections, if there is any relationship between blood-derived methylation of these genes and these behavioral and physiological outcomes, the effect size is too small to be detected reliably with this sample size. These results do not support the hypothesis that blood methylation of *OXT* or *OXTR* is robustly associated with CSF OXT concentration or social behavior in rhesus. It is possible, though, that methylation of these loci in the brain or in cheek epithelia may be associated with central OXT release and behavior. Finally, we consider the limitations of the exploratory nature of this study in the context of statistical power.

3.2 Introduction

The mammalian pro-social repertoire manifests with great variability within species as a consequence of individual differences in social motivation, attention to social cues, and social learning. In humans, the natural variation in affiliative behavior is observed in both healthy and clinical populations, with pronounced social deficits in autism spectrum disorder (ASD), social anxiety, and schizophrenia (Pepper et al. 2018). The precise neurobiological factors explaining individual differences are not clear, but investigating likely predictors in typical populations can identify common pathways that may be disrupted in disorders with social dysfunction.

The neurohormone oxytocin (OXT) and its receptor, OXTR, are well-documented for orchestrating conserved social processes across species (Johnson and Young 2017). Consequently, understanding how variability in the OXT system itself may encode social diversity is critical to understanding its role in disorders with social deficits. Historically, this has led to studying genetic variants, including single nucleotide polymorphisms (SNPs), in the genes encoding OXT and OXTR (*OXT* and *OXTR,* respectively). Such SNPs have been associated with differences in broad aspects of sociality (for review see Tops et al. 2018). Though the directionality and magnitude of the reported effects have been inconsistent (Bakermans-Kranenburg and van Ijzendoorn 2014; LoParo and Waldman 2015), preclinical studies have been critical to informing hypothesis-development about how genetic variants could affect *OXTR* brain expression and, in turn, social behavior. Indeed, in the socially-monogamous prairie vole, for example, a single *OXTR* SNP within a putative cis-regulatory element robustly predicted more than 70% of variability in *OXTR* expression within brain areas critical for social reward (King et al. 2016), demonstrating that a single variant in this gene may have profound consequences.

However, variability in gene expression can also be affected via epigenetic mechanisms, such as DNA methylation (DNAm) which can alter transcriptional activity and, ostensibly, downstream behavior. In mammals DNAm occurs at cytosine-guanine dinucleotide sequences (i.e. CpG sites), and within the oxytocinergic system, methylation of CpG sites in the MT2 region, near the promoter region of *OXTR*, has been associated with lower transcriptional activity *in vitro* (Kusui et al. 2001). Associations of *OXT* or *OXTR* methylation with behavior have been investigated in multiple species including humans in peripheral tissue (Haas et al. 2016; 2018), rodents (Perkeybile et al. 2019), and dogs (Cimarelli et al. 2017). Methylation of *OXTR* is related to a variety of human psychosocial traits and disorders and individual differences in brain activity (see Maud et al. 2018 for review). Increased *OXTR* methylation among individuals with ASD has also been reported in peripheral (Andari et al. 2020) and cortical samples (Gregory et al. 2009).

As the number of studies examining the role of DNAm and social behavior grows, the importance increases of using animal models and direct experimentation to test whether identified associations genuinely reflect changes in the brain. While mechanistic work in rodent models has allowed manipulation of the neural circuitry and genes that affect social behavior (Walum and Young 2018), non-human primate models (NHPs) provide stronger translational value due to their phylogenetic and phenotypic similarity to humans (Phillips et al. 2014), as well as the potential to harvest brain tissue once researchers begin probing the effects of established associations on specific structures within the brain (Putnam et al. 2018).

Socially-housed rhesus macaques, in particular, have been extensively used to study the biological basis of social behavior and provide an ideal model to study the relationship between epigenetic modifications of the OXT system and social phenotypes (Putnam et al. 2018).

Rhesus are similar to humans in brain anatomy, behavior, and cognitive capacity. Unlike rodents, they have well-developed frontal and temporal lobes, which may be critical for complex socio-behavioral interactions lacking in other species (Phillips et al. 2014). Rhesus social behaviors exhibit considerable sophistication and diversity in contrast to rodents; they form reciprocal social alliances, elaborate social hierarchies, and lasting mother-offspring bonds (Altmann 1962; Suomi 2005). Importantly, rhesus and humans both rely on audio-visual social cues in contrast to rodents, which depend more heavily on olfaction (Freeman and Young 2016; Putnam et al. 2018). These species differences are reflected in the neuroanatomical distribution of OXTR, which are more concentrated in visual-auditory processing areas in primates, while in rodents are primarily in olfactory and ventral striatal/reward regions (Freeman, Inoue, et al. 2014; Freeman, Walum, et al. 2014; Freeman and Young 2016). Despite the advantages offered by macaque models, we found only one report of OXT-related epigenetic variation macaques (Baker et al. 2017), and it examined the effects of early experience (maternal deprivation) on H3K4me3 binding (decrease) within *OXTR*. Thus, the effects of epigenetic variation on NHP social behavior remain unexplored, and quantifying these effects was the goal of the present study. We used a moderately-sized sample of socially-housed rhesus monkeys to investigate whether methylation of the *OXT* and *OXTR* genes was related to downstream brain function and behavior. For this, we made use of a unique opportunity to study rhesus living in semi-naturalistic environments, mimicking the social structure and species-typical experiences and behaviors of these animals in the wild (Altmann 1962) and allowing us to capture a dynamic range of social responses. Rhesus form matriarchal, matrilineal societies both in the wild and in captivity, establishing close, lifelong bonds with female-kin (Suomi 2005). When male offspring reach reproductive age, they migrate away as bachelor groups that become incorporated into other troops to become adult breeder males (very few per group 2-5) (Altmann 1962; Suomi 2005). Therefore, here we studied only females owing to (1) the constraints this social structure

places on the number of available males for (epi)genetic studies, since rhesus social groups are predominantly composed of females; (2) the fundamental differences in social relationships among females and males.

In the current study, we explored potential associations between *OXT*/*OXTR* methylation and affiliative behavior, as well as with cerebrospinal fluid (CSF) concentrations of OXT. As the OXT system is also related to aggression (de Jong and Neumann 2018) and anxiety (Naja and Aoun 2017), we also investigated possible associations with these phenotypes.

3.3 Materials and Methods

3.3.1 Subjects and Housing

Subjects assigned to this study were 214 adult female rhesus monkeys of known pedigree living in social groups at the Yerkes National Primate Research Center (YNPRC) Field Station (Lawrenceville, GA). Seven subjects were dropped from the study due to missing behavioral observations or blood samples, leaving 207 subjects for final analyses. Animals were housed in outdoor enclosures (3/4 to 1 acre areas) with access to climate-controlled indoor facilities. Subjects were females of reproductive age, ranging in age from 4-24 years (median: 7 years, IQR: 6-11) and were selected from five social groups consisting of 28-94 adult females, their sub-adult, juvenile, and infant offspring and 2-4 adult males (except for the final mating season of the study, where one social group had 10 adult males). All social groups had a stable matrilineal structure and a linear dominance hierarchy. Female dominance ranks were assessed based on aggression and submission data collected during behavioral observations. Subordinate status was determined by the unequivocal emission of a submissive behavior by one animal to another during dyadic interactions. Animals were provided a standard commercial low-fat, high-fiber diet (Purina Mills International, LabDiets, St. Louis, MO) and water *ad libitum*,

supplemented with seasonal fruits or vegetables twice per day. All procedures were performed in accordance with the Animal Welfare Act and the U.S. DHHS “Guide for the Care and Use of Laboratory Animals” and approved by the Emory IACUC.

Animals were selected from families across all social ranks (high, medium and low social status) and with varying degrees of relatedness (including paternal and maternal half-siblings, full-siblings, cousins, half-cousins, and unrelated subjects), in order to enable heritability analyses in studies parallel to this one.

3.3.2 Behavioral Data Collection

Seven experienced observers conducted focal observations on subjects from observation towers situated above each enclosure between 7:00-11:00AM, when animals are most active. Focal observations were collected in real-time following published protocols (McCormack et al. 2009; Wilson et al. 2013) based on established rhesus monkey ethograms (Altmann 1962). Behaviors were recorded on handheld computers with an in-house program which records the actor, behavior, recipient, and time the behavior occurred (see ethogram in Table 3.1). Inter-rater reliability exceeded 80%. A 20 min observation was collected per week for each animal across 4 consecutive weeks (mean: 80.06 min/animal, SD: 2.94). All observations were collected during the mating season to reduce seasonal variability in behavior.

For this study, we focused on behavioral categories related to pro-social behaviors (time spent in proximity or grooming with others and time sitting alone, to capture lack of social interactions) as well as anxiety-like behaviors (frequencies of self-scratches, yawns, body-shakes, and self-grooming (e.g. Schino et al. 1991)), and aggressive behaviors initiated by the subject. Aggression was coded as frequencies of non-contact aggression (chases, open-mouth threats,

barks, lunges) or contact aggression (biting, slapping, grabbing, pinning down). Durations were converted to percent of time observed engaging in each behavior. Frequency behaviors were kept as count data and adjusted with the addition of an offset term in the final statistical model (which takes into account differences in observation time among subjects).

3.3.3 Physiological Data Collection and Analysis

Blood and CSF samples

Animals were trained and habituated to the experimental procedures in order to facilitate collection of blood and CSF using methods to minimize arousal (Wilson et al. 2013). Each subject was accessed once, between 7:00-9:00am (0-90 minutes after sunrise) during the same season as the behavioral data collection, but never on the same day to avoid effects of handling and anesthesia on the behavioral observations. Animals were quickly anesthetized with Telazol (3 mg/kg, IM) for CSF and blood collection. CSF samples were collected in a subset of the subjects (n=166) to examine central OXT concentrations. A 2 ml CSF sample was collected in each subject from the *cisterna magna* by gravity through a 22 G needle and placed immediately on dry ice. CSF samples were stored at -80ºC for later assay. Following CSF collection, two 3 ml blood samples were collected in EDTA tubes for DNA extraction, immediately placed on ice, and stored at -80ºC until time of processing. CSF samples were processed by the Yerkes National Primate Research Center Biomarkers Core Laboratory using commercially available ELISA kits produced by Assay Designs (Ann Arbor, MI), following manufacturer's recommendations. Sensitivity of the OXT assay was 15.6 pg/ml and the inter- and intra- assay CVs were 7.48% and 10.2%, respectively.

Covariates

The following additional measures were used as covariates in the statistical models to account for their potential confounding effect on behavioral and/or CSF outcomes: (1) social group; (2) social rank (high, middle, low), as it affects measures of aggression and grooming; (3) age; (4) observation year (data were collected across three consecutive years); (5) having an infant (<1 year old); (6) pregnancy status and estimated fetus age, if applicable (if the female was not pregnant at time of access, fetal age was recorded as 0); (7) latency to CSF sample collection from time of disturbance/access; and (8) CSF batch effect (CSF samples were assayed in three batches run at different times).

3.3.4 Epigenetic Analysis

DNAm of OXT and OXTR

DNA was extracted from whole blood using Maxwell 16 Blood DNA purification Kit (Promega) and was quantified with PicoGreen (Quant-iT PicoGreen dsDNA Assay Kit, Thermo Fisher Scientific). Five-hundred ng of DNA were bisulfite-treated (EpiTect Fast 96 Bisulfite Conversion Kit, Qiagen). *OXT* (chr15: 35,896,090 – 35,896,991: on the MacaM reference genome sequence) and *OXTR* (chr3: 140,466,550 to 140,509,583: MacaM reference genome) genes were analyzed using EpiTYPER (MassARRAY system; Agena Biosciences) according to the manufacturer's instructions. DNAm of 20 CpG sites in the promoter region of *OXT* were amplified (chr15: 35,895,833 – 35,896,216: MacaM) using primer pairs we previously used for humans (Haas et al. 2016). DNAm of 27 CpG sites in the promoter region of *OXTR* (Figure 3.1), which contains the MT2 region (Kusui et al. 2001), were also amplified (chr03: 140,497,357 – 140,496,884: MacaM). Primer sequences are detailed in Table 3.2. The primer sets were designed for EpiTYPER using EpiDesigner (Agena Bioscience), and the spectrum characteristics were validated with RSeqMeth (Coolen et al. 2007). One µL of bisulfite converted (BSC) DNA was used in a 5 µL reaction mixture containing 0.4 U of PCR enzyme, 1 X PCR

Buffer with 20 mM $MgCl_2$, 200 µM dNTPs (Agena Bioscience) and 3 pmoles of each primer. Cycling conditions were: denaturation (94°C for 15 min) followed by 50 cycles of amplification (*OXT*: 94°C for 30 s, 62°C for 60 s, and 72°C for 30 s, *OXTR*: 94°C for 30 s, 58°C for 60 s, and 72°C for 30 s) and a final extension step (72°C for 10 min) (GeneAmp® PCR System 9700, ThermoFisher Scientific). Samples were electrophoresed using 2% agarose gel to confirm amplification. The CpG sites were unambiguously interrogated, and their genomic locations are detailed in Figure 3.1.

Additionally, we confirmed the reliability of the *OXT* and *OXTR* methylation assay by using unmethylated (0%) and methylated (100%) genomic DNA standards ("Monkey (Macaca)" low and high methylated genomic DNA, EpigenDx, Inc, MA, USA) to create a standard curve (0%, 25%, 50%, 75%, 100% methylation) and serve as positive controls. Reproducibility and sensitivity of the product of the *OXT* and *OXTR* EpiTYPER assays were assessed using the positive controls run in triplicate and measured on a MALDI-TOF mass spectrometer (Figure 3.2). The mass spectra methylation ratios were generated using EpiTYPER ver. 1.2 (Agena Biosciences). For each subject, percent *OXT* and *OXTR* DNAm values of each CpG unit were retained. Because this technology relies on restriction enzymes to cleave at specific genetic segments, a CpG unit containing single or multiple CpGs is the smallest possible cleaved fragment that can be analyzed by the EpiTYPER system. CpG units with unreliable measurements, such as duplicate CpG units (which have the same mass as other CpG fragments) were excluded (Suchiman et al. 2015). Methylation values at CpG units were used as the criterion variable in subsequent statistical analyses.

Lastly, the existence of polymorphic CpG sites for *OXTR* was checked using the Rhesus Variants Hub corresponding to the UCSC rheMac2 assembly. Our target region contained no frequent polymorphic CpG sites that could interfere with measures of methylation (Table 3.3).

3.3.5. Statistical Analysis

All statistical analyses were performed in the R statistical software package version 3.4.1 (R Project for Statistical Computing, Vienna, Austria). Associations of CpG unit methylation with behavioral and physiological variables (behavior or CSF OXT concentrations) were examined using generalized linear models (GLMs).

First, for each gene, the rank correlations between CpG units were assessed to determine whether CpGs in the target regions were similarly methylated. The correlations were then used to flag overlapping and duplicate units as well as to assess the independence of CpG units, which informed corrections for multiple comparison decisions.

Once the independence of the CpG units was assessed, we inspected the distributions of the dependent variables and chose a statistical model accordingly. Continuous, Gaussian dependent variables were modelled using ordinary least squares regression and log-transformed to improve normality when necessary. Dependent variables that followed a count distribution were inspected for over-dispersion and zero-inflation before selecting between Poisson, negative binomial, and zero-inflated versions of these models. The best fitting model, given the data, was determined with the likelihood ratio test.

Once the appropriate model was selected, we tested the relationship between a CpG unit and an outcome variable with a full model, including all covariates. Covariates with statistical

significance ($P<0.05$) as determined by a likelihood ratio test were retained and applied to the subsequent tests for this outcome. This procedure was repeated for each outcome to determine the appropriate covariates. The final models for each outcome-CpG combination were validated by checking the diagnostics plots to ensure that patterns were not present and that assumptions of the model were not violated. For all statistical models, an uncorrected alpha level of 0.05 was used. To account for multiple comparisons, the alpha level was then adjusted using a Bonferroni correction for the number of CpG units tested per outcome.

3.4 Results

3.4.1 Characteristics of Behavioral and Physiological Measures

For this study, we focused on a subset of behavioral categories related to affiliative, anxiety-like, and aggressive traits due to their relevance to the oxytocin system. Individual behaviors that fell under each category were not strongly correlated with each other (median: 0.01, Q1: -0.04, Q3: 0.06) (Figure 3.3). Thus, we selected individual behaviors for analysis instead of creating composite or sum scores because combining uncorrelated measures into the latter produces a suboptimal representation of the underlying trait (van der Sluis et al. 2010), where the resulting composite score is exceedingly driven by the most frequently occurring behavior.

The behaviors ultimately used in analysis were chosen based on occurrence during focal observations (i.e. low occurrence behaviors were dropped from analyses) and on biological relevance. Because rhesus macaque social structure is matriarchal and matrilineal (i.e. based on affiliation and alliances among adult females (Suomi 2005; Altmann 1962)), we analyzed the percent of time spent in proximity with one or more adult females as our measure of affiliative social behavior, which was also the most frequent affiliative behavior in the ethogram. Self-scratching was selected as the representative anxiety-like behavior due to its higher occurrence

compared to the other anxiety-like behaviors (yawns, body shakes, self-grooming) (Schino et al. 1991). Aggression was less common than other behaviors, with non-contact aggression (chases, barks, open-mouth threats, lunges) occurring more frequently than contact aggression (biting, slapping, pinning-down, etc.). Percent of time spent in proximity to adult females was normally distributed and thus modeled with an ordinary least squares regression, controlling for differences in social group (the only covariate that significantly improved model fit for this behavior). Counts of self-scratching and non-contact aggression displayed overdispersion and were analyzed with a negative binomial model, including social group and rank as covariates in the respective models. No other covariates had effects on our behavioral measures. Descriptive statistics and potential covariates are shown in Table 3.4.

3.4.2 DNAm of OXT and OXTR

Characteristics and descriptive statistics of CpG Units analyzed:

Within our target regions for *OXT* and *OXTR*, methylation at CpG sites were not highly correlated with each other (*OXT* median: 0.08, Q1: 0.02, Q3: 0.10; *OXTR* median: 0.09, Q1: 0.03, Q3: 0.13) (Figure 3.4), with the exception of -902 and -1040/1043 for *OXTR* (Kendall's τ=0.64). The correlation between these two units could be explained by the overlapping mass of these CpG fragments (both fragments had different nucleotide sequences but identical base masses)(Suchiman et al. 2015). Consequently, the DNAm signal for each of these CpGs could not be disentangled and uniquely assigned, resulting in a high correlation value. Descriptive statistics of individual CpG sites for both *OXT* and *OXTR* are listed in Tables 3.5 and 3.6.

Regression analysis revealed no significant associations (α = 0.05 / 10 CpG units = 0.005) between DNAm of *OXT* CpG sites and behavioral measures (Table 3.6). For *OXTR*, greater

methylation at -902 (β=-2.71, z=-2.95, P=0.003) and CpG unit -1040/1043 (β=-2.71, z=-2.92, P=0.003) were both associated with fewer displays of non-contact aggression. The associations of these sites survived a lenient Bonferroni correction (α = 0.05 / 13 CpG units = 0.0038; Table 3.7), but not a stricter correction which accounted for the testing against multiple biobehavioral outcomes (α = 0.05 / (13 CpG units x 4 outcomes) = 0.00096). Additionally, these sites were overlapping CpG fragments, such that they had different nucleotide sequences but the same base mass. Thus, measurements of methylation at -902 and -1040/1043 could not be uniquely assigned.

We did not detect a statistically significant association between the remaining CpG sites and behaviors that survived corrections for multiple comparisons. Before corrections, the largest effects were observed with increased methylation of *OXT* CpG unit -133/130 and decreased pro-social behavior (R^2=0.03, β=-0.15, t(5, 200)=2.52, P=0.011) and methylation of *OXTR* -717, which had both a positive association with pro-social behavior (increased time spent in proximity to others) (R^2=0.03, β=0.12, t(5, 201)=2.52, P=0.013) and a negative association with anxiety-like behavior (self-scratching) (β=-0.45, 1 z=-2.05, P=0.040). The next largest effects in this sample were the following CpG units: (1) -877/879, which had a positive relationship with the amount of time spent in proximity to other adult females (R^2= 0.02, β=0.11, t(5,201)=1.99, P=0.047); and (2) -769, which had a negative relationship with non-contact aggression (β=-2.15, z=-2.17, P=0.03). Again, these effects were not statistically significant after Bonferroni correction for multiple comparisons.

3.4.3 Methylation and OXT in CSF

In a subset of the sample (n=166), CSF OXT concentrations were measured, and regression analyses was used to test the association between with methylation at CpG sites and CSF OXT. CSF OXT measures were log-transformed to normality and modeled with a Gaussian distribution. Social group and assay batch had an effect on CSF OXT levels, so they were included as covariates in the statistical model. No other variables had effects. Measures of CSF OXT were not highly correlated with any behavioral measures in this study (mean correlation: -0.03) (Figure 3.5).

We did not detect a statistically significant association between *OXT* or *OXTR* CpG methylation and CSF OXT concentration that survived corrections for multiple comparisons. The largest observed effects in this sample were a negative relationship between methylation of *OXTR* -1050 and CSF OXT levels (R^2= 0.03, β=-0.39, t(6,152)=-2.19, P=0.03), followed by positive associations between methylation at *OXTR* -717 and OXT levels (R^2= 0.029, β=0.298 t(6,152)=2.14, P=0.034).

3.5 Discussion

Despite weak reported effects, the main contribution of this study is to be the first to map the naturally-occurring variation of *OXT* and *OXTR* DNAm in rhesus, showing the feasibility of capturing this genetic information in the NHP model.

Although methylation of *OXTR* has been associated with decreased expression of the protein in cell culture (Kusui et al. 2001), rodents (Perkeybile et al. 2019), and humans (Gregory et al. 2009), the lack of gene expression data in the present study prevents us from interpreting how methylation at these loci may be regulating expression. Canonically, increased methylation generally represses transcription of the gene, although there are exceptions (e.g. Mamrut et al.

2013). Thus, our finding regarding aggression would suggest that increased expression of *OXTR* may contribute to a more aggressive social phenotype. This is in contrast to other literature. Although complex and context-dependent, most OXT-related aggression studies in rodents show that an upregulated OXT system leads to anti-aggressive effects (de Jong and Neumann 2018), with exceptions for maternal aggression during the perinatal period (Bosch 2013). However, studies in humans report that while OXT serves pro-social functions among members of an in-group, OXT can increase aggression and anti-social tendencies towards outsiders (De Dreu and Kret 2016).

The rhesus social structure revolves heavily around distinguishing others on the basis of group and family membership (Altmann 1962). As such, aggressing out-group members is important and beneficial for the group's social dynamic as it increases social protection/alliances. Though our behavioral data do not identify whether observed aggression was directed towards non-kin, putative increases in *OXTR* expression in rhesus may result in increased sensitivity to circulating OXT, thereby exacerbating aggression towards out-family/group animals. This effect may also be consistent with the behavioral profiles of rhesus as a species, which show high levels of aggression in contrast to other macaque species (Sussman et al. 2013). Aggression, as opposed to pro-social behaviors, may play a disproportionately salient role in shaping rhesus' social relationships. As such, the OXT system may regulate social behaviors in species-specific ways.

Alternatively, the OXT system may not have large effects on the behavioral parameters measured in this study, and therefore the markers analyzed here do not predict most of our outcomes. Indeed, Parker et al. (2018) found that CSF arginine vasopressin (AVP), not OXT, predicted social behavior in rhesus. Furthermore, AVP V1aRs are more widely distributed than

OXTR in the rhesus cortex and limbic system (Freeman and Young 2016). Finally, while OXT-immunoreactive fibers are detected in the cortex of human and chimpanzee brains, they are not detected in the cortex of rhesus macaque (Rogers et al. 2018), raising the possibility that OXT may have more limited effects on social behavior in rhesus. It is also possible that the markers used to assess the OXT system in this study may not accurately reflect variation in OXT processes within the rhesus brain.

3.5.1 Relevance to human studies

With respect to reported findings in the OXTR literature with particular CpGs, we did not find similar relationships with social outcomes. In humans, the CpG site -934 in the MT2 region of *OXTR* received much attention after hypermethylation at this locus (out of five others tested) was associated with ASD status (Gregory et al. 2009). Though, the association of the encompassing MT2 region with ASD has produced mixed results (Elagoz Yuksel et al. 2016; Andari et al. 2020). Andari et al. (2020) examined 21 CpG fragments in the MT2 region and identified one fragment with hypermethylation in adults with ASD compared to neurotypical subjects. However, the fragment including -934 did not show any differences between ASD and neurotypical groups. Nonetheless, the -934 site has been associated with social perception (Jack et al. 2012; Puglia et al. 2015) and attachment (Ebner et al. 2018) in healthy subjects. It has also been linked with psychiatric disorders (Dadds et al. 2014; Bell et al. 2015). However, the direction of the relationship between methylation and outcomes relevant to social processes and function has not been consistent (Jack et al. 2012). While these studies broadly suggest the relevance of this CpG in human social outcomes, we did not observe evidence to support this in female rhesus macaques. There are two ways to determine which CpG in rhesus is analogous to the human CpG -934, either by matching the sequence or by using the absolute distance of bases from the translation start site (TSS). This implicates two rhesus CpG units. Matching the

surrounding sequence, methylation of -877/879 in rhesus had a weak, positive correlation (p=0.047, uncorrected) with affiliative behavior, though this was in the opposite direction of what might be expected based on previous work (Gregory et al. 2009). This site had no associations with other outcomes. Using distance from the TSS, the closest CpG unit in rhesus is -925/932, which had no associations with any outcomes in our study. These inconsistencies may be results of these studies being underpowered (discussed more below).

3.5.2 Is peripheral methylation of *OXTR* a relevant marker?

These results should also be interpreted in the context of the ongoing debate about whether methylation in blood is a useful proxy of methylation in the brain. Several studies have demonstrated that methylation patterns are tissue-specific (Davies et al. 2012; Farré et al. 2015; Beery et al. 2016), which is in line with the fact that blood and brain are derived from different germ layers. Yet moderate to strong correlations in methylation patterns across some tissue types have been reported (Davies et al. 2012; Byun et al. 2009; Perkeybile et al. 2019), suggesting that blood may serve as a predictive surrogate for target tissue that is not easily accessible. While high concordance across tissue-type may be gene-specific (e.g. Di Sante et al. 2018), when limiting analysis to CpGs that are variable and associated with genes relevant to the brain, only a small proportion (7.9%) of sites show significant correlations (Walton et al. 2016). Adding to this complexity, methylation patterns are variable between brain regions themselves (Davies et al. 2012; Farré et al. 2015; Harony-Nicolas et al. 2014). At best, if blood and brain methylation signatures are robustly correlated, we do not know whether that correlation predicts methylation in regions involved in OXT-related behaviors of the species of interest. However, a recent study in voles did find methylation in blood to be correlated with *OXTR* expression in the nucleus accumbens (Perkeybile et al. 2019), but we know little about this relationship in primates. Given the challenges of procuring blood and brain tissue from the

same individuals to investigate methylation concordance, it is unsurprising that the majority of human studies have been constrained by small samples (N≤12) (Byun et al. 2009; Davies et al. 2012; Walton et al. 2016), which makes drawing reliable conclusions about tissue concordance challenging. Additional studies using animal models should prioritize larger samples to replicate and map the patterns between central and peripheral methylation of *OXT*-related genes and expression as well as their impact on brain expression and behavior.

3.5.3 This is an exploratory study with many comparisons – a limitation

Given the exploratory nature of this study, it is important to acknowledge the effect of the number of comparisons on the results we report. While we do detect an association with aggression in our sample after correcting the threshold for statistical significance by the number of *OXTR* CpGs per measure, if we consider the total number of comparisons made, this increases the likelihood that an association between methylation of a CpG and a behavioral or physiological outcome would be detected by chance alone. The total number of comparisons in the paper were 92 (the total number of *OXT* and *OXTR* CpG units X Number of Measures = 92). Using a stringent correction for this many tests results in a much more conservative p-value threshold of $\alpha = 5.43 \times 10^{-4}$. This corrected threshold is based on the assumption that the tests are independent (i.e. uncorrelated), and according to the correlations of CpGs and correlations of behaviors shown in Figures 3.3 and 3.4, this assumption seems to hold. None of the results in this study survive this strict correction, and thus, our finding of an association with aggression should be treated as preliminary given the likelihood of reporting false positive findings when using liberal corrections (Ioannidis 2005).

Rather than suggesting there is no relationship between peripheral methylation and behavior, the lack of associations may also be a consequence of low statistical power, meaning our ability

to detect associations is weak if associations truly exists. Power is a function of both a study's sample size and the underlying magnitude of the effect being probed—in this case, how much of the variance in the behavior or in OXT CSF is explained by single locus' methylation. While identifying the sample size is obvious, identifying the magnitude or size of the effect is more difficult. One must have a realistic estimate about the magnitude of the association or effect being probed.

In our sample of 207 rhesus, the minimum effect size must be greater than R^2=0.092 to gain 80% power, controlling for all CpGs and outcomes analyzed. This means a single CpG must explain more than 9% of the variance in the behavior or CSF concentration in order to cross the threshold of significance. Even when using lenient corrections (correcting for only the number of CpGs for each outcome), a single site must explain more than 6% of the variance in that behavior to reach the threshold of statistical significance.

Some of the observed effect sizes of this study and of others (Cimarelli et al. 2017; Jack et al. 2012) approach these large magnitudes. Consequently, the current study may appear sufficiently powered. However, information from a large body of work is necessary to estimate the correct population effect size. In fact, it has been shown across many scientific disciplines that early studies often report highly-inflated effects and suboptimal power (Ioannidis 2008). Indeed, epigenetic reports of *OXTR* are still in their infancy and methods and findings are variable (Maud et al. 2018). In human genetics of complex traits, where the field has reached critical mass with regard to sample sizes, we can be confident the effect sizes reported are reliable---and they are small (<1% variance explained). If these findings translate to epigenetics, to be well-powered enough to detect smaller, more plausible effect sizes, sample sizes for epigenetic studies would need to be increased substantially (e.g. if the true effect size were

liberally estimated to be half the magnitude reported here (i.e. R^2~ 4.5%), a study with the same number of tests as this one and two predictors (e.g. a single CpG and a single covariate) would require a minimum sample of N~500 to be appropriately powered). An additional, underappreciated consequence of underpowered studies is that the "significant" relationship between the predictor and the outcome is not only likely to be inflated but is also likely to be in the wrong direction (i.e. have the wrong sign)(Gelman and Carlin 2014). Taken together, it is important to interpret all reported effects cautiously. Future studies should focus on well-powered replication attempts, heeding that a replication study will require a larger sample size than the original to achieve sufficient power (Button et al. 2013). Ultimately, meta-analyses may bring us closer to the estimates of the true effects of *OXT* and *OXTR* epigenetic influences on complex social behaviors.

3.5.4 Conclusion

This study is the first to map naturally-occurring variation of DNAm in rhesus within *OXT* and *OXTR.* Our findings show a weak relationship between *OXTR* methylation and aggression and no associations between *OXT* or *OXTR* methylation and other behaviors or CSF OXT levels. These results should be interpreted cautiously given our study is likely underpowered.

We suggest future studies prioritize associations with additional endophenotypes relevant to the OXT system and social behavior, such as receptor expression in the brain, when access to rhesus brain tissue is possible. This has the potential to result in larger effect sizes that may facilitate detection in future studies. Given sufficient sample size and statistical power, the rhesus model provides invaluable translational worth for the possibility of identifying epigenetic markers causally-related to brain expression and variability in downstream social behavior.

Tables and Figures

Table 3.1. Full ethogram used

Variable	Definition	Data Collected
Pro-social Behavior		
Proximity with other(s)	Subject is within 1 foot of one or more other animals (including physical contact), excluding own infants born in the previous year	Duration
Grooming with other(s)	Picking and spreading of the fur, excluding own infants born in the previous year	Duration
Sit-alone	Subject is sitting alone, with no other animal(s) within a 5 meter diameter (lack of pro-social behavior)	Duration
Aggressive Behavior		
Contact aggression	Biting, slapping, grabbing and pinning down of/by another animal	Frequency
Non-contact aggression	Agonistic chases, open-mouth threats, barks, and lunges of/by another animal	Frequency
Submissive Behavior		
Fear grimace	Pulling back of lips in a “smile-like” fashion to display teeth	Frequency
Scream	High-pitched vocalization in response to another animal	Frequency
Lipsmack	Repeated movements of the lips	Frequency
Withdraw	Avoidance of another animal	Frequency
Sexual Behavior		
Follow	Subject follows male, or male follows subject: both move simultaneously together, and within arm’s reach of one another.	Duration
Mount Series	One or several instances in which a male mounts the subject, with intromission, and the pair remains in proximity to each other in between mounts. The mount series starts with the first mount and terminates either when the male ejaculates or whenever the pair separates beyond proximity and are clearly no longer associating with each other.	Duration
Anxiety-like / Solitary Behavior		
Anxiety/self-directed	Self-scratches, yawns, body-shakes, and self-groom episodes	Frequency

For this particular study only behavioral categories for pro-social, anxiety-like, and aggressive traits were examined.

Table 3.2. Details of the primer sequences used to amplify targets of *OXT* and *OXTR*

Name	Sequence
OXT (forward) *	5'- aggaagagagTTTTTTTGTTTTATTTTAGTGGTTTAGG -3'
OXT (reverse) *	5'- cagtaatacgactcactatagggagaaggctTCTTACCTCCCAAAAAACAATTCTA -3'
OXTR (forward)	5'- aggaagagagTTTTTTTGGGGGAATTTTTATTTTA -3'
OXTR (reverse)	5'- cagtaatacgactcactatagggagaaggctATCAAAAACCTCAACCTAACATCAC -3'

*Previously published sequence from Haas et al. (2016)

Table 3.3. Confirmation that in our sample no SNPs were detected at CpG loci for *OXTR*

CpG	Sanger	n	NGS	n
-717	no SNPs	200	no SNPs	207
-925	no SNPs	25	NA	

Three known single nucleotide polymorphisms (SNPs) have been reported for rhesus macaques at the loci for -717 and -925. In each case the alternative allele had a low allelic frequency (<0.4%) [-717: RheMac2, chr2:52232467 C>T and chr2:52232468 G>T; -925: RheMac2, chr2: 52232260 G>A. We confirmed that these variants did not exist within our studied sample via Sanger sequencing. In a subset of the sample, genotyping was also confirmed with Next Generation Sequencing (NGS). No SNPs were detected within our sample at CpG sites.

Table 3.4. Characteristics of Behavioral and Physiological Measures.

Behavioral and Physiological Measures	Characteristics
% Time Spent with one or more Adult Females	mean: 31.86 (SD: 15.28)
Self-scratches (counts/min)	median: 0.08 (Q1: 0.05, Q3: 0.13)
Non-contact aggression (counts/min)	median: 0.01 (Q1: 0, Q3: 0.02)
CSF oxytocin (pg/mL, n=166)	median: 49.18 (Q1: 34.3, Q3: 64.74)
Covariates	
Age (years)	median: 7 (Q1: 6, Q3: 11)
Latency to collect CSF sample (min)	median: 25 (Q1: 21, Q3: 29)
Fetus age (days) at time of sample collection	median: 0 (Q1: 0, Q3: 39.5)
Infant during observation	
Yes	n= 101 (48.79%)
No	n= 106 (51.2%)
Social Group	
A1	n= 81 (39.13%)
D1	n= 36 (17.39%)
D2	n= 22 (10.63%)
BC1A	n= 34 (16.43%)
BC2B	n= 34 (16.43%)
Social Rank	
High	n= 70 (33.82%)
Middle	n= 67 (32.37%)
Low	n= 70 (33.82%)
Observation Year	
Year 1	n= 58 (28.01%)
Year 2	n= 116 (56.04%)
Year 3	n= 33 (15.94%)
CSF oxytocin Assay Batch	
Batch 1	n= 63 (37.95%)
Batch 2	n= 89 (53.61%)
Batch 3	n= 12 (7.23%)

SD: standard deviation; Q1: first quantile; Q3: third quantile. Raw counts of self-scratches and non-contact aggression (and not rates) were used in analysis with an offset term included in the model controls for variable lengths of observation time. For the purposes of producing a summary statistic, these measures are summarized here as rates.

Table 3.5. Details of *OXT* CpGs included in this study.

Rhesus *OXT* *CpG Unit*	CpG IDs	Genomic position (MacaM, chr15)	Location from TSS	Methylation **Median (Q1, Q3)**
-204/202	CpG3	35,895,922	-204	
	CpG4	35,895,924	-202	0.81 (0.68, 0.93)
-188	CpG5	35,895,938	-188	0.88 (0.72, 0.97)
-155	CpG6	35,895,971	-155	0.89 (0.80, 0.94)
-133/130	CpG7	35,895,993	-133	
	CpG8	35,895,996	-130	0.84 (0.72, 0.94)
-49	CpG11	35,896,077	-49	0.92 (0.78, 1.00)
-40	CpG12	35,896,086	-40	0.92 (0.82, 0.97)
-12	CpG14	35,896,114	-12	0.98 (0.77, 1.00)
-6	CpG15	35,896,120	-6	0.79 (0.58, 0.97)
+5	CpG16	35,896,131	+5	0.41 (0.19, 0.58)
+53	CpG20	35,896,192	+53	0.70 (0.42, 0.93)

CpG IDs correspond to IDs used in Figure 3.1. Q1: first quantile, Q3: third quantile. TSS: Translation start site

Table 3.6. Descriptive statistics for *OXTR* methylation

Rhesus *OXTR* *CpG Unit*	CpG IDs	Genomic position (MacaM, chr03)	Location from TSS	Methylation **Median (Q1, Q3)**
-1097	CpG27	140,497,309	-1097	0.02 (0.01, 0.07)
-1062/1064/1068/1074/1079	CpG26	140,497,291	-1079	
	CpG25	140,497,286	-1074	
	CpG24	140,497,280	-1068	
	CpG23	140,497,276	-1064	
	CpG22	140,497,274	-1062	0.23 (0.18, 0.27)
-1050	CpG21	140,497,262	-1050	0.145 (0.06, 0.25)
-1040/1043	CpG20	140,497,255	-1043	OVERLAPPING
	CpG19	140,497,252	-1040	0.25 (0.185, 0.3)
-959	CpG16	140,497,171	-959	0.03 (0.02, 0.05)
-925/932	CpG14	140,497,144	-932	
	CpG13	140,497,137	-925	0.21 (0.18, 0.25)
-902	CpG12	140,497,114	-902	0.16 (0.09, 0.22)
				OVERLAPPING
-877/879	CpG11	140,497,091	-879	
	CpG10	140,497,089	-877	0.43 (0.31, 0.505)
-844	CpG8	140,497,056	-844	0.27 (0.08, 0.4)
-796/803	CpG7	140,497,015	-803	
	CpG6	140,497,008	-796	0.16 (0.14, 0.22)
-769	CpG4	140,496,981	-769	0.01 (0, 0.04)
-749/751	CpG3	140,496,963	-751	
	CpG2	140,496,961	-749	0.05 (0.02, 0.11)
-717	CpG1	140,496,929	-717	0.19 (0.04, 0.3)

Details of *OXTR* CpGs included in this study. CpG IDs correspond to IDs used in Figure 3.1. Q1: first quantile, Q3: third quantile. TSS: Translation start site

Table 3.7. Associations between methylation of *OXT* CpG units and behavioral and physiological measures

OXT CpGs	Affiliative Behavior Proximity to Adult Females			Anxiety-like Behavior Self-scratching		Non-contact Aggression		OXT in CSF		
	R^2	β	p	β	p	β	p	R^2	β	p
-204/202	0.000	0.016	0.785	-0.143	0.605	0.378	0.476	0.004	-0.137	0.460
-188	0.001	-0.021	0.613	-0.147	0.435	0.422	0.259	0.01	-0.148	0.217
-155	0.004	-0.065	0.372	-0.386	0.247	-0.042	0.948	0.005	0.190	0.388
-133/130	0.031	-0.150	0.011	-0.007	0.980	0.560	0.284	0.011	0.227	0.192
-49	0.000	0.010	0.821	0.056	0.794	0.450	0.286	0.014	-0.210	0.135
-40	0.006	0.064	0.282	0.255	0.361	-0.177	0.727	0.003	-0.128	0.479
-12	0.005	-0.044	0.293	0.170	0.372	-0.376	0.274	0.009	0.156	0.236
-6	0.003	-0.03	0.428	0.135	0.447	-0.291	0.373	0.000	-0.002	0.987
+5	0.010	-0.053	0.158	0.004	0.983	-0.038	0.906	0.009	0.133	0.234
+53	0.007	-0.041	0.242	-0.138	0.393	0.199	0.524	0.001	-0.036	0.727

*: Uncorrected p-values shown, $\alpha = 0.05/10$ tests = 0.005.

Table 3.8. Associations between methylation of *OXTR* CpG units and behavioral and physiological measures

OXTR CpGs	Affiliative Behavior Proximity to Adult Females			Anxiety-like Behavior Self-scratching		Non-contact Aggression		OXT in CSF		
	R^2	β	p	β	p	β	p	R^2	β	p
-717	0.03	0.117	0.013	-0.451	0.04	-0.789	0.069	0.029	0.298	0.034
-749/751	0.006	0.093	0.264	0.482	0.189	-0.172	0.815	0.002	0.147	0.570
-769	0.009	-0.102	0.182	-0.121	0.73	-2.152	0.030	0.000	-0.042	0.854
-796/803	0.000	0.017	0.898	0.536	0.37	-0.747	0.529	0.001	0.189	0.634
-844	0.000	-0.006	0.886	0.248	0.219	-0.752	0.063	0.007	-0.138	0.289
-877/879	0.019	0.107	0.047	-0.200	0.419	-0.905	0.058	0.000	-0.030	0.854
-902	0.003	0.083	0.425	0.148	0.756	-2.713	**0.003***	0.000	0.0130	0.967
-925/932	0.002	0.099	0.488	0.656	0.311	-0.481	0.701	0.002	-0.218	0.626
-959	0.011	0.230	0.142	-1.213	0.122	-0.477	0.731	0.01	0.557	0.227
-1040/1043	0.005	0.096	0.334	-0.416	0.36	-2.636	**0.003***	0.001	-0.156	0.656
-1050	0.001	0.023	0.690	0.179	0.491	0.112	0.819	0.030	-0.390	0.030
-1062/64/68/74/79	0.000	0.027	0.797	-0.183	0.700	0.296	0.739	0.007	0.329	0.290
-1097	0.003	0.062	0.453	-0.26	0.504	0.022	0.975	0.009	-0.314	0.233

*:Uncorrected p-values shown, α = 0.05/13 tests = 0.003

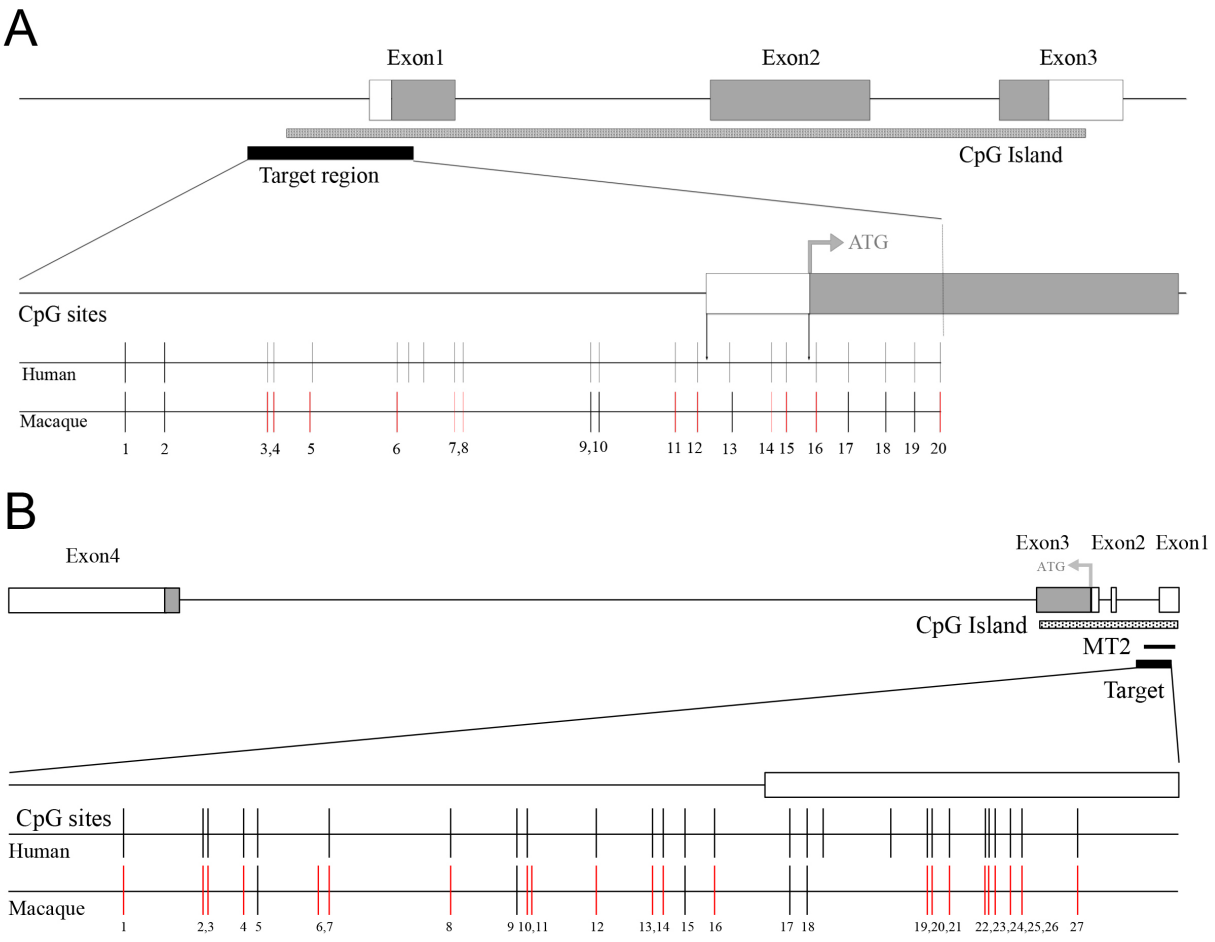

Figure 3.1. Genomic location of CpGs within target regions of OXT (A) and OXTR (B). The location of CpG sites analyzed in this study are indicated in red, whereas those indicated in black had poor spectra quality, had low call rates, or were duplicate CpGs. CpG locations are relative to the translation start site (TSS), which is indicated by 'ATG'. (**A):** *OXT (*MacaM, chr15: 35,896,090 – 35,896,991). Locations: -259 (CpG1), -244 (CpG2), -204/202 (CpG3,4), -188 (CpG5), -155 (CpG6), -133/130 (CpG7,8), -81 (CpG9), -78 (CpG10), -49 (CpG11), -40 (CpG12), -27 (CpG 13), -12 (CpG14), -6 (CpG15), +5 (CpG16), +17/32/43 (CpG17,18,19), +53 (CpG20). (**B):** *OXTR* (MacaM, chr03: 140,478,550-140,497,583) and MT2 region (MacaM, chr03: 140,497,407-140,497,002; Kusui et al., 2001). Locations: -717 (CpG1), -749/751 (CpG2,3), -769 (CpG4), -778 (CpG5), -796/803 (CpG6,7), -844 (CpG8), -867 (CpG9), -877/879 (CpG10,11), -902 (CpG12), -925/932 (CpG13,14), -944 (CpG15), -959 (CpG16), -993 (CpG17), -999 (CpG18), -1040/1043 (CpG19,20), -1050 (CpG21), -1062/1064/1068/1074/1079 (CpG22, 23, 24, 25, 26), -1097 (CpG27).

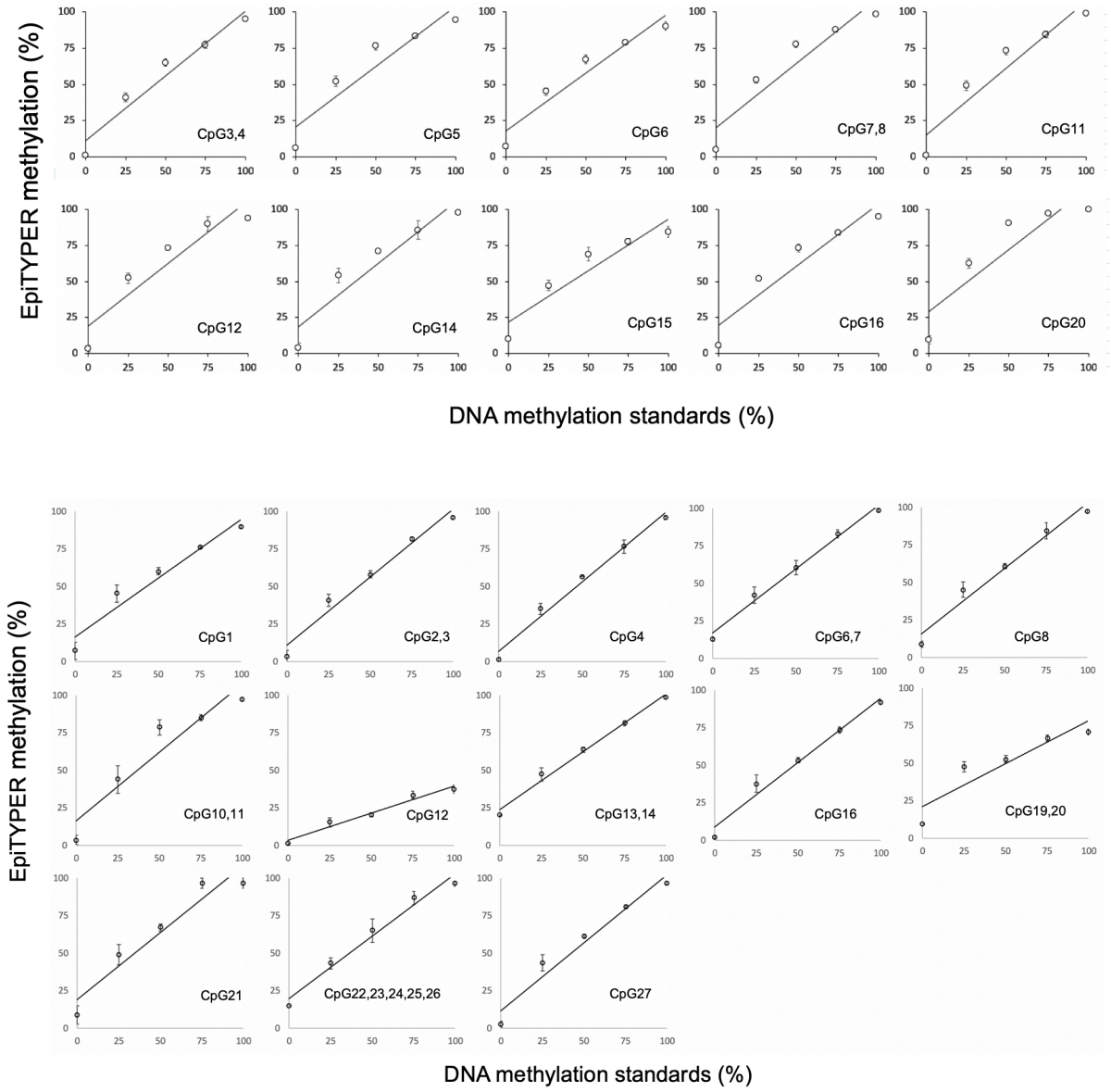

Figure 3.2. Validation of OXT (top) and OXTR (bottom). Linear association between unmethylated (0%), 50%, and 100% methylated positive controls for each CpG unit in the EpiTYPER assay. Although we did not detect any SNPs in our sample, methylation at the site CpG12 with *OXTR* may be explained by the presence of a heterozygous genotype at this locus.

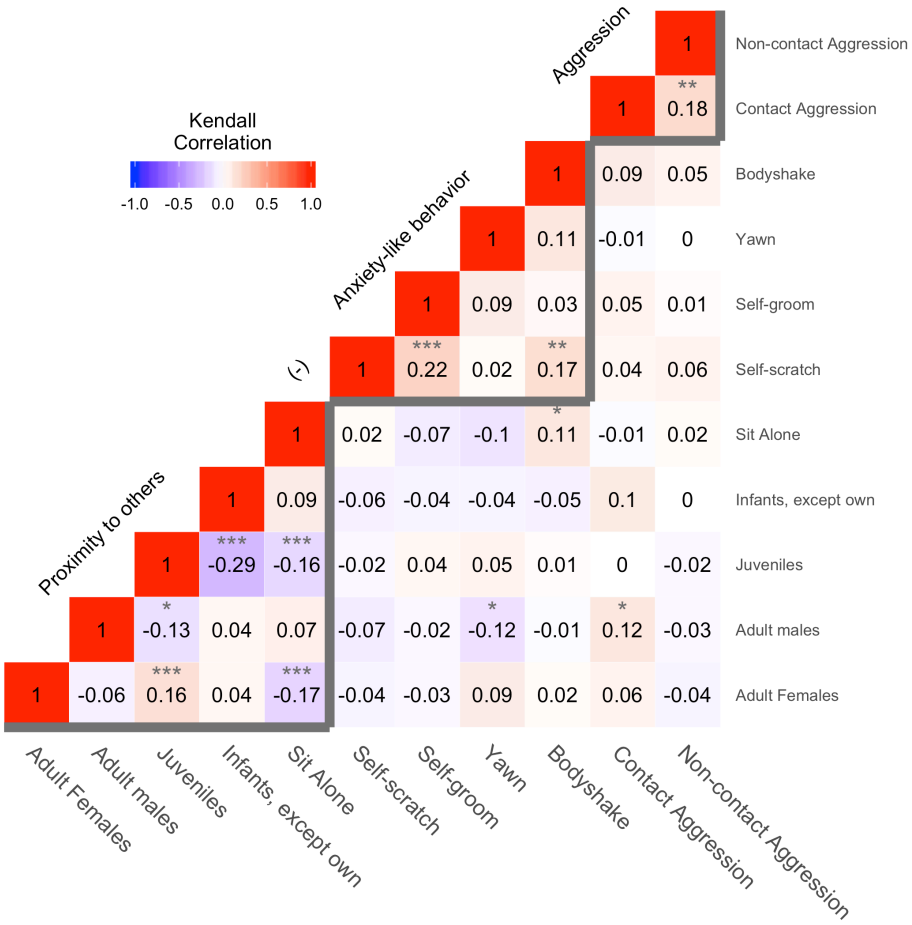

Figure 3.3. Correlation matrix of all behaviors recorded for this study. Individual behaviors were not strongly correlated with each other, as indicated by weak to moderate magnitudes. Correlations that were significantly different from zero are indicated with asterisks: $p < 0.05$ (*), $p < 0.01$ (**), $p < 0.001$ (***).

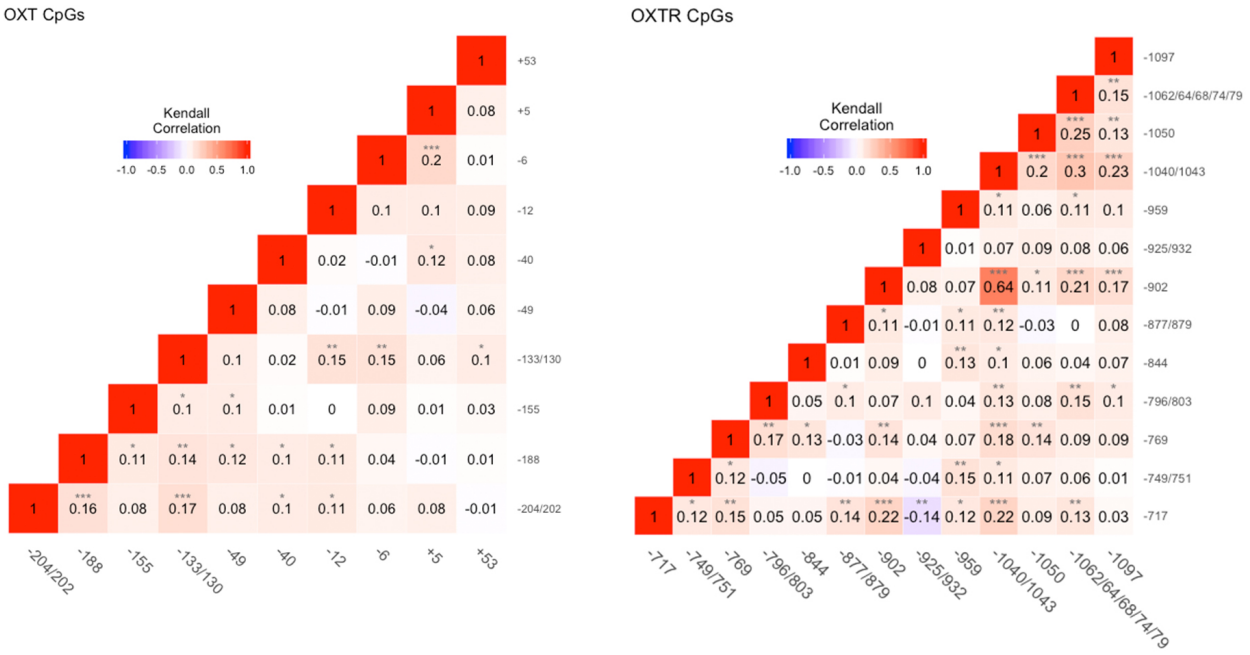

Figure 3.4. Correlations between CpG sites, for OXT (left) and OXTR (right). In general, CpG sites for each gene were not highly correlated (*OXT* mean correlation: 0.07, *OXTR* mean correlation: 0.10). Correlation values that were significantly different from zero are indicated with asterisks: $p < 0.05$ (*), $p < 0.01$ (**), $p < 0.001$ (***).

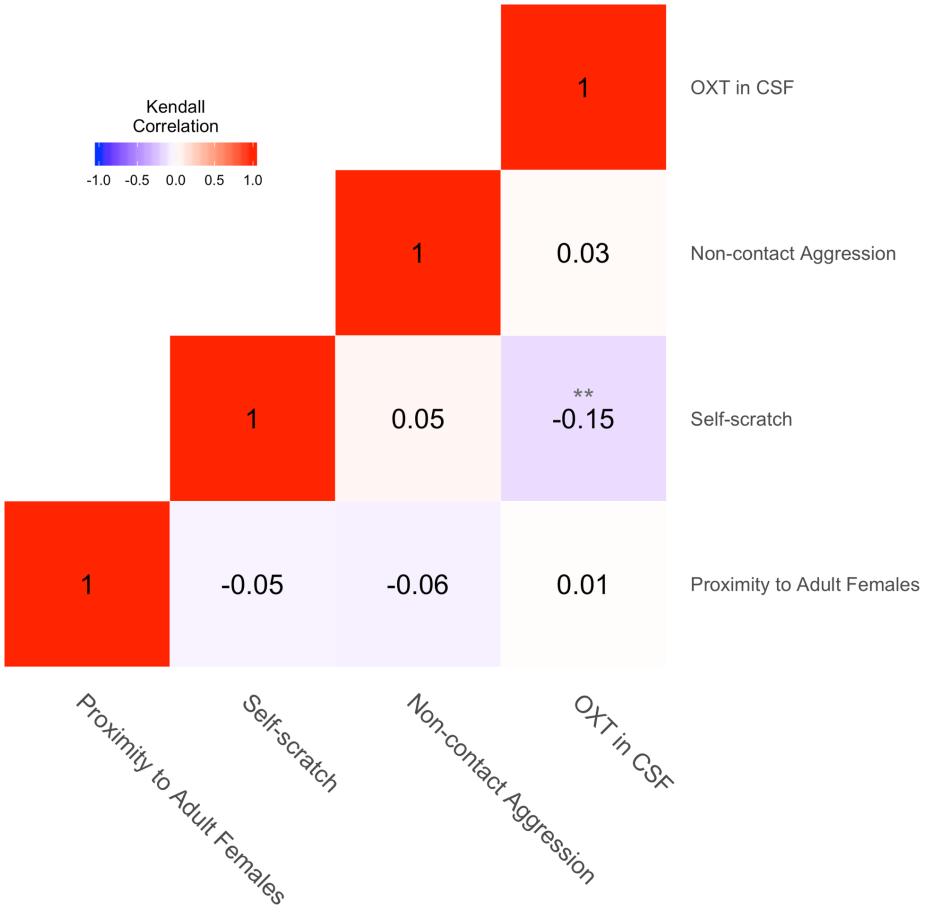

Figure 3.5. Correlations between behavioral measures and OXT in CSF (mean correlation: -0.03). Correlation values that were significantly different from zero are indicated with asterisks: $p < 0.05$ (*), $p < 0.01$ (**), $p < 0.001$ (***). The only pair of outcomes that was significantly distinct from zero was the anxiety like measure of self-scratching versus the concentration of OXT in CSF.

Chapter 4. General Discussion

The evolutionarily conserved neuropeptide OXT has a long history of reports documenting its critical influence on social behavior in a number of species (Johnson and Young 2017). The diversity encoded within *OXT* and *OXTR* genes has the potential to contribute to the breadth and flexibility observed in social behavior within a single species. Identifying the genetic determinants of individual variation in social behavior within typical populations are a first and important step for honing in on pathways which may be altered or disrupted in psychiatric conditions with social deficits.

The work described in this thesis was an exploratory analysis with the goal of identifying how variation in the OXT system of rhesus macaques influences complex social behaviors and central OXT levels. This was done by examining genetic variation of *OXTR* and epigenetic variation of *OXT* and *OXTR* in more than 200 socially-housed rhesus and quantifying their association with pro-social, anxiety-like, and aggressive behaviors, as well as central levels of OXT. Because the rhesus macaque is the most-widely used NHP model in biomedical research, assessing the effects of individual (epi)genetic variants on a behavioral and physiological outcomes carries the potential to complement other work in rhesus and create high translational impact.

Summary of findings

In chapter 2, the goal was to assess the contributions of SNPs in *OXTR* of rhesus on social behavior and central OXT levels. I used behavioral observations from a large, pedigreed sample of adult female rhesus macaques to estimate the heritability of pro-social, anxiety-like, and aggressive behaviors. I also estimated the heritability of OXT in CSF in a subset of animals. Estimates of heritability are useful for two primary reasons. First, they allow for inferences to be made about how much genetic factors (as opposed to environmental influences or other

external factors) contribute to the phenotypic variance of a trait. Identifying how much of the variance of behavioral and physiological traits originates from genetic versus non-genetic sources is important for the central question of this thesis. Second, in SNP association studies carried out in samples comprising related individuals, the quantitative genetic relationships used to assess heritability must also be incorporated into the statistical models to control for shared genetic background. Individuals which are more closely related will share more genes and thus may be more likely to be phenotypically similar on a trait for reasons of sharing a general genetic background and not for reasons of genotype at the specific SNP of interest. As such, it is necessary to include the matrix of quantitative genetic relationships constructed in heritability analyses as a random effect in statistical testing to avoid mis-attributing the influence of many shared genes to a specific SNP on the phenotype of interest.

Heritability estimates for measured traits were moderate in this study, ranging from approximately 20-30%. The credible intervals around these point estimates were wide, indicating a large degree of uncertainty in heritability estimates. However, this is comparable to what has been observed in other reports of heritability for socially-relevant criteria in NHPs. For example, Staes et al. (2016) report bonobo heritability of the sociability personality dimension to be between 1-46%. In our study, the heritability of aggression could not be estimated. This may have been due to issues of “low-information”, in which the occurrence of the behavior was infrequent, and the sample size was not large enough for the given number of kin relationships to provide meaningful estimates for such rare events (Wilson et al. 2010). Other studies that have carried out heritability analyses of other traits in similarly-sized samples of macaques as the ones here have drawn similar conclusions (Chi et al. 2014).

For traits for which heritability could be estimated, no significant effect of individual SNPs were found on OXT CSF concentrations or behaviors that were measured. This included one non-synonymous SNP which has previously been reported to rescue an effect of behavioral deficits in animals that were peer-reared (Baker et al. 2017). The cumulative contribution of all 13 SNPs together did not significantly explain variance in any outcomes either. The main findings from this chapter demonstrate that there are considerable heritable genetic effects of social behavior and OXT CSF, but the genetic contributions of the examined variants within *OXTR* specifically, are likely negligible under these conditions.

In chapter 3, the goal was to assess epigenetic sources of individual variation in behavior and OXT in CSF. I quantified the association between peripheral epigenetic markers near the promoter regions of *OXT* and *OXTR* with the same naturally occurring social behaviors and OXT CSF concentration. Methylation signatures for CpG sites were not significantly heritable (data not shown). Despite other reports that CpG sites within the same genes tend to be similarly methylated (Haas et al. 2016; Perkeybile et al. 2019; Barrera and Peinado 2012), I did not find strong correlations across CpG sites in either *OXT* or *OXTR*. No CpG sites from *OXT* showed a significant association with the social or physiological outcomes I measured. The strongest associations in this particular sample came from *OXTR* epigenetic markers with aggression, but these did not survive correction for multiple comparisons. In summary, peripheral epigenetic markers did not have robust effects on the social behaviors or physiological outcomes measured here. This was the first study of its kind to measure *OXT* and *OXTR* methylation in association with naturally-occurring behavior in adult rhesus. Studies of epigenetic associations are relatively still in their infancy compared to genetic association work with respect to samples sizes and number of published studies (Maud et al. 2018). In humans, decreased methylation of *OXTR* has inconsistently been associated with impaired

social/emotional outcomes, but the existing studies display a great deal of heterogeneity in quantification techniques, types of tissue, CpG sites, and behavioral or emotional outcomes, making it difficult to draw reliable conclusions about true the relationship (Maud et al. 2018). Continued work in this area is merited to gain better estimates of true effect sizes and directionality

It is worth noting that all of the epigenetic marks investigated in this study originate from peripheral samples in blood. There is considerable debate about whether peripheral methylation is a valid proxy for methylation marks in brain tissue, with some studies reporting gene-specific concordance across tissue types at strong correlations between 0.60 to 0.83 (Perkeybile et al. 2019; Provençal et al. 2012; Byun et al. 2009). However, some evidence indicates that the correlation drops considerably when considering only the subset of variably-methylated CpG sites and genes relevant to brain processes (Walton et al. 2016). Studies investigating this relationship in humans have used relatively small samples (Byun et al. 2009; Davies et al. 2012; Walton et al. 2016), so more work in this area in larger samples is merited. Despite the uncertainty in our current understanding of this peripheral measure's value with respect to predicting brain methylation, the continued investigation of blood methylation marks is worthwhile. Even if CpG marks have weak-to-moderate predictive value, having any such biomarker for brain status in easily-accessible tissue would provide an undeniable boon in clinical and diagnostic contexts.

Weak behavioral associations of OXT and OXTR variants in rhesus

Taken together, the findings in chapters 2 and 3 of this thesis do not provide strong support for a robust influence of the selected markers within *OXT* and *OXTR* genes on social behavior or OXT CSF in rhesus. However, the negative findings do not imply that OXT plays no role in

modulating the social or physiological outcomes described. Instead, there are a few possibilities of biological and statistical relevance that could explain the lack of associations. First, it is possible that most of the specific markers we investigated do not have functional downstream consequences. One hypothesis of this thesis was that particular sites of (epi)genetic variation could affect the molecular machinery of the oxytocin system. While this could be via non-synonymous SNPs, which would purportedly lead to the largest effects by resulting in change to the resultant protein, other variants (e.g. synonymous SNPs) in non-coding regions could differentially recruit transcription binding factors or modulate chromatin folding and affect transcription rates or protein concentrations via these indirect mechanisms. Though an informed approach was used to identify variants in gene regions thought most likely to affect transcription or downstream expression, the possibility exists that the SNPs and CpG sites selected here do not functionally impact downstream molecular mechanisms.

Alternatively, there could indeed be a true association between these variants and the behavioral and physiological outcomes measured, but due to random sampling of the subjects and the small sample size in the study this relationship was not captured. Replications attempts with other samples of rhesus would resolve this. However, an additional and more likely explanation is that each marker of genetic and epigenetic variation at these sites does in fact contribute to the complex phenotypes of social behavior, but the individual effects of each are weak. The estimation of the true effect of individual genetic variants in rhesus is difficult to assess because there are not enough studies to conduct a meta-analysis. However, approximations of effect sizes can be pulled from comparisons to human genetics studies. During the advent of association studies in human genetics, variants of large magnitude were optimistically identified for numerous traits and diseases. However, the accumulation of more studies with increasingly larger, and larger samples sizes eventually converged on the

conclusion that these early estimates were inflated (Ioannidis 2008). In the past decade, the human genetics field has largely come to the consensus that complex social behaviors are polygenic and that common variants typically have very small effect sizes (Flint and Munafò 2013).

Specifically relevant to understanding social behavior at the clinical level, relatively weak influence of genetic effects has been reported in GWAS and whole genome sequencing studies of ASD as well. The genetic associations with greatest observed effects sizes appear to be particularly rare, and for these reasons their reliability has been difficult to confirm since identifying large enough cohorts of affected individuals is challenging. For example, the link between the *CHD8* gene and ASD has been one of the most robust findings from ASD whole exome sequencing approaches. The protein produced from this *CHD8* is involved in repressing transcription, and many of its binding targets also happen to be ASD risk genes (Vorstman et al. 2017). Truncation mutations in this gene have been identified in ASD cohorts and have been absent in control cases (Bernier et al. 2014). Despite the large effects observed, the truncation events occurred in less than 0.05% of the nearly 4,000 ASD patients screened (Bernier et al. 2014). Aberrations in other genes related to ASD besides *CHD8* are likely to occur in even smaller proportions. Given this, the recommendation in the ASD-genetics field has been to move away from a focus on single genes and their variants and to focus instead on gene pathways and broader processes in which these genes play roles (Bernier et al. 2014).

As discussed, the effect sizes in human genetic association studies have consistently required very large samples to reliably detect the small effects of these individual markers. However, because the rhesus macaque model provides a controlled experimental context with reduced environmental noise (e.g. controlled diet and environment, living conditions, etc.), a plausible

hypothesis would be that observable effect sizes are larger in rhesus than in humans. This would imply that a smaller number of rhesus subjects would be necessary to reach adequate statistical power for a candidate gene study. If this were the case, this logic could be followed by comparing heritability estimates of comparable social traits in rhesus and in humans. Specifically, one would expect the heritability estimates of rhesus in standardized environments to be greater than those reported in humans. This is because narrow-sense heritability (h^2) is calculated as $h^2 = V_a / (V_a + V_e)$, where V_a is the phenotypic variance due to additive genetic factors and V_e is the phenotypic variance due to all other sources, including environmental factors and/or measurement error. A standardized environment, such as the one in which the rhesus in our studies live, would be expected to decrease the value of V_e. This would leave the variance attributable to genetic factors, V_a, to explain an increasing proportion of the total phenotypic variance --- in other words, increased heritability. However, we observe no such increase in heritability estimates in our sample of rhesus macaques compared to what is observed in humans for various complex traits, including those in social domains (Polderman et al. 2015). Taken together, this suggests that the genetic variants in rhesus likely have similar weak effects on complex traits, as observed in human studies. Thus, a genetic association study of ~200 rhesus macaques would still be underpowered to detect what are likely small effects of variation in *OXT* and *OXTR* genes on complex social behaviors. The only additional study which has also investigated variants of *OXTR (*and the related *AVPR1a*) gene and its relationship to social behavior in a similarly-sized group of rhesus macaques drew similar conclusions (Madlon-Kay et al. 2018). The aforementioned study found effect sizes of *OXTR* SNPs to be close to zero for individual variation on rates of grooming, approaches, passive contact and aggression (Madlon-Kay et al. 2018).

Moving forward with behavioral genetic NHP studies

With respect to the low heritability of these social traits in macaques, where the studies can reduce the variance attributable to environmental factors through high control on factors such as diet, prenatal exposure to drugs, etc., my findings could reflect the high complexity of the social traits studied and the important role of social experiences/environment shaping them (e.g. social rank, quality of maternal care, family/network size, troop social dynamics). Now, it could also be related to the broader challenges of conducting extensive, multi-year studies in rhesus. Because expected genetic effect sizes are small, attempts to quantify genetic associations with behavior are particularly vulnerable to external sources of noise (for example, those related to data collection). While the rhesus model does minimize environmental noise in many ways, data collection in studies of large groups of socially-housed NHPs requires complex coordination and consistency across a large team of people, often over the course of several years. Maintaining a stringent level of consistency throughout the duration of a study is not trivial. For example, inter-rater reliability criteria of 80% or greater are put in place to help standardize the data collection process. However, this minimum threshold may not be sufficient given the sensitivity of genetic association studies to any sources of variation that are non-genetic, including potential bserver bias. Additionally, in these studies, subjects were each observed for an average of 80 minutes across 4 observation periods, but this is likely not long enough to reliably capture a subject's socio-behavioral traits, particularly for behaviors with lower occurrences. Increasing the length of observation time would substantially reduce the influence of random sampling for assessed traits, given that displays of particular behaviors can also be impacted by social group composition, weather, time of day, etc. While accounting for the most influential covariates in the final statistical model can account for these external sources of noise, an exhaustive inclusion of parameters risks the overfitting of the statistical model, deteriorating the inferential value of the study's results (Babyak 2004). In smaller NHP studies of apes, observation time per individual can range on the order of tens of hours (Staes et al. 2016; Koski 2011). Investment in

new technologies which allow behavioral tracking of monkeys through automated processes, some of which are already being developed by investigators at Yerkes (Wallen 2018), may allow for collection and analysis of much larger datasets, while minimizing variability across multiple-person teams of observers.

Future genetic association studies in rhesus macaques should also include a strong focus on outcome measures that are biologically closer to their genetic antecedents, such as receptor expression levels and function in specific brain regions in rhesus. These efforts will yield larger effect sizes that will have a greater likelihood of being statistically well-powered. It should still be noted, however, that examining these more proximate outcomes should not be coupled with a decrease in sample size. Required sample sizes will likely still be large. Electrophysiological endophenotypes, for example, are thought to be influenced by tens of thousands of genetic variants, and identifying the contributions of individual variants on these outcomes would likely still require sample sizes within the hundreds or thousands (Iacono et al. 2017). Brain tissue collection in primates in large numbers is not trivial, but over the course of many years opportunistic banking of brains across multiple primate centers could result in a sizeable collection of samples to be adequately powered.

Lastly, the issue of statistical power is paramount across all of neuroscience, and NHP work is no exception (Button et al. 2013; Nord et al. 2017). Association studies that are underpowered produce effect size estimates that are not only implausibly inflated but are also likely to show the opposite relationship between predictor and outcome (Gelman and Carlin 2014). En masse, unpowered studies can lead to an accumulation of unreliable findings in the field. The most straightforward recommendation for genetic association studies of any kind in NHPs is to move towards increasing sample size and striving for appropriate statistical power. However, a recent

review of major U.S. NHP service providers completed by the NIH Office of Research Infrastructure Programs (NIH ORIP) cites that planned use of rhesus macaques in research-driven or infrastructure-related projects during the fiscal years for 2013-2017 exceeded 40,000 animals, and current NHP centers have already cited shortages in meeting the current demand for rhesus (National Institutes of Health, Office of Research Infrastructure Programs 2018). Furthermore, the demand for rhesus in research is forecasted to increase in the coming five years. The NIH ORIP review also cited that more than half of surveyed investigators working with NHPs report programmatic barriers to their current research progress, including budget constraints such as cuts on funding, NIH policies that place caps on direct costs, rising costs of purchasing NHPs, and caps number of animals permitted for use. Given all of these constraints, it is unlikely that large-enough sample sizes for behavioral genetics work in rhesus will be able to be amassed in the near future through efforts of individual investigators alone. These impediments to research progress would likely need to be addressed by convening of a panel of experts, along with the cooperation of funding agencies and NHP centers, to consider ways that funding for NHP work can be restructured in the future. Encouraging the pooling of animal resources for distinct but complementary investigations is another means of maximizing the use of this particular animal model.

Though they make up less than 0.5% of animals in biomedical research, NHPs are critical animal models that provide a translational bridge from the fundamental work being done in rodents to humans. They are, therefore, an indispensable model for translation to the understanding and treatment of human conditions. The use of the rhesus macaque in research has the potential to produce results with greater translational validity than the rodent model because of the close genetic, cognitive, and physiological resemblance to humans. As such, it is

important that the field optimize translational validity of this model by carrying out well-powered experimental designs in NHPs.

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