Early Life Experience Alters Epigenetic Variation and Expression of the Oxytocin Receptor Gene

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Acknowledgements

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Summary of dissertation

Scientists love to question the world around them, and in this process many have noticed differences in social behavior, leading some to ask "What are the biological mechanisms driving social behavior?" This dissertation aims to provide a few more pieces to this complex puzzle by providing evidence in both humans and in an animal model that early life environment shapes the epigenome at a gene associated with social behavior, the oxytocin receptor gene (human: *OXTR;* animal model: *Oxtr*). We found that the amount of parental care received in early life is associated with significant differences in the epigenome and expression in *Oxtr*.

Our 6 key findings build upon previous work indicating that the amount of parental care received in early life can alter the developmental trajectory of social behavior in both humans and in prairie voles (*Microtus ochrogaster).* In both species, those that received more parental care when they were young tend to develop into adults that displayed more social behavior in comparison to those that received less parental care. For example, children that received more parental care typically developed into adults that experienced fewer feelings of loneliness, depression, low self-esteem, and anxiety. Additionally, children that received more parental care are also more likely to become adults who feel like they have high quality friendships and secure romantic relationships. This suggests that the amount of parental care received in early life may alter the developmental trajectory of emotional function and social behavior in humans. In prairie voles, an animal model of social behavior, pups that received more parental care typically developed into adults that become high care parents when they produced their own offspring,

displayed more partner preference behavior, spent more time in social investigation towards novel prairie voles, and displayed more parental care behavior towards novel pups that they did not produce. In both humans and in animals, if offspring born to low care parents are switched to high care parents early in life, the offspring will typically develop into adults with the social traits of their adoptive high care parents. This indicates that parental care might influence certain aspects of neurobiological and social development more so than genetic inheritance, and this could be associated with the epigenome and expression of the oxytocin receptor gene.

The first chapter of this thesis focuses on exploring the outcome of prairie voles raised by high care parents, with higher amounts of parental care induced by a previously established handling paradigm, in comparison to the control group. Pups raised by high care parents developed into young adults with lower amounts of DNA methylation at select CpG sites (-934_1, -934_2, -924, and -901) within a regulatory region of *Oxtr* and they also had higher levels of *Oxtr* expression in a region of the brain that is associated with social behavior, the nucleus accumbens, in comparison to the control group. We also provide evidence that receiving higher amounts of parental care typically maintains the epigenetic state similar to antepartum pups that did not receive any parental care, as animals that were raised by high care parents had similar levels of DNA methylation in brain tissue of animals collected *in utero* on the expected day of birth. Additionally, in a separate cohort, we provide evidence that the amount of maternal care received correlated with oxytocin receptor density, with animals that received more maternal care in early life corresponding with more oxytocin receptors in the nucleus accumbens. Previous studies indicate that more oxytocin receptors present in this region of the brain correspond with increase display of species typical social behaviors. Collectively, there is strong

evidence that high amounts of parental care received in early life maintains a hypomethylated epigenetic state at select CpG sites in a regulatory region of *Oxtr*, an increase of *Oxtr* expression, a higher amount of oxytocin receptors, and an increase of social behavior. Therefore, to better understand the developmental trajectory of social behavior, we should further explore how various environmental factors influence epigenetic marks in the oxytocin receptor gene.

The second chapter further explores the epigenetic landscape of commonly studied (but poorly understood) regions of *OXTR* in humans, a region known as MT2 and a segment within exon 3. The MT2 region is within the promoter region and previous *in vitro* research indicated that DNA methylation in this region greatly impacts *OXTR* expression. Additionally, studies in humans indicate that DNA methylation in this region is associated with dysregulation of human social behavior. In chapter 1 we determined that DNA methylation in CpG sites -934_1, -934_2, -924, and -901 in the conserved MT2 region in prairie voles correspond with decreases of *Oxtr* expression. However, it remained unknown if DNA methylation at the other CpG sites within the conserved MT2 region could correspond with changes in oxytocin receptor gene expression. Additionally, research in chapter 1 was exclusively done in our animal model and not in human brain tissue. While human brain tissue is difficult to assess, we were able to acquire brain tissue that is associated with social behavior. Therefore, I wanted to expand our understanding of this system by measuring the level of DNA methylation at CpG sites throughout the entire MT2 region and identify which CpG sites were associated with differences in oxytocin receptor gene expression in brain tissue in both humans and in our animal model.

In investigating the MT2 region, I determined that the entire MT2 region is highly conserved between humans and prairie voles, and both have a cluster of CpG sites which are hypermethylation in comparison to the other CpG sites in the MT2 region. The MT2 region is within a CpG island, in the promoter region, and upstream of the transcription start site. Typically, these types of regions have low levels of DNA methylation, yet when DNA methylation does occur, it tends to greatly alter gene expression. Finding a cluster of CpG sites that have higher levels of DNA methylation in the MT2 region suggest that this homologous region may be functionally important in regulating oxytocin gene expression in both humans and in prairie voles.

Also, in both species, this cluster of CpG sites has higher measures of DNA methylation variability in comparison to the rest of the CpG sites within the MT2 region. This is notable since CpG sites with variable levels of DNA methylation tend to be more susceptible to environmental stimuli. Within the same individual in both humans and animals, we found that this cluster of hypermethylated CpG sites, which have more variance in DNA methylation levels, also corresponded to oxytocin receptor gene expression. In humans, CpG sites -934, -924, and -901 are within this notable cluster of CpG sites and increases of DNA methylation at these CpG sites corresponded to decreases in *OXTR* expression. Excitingly, this cluster of CpG sites are directly conserved in prairie voles (-934_1, -934_2, -924, and -901) and increase of DNA methylation at these CpG sites also corresponded to decreases of *Oxtr* expression.

The similarities between the cluster of CpG sites in humans and prairie voles in the MT2 region (which include homologous CpG sites -934, -924, and -901) such as the state of

hypermethylation, higher measures of variance in DNA methylation, and an inverse relationship between DNA methylation and oxytocin receptor gene expression strengthens the choice of using prairie voles as an animal model of human social behavior. This also indicates that our findings in prairie voles may mirror the biological mechanisms influencing social behavior in humans.

In addition to the MT2 region, DNA methylation within a segment of exon 3 in *OXTR* has also been associated with dysregulation of social behavior in humans. Additionally, this exonic segment is conserved in mice, and previous work in a mouse model suggests that increases of DNA methylation at this region are associated with deceases in *Oxtr* expression and social behavior. Yet, no known study has investigated this region in prairie voles. First, I determined that the exon 3 segment previously studied in humans and in mice is highly conserved in prairie voles. The exonic segment of interest is within the coding region, which is a segment of DNA that contains the code for the downstream production of RNA and proteins, and coding regions tend to be evolutionary conserved due to their functional value. The similarity in the exonic region suggest that our findings in prairie voles may parallel the biological mechanisms influencing social behavior in both humans and mice. Second, the DNA methylation landscape across this conserved exonic segment in prairie voles remained unknown. Therefore, I measured the levels of DNA methylation across the CpG sites in the conserved exonic segment within the nucleus accumbens in the same cohort of prairie voles used in the MT2 studies. In comparison to the MT2 region, the exonic region is hypermethylated, which is expected in an exonic region. Additionally, I identified four clusters of CpG sites across the exonic segment which have highly correlated levels of DNA methylation. Increases of DNA methylation at two

CpG sites in cluster 1 (CpGs: 2, 4) and eight CpG sites in cluster 3 (CpGs: 20, 22, 23, 24, 26, 31, 32, 36) correlated with decreases of *Oxtr* expression. Additionally, in cluster 3, I identified two synonymous transition single nucleotide polymorphisms at CpG sites (CpG 25 and CpG 33). In both polymorphic CpG sites, the common variant is GCG and the rare variant is GCA, both are alanine codons. One of these polymorphic CpG sites, CpG 25, might be associated with differences in *Oxtr* expression, although this finding should be explored further with a larger sample size. Our research suggests that DNA methylation at select CpG sites in the exonic region may influence *Oxtr* expression and there are two polymorphic CpG sites in this region.

Following the characterization of the epigenetic landscape in the MT2 region and exonic region, I wanted to further explore my hypothesis that early life environment might shape DNA methylation in the conserved MT2 and exonic region. Therefore, I examined the differences in DNA methylation in the MT2 and exonic region between animals raised by parents that were experimentally manipulated to become high care parents from animals raised in a control condition. In prairie voles, when comparing offspring which received more parental care in early life to the control group, only CpG sites 14, -934_2, -934_1, -924, and -901 in just the MT2 region of *Oxtr* showed a significant difference in DNA methylation following post-hoc analysis, with animals raised by high care parents having significantly lower levels of DNA methylation at these sites. When examining the relationship between DNA methylation and *Oxtr* expression of these CpG sites, only CpG sites -934_2, -924, and -901 following post-hoc analysis had a significant or strongly trending correlation. At these CpG sites, increases in DNA methylation correlate with decreases in *Oxtr* expression. This indicates that out of all the CpG sites in the conserved MT2 and exonic region, homologous CpG sites -934_2, -924, and -901 are

environmentally sensitive to early life parental care, and the state of DNA methylation of these sites are associated with differences in *Oxtr* expression.

Additionally, we provide evidence that DNA methylation at CpG sites -934_1, -934_2, -924, and -901 in the MT2 region of *Oxtr* in brain tissue have a strong correlation to the same epigenetic marks in whole blood within an individual, indicating we can use epigenetic measures in whole blood tissue as a proxy for levels of DNA methylation in the brain at these CpG sites as discussed chapter 1. These CpG sites are highly conserved between prairie voles and humans and they are the CpG sites, which when methylated, primarily effect *OXTR* expression as discussed in chapter 2. This indicates that we can measure DNA methylation in a more accessible tissue in humans, whole blood, and still gather relevant biological markers associated with social function. This finding helped guide our study in which we explored the relationship between human adolescent perception of early life maternal care and the developmental outcome of DNA methylation at key CpG sites in *OXTR* and emotional states associated with social behavior in adulthood as discussed in chapter 3. We measured DNA methylation at CpG sites -934 and -924 within *OXTR* in whole blood, and it was found that adolescents who perceived that they received higher amounts of maternal care developed into adults with lower levels DNA methylation in comparison to adolescents that perceived receiving less maternal care. Additionally, DNA methylation of these CpG sites are predictive of emotional feelings associated with social behavior in adulthood, such as loneliness, depression, anxiety, romantic stress, fatigue, and somatic pain.

Collectively, this dissertation provides evidence for 6 key findings as follows. First, *OXTR* expression is, in part, regulated by DNA methylation at key sites that are homologous between humans and prairie voles. Second, the amount of parental care received in early life of prairie voles corresponds to a distinct pattern of DNA methylation at CpG sites in the conserved MT2 and exonic region of *Oxtr*. Notably, with an increase of parental care significantly corresponding to decreases of DNA methylation at CpG sites 14, -934_1, -934_2, -924, and -901 in the MT2 region. Third, we provide evidence that the more parental care received in early life also corresponds to an increase of *Oxtr* expression. Additionally, DNA methylation at CpG sites -934_2, -924, and -901 in the MT2 region strongly trend or significantly correspond to *Oxtr* expression. Fourth, we discovered that the more maternal care received in early life corresponds with an increase of oxytocin receptor density in the nucleus accumbens. A collection of previous research indicates that both higher amounts of parental and more oxytocin receptors in this region of the brain corresponds to increases of species typical social behavior. Fifth, importantly for future research in humans, we determined that the methylation state of key CpG sites in *Oxtr* is correlated in the brain and blood, which suggest the utility of blood as an important biomarker for the brain. Finally, we provide evidence in humans that the amount of perceived maternal care in adolescence influences DNA methylation at a key CpG site in *OXTR* in adulthood, and DNA methylation of this CpG site is a predictor of emotional functions that are associated with social behavior. Building upon previously published work, I suggest that the amount of parental care received in early life in both humans and in our animal model may shape social behavior through the epigenetic regulation of the oxytocin receptor gene. The research and data analysis in this dissertation provides further evidence for environmental regulation of social behavior through direct actions on the epigenome at the oxytocin receptor gene.

Introduction

The development and biological mechanisms of social behaviors are intriguing to scientists, especially since humans are a highly social species. Yet, if scientists are trying to learn more about human behavior, there are limited types of research that can be done directly with humans. Therefore, it is important to have an animal model in which the biological system of interest acts similarly to humans. A little critter known as the prairie vole has been instrumental in expanding our understanding of the neural mechanisms of social behavior. Voles (genus *Microtus*) most likely originated in Asia and the fossil record indicates that they crossed the Bering Land Bridge into North America 2.1 million years ago (Zakrzewski 1985; Conroy and Cook, 2000), where they quickly diversified. In modern times, voles continue being a diverse species, with over 66 distinct species worldwide (Jones 1997). Prairie voles (*Microtus ochrogaster)* are distributed across north-central North America and live in communal underground nesting areas (Getz and Carter, 1996). Prairie voles are small furry rodents (Figure 1), yet the characteristics that really makes these creatures stand out for science research purposes is their pronounced display of social behaviors that are relatively common in humans, but are very rare in other mammals.

Prairie voles form pair-bonds, a social behavior that brings males and females together in social monogamy (Getz et al., 1981). Pair-bonded mates will raise their young together, with mom and dad taking turns providing for their young (Wang and Insel, 1996). Most animals find the calls of neonates aversive, for example virgin female rats typically take 2-3 days of exposure to stop trying to avoid neonates and 5-7 days of exposure to begin showing maternal care

(Rosenblatt, 1967). Whereas both male and female prairie voles throughout their lifespan, with or without previous exposure to prairie vole pups, will typically start displaying parental care immediately when exposed to a neonate (Oliveras and Novak, 1996; Roberts et al., 1998). Both humans and prairie voles share many of the major components of species typical social behavior, both of these species pair-bond, both mom and dad typically raise their young together, they will take care of the young within their own species that they did not produce, and are highly affiliative, choosing to spend much of their time in the company of others of their own species. Importantly, in choosing prairie voles as a model of social behavior, these social characteristics are rare in other commonly used animal models, with just 3-5% of mammals known to display pair bonding behavior, including humans and prairie voles (Kleiman, 1977).

A) Prairie vole

B) Montane vole

Figure 1: A) Representative image of the prairie vole (*Microtus ochrogaster)* that exhibit typical social behavior such as pair-bonding, biparental care of offspring, alloparental care (display of parental behaviors towards pups that are not offspring), and highly affiliative behaviors. B) Representative image of the montane vole (*Microtus montanus*) in contrast, spend most of their time in isolation and do not display the characteristic social behaviors of prairie voles. Image A: (Ahern, T., Emory University) Image B: (McHugh, T., Science Source).

How did scientists discover this unusual and rare social behavior in prairie voles? It all started in the 1970's as Lowell Getz undertook a population study nearby University of Illinois at Urbana-Champaign in which he started to notice a unique pattern in the prairie voles he trapped (Tucker, 2014). In comparison to other types of voles, he noticed that prairie voles were more likely to be caught in pairs, one male and one female. Following up on his hunch that there must be a reason why prairie voles were more likely to be trapped in pairs, he hooked the animals up to radio collars that allowed him to track their location. He discovered that the pairs, when released back into the wild, spent nearly all of their time together in the same den (Getz et al., 1981). This finding opened up a resounding possibility that prairie voles formed pair-bonds.

Getz shared this finding with one of our collaborators in this dissertation, Sue Carter, and she was one of the first people to be interested in further understanding the neurobiological mechanism of social behavior discovered in prairie voles. As more research was conducted, field observation data indicated there were three common categories of social structures in prairie voles: male-female pairs, communal groups, and single females (Getz et al., 2005). The fundamental social structure of prairie voles typically start with a male-female pair, and then form into communal groups when offspring do not leave the nest and/or unrelated adults join the nest (Getz et al., 1990; Getz et al., 1993; Getz and Carter, 1996). Typically, the majority of single females are survivors of a previous male-female pair, and they will often soon join a communal group following the death of their pair-bonded mate.

The mating system of prairie voles is nuanced and heavily regulated by touch and chemosignaling, and is believed to serve to help prevent incest in communal groups that tend to

be mostly comprised of related animals. Typically, in communal groups there is only one reproducing female who tends to prefer, but not exclusively, mate with her pair bonded male. Estrus needs to be induced in females, and it is induced by chemosignaling derived from male urine and male naso-genital sniffing (Carter et al., 1989). Typically, females will only allow non-familiar or pair-bonded males to participate in naso-genital sniffing, and although prairie voles are socially monogamous, there is a range of mate guarding and sexual fidelity behavior (Carter et al., 1980; Ophir et al., 2008). It is common for prairie voles to also mate with wandering non-related animals from different nests, resulting in communal groups with offspring of mixed parents.

Prairie voles mate year round, with a gestation period of 21 days, and typically produce 3-6 young per litter (Getz and Carter, 1996). Offspring reach independence at around 3 weeks old, reach sexual maturity between 30-45 days old, and are considered fully developed adults at 60 days old (Getz and Carter, 1996). The majority of the offspring will remain in the nest that they are born into and will often help in raising new litters of pups, a behavior referred to as alloparental care (Getz and Carter, 1996). The observation that pair bonds and the following communal groups are the default social structure of prairie voles indicate that these animals have evolved to rely on social traits in order to achieve more fitness. It is proposed that the social traits of monogamy, mate guarding, philopatric offspring, communal nesting, and territorial characteristics are a result of evolving in the low food habitats, high nest predation, and low population densities (Getz et al., 1993). In environments with low food, high predation, and few mate potentials, there is a benefit to pair bonding in which little energy would be needed in searching for mates. The additional characteristic social traits of prairie voles are believed to be

originally tied to pair bonding behavior, but the exact environmental pressures shaping the current complex social behaviors of this species of animals are still unknown. However, much of what we do understand about their social behavior was gathered from laboratory experiments and observations, as there are many limitations to studying prairie voles in the wild.

Sue Carter brought these critters into the lab to further study prairie vole social behavior, and excitingly she discovered that the pair bonding, mating, and affiliative behaviors typical for this species continued to be displayed in a lab setting. However, certain conditions in the lab differ from wild. The lifespan of a prairie vole in the wild is often short, as they typically do not live past 60 days because of predation (Getz et al., 1997). Yet in the lab, typically the colony breeders remain in steady pair-bonded relationship for up to three years, with offspring weaned every 21 days. Experimental animals are typically kept in same-sex sibling pairs following weaning, and following postnatal day 60, animals either remain with their siblings or are paired with a similarly aged animal of the opposite sex. Bringing these animals into the lab has allowed us to study social behavior following various experimental manipulations, which allowed scientists to further explore the neurobiology of social attachment in a model organism that displays social behavior that is more similar to humans in comparison to other commonly used rodent species.

Many insights into the biological mechanisms of social behavior have been made from contrasting the behavior and biological systems of prairie voles to a near-identical species, the montane vole (*Microtus montanus*) (Figure 1). Many types of voles, like the montane vole, do not form pair-bonded relationships, do not share parental responsibilities, do not show parental

behavior to strange pups, and live most of their lives in isolation (Oliveras and Novak, 1996). Prairie voles and montane voles have vastly different social behaviors, but intriguingly, they share very similar genomes (Young, 1999). If their DNA code is similar, what other biological factors could be causing such stark differences in social behavior between these two species? One of the first hypotheses that was put to the test was to see if the oxytocin system was different between prairie voles and montane voles.

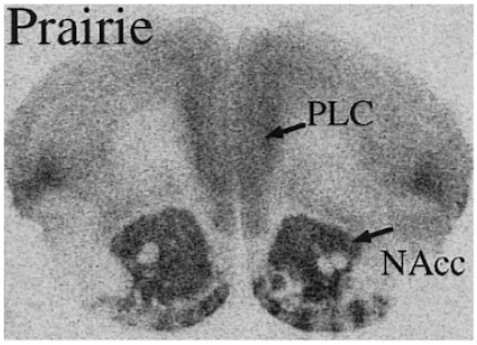
Oxytocin is a neuropeptide consisting of nine amino acids, which are in a ring structure with a tail. Oxytocin is produced almost exclusively in mammals, although nearly all terrestrial vertebrates have an oxytocin-like mesotocin molecule (Gimpl and Fahrenholz, 2001). Oxytocin is primarily synthesized in the central nervous system, in the paraventricular nucleus and supraoptic nucleus of the hypothalamus (Sawchenko and Swanson, 1982). The two known cell types to produce oxytocin in the hypothalamus are magnocellular and parvocellular neurons (van de Pol, 1982). Magnocellular cells send axons to the posterior pituitary, which can transmit oxytocin outside of the central nervous system through the portal circulation system (Gainer and Wary, 1994). These cells can also release oxytocin into the central nervous system through somatodendritic release into the circulating cerebrospinal fluid, resulting in paracrine signaling. The parvocellular neurons send direct axonal projections to target regions of the nervous system, many of which project to regions of the brain associated with social behavior such as the nucleus accumbens, amygdala, hippocampus, striatum, suprachiasmatic nucleus, bed nucleus of stria terminalis and brainstem (Otero-Garcia et al., 2016).

Oxytocin has been implicated in numerous social behaviors in mammals, including increased social interaction, pair-bonding behavior, sexual behavior, and maternal and biparental care (Witt et al., 1991; Witt et al., 1992; Williams et al., 1992; Pedersen et al., 1994; Insel, 1997; Carter, 1998; Cho et al., 1999, Bales and Carter, 2003, Bales et al., 2007). Due to the association between oxytocin and social behavior in many mammalian species, scientists hypothesized that perhaps the differences in social behavior between prairie voles and montane voles could be due to the differences in the typical levels of oxytocin in regions of the brain associated with social behaviors. Yet, surprisingly, the levels of oxytocin in prairie vole and montane voles were similar in regions of the brain associated with social behaviors (Wang et al., 1996; Insel et al., 1992). If the amount of oxytocin produced in these species is similar, could there be another aspect of the oxytocin system that differed between these two species?

Could it be that the effect of oxytocin on social behavior could be dependent on binding to its one known receptor, the oxytocin receptor? Perhaps the amount of oxytocin receptors was different between prairie voles and montane voles in regions of the brain associated with social behaviors? The oxytocin receptor is a G protein-coupled seven transmembrane domain receptor, and using the technique of autoradiography allowed scientists to visualize the amount of oxytocin receptor present across regions of the brain in different species. It should be noted that other commonly used techniques to quantify receptors lack a commercially available antibody that is sensitive or specific enough for the oxytocin receptor. Therefore, even though autoradiography lacks cellular resolution, it is the best technique to determine oxytocin receptor density at this time. Using this method, a clear and dramatic difference in the amount of oxytocin

receptor in regions of the brain associated with social behaviors was observed between prairie voles and montane voles (Insel and Shapiro, 1992; Ross and Young, 2009). In prairie voles, brain regions such as the nucleus accumbens, amygdala, medial preoptic area, bed nucleus of the stria terminalis, paraventricular nucleus are associated with social behavior and are rich in oxytocin receptors (Figure 2). In comparison, these regions of the brain are low in oxytocin receptor density in montane voles (Figure 2).

A)



B)

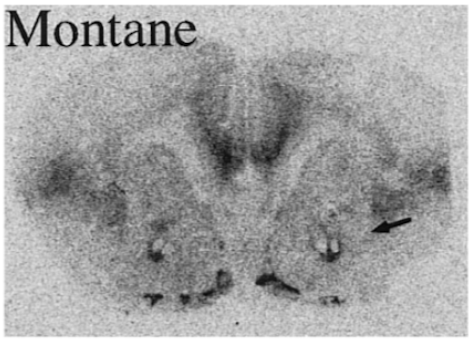


Figure 2: Representative autoradiograms of oxytocin receptor density in (A) prairie voles and (B) montane voles, in which darker shades within the brain slices represent areas of higher oxytocin receptor density. Regions associated with social behavior, such as the nucleus accumbens (NAcc), are rich in oxytocin receptors in prairie voles in comparison to montane voles. The amount of oxytocin receptors present in regions of the brain associated with social behaviors is a key neurological difference between a species that displays pair-bonding and is highly social (prairie voles) and a species that is polyamorous and solitary (montane voles). NAcc = nucleus accumbens; PLC = prelimbic cortex. (Insel and Shapiro, 1992).

Two similar species, the prairie vole and montane vole, have drastically different displays of species typical social behavior, and one of the major neuronal differences between these species is the amount of oxytocin receptor present in brain regions associated with social behavior. This strong piece of evidence supports the hypothesis that the oxytocin receptor may

be a key factor in social behavior. This idea was further strengthened when scientists were able to induce an increase of oxytocin receptor through a viral vector in the nucleus accumbens of prairie voles and observed an increased display of pair-bonding behavior (Ross et al., 2009). Additionally, central injections of oxytocin increases species typical social behavior in prairie voles but does not alter social behavior in montane voles, indicating the actions of oxytocin on social behavior are mediated by its receptor (Wang et al., 1996, Insel and Shapiro, 1992; Insel et al., 1995). Also, an intracerebroventricular infusion of oxytocin receptor antagonist, which acts to block the oxytocin receptor from functioning, corresponds with decreases of species typical social behaviors in prairie voles (Williams et al., 1994; Bales and Carter, 2003; Bales et al., 2004; Bales et al., 2007). These studies suggest that the actions of oxytocin on social behavior are dependent on oxytocin receptors in the central nervous system. Therefore, if we want to better understand the biological mechanisms of social behavior, the collective evidence was pointing towards further researching the role of the oxytocin receptor.

Additionally, in conducting research on prairie voles it was observed that different individual prairie voles displayed a spectrum of social behaviors, with some animals naturally performing more species typical social behaviors in comparison to other prairie voles.
A collection of studies indicated that there were differences in the amount of oxytocin receptors between *different* species, but could there also be differences *within* the same species? Could this spectrum of social behaviors observed in prairie voles correspond to the amount of oxytocin receptor present in key regions of the brain? In fact, it was discovered that those animals which naturally displayed more social behaviors also had corresponding higher amounts of oxytocin receptors in regions of the brain associated with social behavior, such as the nucleus accumbens

(Olazabal and Young, 2006; Ross et al., 2009)(Figure 3). This provided more evidence that the amount of oxytocin receptor present in regions of the brain associated with social behavior corresponded with the amount of social behavior an individual would likely display. This lead scientists to wonder, what could be influencing this spectrum of oxytocin receptor density and corresponding connection to downstream social behaviors in prairie voles?

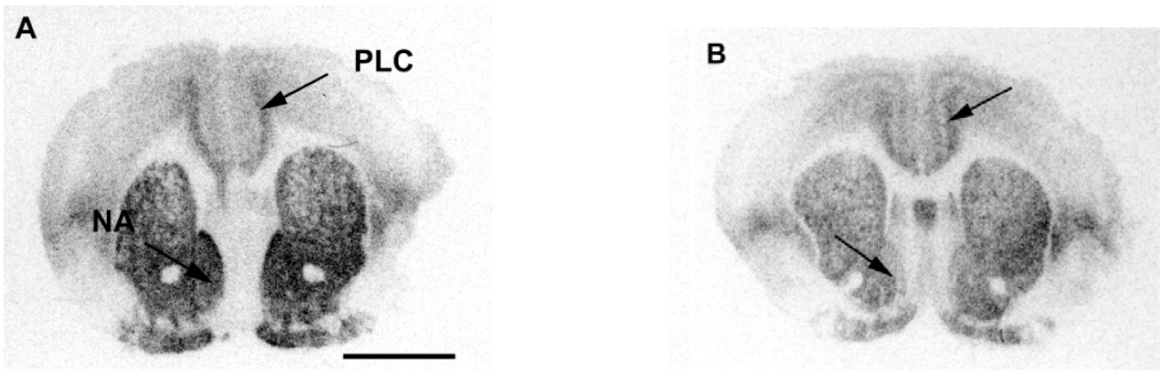


Figure 3: Representative autoradiograms of oxytocin receptor density of (A) naturally high maternal care female prairie vole and (B) naturally low maternal care prairie vole. Prairie voles which spontaneously display more social behavior have more oxytocin receptors in regions on the brain associated with social behavior, such as the nucleus accumbens. NA = nucleus accumbens; PLC = prelimbic cortex. (Olazabal and Young, 2006).

The oxytocin receptor is coded by one known gene, the oxytocin receptor gene (human: *OXTR*, prairie vole: *Oxtr),* and researchers turned to the genome to better understand how there could be differences in oxytocin receptor density in the brain. Historically, genetic research primarily has focused on deoxyribonucleic acid (DNA) as the heritable chemical compound that encodes biological information from parents to offspring. The principles of inheritance traditionally focus on the mechanism of how genes are passed to subsequent generations and

how an organism's phenotype is influenced by factors within their genotype. Occasionally, mutations can cause permanent, heritable changes in genetic information. However, mutations are random, a majority of mutations do not pass to the genome of offspring, and the outcome of these mutations can range from beneficial, harmful, or neutral to the organism. While genetic alterations and DNA sequences are essential in regulating heritable biological functions, an additional set of biological processes, known as epigenetics, can occur throughout the lifespan of organisms that alter gene expression.

The beginnings of the field of epigenetics are linked with the study of evolution and development. Up until the 1950s the word epigenetics was used broadly to categorize all the developmental events leading from a fertilized zygote to a mature organism, and it was a term that once incorporated all of the biological processes that provided the information to synthesize the components of an organism through the synchronized coordination of gene expression throughout development. Currently, the concept and definition of epigenetics were updated and restricted to "the study of mitotically heritable alterations in gene expression that are not caused by changes in DNA sequence," indicating that epigenetic processes modify the expression of genes without changing the underlying DNA sequence itself (Waterland, 2006). Many aspects of gene expression are influenced by epigenetic mechanisms, including transcription, translation, imprinting, silencing transposons, genomic imprinting, and X-chromosome inactivation (Gibney & Nolan, 2010). Many processes of epigenetics are tied to the early development of an organism, but one additional exciting finding of epigenetic research is the implication that an individual can postnatally adapt to their environment through modifications of DNA function via epigenetic regulation, rather than have their species adapt through the slow processes of adaptation through

genetic mutation and evolution. Epigenetic processes can provide mechanisms for an organism to quickly adapt by fine tuning various biological systems in response to the environment.

It was observed that the compaction of DNA can fluctuate, and when DNA is highly compact it creates an inactive state that is associated with a decrease in gene expression. Vice versa, when DNA is in a looser and open state it corresponds to an increase of gene expression. These observations were connected to the epigenetic regulation of DNA, and the two main categories of epigenetic marks are DNA methylation and histone modifications. For the purposes of this dissertation, the epigenetic marker of interest is DNA methylation. The addition of a methyl group directly occurring on DNA is known as DNA methylation, a stable epigenetic mark which can influence gene expression (Newell-Prince et al., 2000). DNA is comprised of four nucleotides --adenine, thymine, cytosine, and guanine. Cytosine can undergo an epigenetic modification with the addition of a methyl (CH_3) on this nucleotide, typically in the context of a cytosine (C) upstream and next to a guanine (G). The sequence of DNA in which this can occur is commonly referred to as a CpG site, with the "p" referring the phosphate bond that is formed between the cytosine and guanine nucleotides. DNA becomes methylated by a family of enzymes referred to as DNA methyltransferases, which serve to lay down new DNA methylation marks or to maintain DNA methylation during mitosis (Weber & Schubeler, 2007). When CpG sites are methylated, the DNA typically becomes more compact (Newell-Prince et al., 2000) (Figure 4). Therefore, increases in DNA methylation usually correspond with decreases in gene expression, and decreases of gene expression are typically associated with decreases in the downstream process of ribonucleic acid (RNA) and protein production.

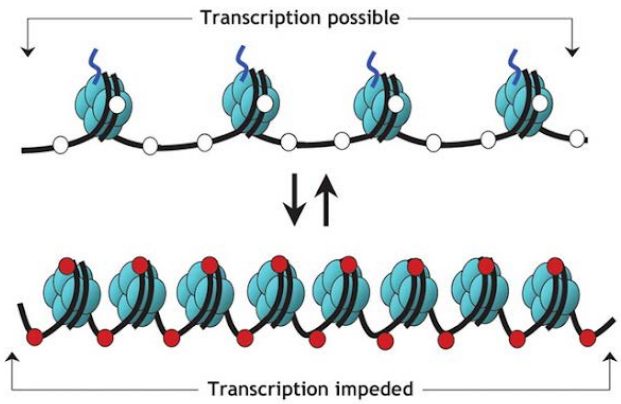


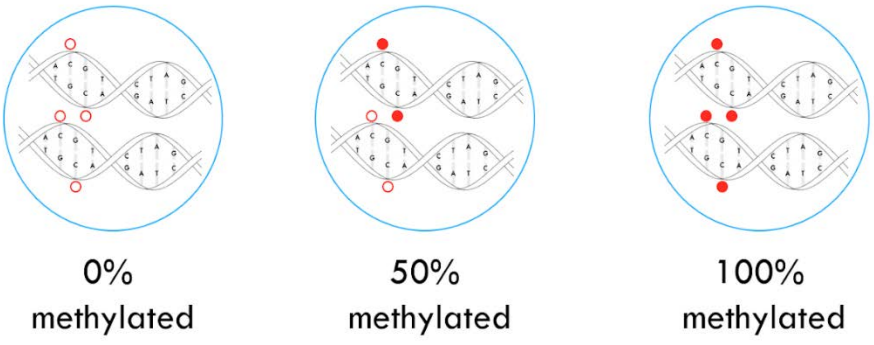
Figure 4: Schematic of epigenetic regulation through DNA methylation. Strands of DNA are wrapped around histone proteins, forming nucleosomes, which is organized into chromatin. DNA methylation forms directly on cytosine nucleotides, typically when the cytosine is adjacent to and upstream of a guanine (CpG sites). DNA methylation is an epigenetic mark which can regulate chromatin structure and gene expression. Unmethylated CpG sites are associated with an open chromatin state and a transcriptionally active gene (white circles = unmethylated CpG sites). In contrast, methylated CpG sites are associated with a compact chromatin state and a transcriptionally inactive gene (red circles = methylated CpG sites). Image: (cnx.org).

In this dissertation, I report on levels of DNA methylation at CpG sites in tissue samples. Tissues are comprised of many cells, and each cell can only have three states of DNA methylation per CpG site -- 0% methylated, 50% methylated and 100% methylated. Humans and prairie voles are diploid organisms, and offspring receive one complete copy of their genetic material from each parent. A typical cell would have two alleles, and a CpG site at each allele could either be methylated or unmethylated. If both alleles are methylated at a single CpG site, then the cell would have 100% methylation at that site; if one allele is unmethylated and one is methylated at a single CpG site then the cell would have an average of 50% methylation; and if both alleles are unmethylated at a single CpG site then the cell would be 0% methylated. I will

be reporting the average DNA methylation levels at specific CpG sites in a collection of cells, also known as tissues, and therefore our range will be between 0-100% DNA methylation. For example, if our tissue was a collection of 5 cells, there would typically be 10 alleles, and if 7 of these alleles were methylated and 3 were unmethylated at a specific CpG site, that CpG site would have an average of 70% methylation (Figure 5).

Also, it should be noted that DNA is double stranded and when there is a CpG site on one strand, the reverse strand will also contain a CpG site. In a developed cell, when DNA methylation occurs at one strand of DNA, typically the reverse strand will also be methylated (Figure 5). In cellular replication, the DNA becomes hemimethylated, in that the mother strand retains its methyl marks and the daughter strand is without methyl marks. A DNA methyltransferases enzyme (Dnmt1) is attracted to hemimethylated DNA, and will lay down a methyl mark on the unmethylated CpG sites in the daughter strand (Svedruzic, 2011). The methyl mark is stably passed down through cellular division, and the result is that developed cells carry the epigenetic mark of DNA methylation at CpG sites on both strands of DNA.

A)



B)

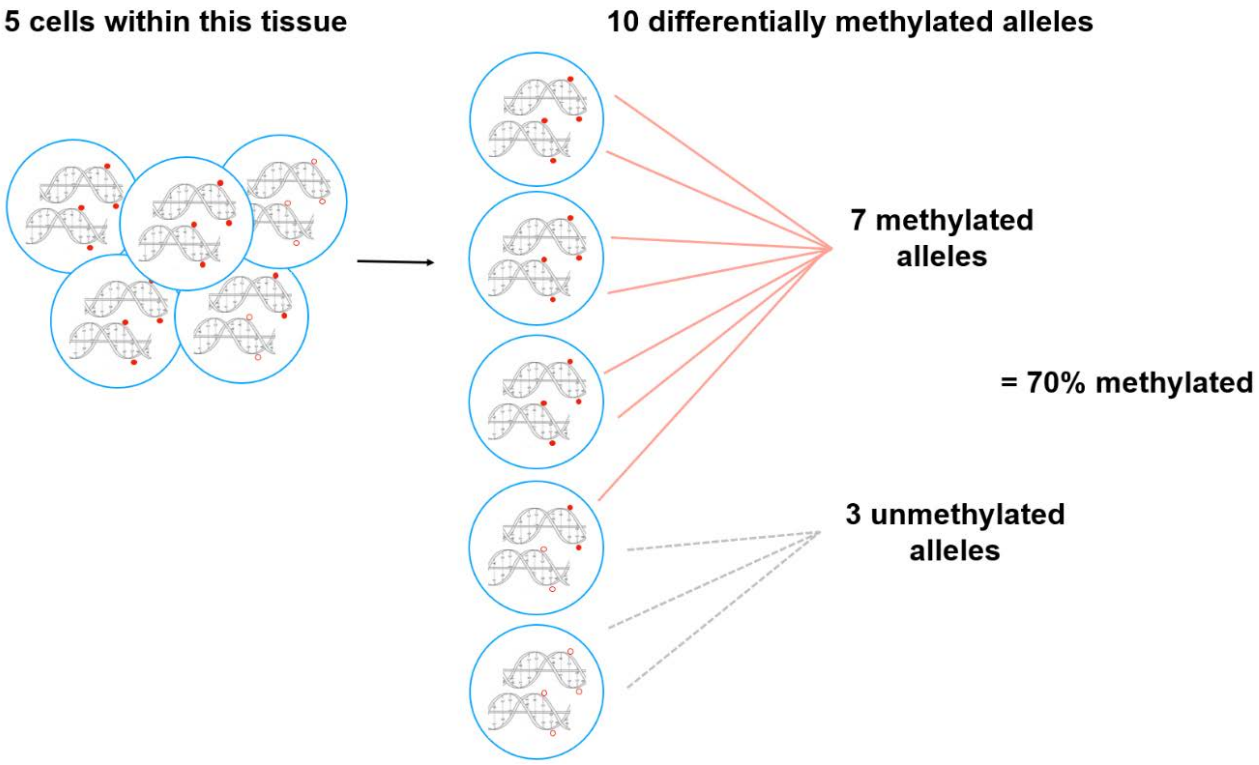


Figure 5: DNA methylation in cells and tissues. (A) Cellular schematic of the three possible states of DNA methylation per CpG site. Both alleles can be unmethylated at a single CpG site (0% DNA methylated), one allele can be methylated and one allele can be unmethylated at a single CpG site (50% methylated), or both alleles can be methylated at a single CpG site (100% methylated). White circles = unmethylated CpG; Red circles = methylated CpGs. It should be noted that DNA methylation typically occurs on both strands of DNA in differentiated cells. (B) Tissue schematic of DNA methylation, which can range from 0-100% methylated. Assays can distinguish methylated or unmethylated sites in the collection of alleles within a tissue. In this representative example, a tissue has 5 cells for a total of 10 alleles, which 7 of those alleles are methylated and 3 are unmethylated, therefore this tissue has an average of 70% methylation at the CpG site of interest. (Image: Katie Krol and Kelly Wroblewski, unpublished.)

Discovering the impact DNA methylation has on gene expression lead scientists to wonder if DNA methylation at certain areas along DNA sequences affect gene expression more than other regions. In researching the epigenetic landscape, scientists found general patterns of DNA methylation. Most regions are highly methylated (85%-100%) except for CpG islands which are typically unmethylated (0-5%) (Staussman et al., 2009; Laurent et al., 2010). Two regions which I would like to highlight for this dissertation are exonic regions and promoter regions. Exons are regions of genes which include the code leading to the downstream production of RNA and then proteins, and these regions tend to have high levels of DNA methylation. The other region of interest in this dissertation, promoter regions, surround the transcription start site and are often within CpG islands, which are regions larger than 200 base pairs, a CG content more than 50%, and an observed/predicted CpG ration of more than 0.6, and typically have very low levels of DNA methylation (Gardiner-Garden and Frommer, 1987).

In mammals most CpG sites are methylated, but CpG sites in CpG islands within promoter regions tend to remain mostly unmethylated because they are typically protected from de novo DNA methyltransferases (Dnmt3a and Dnmt3b) (Bird et al., 1985). There are multiple mechanisms involved in protecting CpG sites from de novo DNA methylation, often the protection from DNA methyltransferases can occur through the actions of select proteins with CXXC domains (ex. TET1/TET3, Cfp1, and Kdm families) which are recruited to bind to unmethylated CpG sites (Ooi et al., 2007; Ceder and Bergman, 2009, Blackledge et al., 2013). An unmethylated CpG state is associated with the recruitment of proteins that typically corresponds with epigenetic histone modifications (ex. acetylation), that also protects the CpG site from DNA methylation (Bird and Wolffe, 1999). Additionally, many types of transcription

factors are attracted to CG rich regions, such as CpG islands, and when the chromatin state is open it increases the probability of transcription factor binding, which is associated with stabilizing an open chromatin environment and an increase of gene transcription (Deaton and Bird, 2011; Jones, 2012).

Even though DNA methylation in promoter regions tends to be low, when DNA methylation does occur, it tends to greatly impact gene expression (Razin, 1998). DNA methylation projects into the major groove of DNA and acts directly to physically prevent the binding of transcription factors to their recognition motifs (Watt and Molloy, 1988). Methylated CpGs can also act indirectly by attracting methyl binding proteins and co-repressor complexes, which is associated with the recruitment of enzymes that modify histone modifications, such as histone deacetylases enzymes which remove acetyl groups from histone proteins (Meehan et al. 1998; Bird and Wolffe, 1999, Jones et al., 1998). The removal of histone acetylation is associated with the recruitment of methyl-CpG-binding proteins which act to protect the DNA methylation mark from being oxidized by TET enzymes, the beginning step in a cytosine returning to an unmethylated state (Meehan et al. 1998, Jones et al., 1998. Tahiliani et al., 2009, Ito et al., 2010). The end result is that DNA methylation is typically a stable mark that corresponds with compressed chromatin and reduced transcription.

Additionally, in this dissertation, I present evidence that two CpG sites in the exonic region of prairie voles are synonymous transition single nucleotide polymorphisms. The common variant is GCG and the rare variant is GCA, both are alanine codons. The reverse complement of a CG → CA mutation is CG → TG, and previous research indicates that cytosine to thiamine

mutations are common at methylated cytosine (Cooper and Youssoufian, 1988). The ability of DNA methylation to silence potentially harmful gene sequences, such as retrotransposons, is essential for the maintenance of the genome, proper development, and maintaining cellular identity. However, CpG sites are also hotspots for point mutation, and this type of mutation is one of the most frequent single causes of human disease (Cooper and Youssoufian, 1988). Unmethylated cytosine can spontaneously turn to uracil, but since this is a base pair not associated with DNA, repair mechanisms typically identify this error and repair the sequence back to a cytosine. However, when a CpG site is methylated, the cytosine can spontaneously deaminate and form thymine (Lutsenko and Bhagwat, 1999). Since thymine is a natural DNA base, the repair is not as efficient. Therefore, cytosine to thymine mutations have a higher chance of being passed down in cell divisions and through the germ line (Lutsenko and Bhagwat, 1999). Roughly ⅓ of all point mutations in humans are cytosine to thymine mutations at CpG sites, and there is an underrepresentation of CpG sites in mammalian genome indicating the evolutionary instability of CpG sites (Copper and Youssouflain, 1988; Bird, 1980).

Investigating how DNA methylation impacts gene expression is common in epigenetic research, but surprisingly examining the relationship between DNA methylation in the promoter and exonic regions in relation to oxytocin receptor gene expression in humans and in prairie voles has not been well studied. However, one pivotal study was conducted by Kusui and colleagues, in which they investigated if DNA methylation within a CpG island in *OXTR* influenced transcription. Using a luciferase reporter gene assay, they showed that DNA methylation of gene constructs without the CpG island reduced transcription by 19% whereas the gene construct with the CpG island was suppressed by 70% (Kusui et al., 2001). This indicates

that DNA methylation of the CpG island functionally suppresses *OXTR* transcription. Additionally, Kusui *et al.* identified a region termed MT2 that was primarily responsible for this DNA methylation dependent transcription repression, since when the MT2 region was deleted it led to a rescue of transcriptional activity of the gene constructs. This study indicates that DNA methylation in the MT2 region could greatly impact *OXTR* expression, but the specific CpG sites corresponding to this outcome remained unknown. I address this in both human and prairie voles in this dissertation.

Additionally, previous work indicates that the amount of maternal care received in early life can alter the DNA methylation state of a gene postnatally (Weaver et al., 2004). Michael Meaney, Moshe Szyf, and collaborators performed an experiment which provide evidence for the first time that the amount of maternal care offspring receive could alter the epigenome of the glucocorticoid receptor gene postnatally, and changes in offspring epigenome are associated with behavioral and physiological characteristics (Weaver et al., 2004). Work in this dissertation further explores the role of early life parental care and corresponding changes in DNA methylation, but within the oxytocin receptor gene in both humans and in prairie voles. Our goal is to better understand the biological mechanisms of social behavior, the findings in Kusui *et al*. indicate that exploring the effect of DNA methylation within the MT2 region may correspond to differences in the oxytocin receptor gene expression, and the work conducted by Weaver *et al*., suggests that early life parental care may impact the epigenome, receptor density, and social behavior.

Also, previous studies in our animal model of social behavior, the prairie vole, indicate that the relationship between oxytocin receptor density and subsequent social behavior is more dependent on the environment than genetic inheritance (McGuire, 1988; Ahern and Young, 2009; Stone and Bales, 2010, Perkeybile et al., 2013, 2015). This was determined through cross-fostering experiments in which prairie vole pups born from high care parents were brought into the care of low care parents right after birth, and vice versa with a pup derived from a low care family being brought into the care of a high care family. When these pups developed into adults, they showed the neuroanatomy and social characteristics of the family they were raised by, and not the family they were born from (Perkeybile et al., 2015). Therefore, a pup with the genes of a high care family, but raised by a low care parents, typically have lower amounts of oxytocin receptors in regions of the brain associated with social behavior and display less amounts of species typical social behavior (Perkeybile et al., 2015). Whereas a pup born of a low care family, but raised by high care parents, would typically have more oxytocin receptors in brain regions associated with social behavior and display more pair-bonding, parental care behavior, and gregarious behavior (Stone and Bales, 2010; Perkeybile et al., 2015). Cross-fostering experiments provide evidence that the environment, which can impact the epigenetic landscape, may influence the biological mechanisms regulating the oxytocin receptor system and downstream effects on social behavior. Yet, the relationship between the amounts of parental care received in early life with DNA methylation in *Oxtr* is a gap of knowledge that is important in our understanding of social behavior, and is addressed in this dissertation.

In this dissertation I investigate how early life parental care in prairie voles could alter the epigenetic landscape by measuring DNA methylation at CpG sites across select areas of the

highly conserved MT2 and exonic regions within *Oxtr*. In a separate cohort, we measured the amount of oxytocin receptor binding present in brain tissue of animals that received different amounts of parental care in early life. In previously published research, differences in the amount of parental care received in early life was associated with differences in social and stress behavior in offspring, with offspring which received more parental care typically displaying more species typical social behavior and less of a stress response in adulthood in comparison to the offspring in the control group (Bales et al., 2007; Stone and Bales, 2010; Bales et al., 2011, Perkeybile et al., 2013). Scientists are currently trying to determine if discoveries involving early life environment in animal models transfer to humans. Developments in behavioral epigenetics in suggest that psychosocial experiences in early life could alter the composition of the epigenome, which can result in behavioral differences later in life. I propose that mechanisms discovered in prairie voles have a similar function in influencing differences in social behavior in human behavior. Previous work suggests the behavioral and physiological outcomes of prairie voles raised by low care parents have similar characteristics to humans raised in socially restrictive early life environments.

Studies of early life experience and the resulting outcome on the developmental trajectories of human behavior and psychopathology indicate that growing up in negligent or abusive environments may increase vulnerability in later life with a wide array of negative symptoms. Longitudinal studies indicate infants that live in abusive and negligent environments have an increased risk of developing cognitive impairments, mental pathologies, elevated stress response, increased immune response, and have social and emotional difficulties (Trickett & McBride-Chang, 1995; Danese et al., 2007). Also, humans raised in low care environments tend

to have difficulties with various aspects of social behavior and internalizing emotions (i.e. loneliness, depression, anxiety), and this can interfere in the processes through which social relationships could influence health.

It is hypothesized that social relationships can help people feel buffered from stressors from life events, promote healthy life choices, and provide a sense of belonging (Holt-Lunstad et al., 2010). A meta-analytic review examined the extent to which social relationships influence risk of mortality and determined that people who perceive themselves as lonely and having less social connections are at a higher risk of early mortality (Holt-Lunstad et al., 2010). Strikingly, perception of social support influenced mortality rate more so than smoking, excessive alcohol drinking, obesity, exercise, and air pollution (Holt-Lunstad et al., 2010). When examining how environmental factors can influence health and quality of life, health professionals and the public take risk factors such as smoking, diet, and exercise seriously. Yet, there is a growing body of evidence that social relationships and perception of loneliness are equally, or even more important, in extending the quantity and quality of life.

The mechanisms driving these behavioral and physiological outcomes remain unknown, however epigenetic studies in humans and in animal models have expanded our understanding of how social environments can alter adult phenotypes. However, unlike in animal models, it remains unclear if epigenetic alterations in humans occur during early childhood or if they were present in the germline or introduced during embryogenesis. Yet, there are intriguing correlations between DNA methylation in the MT2 region and exonic region of *OXTR* to behavioral outcomes in humans. Previous studies indicate that altered levels of DNA methylation

in *OXTR* are a risk factor for several psychiatric disorders, including autism spectrum disorder (Gregory et al., 2009; Rijlaarsdam et al., 2017), postpartum depression (Bell et al., 2015; Kimmel et al., 2016), major depressive disorder (Reiner et al., 2016, Chagnon et al., 2015), anxiety disorders (Ziegler et al., 2015; Cappi et al., 2016), anorexia nervosa (Kim et al., 2014), callous-unemotional traits and psychopathy (Dadds et al., 2014; Cecil et al., 2014), and psychosis (Rubin et al., 2016). It has also been shown that *OXTR* DNA methylation is related to neural endophenotypes of social perception (Jack et al., 2012; Puglia et al., 2015), which are commonly dysregulated in the above mentioned disorders.

We wanted to explore if the amount of parental care received in early life in humans would correspond with differences in DNA methylation in *OXTR* and subsequent social behavior. In this dissertation, our research indicates that when teenagers reported on the amount of maternal care received in childhood, those which perceived that they received less maternal care appear to follow a different developmental trajectory in comparison to teenagers which felt like they received more maternal care. Those that perceived that they received less maternal care developed into adults with higher levels of DNA methylation at key CpG sites in *OXTR*, and importantly these biological findings corresponded with feelings of romantic stress, loneliness, depression, anxiety, withdrawn symptoms, fatigue, and somatic pain. As previously mentioned, people who feel lonely and perceive having less social support tend to die sooner and have a lower quality of life (Holt-Lunstad et al., 2010). Collectively, there is growing evidence that the amount of care received in early life can epigenetically fine-tune systems associated with social behavior and stress, which can alter the trajectory of social behavior and mental health in later development, which could influence risk factors of morality and quality of life.

As we gain a better understanding of how the environment can induce epigenetic changes, we may be able to design behavioral strategies and interventions to prevent or decrease negative health outcomes. Research in animal models suggests that when adolescent animals raised by low care moms are placed in an enriched environment with many other animals, their social behavior in adulthood is more similar to animals raised by high care moms. (Champagne and Meaney, 2007). This suggests that offspring which faced early life adversity can still fine-tune the biological mechanisms of social behavior through other sources of environmental stimuli and social interaction. In humans, at-risk youths often experience stressful family environments, yet those with a mentor have a reduction in depression systems and an increase perception of social acceptance (Herrera et al., 2013). The association between the epigenome and health outcomes has spurred research focusing on the plasticity of epigenetic markers, leading to a better understanding of pharmacological and therapeutic treatments aimed at reversing epigenetic profiles associated with poor physiological outcomes and mental health. Moreover, exploring the role of epigenetics on biological systems may hold the key in developing novel prevention programs and acquiring a more integrated understanding of health and behavior to better enhance quality of life in the future.

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Chapter 1

Early nurture epigenetically tunes the oxytocin receptor

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Early nurture epigenetically tunes the oxytocin receptor

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ABSTRACT

Mammalian sociality is regulated in part by the neuropeptide oxytocin. In prairie voles, subtle variation in early life experience changes oxytocin receptor-mediated social behaviors. We report that low levels of early care in voles leads to *de novo* DNA methylation at specific regulatory sites in the oxytocin receptor gene (*Oxtr*), impacting gene expression and protein distribution in the nucleus accumbens. DNA methylation state of the blood predicts expression in the brain indicating the utility of the blood as a biomarker for the transcription state of the brain. These experience-sensitive CpG sites are conserved in humans, are related to gene expression in the brain, and have been associated with psychiatric disorders and individual differences in neural response to social stimuli. These results identify a mechanism by which early care regulates later displays of typical prairie vole social behavior and suggest the potential for nurture driven epigenetic tuning of *OXTR* in humans.

1. Introduction

Experiences early in life have the potential to alter social behavior and emotion regulation across the lifespan (Levine, 1957; Harlow et al., 1965; Bowlby, 1969). One of the most critical relationships in early life is the parent-offspring dyad, where variation in experience can permanently alter the developmental trajectory of the offspring. Variability in early care may be adaptive, serving to prepare offspring for their likely future environment, and is also a key mechanism allowing for the development of individual differences in behavior (Denenberg et al., 1962; Levine et al., 1967; Francis et al., 1999).

In the prairie vole (*Microtus ochrogaster*), variability in the early life environment is linked to later differences in the expression of species-typical social behavior. Naturally occurring high levels of early biparental care lead to offspring who engage in high levels of alloparental behavior towards unrelated infants and an increase in the propensity to form a selective partner preference with an opposite sex mate (Perkeybile et al., 2013, 2015; del Razo and Bales, 2016). Likewise, a single episode of handling on the first day of life results in later increases in both alloparental care and partner preference formation (Bales et al., 2007). Both alloparenting and pair bonding behaviors are regulated by oxytocin receptor (OXTR) expression in the nucleus accumbens. Prairie voles with higher levels of OXTR binding in this region engage in a greater amount of both of these social behaviors (Liu and Wang, 2003; Olazabal and Young, 2006a; Ross et al., 2009), and artificially upregulating or downregulating OXTR expression acts to increase or decrease these behaviors, respectively (Ross et al., 2009; Keebaugh and Young, 2011; Keebaugh et al., 2015).

The expression of OXTR in the nucleus accumbens is remarkably variable both between and within species, although levels of the oxytocin peptide are less variable (Insel and Shapiro, 1992; Young, 1999; Gimpl and Fahrenholz, 2001; Olazabal and Young, 2006b). Mechanisms regulating expression of the receptor, rather than oxytocin itself, may provide an improved explanation for the variability in social behavior seen after differing early experiences. Here we explore one such mechanism by which early experience results in changes to OXTR-dependent social behaviors in the prairie vole. Previous work has demonstrated that both early parenting and early handling alter alloparental behavior and pair bonding behavior, both of which are dependent on OXTR expression in the nucleus accumbens. Using a candidate gene approach, we propose an epigenetic mechanism where early experience regulates DNA methylation of the oxytocin receptor

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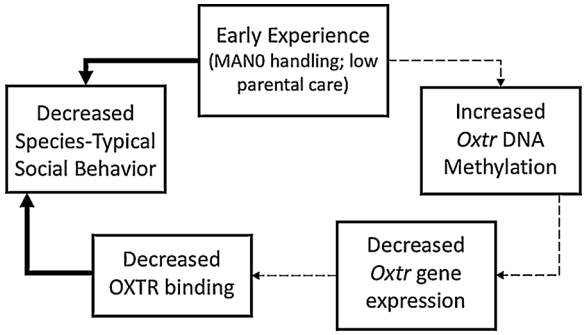

Fig. 1. Proposed epigenetic mechanism for early experiences to impact oxytocin receptor-dependent social behavior. Early experiences act to regulate *Oxtr* DNA methylation in the nucleus accumbens, which alters *Oxtr* gene expression and eventually OXTR protein distribution in this region. This change in OXTR protein is responsible for the changes in social behaviors seen after varying early experiences. Known relationships are indicated by solid lines. Proposed mechanisms are indicated by dashed lines.

gene, *Oxtr*, leading to downstream consequences for *Oxtr* gene expression and eventually for OXTR protein levels. We hypothesize that low levels of early handling and early parental care lead to increased *Oxtr* DNA methylation, decreased *Oxtr* gene expression, and low levels of OXTR binding in the nucleus accumbens, which is known to predict decreases in the social behaviors impacted by our early experience models (see Fig. 1).

2. Materials and methods

2.1. Animal model

Subjects were laboratory-bred prairie voles (*Microtus ochrogaster*), descendants of a wild-caught stock captured near Champaign, Illinois. Breeding pairs were housed in large polycarbonate cages (44cmx22 cm x 16 cm) and same sex offspring pairs were housed in smaller polycarbonate cages (27cmx16 cm x 16 cm) after weaning on postnatal day (PND) 20 (date of birth: PND0). Animals were given food (high-fiber Purina rabbit chow) and water *ad libitum*, cotton nestlets for nesting material in breeding cages, and were maintained on a 14:10 light:dark cycle.

Procedures involved in measuring the effects of early handling on parental behavior were reviewed and approved by the Institutional Animal Care and Use Committee (IACUC) at the University of Illinois, Chicago. Procedures involved in generating tissue for the analysis of DNA methylation, gene expression, and OXTR protein following handling and naturally occurring parental care variation were reviewed and approved by the IACUC at the University of California, Davis. Procedures involved in collection of embryonic tissue were reviewed and approved by the IACUC at Northeastern University.

In all cases, animals were euthanized via cervical dislocation and rapid decapitation under deep isoflurane anesthesia. Brains were extracted, flash frozen on dry ice, and stored at −80 °C until analysis. Trunk blood samples were immediately frozen and stored at −80 °C until analysis.

2.2. Early handling manipulation (the MAN paradigm)

Within 24 h of giving birth, breeding pairs underwent a single treatment experience, herein termed MAN0 (no direct handling manipulation) or MAN1 (direct handling manipulation) handling. MAN0 litters were picked up for 30 s in a clear plastic cup (no direct handling). If animals were sitting, they were scooped up in the cup. If they were walking around the cage, the cup was positioned in front so that they would walk into it. In this condition, pups were supported by the cup. Pairs in the MAN1 condition were picked up by the scruff of the neck by a gloved hand for 30 s (direct handling). Prairie vole pups have milk teeth to attach to the mother's nipple, so in this condition, pups hung unsupported from the mother and were therefore not touched by the experimenter (if pups were unattached, researchers waited to perform the handling). Both the mother and father of each pair were handled in the same manner. Pups were only manipulated during the handling of the mother because they were attached to her.

2.3. Quantification of parental behavior following early handling

Maternal and paternal pup directed care were characterized and scored for 60 min immediately after handling on PND1, and again on PND8 and PND15 (with no further manipulation) between 17:00 and 20:00. Parental pup-directed care was video recorded and later scored by two trained observers using Observer 5.0 behavior tracking software (Noldus Inc., Wageningen, Netherlands). Each observation period was divided into 15-second segments (240 segments total) and behaviors were recorded in a one-zero fashion for each segment. Observations were conducted while the animals were in their home cage and they were not disturbed throughout the observation period. Behaviors observed included active nursing (maternal only), huddling, lateral contact with pups, hunched contact with pups, sniffing and licking/grooming of pups, anogenital licking/grooming of pups, rearranging pups, and retrieval or removal of pups from the dam. Behavioral score reflects the percentage of segments active pup-directed behaviors were observed during the observation period (MAN0, n = 7; MAN1, n = 6). During each observation, parents were distinguished from one another by distinct fur color and markings, body size, and the presence of pups visibly attached to the nipple. Inter-rater reliability was 0.89. Intra-rater reliabilities were 0.95 for the primary coder and 0.96 for the secondary coder using the kappa statistic.

2.4. Identifying conserved MT2 region in prairie vole

The Multiz Alignment of 100 Vertebrates track in the UCSC genome database (Blanchette et al., 2004) was used to identify a 71 base pair alignment block that contained similar sequence in rats, mice, and prairie voles. To assess the significance of this similarity between human and prairie vole we used the UVa FASTA server (http://fasta.bioch.virginia.edu/) and PRSS (DNA:DNA) to shuffle the prairie vole sequence 200 times and estimate the statistical significance of the shuffled scores. This analysis identified a conserved 62 base pair region between human and prairie vole with 72.6% identity. PRSS output is provided in Supplemental Fig. 2C.

2.5. Tissue collection for epigenetic analyses following early handling

An additional cohort of offspring underwent early handling procedures as described above (13 male/female sibling pairs; MAN0: 7 females, 7 male; MAN1: 6 females, 6 male). On PND24, brain and blood tissues were collected, immediately frozen on dry ice, and then stored at −80 °C until DNA and RNA isolation. Brains were equilibrated to −20 °C for two hours prior to sectioning. Brain samples were dissected by 1) a coronal cut to remove the olfactory bulbs, 2) a coronal cut at the nerve chiasma just rostral to the hypothalamus and bregma and, 3) bilateral punches (1 mm in diameter, 2 mm in depth) to isolate nucleus accumbens tissue. Following sectioning, nucleus accumbens tissue was placed in a DNAse/RNAse free microcentrifuge tube and flash frozen with liquid nitrogen. Brain tissue was then crushed using a mortar and pestle in preparation for DNA/RNA isolation.

2.6. Prenatal pup brain collection

Adult males and females were mated using a timed mating paradigm to accurately predict birth. Males and females were initially

paired for 24 h in a large polycarbonate cage. Prairie vole females experience estrus induction 48–72 hours after exposure to a male and will typically not allow mating within the first 24 h of pairing. Therefore, a divider was placed in the cage 24 h after pairing to allow for olfactory and auditory interaction but minimal physical contact and no mating. The divider was removed after 72 h, at which point pairs were observed for mating. Pairs were then left undisturbed throughout gestation except for routine cage changes. On the expected day of birth, pregnant females (n = 9) were euthanized and pups were immediately removed from the uterine horns via caesarean section. Male/female sibling pairs (n = 18; 9 females, 9 male) were used for forebrain (which includes the nucleus accumbens) *Oxtr* DNA methylation analysis, and only pups that weighed 1.8 g or greater were included in analysis.

2.7. Quantification of natural variation in parental care

Following methods previously established by this laboratory (Perkeybile et al., 2013; Perkeybile and Bales, 2015), the type and amount of naturally occurring parental care directed toward offspring was observed between PND 1–3 in real time by a trained observer using Behavior Tracker software (www.behaviortracker.com) for 30 established breeding pairs. Behavior observations were conducted in the same manner for two separate litters for each breeding pair. Each parent was observed for 20 min in the morning and 20 min in the afternoon two days between PND 1–3 for a total of 4 maternal care and 4 paternal care observations per litter. Parents were distinguished from one another based on individual characteristics such as body size, fur color and markings, or the presence of pups visibly attached to the nipple.

When ranking breeder pairs in relation to one another, total contact times for pup-directed behaviors were summed across each of the 4 observations for the mother and the father. A mean was then calculated for pup-directed behavior for these 4 summed scores for both the mother and the father, and these two means were then summed to produce an average total contact score for the breeder pair for a single litter. These scores for all 30 breeding pairs were then rank-ordered and split into approximate quartiles. Parental care of a second litter was characterized in the same way to determine if breeder pairs ranked in the same quartile for a subsequent litter. In all cases, pairs fell into the same quartile for both litters and were normally distributed, replicating previous findings (Perkeybile et al., 2013). The top quartile breeder pairs (n = 8) produced high contact offspring (n = 16), the middle two quartiles (n = 14) produced medium contact offspring (n = 90), and the bottom quartile breeder pairs (n = 8) produced low contact offspring (n = 49). The number of offspring born to breeder pairs in each contact group does not vary significantly across groups, nor does offspring mortality. Rather, the variation in number of offspring used here for each contact group is a result of animal availability due to other ongoing research at the time of this study.

Observations were done with the animals in their home cage; animals were not disturbed during the observations. Behaviors quantified included maternal and paternal huddling, non-huddling contact, licking/grooming, retrievals, and non-pup directed behaviors, such as nest building, eating and drinking. Maternal nursing postures were also recorded, including neutral, lateral, and active nursing. Behaviors recorded were based on an ethogram presented in Stone and Bales (Stone and Bales, 2010). Following weaning, offspring were housed with a same-sex sibling until euthanasia. Trunk blood samples were taken between PND 48–52 for DNA isolation.

In a separate cohort of 10 breeding pairs, naturally occurring early parental care was observed using the same behavioral ethogram. In this case, pairs were not ranked; instead care was used as a continuous variable. Offspring were euthanized between PND 24–26 and brains were collected for oxytocin receptor autoradiography (n = 32).

2.8. Oxtr DNA methylation analysis

Extraction of DNA was done using the Qiagen AllPrep DNA/RNA Mini Kit (Qiagen, Valencia, CA) following manufacturer instructions. Samples included DNA and RNA isolated from the nucleus accumbens and DNA from whole blood from animals that experienced early handling (i.e. MAN1, MAN0); and DNA from whole blood from animals experiencing naturally varying amounts of early parental care. One hundred nanograms (ng) of DNA from early manipulation subjects (due to low DNA extraction volumes for 2 subjects) or 200 ng from the natural variation in parenting subjects were subject to bisulfite treatment (Kit MECOV50, Invitrogen, Carlsbad, CA) per manufacturer instructions. Bisulfite conversion allows for the detection of methylated cytosines by sequencing. Twelve nanograms of bisulfite converted DNA was used as a template for PCR using a Pyromark PCR kit (Qiagen, Valencia, CA) and 0.2 u M of primers TSL201 F 5′-GGGGATAGGATGG TTAGTTAGTATT-3′ and TSL201R 5′−CCAACAACCTCAAAACTCT ACT-3′. Samples were amplified in triplicate on three identical PCR machines (S1000 Thermal Cycler, Bio-Rad, Hercules, CA.) The following cycling conditions were used for amplification of the target *Oxtr* fragment, which included CpG sites -934_1, -934_2, -924 and -901: [Step 1: (95 °C/15 min)/1 cycle, Step 2: (94 °C/30 s, 58 °C/30 s, 72 °C/30 s)/50 cycles, Step 3: (72 °C/10 min)/1 cycle, Step 4: 4 °C hold]. Standard controls of 0% and 100% methylated DNA, as well as a no DNA control and a positive control vole standard were included for each PCR plate. Pyrosequencing was performed using two primers: TSL201S 5′-GAGGGAAGGTTTTGGAGTTTTTTATAT-3′ and TSL201S2 5′-AGGGATTGAAAAGTGA-3′ on a Pyromark Q24 using PyroMark Gold Q24 Reagents (Qiagen, Valencia, CA) per the manufacturer protocol. Epigenotypes reported are an average of the three replicates. On average, nucleus accumbens samples deviated from the mean by, -934_1: 1.12%; -934_2: 1.37%; -924: 1.13%, -901: 1.63%. On average, whole blood samples deviated from the mean by, -934_1: 0.95%; -934_2: 1.02%; -924: 0.95%, -901: 0.92%.

2.9. Oxtr gene expression analysis

Extraction of RNA was done using the Qiagen AllPrep DNA/RNA Mini Kit (Qiagen, Valencia, CA) following manufacturer instructions. RNA was processed for cDNA synthesis following the protocol provided in the iScript cDNA Synthesis kit (Bio-Rad, Hercules, CA). Real-time PCR was conducted using a 7500 Fast Real-Time PCR System (Applied Biosystems) using *Power* SYBR Green (Applied Biosystems No. 4367659). The cycling conditions are as follows: for *Oxtr* [Step 1: (95 °C/10 min) 1 cycle, Step 2: (95 °C/15 s, 63.4 °C/60 s) 35 cycles], for *Pgk1* [Step 1: (95 °C/10 min) 1 cycle, Step 2: (95 °C/15 s, 65.3 °C/60 s) 35 cycles]. All reactions were run in triplicate (replicate standard deviation was < 0.05) and their specificity verified by melting curve analysis and separation on a 2% agarose gel. Primers performance was evaluated using standard serial dilution and both primer sets performed within acceptable range for efficiency > 90% (*Oxtr* efficiency = 99.7%, R^2 = 0.993; *Pgk1* efficiency = 93.5%, R^2 = 0.998). The primer sequences used for *Oxtr* are TSL401_F 5′-GCCTTTCTTCTTCGTGCAG ATG-3′ (Fwd) and TSL401_R 5′-ATGTAGATCCAGGGGTTGCAG-3′ (Rev); for *Pgk1* TSL402_F 5′-TTGCCCGTTGACTTTGTCAC-3′ (Fwd) and TSL402_R 5′-GCCACAGCCTCAGCATATTTC-3′ (Rev). Relative gene expression is presented using the comparative Ct method, $2^{-\Delta Ct}$. *Pgk1* was chosen as a reference based on data in mouse brain showing its reliability across brain regions and developmental time points (Boda et al., 2009).

2.10. Oxtr SNP analysis

Ten nanograms of DNA was used as a template for PCR using a Pyromark PCR kit (Qiagen, Valencia, CA) and 0.2 u M of primers TSL202 F 5′- CAGGGACGTTCACGTTACATG-3′ and TSL202R 5′- GACA

GAGTCTCCAGCCAAGAAG-3′. The following cycling conditions were used for amplification of the target *Oxtr* fragment, which included SNP NT213739: [Step 1: (95 °C/15 min)/1 cycle, Step 2: (94 °C/30 s, 56 °C/30 s, 72 °C/30 s)/45 cycles, Step 3: (72 °C/10 min)/1 cycle, Step 4: 4 °C hold]. A single product corresponding to 99 base pairs was identified via gel electrophoresis. Pyrosequencing was performed to detect the C/T SNP using primer: TSL202S 5′- GAATCATCCCACCGT-3′ on a Pyromark Q24 with PyroMark Gold Q24 Reagents (Qiagen, Valencia, CA) per the manufacturer protocol.

2.11. Oxytocin receptor autoradiography

Oxytocin receptor autoradiography was performed following previously established methods (Bales et al., 2007; Perkeybile et al., 2015). Brains were sectioned at 20 µm into six series, mounted onto Super-frost slides, and stored at −80 °C until assayed. Slides were allowed to thaw at room temperature and were then fixed in 0.1% paraformaldehyde (7.4 pH) for 2 min. Slides were washed two times for 10 min in 50 mM Tris-HCl buffer solution (7.4 pH), then incubated at room temperature for 60 min in tracer buffer (50 mM Tris-HCl buffer with 10 mM $MgCl_2$, 0.1% bovine serum albumin, and 50 pM of radiotracer [^{125}I]-ornithine vasotocin analog [(^{125}I)OVTA] [vasotocin, d(CH_2)$_5$[Tyr(Me)^2, Thr^4, Orn^8, (^{125}I)Tyr^9-NH_2]; 2200 Ci/mmol]). Following incubation, slides were rinsed in 50 mM Tris-HCl buffer with 10 mM $MgCl_2$ at 4 °C four times for 5 min each, followed by a 30 min wash in the same solution at room temperature while agitating. Sections were briefly dipped in 4 °C dH_2O and then rapidly dried with a stream of cool air. The following day slides were exposed to Kodak Bio Max MR film (Kodak, Rochester, NY, USA) with ^{125}I microscale standards (American Radiolabeled Chemicals, Inc., St. Louis, MO, USA) for 168 h. Receptor binding was quantified from film using NIH ImageJ. The ^{125}I microscale standards were used to convert uncalibrated optical density to disintegrations per minute (DPM). Nucleus accumbens was quantified in both hemispheres and sides were compared for any differences, of which none were found. A measure of non-specific binding (NSB) was taken for each section by quantifying binding in an area of cortex with no oxytocin receptors. The NSB value was subtracted from the binding value for each section and a mean was then calculated for each section, followed by a mean for the entire area for each subject. Area means were used in data analysis and are referred to as normalized DPM.

2.12. Experimental design and statistical analysis

Statistical analyses were conducted using GraphPad Prism 6.0. For each analysis, $p < 0.05$ was regarded as statistically significant. Parental care following early handling manipulation was analyzed using a 2-way parent x handling condition analysis of variance (ANOVA). *Oxtr* methylation in nucleus accumbens and whole blood in the prairie vole was analyzed using a 2-way CpG site x handling condition ANOVA with exploratory post-hoc testing with Bonferroni multiple comparisons correction. One set of male/female sibling pairs per unique parenting pair were included for analysis. Offspring from three MAN0 pairs and four MAN1 pairs were excluded from analyses because the litters had only female offspring. Levels of *de novo Oxtr* DNA methylation following an early handling manipulation were analyzed using unpaired two-tailed t tests. *Oxtr* expression in the nucleus accumbens was assessed with an unpaired two-tailed t test. A Partial Least Squares (PLS) regression was used to explore how *Oxtr* methylation within each of the four assayed CpG sites in each tissue correlated with *Oxtr* expression. Given the high correlations in methylation values between these sites (see Supplemental Table 1), ordinary least squares regression is not appropriate due to multicollinearity. We therefore used PLS regression which operates on the entire data structure at once to find latent components which maximize the variability of predictors and have maximum correlation with the response. Model fitting and selection was performed with leave-one-out cross-validation using the PLS package (Mevik et al., 2016) in R (Team, 2017). The optimal number of components to retain for each analysis was determined by selecting the first local minimum in the root mean squared error of prediction curve. For both tissue types, a 1-component model emerged as optimal, indicating that all four CpG sites show a similar relationship to *Oxtr* expression and can be represented by a single latent methylation component. For visualization purposes only, we plotted trend lines from linear models predicting methylation from expression. *Oxtr* polymorphism impact on gene expression was analyzed using a 2-way handling condition x SNP ANOVA. Correlation was used to examine the relationship between *Oxtr* DNA methylation in central and peripheral prairie vole tissue. Normality was assessed by Kolmogorov-Smirnov (K–S) test. Spearman's rank correlation was used when data were not normally distributed, whereas Pearson's correlation was used when data were normally distributed. *Oxtr* methylation after experiencing naturally varying early parental care was analyzed with a nested ANOVA with a post-hoc Bonferroni multiple comparisons correction to account for the use of multiple offspring of each sex from a single breeding pair in the DNA methylation analysis. One animal from the medium contact group was removed due to pyrosequencing failure. The relationship between early care and oxytocin receptor protein levels in offspring was determined using Spearman's rank correlation.

3. Results

3.1. Early handling increases parental care

Prairie voles reared under conditions in which they are left relatively undisturbed, with no direct handling, for the first week of life (MAN0), display marked disruptions later in life in alloparenting, pair bonding, and parenting, likely as a result of increased anxiety (Bales et al., 2007, 2011). In contrast, offspring that experience a brief direct handling manipulation (MAN1) on postnatal day (PND) 1, in which mother, father, and pups are lifted by an investigator's gloved hand for 30 s, show later adult social behaviors typical of this species. In rats, brief early handling leads to similar changes in adult offspring behavior (Levine, 1957; Denenberg et al., 1962), likely because dams increase pup licking and grooming immediately after the handling episode (Smotherman et al., 1977; Boccia and Pedersen, 2001). Alternately, rats receiving little to no early life handling disturbance had increased anxiety-like behavior in adulthood (Levine et al., 2002).

We observed maternal and paternal behaviors immediately following the MAN1 handling or in unmanipulated (MAN0) voles at comparable times on PND1. Total pup-directed behavior rather than a single behavior (as typically done in rats) was quantified to more accurately characterize the total biparental stimulation experienced by prairie vole pups in early life. MAN1 parents were more attentive to their pups ($F_{(1,\ 20)} = 7.24$, $p = 0.014$) with higher amounts of pup directed care being provided by the mother ($F_{(1,\ 20)} = 13.76$, $p = 0.0014$; Fig. 2A). No differences in parental behavior were found on PND8 or PND15 (Supplemental Fig. 1) indicating that the effects of manipulation are transient. The increase in care after direct (MAN1) handling may reflect conditions experienced by offspring in the field. During maternal foraging bouts, offspring are briefly separated from the mother. Upon her return to the nest, she typically licks and grooms offspring for a short time. MAN1 handling appears to mimic this condition. We hypothesized that, as in other models (Weaver et al., 2004), this transient increase in early care would lead to experience-based differences in DNA methylation impacting both gene and protein expression. We focused our investigation on *Oxtr* because the social behaviors influenced by early handling are heavily oxytocin-dependent.

3.2. Early handling alters DNA methylation of the oxytocin receptor in both brain and blood

In order to identify CpG sites in the prairie vole *Oxtr* that may be

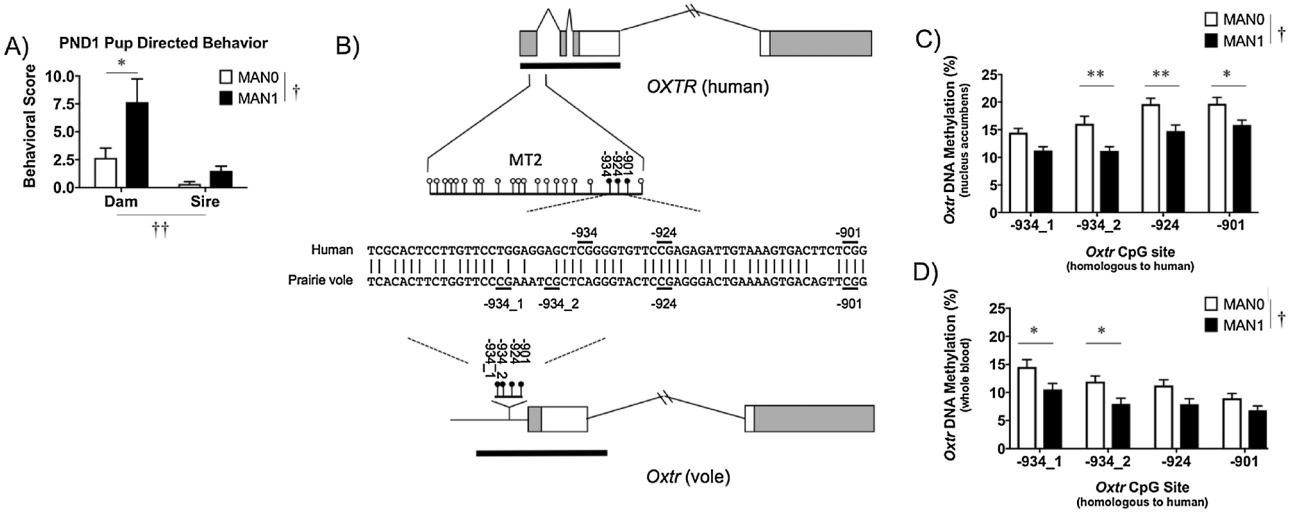

Fig. 2. Early handling increases parental behavior directed toward offspring and alters DNA methylation in the brain and blood at conserved CpG sites in the promoter region of *Oxtr* in the prairie vole. (A) Direct handling manipulation (MAN1) increases parental care of offspring on PND 1, specifically increasing maternal pup-directed behavior (2-way parent x handling condition ANOVA; MAN0, n = 7; MAN1, n = 6; † main effect of handling; †† main effect of parent; *p < 0.05; error bars indicate standard error). (B) DNA methylation at CpG sites -934, -924, and -901 in *OXTR* MT2 region of the human *OXTR* are conserved in prairie vole *Oxtr*. Exons are displayed as boxes (coding, white; untranslated, grey) and introns are solid lines. CpG islands are indicated with black bars. Specific CpG dinucleotides analyzed here are indicated with sticks and circles, with closed circles indicating sites that exhibit significant association with expression. Partial alignment of conserved human and prairie vole gene sequence is indicated, conserved nucleotides are represented with a vertical line (|) and CpG sites are highlighted with a horizontal line (-). (C) Offspring receiving less maternal attention (MAN0) have higher levels of *Oxtr* DNA methylation in the nucleus accumbens and (D) in whole blood at the CpG sites conserved from the human *OXTR* (2-way CpG site x handling condition ANOVA; 13 male/female sibling pairs; MAN0: n = 7 females, 7 males; MAN1: n = 6 females, 6 males; † main effect of handling; Bonferroni correction, *p < 0.05, **p < 0.01; error bars indicate standard error).

sensitive to early life experience and impact the expression of the gene, we used a region of the human *OXTR* promoter that contains a DNA methylation specific regulatory region (termed MT2, hg38: chr3:8,769,033-8,769,438) to probe the prairie vole genome (Kusui et al., 2001). First, we identified a 71 base pair alignment block in the UCSC genome browser (Kent et al., 2002) that contains a portion of the MT2 region in several rodent species including the prairie vole (Supplemental Fig. 2). Next, we estimated statistical significance of the human and vole alignment by shuffling and identified significant homology (200 shuffles, z = 239.9; bits = 48.3; E (10,000) = 1.5×10^{-11}) (Pearson, 2013). Remarkably, this region in the human contains four CpG sites (−959, −934, −924, −901) which when methylated are associated with decreased transcription in the human brain (Gregory et al., 2009) and variability in DNA methylation of one of these sites (-934) has been associated with several psychiatric disorders (24–28) as well as individual variability in the brain's response to social perception (22, 23). Two of these CpG sites, −924 and −901 were conserved in the prairie vole and the third site, −934, may be functionally related to −934_1 and −934_2, (Fig. 2B). For ease in highlighting CpG site conservation with the human sequence, we named the prairie vole CpG sites after the sites that have been previously reported in the human. Though conservation does not imply function, it is interesting to note that this CpG site conservation was not present in the reference genome of the laboratory rat and only one site, −924, was conserved in the mouse (Supplemental Fig. 2). Identification of these conserved sites specifically in the prairie vole provides a starting point for DNA methylation analysis.

To examine the hypothesis that differential early care alters DNA methylation of *Oxtr* in the brain, we subjected prairie vole pups to the early handling manipulation paradigm described above. On PND24, brain tissue was obtained from the nucleus accumbens, a high OXTR expressing region that has been implicated in several social behaviors including pair bonding and alloparenting (13 male/female siblings; MAN0, n = 7 females, 7 males; MAN1, n = 6 females, 6 males). DNA methylation analysis was targeted to the conserved region containing CpG sites −934_1, −934_2, −924, and −901. Confirming our prediction, offspring receiving less early care (MAN0) had higher levels of *Oxtr* DNA methylation in the nucleus accumbens (main effect of handling, $F_{(1, 96)} = 34.22$, $p = < 0.0001$; main effect of CpG site, $F_{(3, 96)} = 34.22$, $p = < 0.0001$; no interaction; Bonferroni post hoc analysis, −934_1: $p = 0.108$; −934_2: $p = 0.004$; −924: $p = 0.004$; -901: $p = 0.037$; Fig. 2C). A higher level of *Oxtr* DNA methylation in MAN0 compared to MAN1 offspring was also observed in whole blood of the same animals (main effect of handling, $F_{(1, 96)} = 22.03$, $p < 0.0001$; main effect of CpG site, $F_{(3, 96)} = 7.16$, $p = 0.0002$; no interaction; Bonferroni post hoc analysis, −934_1: $p = 0.025$; −934_2: $p = 0.027$; −924: $p = 0.092$; −901: $p = 0.546$; Fig. 2D; Supplemental Table 1). Sex differences were not observed for either methylation analysis. The higher levels of DNA methylation found in the nucleus accumbens of MAN0 offspring indicate experience sensitive differences in the epigenetic state of *Oxtr* in a brain region important in the control of social attachment. That the same pattern of DNA methylation after early handling was found in the whole blood of offspring suggests that blood DNA may be used as a biomarker of this change.

3.3. Heightened early care prevents de novo methylation of Oxtr

It was not clear if high amounts of early care, induced here by postnatal handling, act to decrease methylation or if less early care leads to *de novo* DNA methylation. To determine this, we collected the forebrain (which contains the nucleus accumbens) from embryonic day (ED) 21 offspring dissected from the uterus on the expected day of birth and prior to the receipt of any parental care to compare *Oxtr* DNA methylation levels in these ED21 offspring to levels observed in PND24 offspring in the above handling experiment. Compared to ED21 offspring, animals that received less early care (MAN0) had significant increases in DNA methylation across all of the conserved CpG sites (−934_1: t(30) = 2.72, $p = 0.011$; −934_2: t(30) = 5.14, $p < 0.0001$; −924: t(30) = 4.27, $p = 0.0002$; −901: t(30) = 2.80, $p = 0.009$), while methylation levels of MAN1 offspring that received high early care tended to not differ from ED21 offspring (−934_1: t(28) = 0.524, $p = 0.605$; −934_2: t(28) = 2.56, $p = 0.016$; −924: t(28) = 0.78,

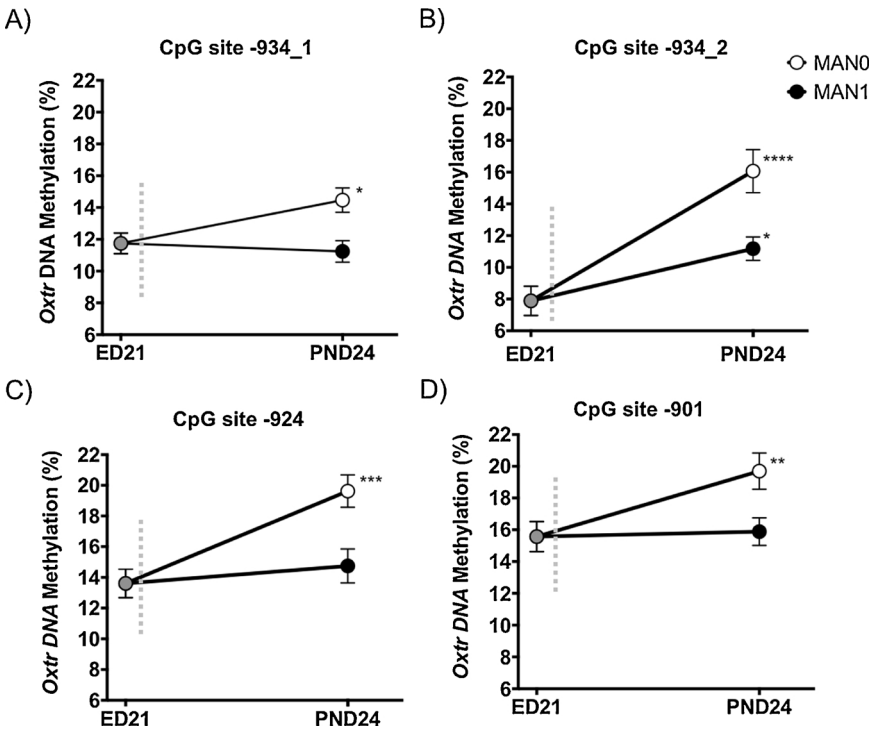

Fig. 3. Early handling prevents de novo DNA methylation of Oxtr. *Oxtr* DNA methylation levels at CpG sites **(A)** −934_1, **(B)** −934_2, **(C)** −924, and **(D)** −901 in the forebrain of ED21 offspring (n = 18, 9 male/female sibling pairs) are similar to MAN1 offspring that received increased early parental care, whereas MAN0 offspring that received low amounts of early care have a significant increase in postnatal *de novo* DNA methylation in the nucleus accumbens (unpaired two-tailed t test; *p < 0.05, **p < 0.01, ***p < 0.001, ****p < 0.0001; error bars indicate standard error).

$p = 0.444$; −901: $t(28) = 0.23$, $p = 0.821$, Fig. 3). High amounts of early care, then, prevent *de novo* DNA methylation of *Oxtr*.

3.4. Heightened early care increases Oxtr gene expression

To establish that differences in the amount of early care lead to changes in gene expression in our model, we measured *Oxtr* gene expression in the nucleus accumbens of the same PND 24 animals. Offspring receiving less early care and also displaying increased levels of *Oxtr* DNA methylation (MAN0) exhibited decreased *Oxtr* gene expression compared to MAN1 animals ($t(24) = 2.28$, $p = 0.032$, Fig. 4A). We then explored how *Oxtr* DNA methylation within the four assayed CpG sites correlated with *Oxtr* expression. Using a Partial Least Squares (PLS) regression, a 1-component model emerged as optimal, indicating that all four CpG sites show a similar relationship to *Oxtr* expression and can be represented by a single latent methylation component. This 1-component model was capable of explaining 79.07% of the variance in *Oxtr* DNA methylation and 26.27% of the variance in

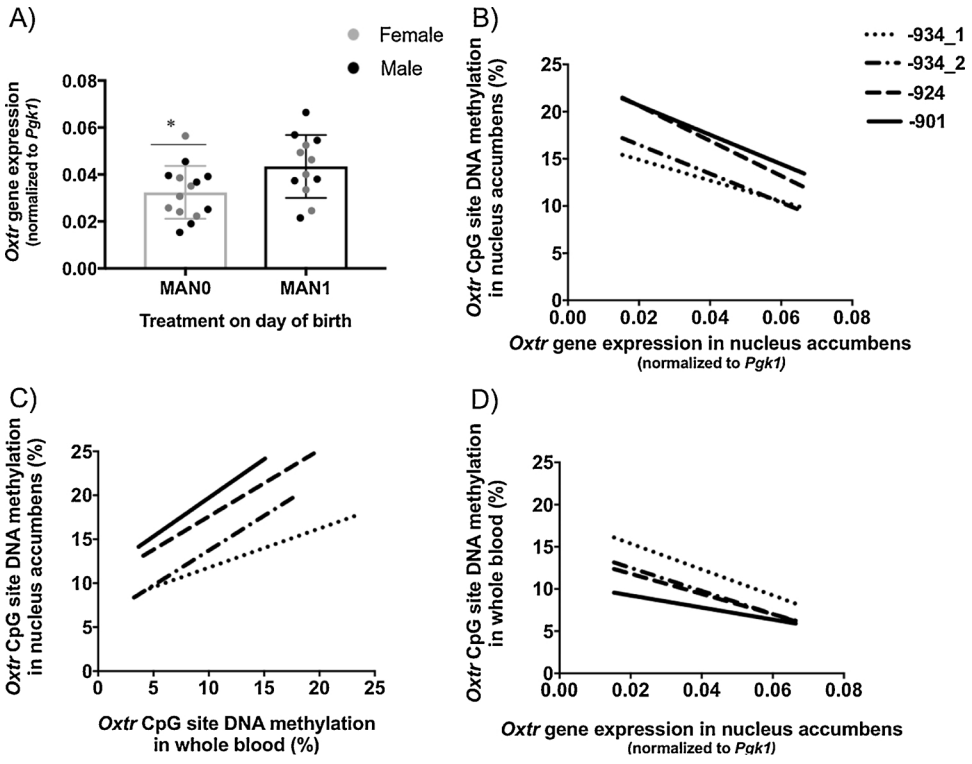

Fig. 4. Experience sensitive *de novo* DNA methylation of *Oxtr* decreases gene expression in the brain, is reflected in the blood and can be used as a biomarker of the transcription state of the nucleus accumbens. Offspring receiving less maternal attention (MAN0) (A) have decreased *Oxtr* gene expression in the nucleus accumbens (unpaired two-tailed t test). (B) *Oxtr* DNA methylation is negatively correlated with *Oxtr* gene expression in the nucleus accumbens (Pearson's r). (C) DNA methylation in whole blood is positively correlated with DNA methylation levels in the nucleus accumbens and (D) negatively correlated to *Oxtr* gene expression in the nucleus accumbens (Pearson's r). Trend lines are plotted predicting methylation from expression for data visualization purposes only (n = 26, 13 male/female sibling pairs; MAN0 = 14, MAN1 = 12; unpaired, two tailed t-test, *p < 0.05; error bars indicate standard error).

Table 1
Oxtr DNA methylation in the nucleus accumbens and in whole blood is negatively associated with *Oxtr* gene expression in the nucleus accumbens. All methylation sites significantly negatively load onto the single CpG site component, indicating a significant negative association between *Oxtr* methylation and *Oxtr* expression in Nacc. In a separate analysis, all methylation sites significantly negatively load onto the single CpG site component, indicating a significant negative association between *Oxtr* methylation in whole blood and *Oxtr* expression in Nacc. Loadings, regression coefficients, and jackknife approximate t tests of regression coefficients for each model are listed in Table 1.

Model	Site	Loading	Estimate	Std. Error	Df	t value	p value
Nucleus Accumbens	934_1	−0.364	−3.10E-04	1.09E-04	25	−2.84	0.009
	934_2	−0.567	−4.29E-04	1.67E-04	25	−2.57	0.017
	924	−0.56	−5.22E-04	1.62E-04	25	−3.22	0.004
	901	−0.489	−4.36E-04	8.89E-05	25	−4.91	< .0001
Whole Blood	934_1	−0.599	−4.52E-04	1.71E-04	25	−2.64	0.014
	934_2	−0.515	−4.04E-04	1.61E-04	25	−2.52	0.019
	924	−0.499	−3.53E-04	1.45E-04	25	−2.43	0.022
	901	−0.367	−2.10E-04	1.04E-04	25	−2.03	0.053

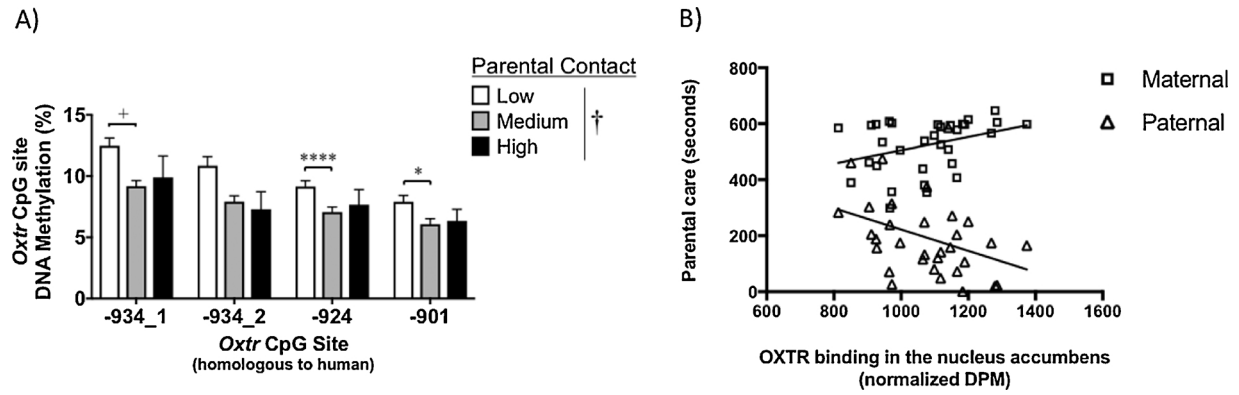

Fig. 5. Offspring reared by parents who exhibit naturally low levels of parental care exhibit increased *Oxtr* DNA methylation in the blood and decreased OXTR distribution in the nucleus accumbens. (A) Offspring of low contact parents (n = 49) have higher levels of *Oxtr* DNA methylation in whole blood compared to offspring of medium (n = 89) or high (n = 16) contact parents (2-way CpG site x parenting ANOVA; † main effect of handling; *p < 0.05, ****p < 0.0001, +p = 0.06). (B) High levels of early maternal care and low amounts of early paternal care are associated with increased OXTR binding in the nucleus accumbens of adolescent offspring (Spearman's rho; n = 32).

Oxtr expression in the nucleus accumbens. All methylation sites significantly negatively loaded onto the component, indicating a significant negative association between *Oxtr* DNA methylation and *Oxtr* expression in this region (Table 1). Trend lines plotted to visualize the data support conclusions of a negative relationship between *Oxtr* DNA methylation and *Oxtr* expression in the nucleus accumbens (Fig. 4B). Sex differences were not observed, nor was there a relationship with a previously associated *Oxtr* polymorphism (King et al., 2016) (Supplemental Fig. 3). These data suggest that methylation at these CpG sites may be relevant in regulating transcription of *Oxtr* and also provide a mechanism through which individual differences in social behavior may develop following differences in early care.

3.5. Peripheral measures of DNA methylation predict central levels

Data in humans suggest a positive association between levels of *OXTR* DNA methylation in central and peripheral tissues (Gregory et al., 2009). Here we found a strong positive correlation between *Oxtr* DNA methylation in whole blood and the nucleus accumbens at PND 24 at all four conserved CpG sites (−934_1: r(26) = 0.684, p = 0.0001; −934_2: r(26) = 0.677, p = 0.0001; −924: r(26) = 0.650, p = 0.0003; -901: r(26) = 0.654, p = 0.0003, Fig. 4C). The methylation state of the blood was also associated with the level of transcription in the brain at three of the four CpG sites. Using PLS regression, the 1-component model for whole blood was capable of explaining 94.92% of the variance in *Oxtr* DNA methylation and 18.20% of the variance in *Oxtr* expression. All methylation sites significantly negatively loaded onto the component, indicating a significant negative association between whole blood *Oxtr* DNA methylation and *Oxtr* expression in the nucleus accumbens (Table 1). Trend lines plotted to visualize the data support conclusions of a negative relationship between *Oxtr* DNA methylation in whole blood and *Oxtr* expression in the nucleus accumbens (Fig. 4D). Peripheral measures of *Oxtr* DNA methylation, therefore, provide useful information on central epigenetic markers, at least in the nucleus accumbens.

3.6. Naturally varying early care alters DNA methylation

To validate findings from the handling model, we assessed naturally occurring variation in early care in prairie voles and *Oxtr* DNA methylation in the blood since variation in early care impacts adult social behavior (Perkeybile et al., 2013, 2015; del Razo and Bales, 2016). Whole blood was collected from low contact (LC; n = 49), medium contact (MC; n = 90), and high contact (HC; n = 16) offspring in young adulthood (PND48–52) and assessed for DNA methylation at the conserved CpG sites. LC offspring who received the lowest levels of early care had increased levels of *Oxtr* DNA methylation at two of the four conserved CpG sites and trended toward increased DNA methylation at a third CpG site (main effect of parental contact, $F_{(2,\ 604)} = 22.05$, $p < 0.0001$; main effect of CpG site, $F_{(3,\ 604)} = 12.34$, $p < 0.0001$; no interaction; Bonferroni post hoc analysis, −934_1: p = 0.0605; −934_2: p = 0.1227; −924: $p <$ 0.0001; −901: p = 0.0376; Fig. 5A) compared to MC offspring. These results support findings of the impact of early handling, where MAN0 offspring experienced lower amounts of early care and had increased *Oxtr* DNA methylation levels. This offers further support for the hypothesis that the epigenetic modifications seen here are a result of the amount of total care received in the early postnatal period and that the blood can be used as a biomarker

of this change.

3.7. Heightened early care increases OXTR protein

For variation in early care to impact later life social behavior and attachment in offspring through pathways involving oxytocin, the changes seen in *Oxtr* DNA methylation and gene expression need to correspond to changes in receptor protein in the same region. We tested this using autoradiography methods to visualize OXTR in the nucleus accumbens in an additional cohort of PND 24–26 (n = 32) animals following naturally varying early care. Increased binding of OXTR in this region was associated with increased early maternal care ($\rho(32) = 0.388$, $p = 0.028$) and with decreased early paternal care ($\rho(32) = -0.4263$, $p = 0.015$, Fig. 5B). Maternal care was also inversely related to paternal care (Supplemental Fig. 4), which matches previous data from our lab (Perkeybile et al., 2013) as well as data from parental care after early handling presented above. That lower early maternal care is associated with low OXTR protein binding in the nucleus accumbens provides additional support that differences in these epigenetic markers are altering the function of oxytocin pathways via changes at the receptor level.

4. Discussion

Our findings provide strong evidence that early parental care directly impacts functioning of oxytocin pathways in the prairie vole through altering DNA methylation of *Oxtr* at four CpG sites conserved from the human sequence. This highlights for the first time a mechanism through which early experience can create individual differences in oxytocin-dependent behaviors. Whether naturally occurring or experimentally induced via reduced early handling, decreased early care in the prairie vole leads to decreased alloparenting in adolescence and adulthood (Bales et al., 2007, 2011; Perkeybile et al., 2013; del Razo and Bales, 2016) and these effects are passed to a subsequent generation through non-genomic transmission (Stone and Bales, 2010; Perkeybile et al., 2015). Alloparental behavior in prairie voles is regulated by oxytocin receptors in the nucleus accumbens (Olazabal and Young, 2006b, a; Kenkel et al., 2017); increased oxytocin receptor density in this region is positively correlated with alloparenting within individual prairie voles and between vole species. Similarly, formation of pair bonds, a behavior that is also linked to distribution and density of oxytocin receptors (Insel and Shapiro, 1992; Young, 1999; Ross et al., 2009; Ophir et al., 2012; Keebaugh et al., 2015; Johnson et al., 2016), is reduced following decreased early care (Bales et al., 2007; del Razo and Bales, 2016). That we find increased *Oxtr* DNA methylation in two models of decreased early parental care, then, helps to explain previously seen decreases in alloparenting and pair bonding. Higher levels of *Oxtr* DNA methylation work to decrease *Oxtr* gene expression, theoretically resulting in decreased oxytocin receptor density. Our results support this, with low levels of early parenting being associated with both increased *Oxtr* DNA methylation and decreased OXTR receptor density in the nucleus accumbens. We hypothesize that early care alters OXTR in this region by altering DNA methylation patterns. This lower receptor density would then work to decrease expression of species-typical alloparenting and pair bonding. This provides a mechanism by which early experiences can alter an organism's developmental trajectory and create individual differences in social behavior and adds to the growing literature on the role epigenetic markers play in shaping variation in behavior.

Site-specific analyses in humans have revealed associations between CpG DNA methylation levels and both psychiatric diagnosis and neural functioning in neurotypical populations. Increased levels of *OXTR* methylation at CpG site -934 measured in blood have been linked to postpartum depression (Bell et al., 2015), autism (Gregory et al., 2009), increased neural activity in emotion processing regions (Puglia et al., 2015), and increased activity in brain regions responsible for social perception in response to socially ambiguous stimuli (Jack et al., 2012). Our work here demonstrates the need for further investigation of the impacts of the early life environment on *OXTR* DNA methylation in both neurotypical and atypical individuals. This focus will allow us to gain a better understanding of the consequences of early life experience on oxytocin system regulation and its link to psychiatric health and well-being.

Early life experiences, particularly those occurring within the parent-infant dyad, have long been known to alter the developmental trajectory of offspring. This has now been demonstrated with regard to epigenetic regulation of *OXTR*. In humans, low amounts of childhood maternal care are associated with increased levels of *OXTR* DNA methylation in adults (Unternaehrer et al., 2015) and childhood abuse interacts with *OXTR* DNA methylation to predict depression and anxiety (Smearman et al., 2016). Our results support these findings, where we see higher levels of CpG site-specific DNA methylation at sites that are associated with psychiatric disorder in humans. Therefore, future work investigating these specific CpG sites in humans following varying early life experience would be useful to support our and others' previous findings. This may help to uncover new pathways via which early life neglect influences late mental health outcomes and allow for the development of new interventions to counteract these outcomes.

The strong relationship shown here between central and peripheral *Oxtr* DNA methylation levels verifies the usefulness of peripherally obtained DNA as we seek to understand epigenetic mechanisms regulating social behavior. As more research aims to understand the role of epigenetic markers on a range of human outcomes, it will be necessary to have access to biological samples that can serve as a useful proxy for central tissue. Our results indicate easily accessible blood samples may be a useful tool for this. The ability to use peripheral sampling techniques also opens the door for lifespan development animal studies with multiple sampling time points in a single animal that previously were not possible.

Our findings provide strong evidence for a mechanism by which early experiences can alter an organism's developmental trajectory and create individual differences in social behavior and adds to the growing literature on the role epigenetic markers play in shaping variation in behavior. Future work should combine detailed behavioral measures with analyses of epigenetic markers to establish a direct link between the two outcomes following varying early experiences. As more research aims to understand the role of epigenetic markers on a range of human outcomes, it will be vital to have a comparable animal model. Our findings suggest the prairie vole will be useful in this regard, allowing for an expanded understanding of the role of epigenetic markers in controlling oxytocin pathways and impacting complex social behavior that can then inform and guide work on human conditions.

Author contributions

A.M.P., C.S.C., K.L.B., and J.J.C. were responsible for designing research. A.M.P., W.M.K., T.S.L., T.K., N.M., and L.E. conducted the studies. A.M.P., M.H.P., C.S.C., K.L.B., and J.J.C. analyzed and interpreted the data collected. A.M.P., C.S.C., K.L.W., S.G.G., K.L.B., and J.J.C. wrote the manuscript. All authors have approved of the final submission of this manuscript.

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Conflicts of Interest

The authors declare no competing financial interests.

Appendix A. Supplementary data

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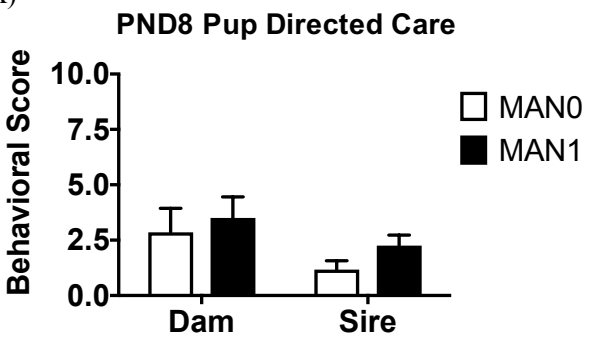
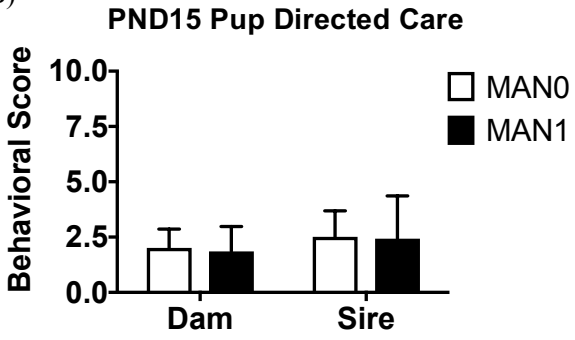
Supplemental Materials

Supplemental Figure 1.

A)



B)



Supplemental Figure 2.

A)

Human: >hg38_dna range=chr3:8769078-8769148

GTCTCAATCCCGAGAAGTCACTTTACAATCTCTCGGAACACCCCGAGCTCCTCCAGGAACAAGGAGTGCGA

-901 -924 -934

Mouse: >mm10_dna range=chr6:112490620-112490691

ACCTTCGTCCAAGCTGTCACTTTTCAGTCTCTCTCGGTACACCCTGAGCACCTCCCGGAACCAGAGACGTGA

-924

Rat: >rn6_dna range=chr4:144416943-144417009

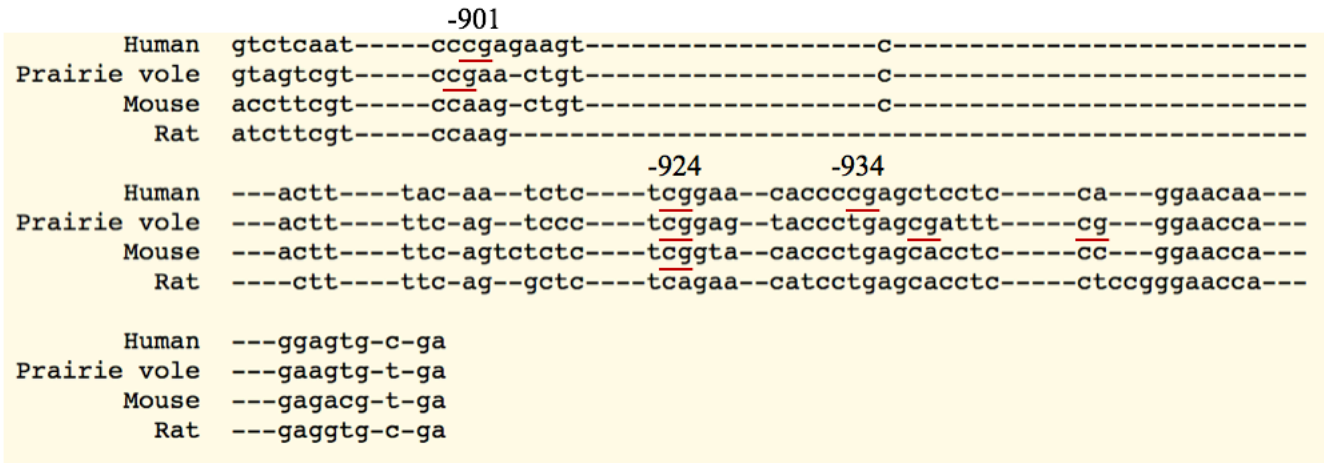
ATCTTCGTCCAAGCTTTTCAGGCTCTCAGAACATCCTGAGCACCTCCTCCGGGAACCAGAGGTGCGA

Prairie vole*

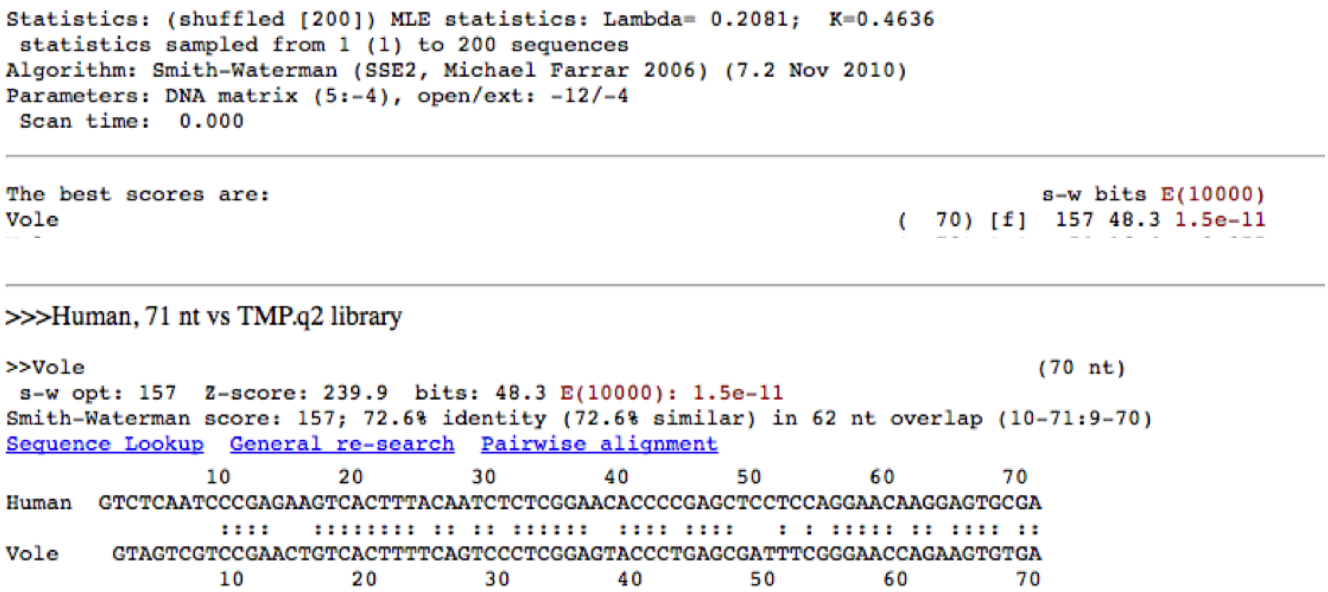
GTAGTCGTCCGAACTGTCACTTTTCAGTCCCTCGGAGTACCCTGAGCGATTTCGGGAACCAGAAGTGTGA

-901 -924 -934_2 -934_1

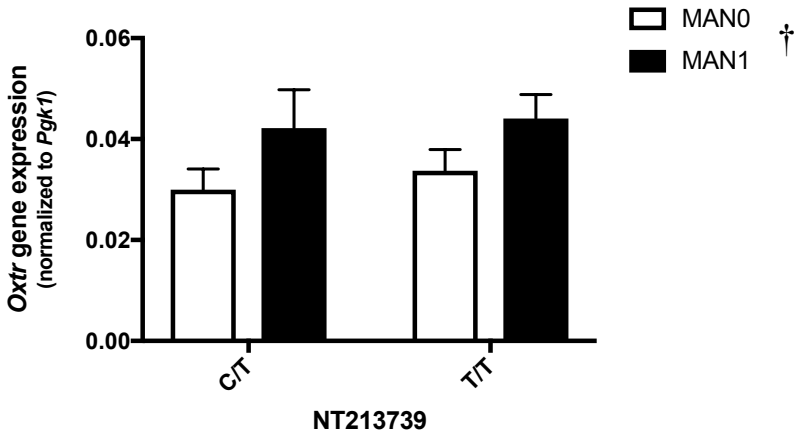
B)



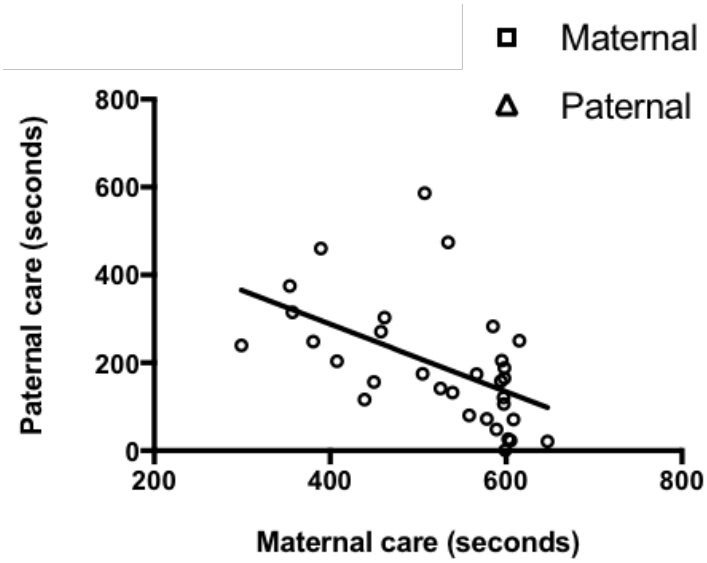
C)



Supplemental Figure 3.



Supplemental Figure 4.



Supplemental Table 1.

	1	2	3	4	5	6	7	8
1. Blood -934_1	1.00							
2. Blood -934_2	0.95	1.00						
3. Blood -924	0.96	0.95	1.00					
4. Blood -901	0.86	0.88	0.92	1.00				
5. Nacc -934_1	0.68	0.70	0.59	0.53	1.00			
6. NAcc -934_2	0.60	0.68	0.49	0.42	0.90	1.00		
7. NAcc -924	0.71	0.75	0.65	0.57	0.91	0.88	1.00	
8. NAcc -901	0.76	0.80	0.71	0.65	0.83	0.81	0.89	1.00

Supplemental Figure and Table Legends

Supplemental Figure 1. Differences in parental care in MAN model are transient. Parental care of offspring following early manipulation did not differ between MAN0 and MAN1 groups on **(A)** PND 8 or **(B)** PND 15.

Supplemental Figure 2. A portion of the human MT2 region in *OXTR* is conserved in prairie voles. **(A)** Genome position and sequences of similar *OXTR* regulatory region in humans and rodents. Prairie vole sequence from GenBank: DP001214.1 **(B)** Partial output of UCSC Multiz alignment highlighting conserved CpG sites of putative *OXTR* regulatory region in multiple species. **(C)** Output from PRSS found at http://fasta.bioch.virginia.edu/.

Supplemental Figure 3. Polymorphism NT213739 does not impact *Oxtr* gene expression in this sample. A polymorphism (NT213739) in the prairie vole *Oxtr* gene was previously reported (King et al, 2016) to impact transcription of the gene. We did not identify C/C homozygotes in the sample we studied and there was no significant difference in transcription between C/T (MAN0, n=5; MAN1, n=4) and T/T (MAN0, n=9; MAN1, n=8) genotypes in our sample. (2-way Handling Condition x SNP ANOVA, † main effect of handling, $F_{(1, 22)}$ =4.559, p=0.0441; no effect of SNP, $F_{(1,22)}$ =0.2909, p=0.5950; error bars indicate standard error).

Supplemental Figure 4. Relationship between maternal and paternal behavior. Naturally varying levels of maternal and paternal care are inversely related.

Supplemental Table 1. Correlation of DNA methylation levels between *Oxtr* CpG sites. Methylation values are strongly correlated between CpG sites both within and across tissue types.

Chapter 2

Oxytocin receptor is epigenetically sensitive at select homologous CpG sites between humans and prairie voles

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Abstract

The neuropeptide oxytocin is involved in the regulation of complex social behaviors. The actions of this neuromodulator are dependent on the oxytocin receptor, which is encoded by the oxytocin receptor gene (*OXTR*). There is growing interest in examining the relationship between epigenetic programing of *OXTR* and social behavior in humans. Current studies have focused on DNA methylation at a region within exon 1 and intron 1 (MT2 region) and a segment within exon 3. Of the 22 CpG sites measured across the MT2 region in human brain tissue, only CpGs -934, -924, and -901 had a strong correlation with increases of DNA methylation corresponding to decreases in *OXTR* expression (-934: $\rho(36) = -0.78$, $p = 0.006$; -924: $\rho(36) = -0.68$, $p = 0.024$; -901: $\rho(36) = -0.65$, $p = 0.032$). Yet, as is common in emerging fields, there is not enough published evidence to draw definitive conclusions on the role of DNA methylation on human social behavior. Therefore, we turned to a commonly used animal model of social behavior, the prairie voles *(Microtus ochrogaster)* for further analysis. We found that only a small number of CpGs within the conserved MT2 and exon 3 region have a strong inverse relationship between DNA methylation and *Oxtr* expression. Additionally, to determine if there are environmental drivers of DNA methylation, we investigated the role of parental care in early life. We discovered that the amount of parental care received within the first week of life influences DNA methylation across the conserved MT2 and exon 3 region (2-way ANOVA, $p = <0.0001$). Following multiple comparison corrections, only 5 CpG sites, all within the conserved MT2 region, had significant differences in DNA methylation. We investigated the relationship between DNA methylation and *Oxtr* expression of these 5 CpG sites and discovered that only homologous CpGs -934_2, -924, and -901 had a strong trending or significant correlation, with increased *Oxtr* DNA methylation corresponding to decreases in *Oxtr* expression (-934_2: $\rho(26) = -0.47$, $p = 0.058$; CpG -924: $\rho(26) = -0.50$, $p = 0.043$, CpG -901: $\rho(26) = -0.47$, $p = 0.058$). Collectively, our findings demonstrate that *Oxtr* is epigenetically sensitive to early life experience, particularly at CpG sites that are primarily regulating *Oxtr* expression (-934_2, -924, -901). We show that these CpG sites are homologous in humans, and as we move forward in this emerging field, our findings point to narrowing our region of interest to the homologous CpGs -934, -924, and -901 within the MT2 region in both humans and prairie vole studies.

Introduction

Oxytocin is a neuropeptide that has been implicated in the development and display of social behavior. Oxytocin modulates various behaviors through its association with its receptor (OXTR). Factors that regulate the expression of the oxytocin receptor, rather than oxytocin itself, may provide an improved, more informed explanation for the variability in social behavior. The oxytocin receptor is encoded by the oxytocin receptor gene (*OXTR)* and previous studies in humans indicate that altered levels of DNA methylation in *OXTR* are a risk factor for several psychiatric disorders, including autism spectrum disorder (Gregory et al., 2009; Rijlaarsdam et al., 2017), postpartum depression (Bell et al., 2015; Kimmel et al., 2016), major depressive disorder (Reiner et al., 2016, Chagnon et al., 2015), anxiety disorders (Ziegler et al., 2015; Cappi et al., 2016), anorexia nervosa (Kim et al., 2014), callous-unemotional traits and psychopathy (Dadds et al., 2014; Cecil et al., 2014), and psychosis (Rubin et al., 2016). It has also been shown that *OXTR* DNA methylation is related to neural endophenotypes of social perception (Jack et al., 2012; Puglia et al., 2015), which are commonly dysregulated in the above mentioned disorders.

Most human studies investigating the relationship between *OXTR* DNA methylation and social phenotypes have focused on a segment within the promoter region located within exon 1 and intron 1, known as the MT2 region. This region has been identified as functionally significant, with DNA methylation dependent regulation of *OXTR* expression (Kusui et al., 2001) and increases of DNA methylation of specific CpG sites in this region corresponding to decreases in *OXTR* expression in human blood and brain tissue (Gregory et al., 2009). A majority of *OXTR*

studies indicate that increases in DNA methylation within the MT2 region correspond with psychiatric disorders and increased brain activity evoked by social tasks (Gregory et al., 2009; Jack et al., 2012; Kim et al., 2014; Dadds et al., 2014; Cecil et al., 2014; Bell et al., 2015; Puglia et al., 2015, Rubin et al., 2016; Rijlaarsdam et al., 2017).

An additional genomic region in *OXTR* that has been studied is a segment in exon 3, with differences in DNA methylation corresponding to human social behavior and early life stress (Ziegler et al., 2015; Chagnon et al., 2015; Unternaehrer et al., 2015, 2016; Smearman et al., 2016; Cappi et al. 2016). Initial studies examining the relationship between DNA methylation within the exon 3 segment and biological outcomes were not hypothesis driven, as the target region was designed in an exploratory manner. Also, no study has examined the relationship between DNA methylation in exon 3 and *OXTR* expression in humans. Additionally, the data presented in the exon 3 literature are convoluted, as increases and decreases of DNA methylation in this region have been associated with adverse phenotypes. The DNA methylation data is also difficult to interpret since it is typically presented as log-transformed differences of DNA methylation and relies on data driven models, which does not necessarily translate well to biological conclusions. Also, statistical significance is often only achieved when DNA methylation is averaged across selected CpG sites, indicating that data was excluded or combined post-hoc.

This study sets out to clarify the relationship between DNA methylation in the MT2 and exon 3 region on oxytocin receptor gene expression by using an animal model, the prairie vole (*Microtus ochrogaster*). The prairie vole is a rodent model often employed to study of the impact

of oxytocin on the formation of complex social dynamics that may be relevant to our understanding of human behavior. Prairie voles display social characteristics that are common in humans, but relatively rare in mammals, such as forming socially monogamous relationships characterized by a high degree of selective pair bonding behavior, biparental care of young with pair bonded partner, and alloparental care to both related and unrelated young pups (Williams et al., 1992; Solomon, 1993; Oliveras and Novak, 1986; Getz et al., 1981; Carter et al., 1995, 1998; Roberts et al, 1998; Young et at., 1998; Insel and Young, 2001). The activation of the oxytocinergic system through the oxytocin receptor rich nucleus accumbens is particularly important for the formation of these behaviors. In prairie voles, exposure to an oxytocin antagonist in the nucleus accumbens results in the inhibition of pair-bonding and alloparenting (Young et al., 2001; Olazábal and Young, 2006), overexpression of oxytocin receptors and oxytocin infusion in the nucleus accumbens increases partner preference (Ross et al., 2009; Liu and Wang, 2003) and increased binding of oxytocin receptors correlates with an increase of parental behavior in females (Olazábal and Young, 2006b). This guided us to examine the nucleus accumbins in the prairie vole and to perform a targeted study about the relationship between *Oxtr* DNA methylation and *Oxtr* expression at CpG sites in the conserved MT2 and exonic regions to gain a better understanding of regions commonly examined in human *OXTR* studies.

In humans, aversive social environment in early life increases the vulnerability of developing numerous pathologies in adulthood (Power et al., 2007). Longitudinal studies indicate that children exposed to negligent environments have an increased risk of developing cognitive impairments, mental pathologies, social impairments, and emotional difficulties (Trickett and McBride-Chang, 1995; Heim and Nemeroff, 2001). We hypothesize that the DNA methylation

state of *Oxtr* is modulated by early life environment, setting up individual variability of expression of this gene and downstream differences in social behavior. Specifically, we examined the role of amount of parental care received early in life plays in modulating DNA methylation in the conserved MT2 and exonic region in prairie voles. In prairie voles, as in other rodent models, small manipulations created by differences in handling parents during the postnatal period can result in lifelong behavioral effects in offspring.

A series of experiments have shown that a mild disturbance of the prairie vole family on postnatal day (PND) 1 lead to increases in parental interaction with the young, especially from the dam (Bales et al., 2007; Carter et al., 2008, 2009; Stone and Bales, 2010; Bales et al., 2011; Perkeybile et al., 2013; Perkeybile et al. 2019). Behavioral testing conducted in the juvenile period (PND 21-23) and adulthood (PND 60-90) indicates that the amount of parental care received in early life impacts the offspring's later life social behavior. Offspring raised by high care parents display increases in parental behavior, pair bonding, alloparental care, as well as increases in anxiety behavior, along with a longer return time to baseline corticosterone following a stressor (Stone and Bales, 2010; Bales et al., 2007). The differences in social behavior in offspring are mediated by the behavior of the parent (non-genomic transmission), and parental behavior transfers across generations (Stone and Bales, 2010; Perkeybile et al., 2015).

Additionally, increases of parental care received in early life corresponds to decreases of DNA methylation and increases in *Oxtr* expression in homologous CpG sites between humans and prairie voles (Perkeybile et al., 2019*).* Within the same cohort of animals, increases in OXTR binding within the nucleus accumbens also correspond to increases of amount of maternal care

received early in life (Perkeybile et al., 2019*).* However, this study only focused on 4 CpG sites within *Oxtr* in prairie voles. Our current study furthers examines how the amount of parental care received in early life can influence the relationship between DNA methylation and gene expression in all CpG sites within the conserved MT2 and exonic regions, regions which have been previously implicated the epigenetic programing of *OXTR* and social and emotional behavior in humans. Therefore, findings in our animal model will further our understanding of the relationship between DNA methylation in conserved MT2 and exonic regions and *Oxtr* expression and how parental care can act as an environmental driver of epigenetic programming in conserved regions of *Oxtr* that have been implicated in human social behavior and dysfunction.

Methods

Human brain DNA and RNA samples

Genomic DNA and RNA isolated from the temporal cortex (BA 41/42) was obtained from the Maryland National Institute of Child Health and Human Development Brain Tissue Center and the Harvard Brain Tissue Resource Center (n= 11, male, 11-30 yrs). Genomic DNA was analyzed for *OXTR* DNA methylation at CpG sites in the MT2 region of the promoter and gene expression was evaluated as previously described (Gregory et al., 2009).

Animal model

Subjects were laboratory-bred prairie voles (*Microtus ochrogaster*), descendants of a wild-caught stock captured near Champaign, Illinois. Breeding pairs were housed in large polycarbonate cages (44cmx22cm x 16 cm) and same sex offspring pairs were housed in smaller

polycarbonate cages (27cmx16 cm x 16 cm) after weaning on postnatal day (PND) 20 (date of birth: PND0). Animals were given food (high-fiber Purina rabbit chow) and water *ad libitum*, cotton nestlets for nesting material in breeding cages, and were maintained on a 14:10 light:dark cycle. Procedures involved in measuring the effects of early handling on parental behavior were reviewed and approved by the Institutional Animal Care and Use Committee (IACUC) at the University of Illinois, Chicago. Procedures involved in generating tissue for the analysis of DNA methylation and gene expression following handling were reviewed and approved by the IACUC at the University of California, Davis. Animals were euthanized via cervical dislocation and rapid decapitation under deep isoflurane anesthesia. Brains were extracted, flash frozen on dry ice, and stored at −80 °C until analysis.

Identifying conserved MT2 and exonic region in prairie voles

The MT2 region of *OXTR* in humans as identified by Kusui *et al.*, 2001 (hg38, chr3:8,769,033-8,769,438) was compared to the *Oxtr* sequence in prairie voles (GenBank: DP001214.1) and with the NCBI BLAST program, and a region in prairie voles was identified as having a highly similar sequence of DNA. To assess the significance of this similarity of the MT2 region between human and prairie vole, we used UVa FASTA server (http://fasta.bioch.virginia.edu/) and PRSS (DNA: DNA) to shuffle the prairie vole sequence 200 times and estimate the statistical significance of the shuffled scores. For the MT2 region, this analysis identified a conserved region between human and prairie vole with 64.3% shared identity (Figure 1B; Smith-Waterman score: 566; 64.3% similar; Z-score: 120.2; bits: 120.2; E(1000): 1^{-31}).

The exonic region was identified from the BS3 region in Towers *et al.*, 2018, and the conserved human (hg38, chr3:8,767,386-8,767,963) and prairie vole sequence (GenBank: DP001214.1) was aligned with the NCBI BLAST program and again the significance of similarity was analyzed with UVa FASTA server and PRSS (DNA: DNA) to shuffle the prairie vole sequence 200 times and estimate the statistical significance of the shuffled scores. For the exonic region, this analysis identified a conserved region between human and prairie vole with 88.4% identity (Fig. 1C; Smith-Waterman score: 2275; 88.4% similar; Z-score: 1966.2; bits: 373.7; E(1000): $1^{\wedge -107}$).

Cohort for mechanistic studies

We used a cohort of breeding pairs to investigate the effects of early parental handling on *Oxtr* DNA methylation and gene expression. The experimental manipulation occurred on PND1 as previously described by Perkeybile *et al.*, 2019 (MAN0 = offspring received natural variances of parental care, MAN1 = offspring received an increase of parental care within the first week of life). On PND24 offspring were anesthetized with isoflurane and euthanized via cervical dislocation and rapid decapitation for tissue collection. Brains were extracted and flash frozen in liquid nitrogen and stored at -80 °C. Only male/female sibling pairs were included in the analysis of *Oxtr* DNA methylation and *Oxtr* expression (MAN0: n= 7 per sex; MAN1: n= 6 per sex).

Sectioning of the nucleus accumbens

Whole brains were stored at -80 °C and equilibrated to -20°C for two hours prior to sectioning. Following sectioning, nucleus accumbens tissue was placed in a DNA/RNA free

microcentrifuge tube and flashed frozen with liquid nitrogen. Brain tissue was crushed using a mortar and pestle in preparation for DNA/RNA isolation.

Oxtr DNA Methylation analysis

Extraction of DNA from nucleus accumbens tissue was done using the Qiagen AllPrep DNA/RNA Mini Kit (Qiagen, Valencia, CA) following manufacturer instructions. Two hundred nanograms of DNA was subject to bisulfite treatment (Kit MECOV50, Invitrogen, Carlsbad, CA), which allows for the detection of methylated cytosines by sequencing. PCR was performed using a Pyromark PCR kit (Qiagen, Valencia, CA), and each PCR reaction was amplified in triplicate on three identical PCR machines (S1000 Thermal Cycler, Bio-Rad, Hercules, CA.). Standard controls of 0% and 100% methylated DNA, as well as a no DNA control standard were included for each PCR plate. All samples were randomized for pyrosequencing to account for plate and run variability. Pyrosequencing was performed on a Pyromark Q24 using PyroMark Gold Q24 Reagents (Qiagen, Valencia, CA) per the manufacturer's protocol. Epigenotypes reported are an average of three replicates. PCR primers, PCR cycling conditions, pyrosequencing primers, and mean deviation per CpG site measured are in Tables 4, 5, 6, and 7.

Oxtr expression analysis

Extraction of RNA was done using the Qiagen AllPrep DNA/RNA Mini Kit (Qiagen, Valencia, CA) following manufacturer instructions. RNA was processed for cDNA synthesis following the protocol provided in the iScript cDNA Synthesis kit (Bio-Rad, Hercules, C.A.). Real-time PCR was conducted using a 7500 Fast Real-Time PCR System (Applied Biosystems)

using *Power* SYBR Green (Applied Biosystems No. 4367659). The cycling conditions are as follows: for *Oxtr* [Step 1: (95 °C/10 min) 1 cycle, Step 2: (95 °C/15 s, 63.4 °C/60 s) 35 cycles], for *Pgk1* [Step 1: (95 °C/10 min) 1 cycle, Step 2: (95 °C/15 s, 65.3 °C/60 s) 35 cycles]. All reactions were run in triplicate (replicate standard deviation was <0.05) and their specificity verified by melting curve analysis and separation on a 2% agarose gel. Primers performance was evaluated using standard serial dilution and both primer sets performed within acceptable range for efficiency >90% (*Oxtr* efficiency = 99.7%, $R^2 = 0.993$; *Pgk1* efficiency = 93.5%, $R^2 = 0.998$). The primer sequences used for *Oxtr* are TSL401_F 5′-GCCTTTCTTCTTCGTGCAGATG-3′ (Fwd) and TSL401_R 5′-ATGTAGATCCAGGGGTTGCAG-3′ (Rev); for *Pgk1* TSL402_F 5′-TTGCCCGTTGACTTTGTCAC-3′ (Fwd) and TSL402_R 5′-GCCACAGCCTCAGCATATTTC-3′ (Rev). Relative gene expression is presented using the comparative Ct method, $2^{-\Delta Ct}$. *Pgk1* was chosen as a reference based on data in mouse brain showing its reliability across brain regions and developmental time points (Boda et al., 2009).

Statistical analysis

Statistical computing and graphics conducted using GraphPad Prism 8.0 and R. For each analysis, $p < 0.05$ was regarded as statistically significant. The correlation matrices and variance of DNA methylation in the MT2 region and exonic regions in humans and in prairie voles was determined using R (Team, 2019). In both humans and in prairie voles, Spearman's rank correlation was used to determine the relationship between DNA methylation and gene expression and uncorrected p-values are reported for human MT2 region, prairie vole MT2 region, and prairie vole exonic region. The Spearman's Rho correlations, 95% confidence interval, and *p*-values for each CpG site measures in the human MT2 region, prairie vole MT2 region, and prairie vole exonic region can be found in Table 1, 2, and 3. Additionally, for

visualization purposes, we plotted trend lines from linear regression models predicting DNA methylation from expression for the human MT2 region, prairie vole MT2 region, and prairie vole exonic region. The effect of handling manipulation on *Oxtr* DNA methylation in the prairie vole was analyzed using a 2-way CpG site DNA methylation x handling condition ANOVA, with exploratory post-hoc testing with Sidak multiple comparisons correction. Only 5 CpGs (MT2: CpGs 14, -934_1, -934_2, -924, -901) had a significant difference in DNA methylation by handling condition. Spearman's rank correlation was used to determine the relationship between DNA methylation and gene expression of these 5 CpG sites, following post-hoc Holm-Sidak multiple comparisons CpG sites -934_2, -924, and -901 had a significant or strongly trending outcome. *Oxtr* polymorphisms association with DNA methylation was analyzed using a 2-way DNA methylation x SNP ANOVA, and the *Oxtr* polymorphisms association to gene expression was analyzed using a 2-way gene expression x SNP ANOVA. One set of male/female sibling pairs per unique parenting pair was included for analysis. Offspring from three MAN0 pairs and four MAN1 pairs were excluded from analyses because the litters had only female offspring.

Results

Significant Conservation found within the oxytocin receptor gene

In both humans and prairie voles, our analysis found significant conservation within the oxytocin receptor gene. Kusui *et al.* previously established DNA methylation at a region within the first exon and the first intron (MT2 segment, hg38, chr3:8,769,033-8,769,438) in *OXTR* greatly impacts gene expression in human tissue (Fig. 1A; Kusui et al., 2001). We compared the MT2 sequence between humans and the *Oxtr* sequence in prairie voles (GenBank: DP001214.1)

through the NCBI BLAST program to identify regions of similarity (Fig 1B). The MT2 region between humans and prairie voles was determined to have significant conservation through a PRSS shuffle analysis, with a 64.3% shared identity (Fig. 1B; Smith-Waterman score: 566; 64.3% similar; Z-score: 120.2; bits: 120.2; E(1000): 1^{-31}). Importantly, CpG sites -934, -924, and -901 are homologous between humans and prairie voles (Fig. 1B).

A region in exon 3 (BS3 region, mm9, chr6:112439019-1124395) was reported to be hypermethylated in mice, with DNA methylation in the BS3 region associated with altered *Oxtr* expression (Towers et al., 2018). We used the genomic boundaries established in the Towers *et al.,* study because it is the only known study which provides evidence that DNA methylation in this exonic region may influence *Oxtr* expression. Additionally, the BS3 region corresponds to the exon 3 region, which have been examined in human studies. The corresponding BS3 exonic region in humans (hg38, chr3:8,767,386-8,767,963) was compared to the *Oxtr* sequence in prairie voles (GenBank: DP001214.1) through the NCBI BLAST program to identify regions of similarity (Fig 1C). The exonic region between humans and prairie voles was determined to have significant conservation through a PRSS shuffle analysis, with a 88.4% identity (Fig. 1C; Smith-Waterman score: 2275; 88.4% similar; Z-score: 1966.2; bits: 373.7; E(1000): $1^{\wedge -107}$).

Epigenetic similarity in MT2 region

The human *OXTR* contains a DNA methylation specific regulatory region (MT2 region) within its promoter, where increased DNA methylation leads to decreased gene expression *in vitro* (Kusui et al., 2001). Additionally, DNA methylation at CpG sites within the MT2 region have been shown to be associated with endophenotypes of social perception in humans (Jack et

al. 2012; Puglia et al., 2015) and psychiatric disorders (Gregory et al. 2009, Cecil et al., 2014, Bell et al., 2015, Dadds et al., 2014, Kim et al., 2014, Kimmel et al. 2016, Rubin et al., 2016, Rijlaarsdam et al., 2017). To identify the critical CpG sites that may regulate this process, we assessed the relationship of the DNA methylation state of individual CpG sites within this region with *OXTR* gene expression using DNA and RNA extracted from human temporal cortex (n= 11, male, 11-30 yrs). Of the 22 CpG sites studied in MT2, a cluster of sites are hypermethylated and have a higher amount of variance (Fig. 2A, C, E, G). Within this cluster, three sites were related to *OXTR* expression, sites -934, -924, and -901 (Fig 2G; -934: $\rho(11) = -0.78$, $p = 0.006$; -924: $\rho(11) = -0.68$, $p = 0.024$; -901: $\rho(11) = -0.65$, $p = 0.032$; uncorrected *p*-values). We performed a comparable set of experiments and analysis in the conserved MT2 region (Fig.1B) in the nucleus accumbens tissue of prairie voles and discovered a similar epigenetic landscape, with a cluster of hypermethylated and variable CpG sites resembling our findings in humans (Fig. 2B, D, F). Homologous CpG sites -934_1, -934_2, -924, and -901 have an inverse relationship to *Oxtr* expression, with increases of DNA methylation corresponding to decreases of *Oxtr* expression (Fig. 2H; -934_1: $\rho(36) = -0.34$, $p = 0.042$; -934_2: $\rho(36) = -0.47$, $p = 0.0043$; -924: $\rho(36) = -0.48$, $p = 0.0031$; -901 $\rho(36) = -0.47$, $p = 0.0042$; NAcc: PND24 harvest, uncorrected *p*-values).

These CpG sites, which are associated with *OXTR* expression and social behavior in humans and are conserved in prairie voles, are not as prevalent in other commonly used rodent models (Perkeybile et al., 2019), which further strengthens the choice of prairie voles as an animal model as we explore the biological mechanisms of social behavior. Additionally, promoter regions upstream of the transcription start site typically have low levels of DNA methylation, but

when methylation does occur, it tends to greatly impact gene expression (Razin, et al., 1998). Our findings in the MT2 region adhere to this expectation in both humans and in prairie voles (Fig. 2). Statistical information on values for Spearman's Rho correlations, 95% confidence intervals, and p-values for 22 CpG sites within the human MT2 region and the 24 CpG sites in the conserved MT2 region in prairie voles are in Tables 1 and 2. This data indicates the potential functional relevance for CpG sites -934, -924, and -901 in both human and prairie vole oxytocin receptor gene expression within the MT2 region.

Exonic epigenetic characteristics

In humans, no study has investigated the relationship between exon 3 DNA methylation and *OXTR* expression. Yet, DNA methylation patterns in the exon 3 region correspond to differences in social behavior and early life stress (Ziegler et al., 2015; Chagnon et al., 2015;, Unternaehrer et al., 2015, 2016; Cappi et al., 2016; Smearman et al., 2016). Using the prairie vole as an animal model, we examined the epigenetic landscape of the conversed exonic region and the relationship between DNA methylation and *Oxtr* expression in the conserved exonic segment in the nucleus accumbens to determine critical CpG sites influencing gene expression (Fig. 1C). Exons characteristically have high levels of DNA methylation, and the exonic region examined in prairie voles was hypermethylated (Fig. 3A) in a similar pattern found other rodent species (Towers et al., 2018). Additionally, two CpG sites, CpG 25 and CpG 33, where determined to be synonymous transition single nucleotide polymorphisms, in which the common variant is GCG and the rare variant is GCA, both are alanine codons. Both CpG 25 and CpG 33 are CG to CA polymorphisms, indicating the reverse strain is a CG to TG mutation, which is a common mutation that occurs at methylated cytosine (Copper and Youssouflain, 1988; Bird, 1980). The

polymorphism at CpG 25 might influence *Oxtr* expression, however due to the small sample size this should be further studied (Fig. 5A). The polymorphism at CpG 33 is not associated with differences in *Oxtr* by genotype in our sample (Fig. 5B).

Additionally, we identified four clusters of CpG sites which have highly correlated levels of DNA methylation and similar patterns of variance in the exonic region (Fig. 3B, 3C). Two of the CpG sites fall in cluster 1 and eight of the CpG sites are within cluster 3, a notable relationship between increases of DNA methylation corresponding to decreases in *Oxtr* expression (Fig. 3D; CpG 2: ρ (36) = -0.37, p = 0.025; CpG 4: ρ (36) = -0.41, p = 0.014; CpG 20: ρ (36) = -0.34, p = 0.044; CpG 22: ρ (36) = -0.39, p = 0.019; CpG 23: ρ (36) = -0.39, p = 0.017; CpG 24: ρ (36) = -0.37, p = 0.026; CpG 26: ρ (36) = -0.38, p = 0.028; CpG 31: ρ (36) = -0.34, p = 0.041; CpG 32: ρ (36) = -0.36, p = 0.031; CpG 36: ρ (36) = -0.40, p = 0.016, NAcc: PND24 harvest, uncorrected p-values). Statistical information on values for Spearman's Rho correlations, 95% confidence intervals, and p-values of the 42 CpG sites in the conserved exonic region in prairie voles are in Table 3. This data indicates that only a select few CpG sites within the exonic region may influence *Oxtr* expression, and nearly all of these sites are clustered together within a distinct region (cluster 3) with highly correlated values of DNA methylation.

Increased parental care corresponds with decreases of *Oxtr* DNA methylation

Previous work has shown that a single episode of direct handling of prairie vole parents on PND1 produces a temporary increase of parental care (Bales et al, 2007, 2011; Stone and Bales, 2010; Perkeybile et al., 2019). Higher amount of parental care received in early life is associated with an increase of species-typical social behaviors, as well as changes to the oxytocin

system in offspring (Bales et al., 2007, 2011; Stone and Bales, 2010), including an increase of oxytocin receptors in a brain region associated with social behaviors (Perkeybile et al., 2019). In this study, offspring that had parents handled on PND1 received a temporary increase of parental care within the first week of life (MAN1) in comparison to control group offspring (MAN0) as described in Perkeybile *et al.*, 2019.

We sought to investigate the changes in DNA methylation in the conserved MT2 and exonic regions in prairie voles which received different amounts of parental care in early life (MAN1 offspring vs. MAN0 offspring). We examined DNA methylation in adolescent (PND24) offspring at CpG sites across the conserved MT2 and exonic regions in the nucleus accumbens, and discovered increases of parental care received in early life (MAN1 offspring) are associated with decreases of DNA methylation in both MT2 and exonic regions (Fig. 4A, 4B; MT2: 2-way ANOVA, main effect of handling p= <0.0001; exonic: 2-way ANOVA, main effect of handling p= <0.0001; MAN0 = 7 sibling pairs, MAN1 = 6 sibling pairs). Following post-hoc multiple comparisons analyses, only CpG sites 14, -934_1, -934_2, -924, and -901 within the conserved MT2 region showed significantly lower levels of *Oxtr* DNA methylation in MAN1 offspring, which received higher amounts of parental care in early life, in comparison to the control group (Fig. 4A, 4B; MT2: CpG 14: p= 0.0027; -934_1: p =0.0005; -934_2: p =<0.0001; -924: p =0.0004; -901: p =0.0015; Sidak post-test by site, MAN0 = 7 sibling pairs, MAN1 = 6 sibling pairs).

DNA methylation landmarks correlate to *Oxtr* expression

Of the 24 CpG sites in the conserved MT2 region and the 42 CpG sites in the conserved exonic regions, only 5 CpG sites within the MT2 region had a significant and corrected difference in DNA methylation levels based on early life experience. Offspring that received higher amounts of parental care within the first week of life (MAN1) had lower levels of DNA methylation in CpG sites 14, 934_1, -934_2, -924, and -901 within the conserved MT2 region (Fig. 4A, 4B). To determine if DNA methylation at these sites correspond to changes in *Oxtr* expression, we measured *Oxtr* expression in the nucleus accumbens within the same set of animals used in the DNA methylation data set. DNA methylation inversely correlated with *Oxtr* expression, and three CpG sites -934_2, -924, and -901 have a strong trending or significant relationship following multiple comparison testing (Fig 4C; MT2: CpG 14 $\rho(26)= -0.390$, $p= 0.096$, CpG -934_1: $\rho(26) = -0.382$, $p=0.096$; CpG -934_2: $\rho(26) = -0.473$, $p= 0.058$; CpG -924: $\rho(26) = -0.503$, $p=0.043$; CpG -901: $\rho(26) = -0.471$, $p=0.058$; Holm-Sidak adjusted *p*-values; NAcc: PND24 harvest, sibling pairs = 13). Collectively, this data indicate that parental care alters DNA methylation in the conserved MT2 and exonic region and *Oxtr* expression in the nucleus accumbens, and this study provides further evidence that the homologous CpG sites -934_2, -924, and -901 in the MT2 region may be particularly important in regulating the transcription levels of *Oxtr*.

Discussion

We are the first to provide evidence indicating the following 6 findings about the relationship between DNA methylation and oxytocin gene expression in both human brain tissue and with the prairie vole, an animal model commonly used in social behavior studies.

Specifically, (1) Increases in DNA methylation of specific *OXTR* CpG sites (-934, -924, and -901) in the MT2 region correspond to decreases in *OXTR* expression in the human brain. (2) The epigenetic landscape of the MT2 region is similar between humans and prairie voles. (3) The amount of parental care received in early life in prairie voles significantly impacts DNA methylation at CpG sites 14, -934_1, -934_2, -924, and -901, all just within the MT2 region. (4) Of these CpG sites, only homologous CpG sites -934_2, -924, and -901 have a strong trending or significant relationship with *Oxtr* expression, with increase of DNA methylation corresponding to decreases in *Oxtr* expression. (5) The conserved exonic region has 4 clusters of highly correlated DNA methylation domains, and two CpG sites within cluster 1 and eight CpG sites within cluster 3 correspond to *Oxtr* expression. (6) Two polymorphic CpG sites within the conserved exonic region and the polymorphic site at CpG 25 correspond to differences in *Oxtr* expression within our population of prairie voles.

This is an emerging field of inquiry, with an interest in gaining a better understanding of epigenetic programing and development of social behaviors in both humans and animals. The oxytocin system plays a central role in a broad range of social outcomes, and DNA methylation in the MT2 and exon 3 region of *OXTR* in humans has been assessed, but there is not sufficient evidence to draw conclusions about the role of *OXTR* DNA methylation on human social and emotional behavior. We provide evidence of the relationship between DNA methylation and oxytocin gene expression in both human brain tissue and with an animal model commonly used in social behavior studies, the prairie vole.

The MT2 region is significantly conserved between humans and prairie voles, and strikingly the CpG sites which have a definitive inverse relationship between DNA methylation and *OXTR* expression in humans (MT2: CpGs -934, -924, -901) are homologous in prairie voles. Within our animal model, CpGs -934_1, -934_2, -924, and -901 within the conserved MT2 region also have a notable inverse relationship between DNA methylation and *Oxtr* expression. These sites are poorly conserved in other commonly used rodent models, with rats lacking all of the sites previously mentioned and mice only containing CpG site -924, indicating the regulatory importance of this region of the gene may be conserved in prairie voles, but not in other rodent models of social behavior (Perkeybile et al., 2019). Additionally, a targeted segment within exon 3 is also significantly conserved between humans and prairie voles, indicating that our findings in our prairie vole animal model are likely to be applicable to humans. Selected CpG sites in the exonic region in prairie voles have a detectable inverse relationship between DNA methylation and *Oxtr* expression, with nearly all of these CpG sites laying within cluster 3 (exonic CpGs 2, 4, 20, 22, 23, 24, 26, 31, 32, 36).

Our findings also provide evidence that early parental care affects the function of the oxytocinergic system by altering DNA methylation of *Oxtr*, providing a mechanism by which early experience can alter an organism's developmental trajectory and create individual differences in social behavior. Previous work has found that a single direct handling manipulation of prairie voles parents (MAN1) on PND1 results in a transient increase of parental behavior, which subsequently protects offspring against de novo *Oxtr* DNA methylation, resulting in overall higher levels of *Oxtr* gene expression and OXTR density in the nucleus accumbens, increases oxytocin production, and increases of species typical social behaviors

(Bales et al., 2007, 2011; Cater et al., 2008; Stone and Bales, 2013; Perkeybile et al., 2013, 2019). In our study, we expanded the quantification of DNA methylation across CpG sites in the conserved MT2 and exonic regions in relation to the amount of parental care received in early life. Increases of parental care (MAN1) corresponded with offspring displaying overall lower levels of DNA methylation across the MT2 and exonic region, yet only a handful of CpG sites survived multiple comparison testing. The sites that have a significant difference of DNA methylation between groups are all within the conserved MT2 region and are CpG sites 14, -934_1, -934_2, -924, and-901. Of those CpG sites, -934_2, -924, and -901 in the conserved MT2 region have a significant or a strong trending inverse relationship between levels of DNA methylation and *Oxtr* expression, with increases of DNA methylation of these CpG sites corresponding to decreases in *Oxtr* expression. Furthermore, results in human studies have linked increases of DNA methylation at these homologous *OXTR* sites to social cognition and emotion processing, as well as several psychiatric disorders, suggesting that an increase of DNA methylation at CpG sites -934, -924, and -901 may correspond to a decrease in the function of the endogenous oxytocin system (Gregory et al., 2009; Jack et al., 2012; Kim et al., 2014; Bell et al., 2015; Puglia et al., 2015; Rubin et al; 2016).

Conclusion

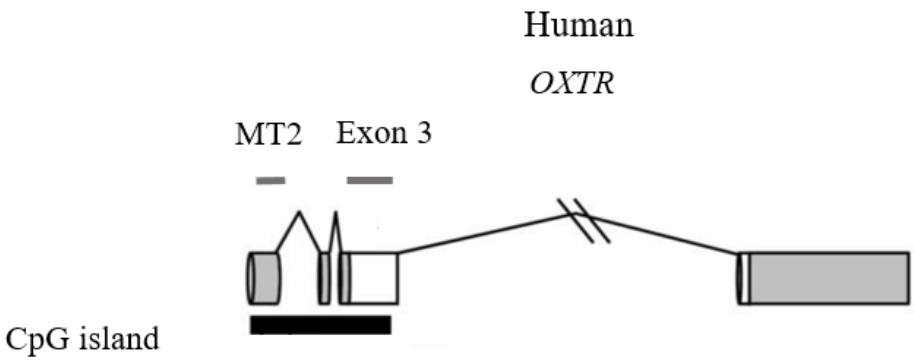
As more research aims to understand the role of epigenetic markers on a range of human outcomes, it will be vital to have a comparable animal model that will allow for more in-depth analysis. Our findings suggest the prairie vole will be useful in this regard, allowing for an expanded understanding of the role of epigenetic markers in controlling oxytocin pathways and impacting complex social behavior that can then better inform and guide work in humans. In

prairie voles, we were able to show that five specific CpG sites are particularly sensitive to postnatal DNA methylation modulation, all of which are in the MT2 region (CpGs 14, -934_1, -934_2, -924, and -901) and that can be influenced by the amount of parental care received in early life. Of these CpG sites, only three of them (CpGs -934_2, -924, and -901) have a strong trending or significant association between DNA methylation and *Oxtr* expression. These sites are homologous between humans and prairie voles, and we show for the first time that DNA methylation at CpG sites -934, -924, and -901 in *OXTR* of human brain tissue have a notable correlation with *OXTR* expression. As we continue investing the role of DNA methylation on oxytocin receptor gene expression, and its subsequent modulation of social behavior in both human and animal models, our findings indicate that homologous CpG sites -934, -924, and -901 are particularly important in epigenetically tuning the oxytocin system and can help to better target future research in this area.

Contribution of work: Wroblewski contributed to designing the research, conducting the epigenetic studies, analyzing and interpreting the data collected, and writing the manuscript. Graves contributed to conducting analysis requiring the computer program R. Lillard contributed to conducting epigenetic studies. Carter contributed with animal studies. Connelly contributed to designing the research and writing the manuscript.

Figures

A.


Human
OXTR
MT2
Exon 3
CpG island

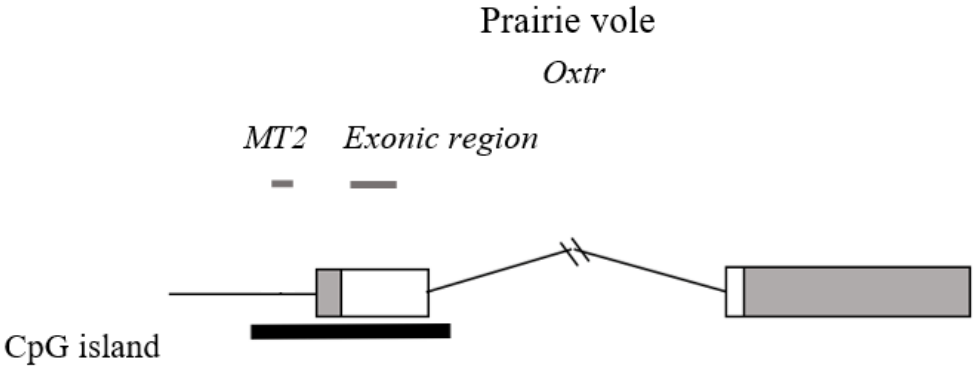
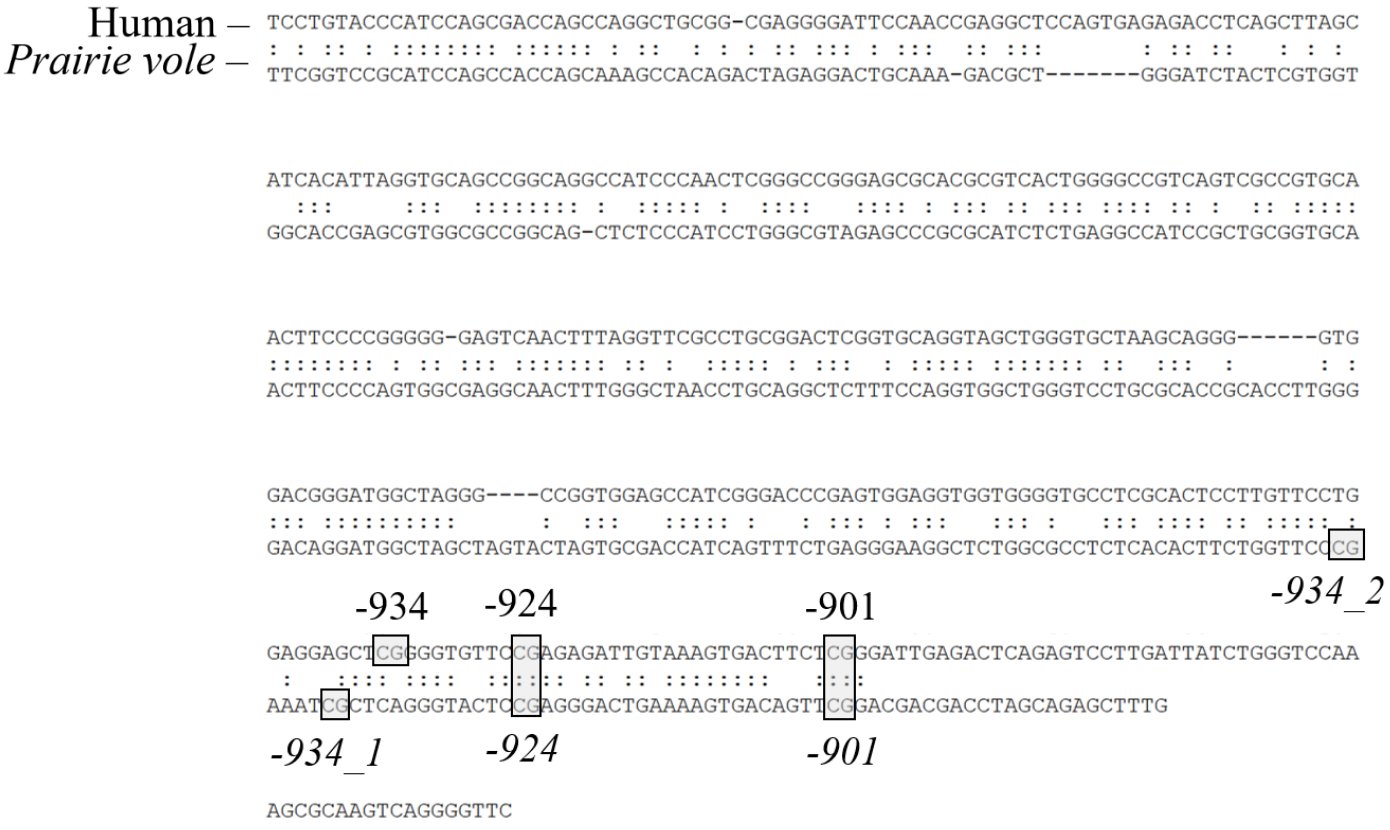

Prairie vole
Oxtr
MT2
Exonic region
CpG island

Figure 1A. Oxytocin receptor gene schematic in humans and in prairie voles. A) Exons are displayed as boxes (coding, white; untranslated, grey) and introns are solid lines. CpG islands are indicated with black bars. The grey bars above the oxytocin receptor gene schematics are representative of the MT2 region and a targeted segment of the exon 3 region examined in this study.

B.

MT2



C.

Exonic region

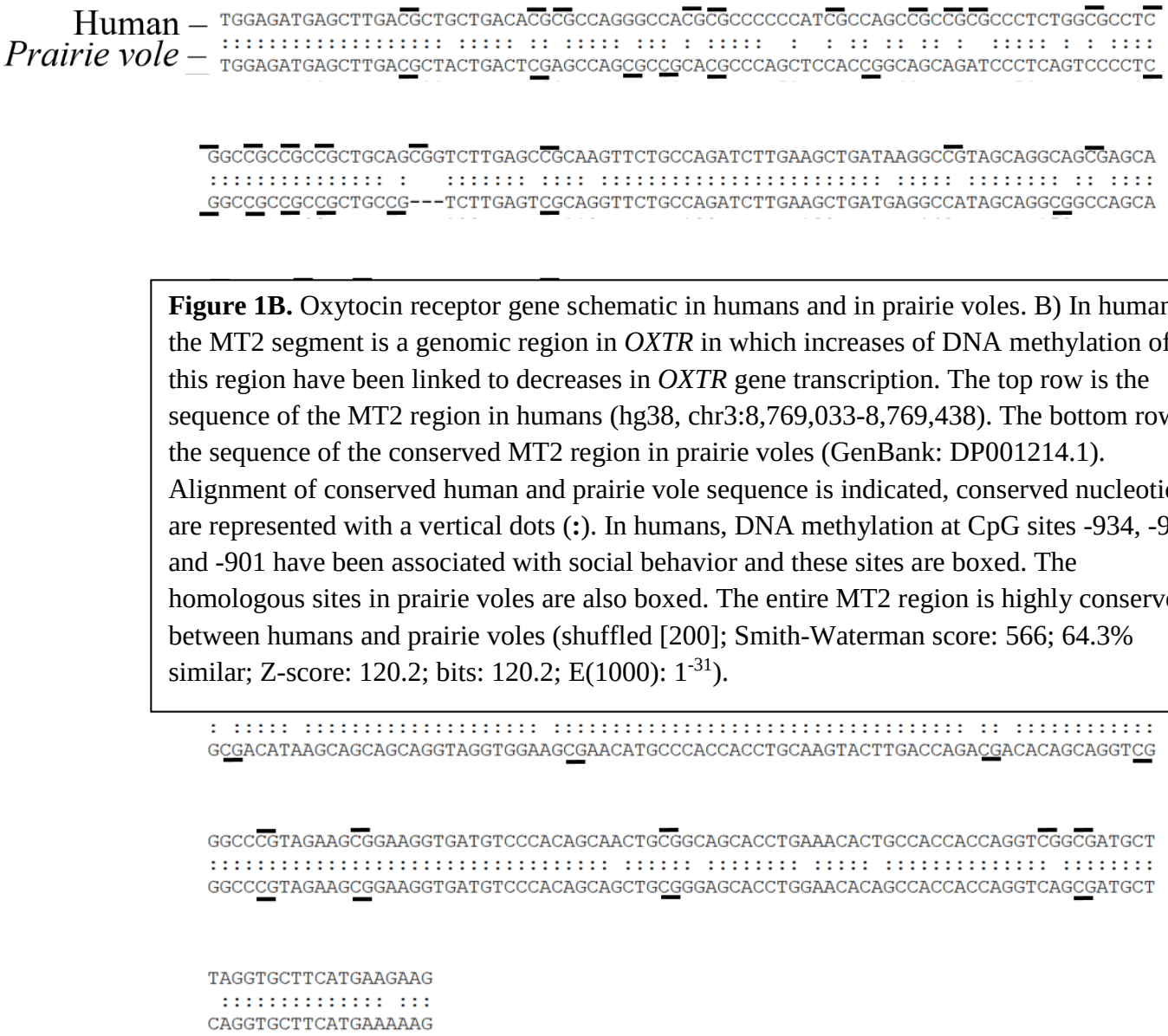


Figure 1B. Oxytocin receptor gene schematic in humans and in prairie voles. B) In humans, the MT2 segment is a genomic region in *OXTR* in which increases of DNA methylation of this region have been linked to decreases in *OXTR* gene transcription. The top row is the sequence of the MT2 region in humans (hg38, chr3:8,769,033-8,769,438). The bottom row is the sequence of the conserved MT2 region in prairie voles (GenBank: DP001214.1). Alignment of conserved human and prairie vole sequence is indicated, conserved nucleotides are represented with a vertical dots (**:**). In humans, DNA methylation at CpG sites -934, -924 and -901 have been associated with social behavior and these sites are boxed. The homologous sites in prairie voles are also boxed. The entire MT2 region is highly conserved between humans and prairie voles (shuffled [200]; Smith-Waterman score: 566; 64.3% similar; Z-score: 120.2; bits: 120.2; E(1000): 1^{-31}).

Figure 1C. Oxytocin receptor gene schematic in humans and in prairie voles. C) The top row is the gene sequence of a targeted segment of the exon 3 region in humans (hg38, chr3:8,767,386-8,767,963). The bottom row is same conserved exonic sequence in prairie voles (GenBank: DP001214.1). Conserved nucleotides are represented with a vertical dots (**:**). CpG sites are indicated with a black bar above human sites and below for prairie vole sites. The targeted segment of the exon 3 region is highly conserved between humans and prairie voles. (Shuffled [200]; Smith-Waterman score: 2275; 88.4% similar; Z-score: 1966.2; bits: 373.7; E(1000): 1^-107).

A.

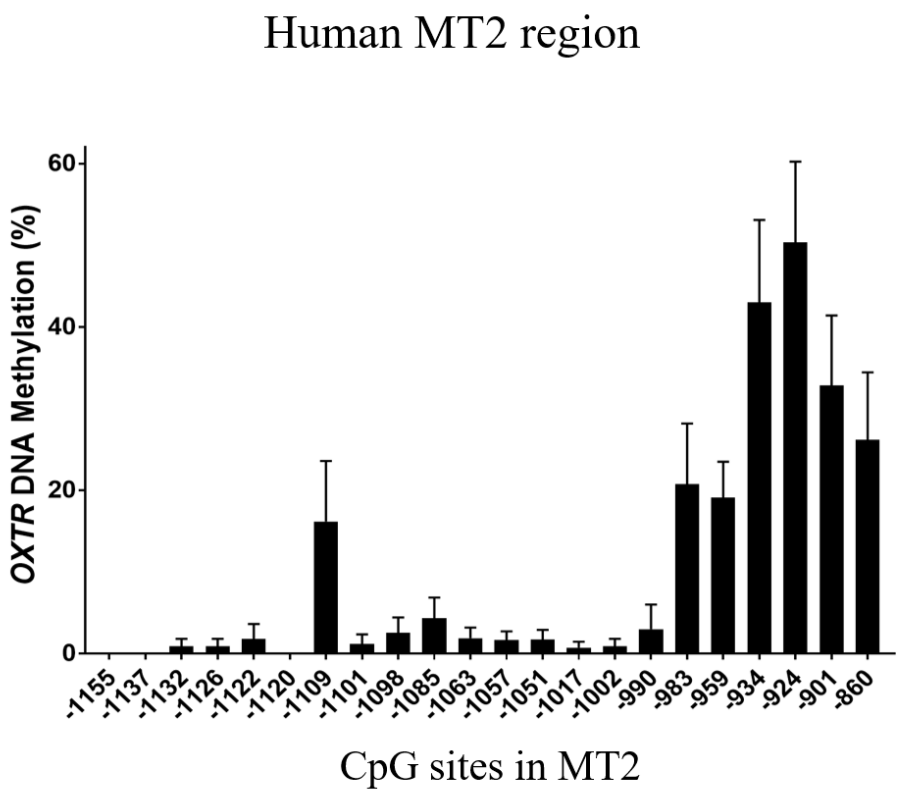


Figure 2A Individual CpG sites have differentiated levels of DNA methylation across MT2 region in humans, per findings in Kusui *et al.* that indicate DNA methylation in this region significantly impacts *OXTR* expression. A) CpG DNA methylation profiles of 22 CpG sites within the MT2 region in humans. A cluster of CpG sites that exhibit highest levels of DNA methylation in this region are common in humans and prairie vole and contain previously associated sites -934, -924, and -901. (Error bars presented as mean +/- SEM; superior temporal gyrus, Males ages: 18-30; n=11.)

B.

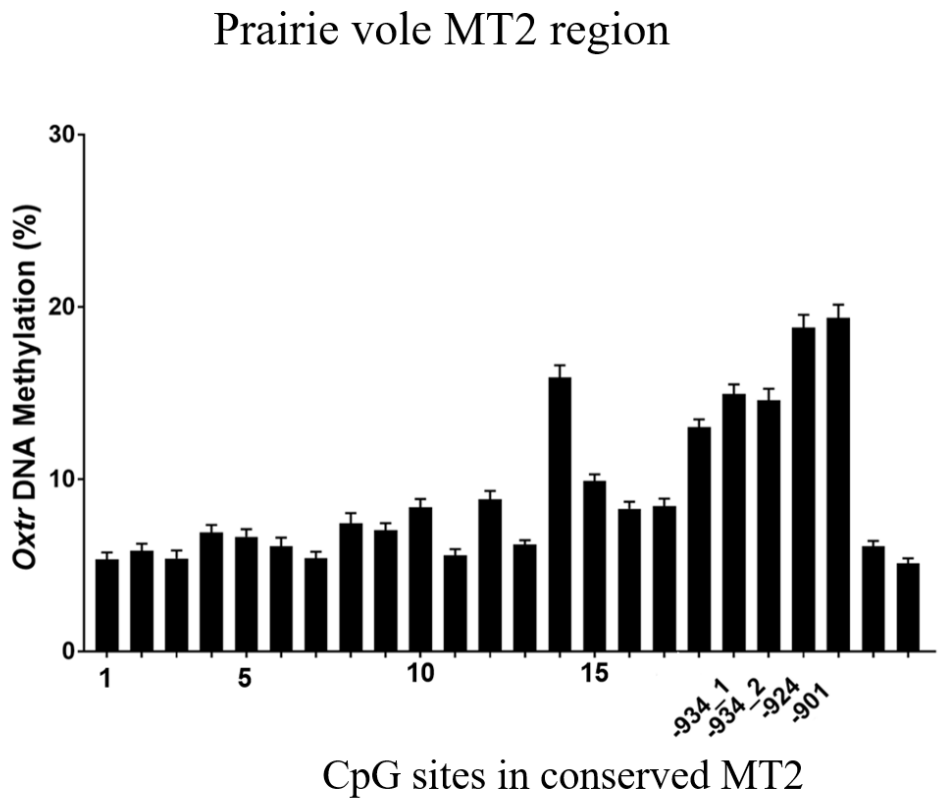
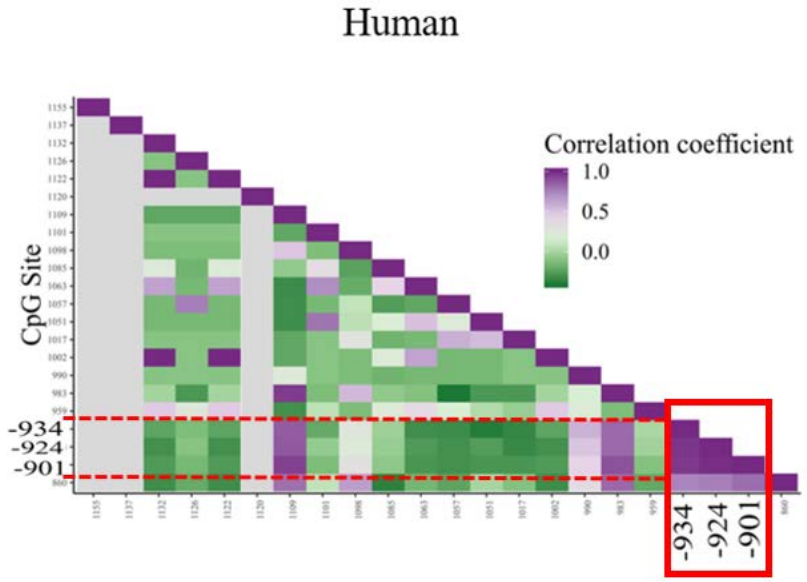


Figure 2B Individual CpG in the conserved MT2 region in prairie voles B) CpG methylation profiles of the 24 CpG sites in the conserved MT2 region in prairie voles. A majority of CpG sites with variable and high levels of DNA methylation are group together, and include conserved sites -934_1, -934_2, -924, and -901. (Error bars presented as mean +/- SEM; NAcc: PND24 harvest; sibling pairs = 18.)

C.



D.

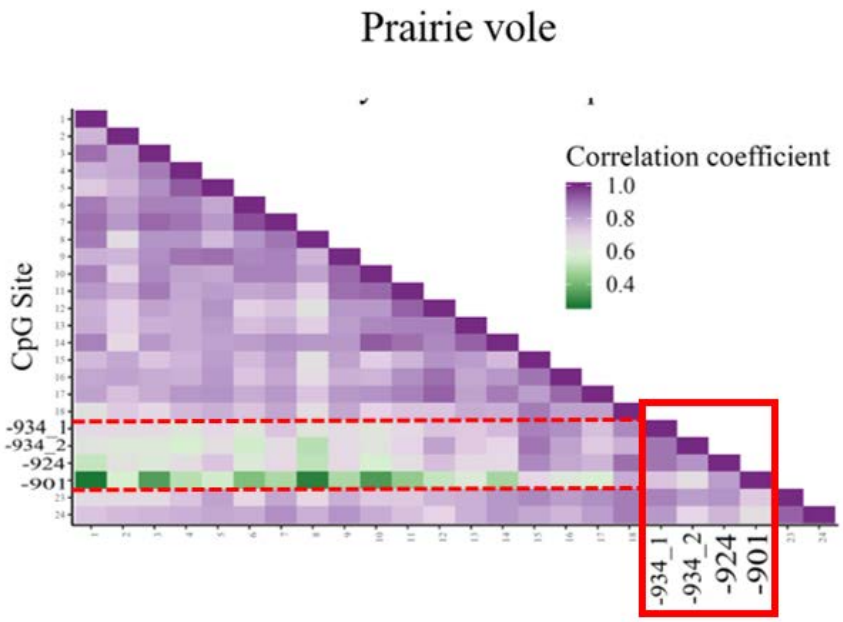
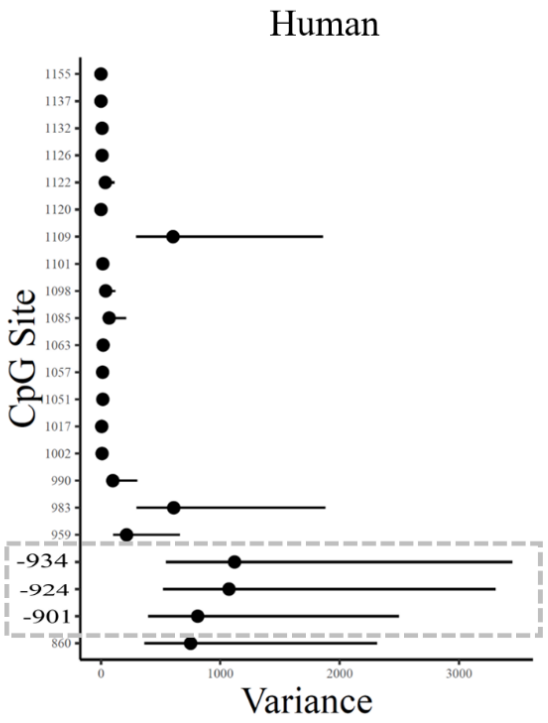


Figure 2C, 2D. Correlation of DNA methylation levels between oxytocin receptor gene (CpG sites in MT2 region C-D) and CpG sites with DNA methylation levels that have the lowest levels of correlation in comparison to other CpG sites, but are highly correlated with each other in the MT2 regions are grouped together and include CpG sites -934, -924, -901 for humans and the conserved CpGs -934_1, -934_2, -924, -901 in prairie vole. The CpG sites in the MT2 region are on the y axis, and the darker shades indicate a higher level of correlation within the matrix. Columns in grey represent CpG sites with 0% DNA methylation in all samples.

E.



F.

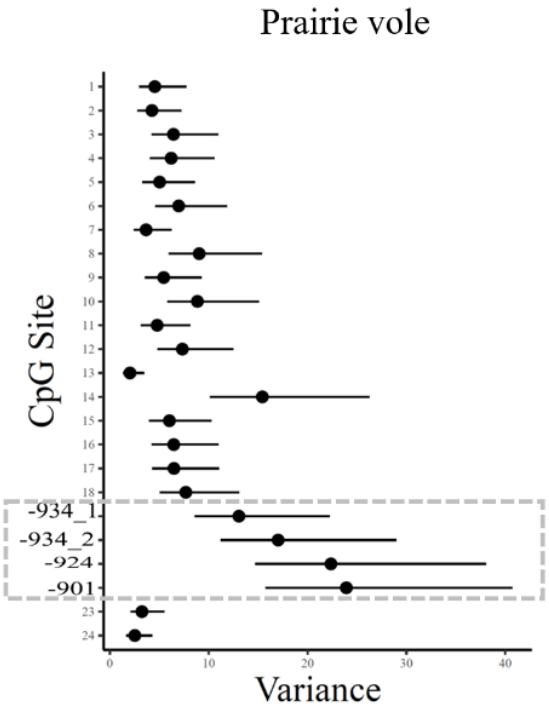
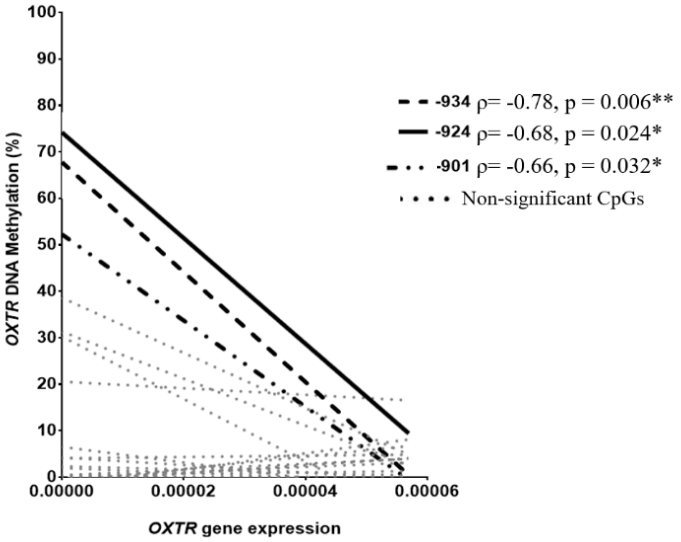


Figure 2E, 2F. Variance of DNA methylation levels between oxytocin receptor gene CpG sites in MT2 region, CpGs with largest amount of variance are clustered. E) The CpG sites that have the largest amount of variation in DNA methylation across the MT2 region in humans include CpG sites -934, -924, and -901. The bar represents the range of variance in DNA methylation and the dot is the mean variance in DNA methylation per CpG site across the sample. F) The CpG sites that have the largest amount of variation in DNA methylation across the conserved MT2 region include CpG 14, -934_1, -934_2, -924, and -901. The CpG sites in the conserved MT2 region are on the y axis, and the bar represents the range of variance and the dot is the mean variance in DNA methylation per CpG site.

G.



H.

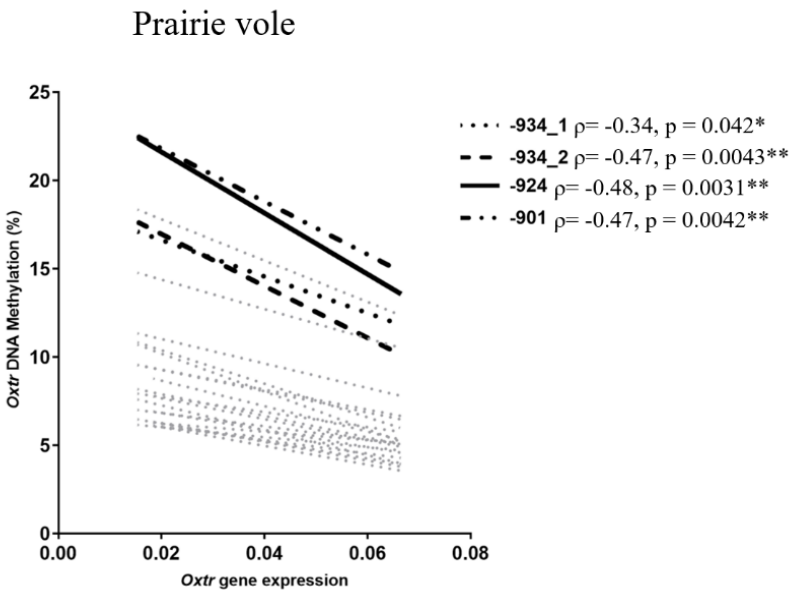


Figure 2G, 2H. DNA methylation and oxytocin receptor gene expression are correlated in the MT2 region in select CpG sites in humans and the conserved MT2 region in prairie voles. G) The relationship between DNA methylation and *OXTR* expression in 22 CpG sites within the MT2 region in humans. Select CpG sites -934, -924, and -901 have been previously implicated in the regulation of human social behavior, and have an inverse relationship between DNA methylation and *OXTR* expression. Significant sites are highlighted in black, and non-significant sites are in grey dotted lines (-934: ρ(11) = -0.78, *p* = 0.006; -924: ρ(11) = -0.68, *p* = 0.024; -901 ρ(11) = -0.65, *p* = 0.032). H) The relationship between DNA methylation and *Oxtr* expression in the 24 CpG sites within the conserved MT2 region in prairie voles. The conserved CpG sites -934_1, -934_2, -924, and -901 also have a inverse relationship between DNA methylation and *Oxtr* expression and are highlighted in black (-934_1: ρ(36) = -0.34, *p* = 0.042; -934_2: ρ(36) = -0.47, *p* = 0.0043; -924: ρ(36) = -0.48, *p* = 0.0031; -901 ρ(36) = -0.47, *p* = 0.0042; NAcc; PND 24 harvest). Spearman's Rho for 22 CpG sites within the human MT2 region and the 24 CpG sites in the conserved MT2 region in prairie voles are in Tables 1 and 2.

A.

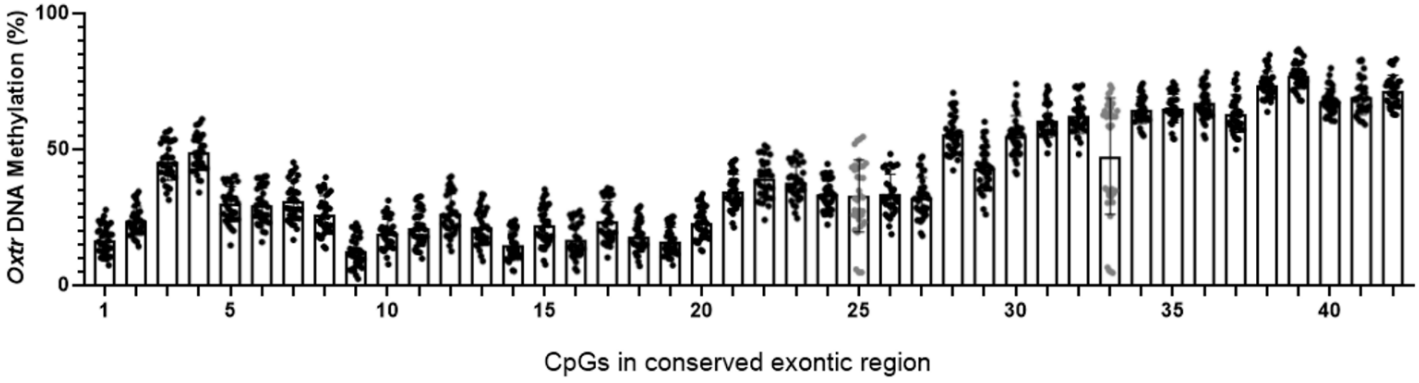


Figure 3A. DNA methylation across the conserved exon 3 region in prairie voles. A) CpG methylation profiles of 42 CpG sites within the conserved exon 3 region in prairie voles. The DNA methylation patterns in CpG site 25 and 33 indicate a polymorphic CpG sites, and are highlighted in grey dots. (Error bars presented as mean +/- SEM, NAcc; PND 24 harvest; sibling pairs = 18.)

B.

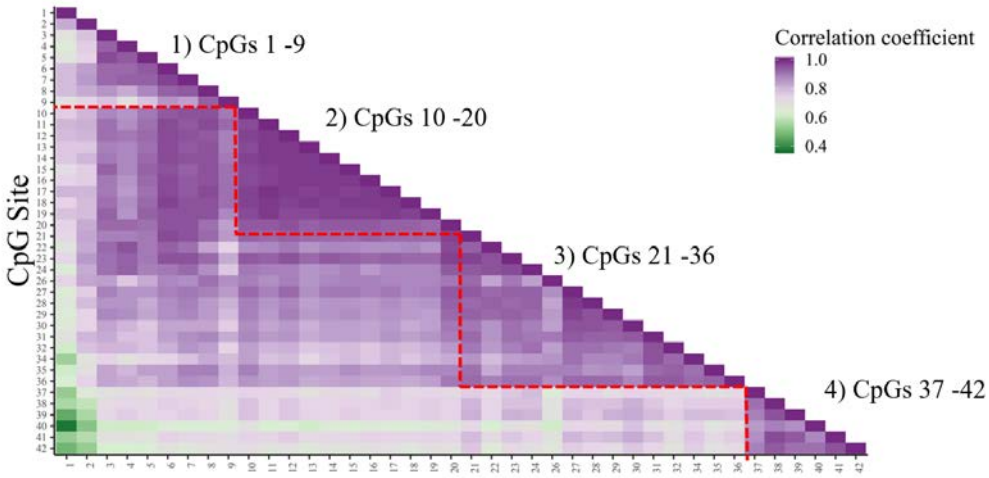


Figure 3B. Correlation of DNA methylation levels between *Oxtr* CpG sites in conserved exonic region. B) CpG sites that have highly correlated levels of DNA methylation fall within 4 clustered domains. Cluster 1 contains CpGs 1-9, cluster 2 contains CpGs 10-20, cluster 3 contains CpGs 21-36, and cluster 4 contains CpGs 37 and 42. To add in visualization, these clusters have been marked in red dotted lines. Polymorphic CpGs 25 and 33 have been removed as outliers in the correlation matrix. The CpG sites in the conserved exon 3 region are on the y axis, and the darker shades indicate a higher level of correlation within the matrix. (PND 24 harvest; sibling pairs = 18.)

C.

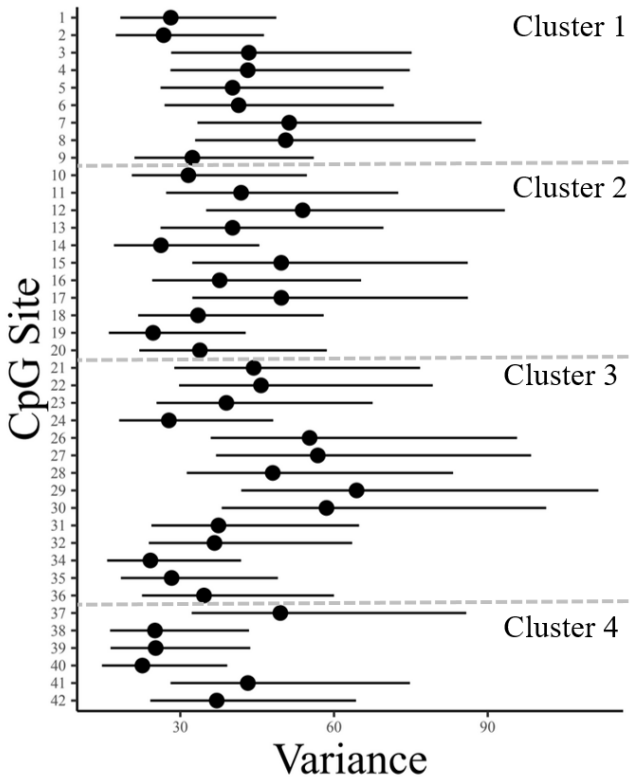


Figure 3C. Variance of DNA methylation levels between *Oxtr* CpG sites in conserved exonic region. C) The CpG sites within each cluster have similar levels of variance. The bar represents the range of variance in DNA methylation and the dot is the mean variance in DNA methylation per CpG site across the sample. Polymorphic CpGs 25 and 33 have been removed as outliers. (PND 24 harvest; sibling pairs = 18.)

D.

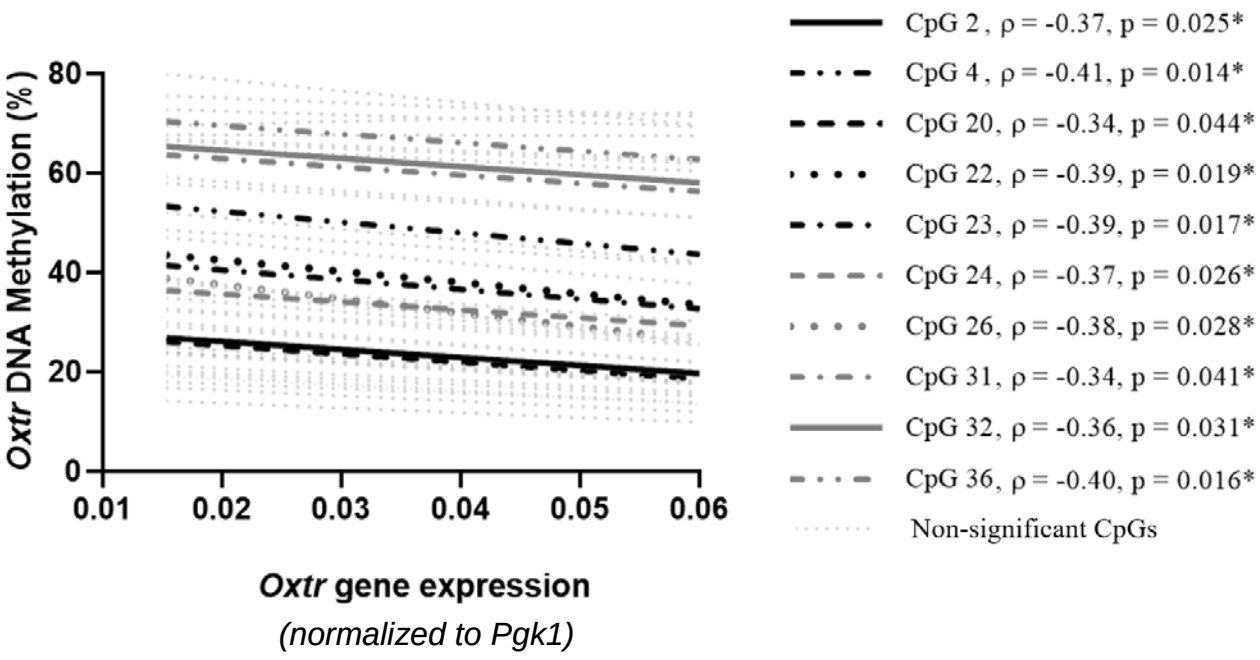
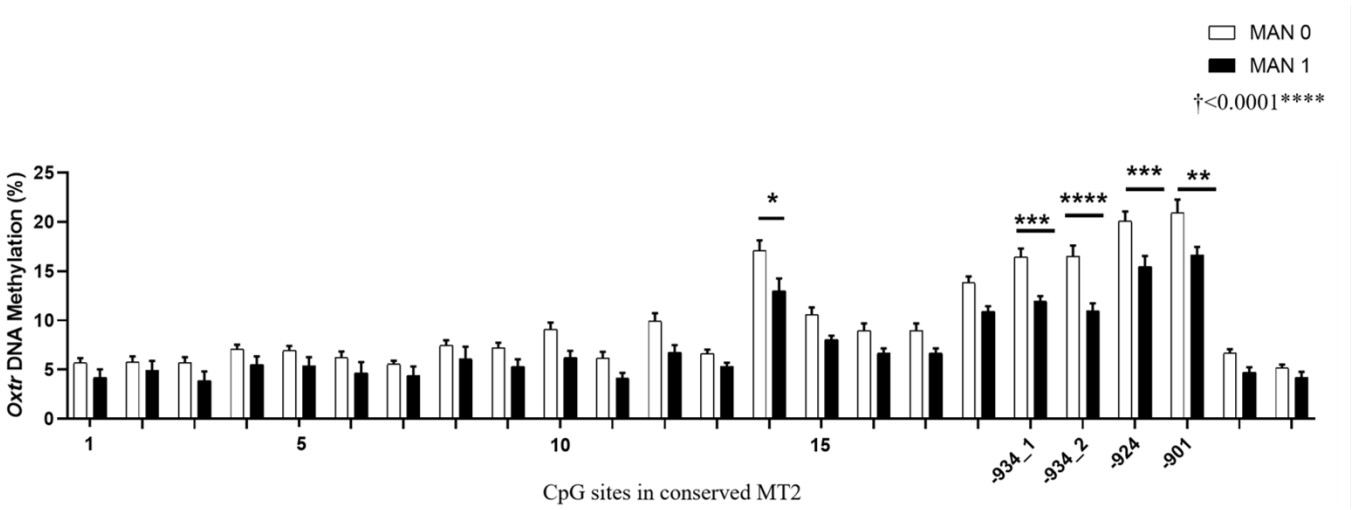


Figure 3D. DNA methylation and oxytocin receptor gene expression are correlated in select CpG sites the conserved exonic region in prairie voles. G) The relationship between DNA methylation and *Oxtr* expression in 42 CpG sites within the conserved exonic region. Select CpG sites that have a significant inverse relationship between DNA methylation and *Oxtr* expression fall predominately in cluster 3. They are highlighted in black and dark grey lines, and non-significant sites are in light grey dotted lines (CpG 4 : ρ(36) = -0.41, *p* = 0.014; CpG 20 : ρ(36) = -0.34, *p* = 0.044; CpG 22 : ρ(36) = -0.39, *p* = 0.019; CpG 23 : ρ(36) = -0.39, *p* = 0.017; CpG 24 : ρ(36) = -0.37, *p* = 0.026; CpG 26 : ρ(36) = -0.38, *p* = 0.028; CpG 31 : ρ(36) = -0.34, *p* = 0.041; CpG 32 : ρ(36) = -0.36, *p* = 0.031; CpG 36 : ρ(36) = -0.40, *p* = 0.016; PND 24 harvest; sibling pairs = 18; uncorrected p-values). Polymorphic CpGs 25 and 33 have been included.

A.



B.

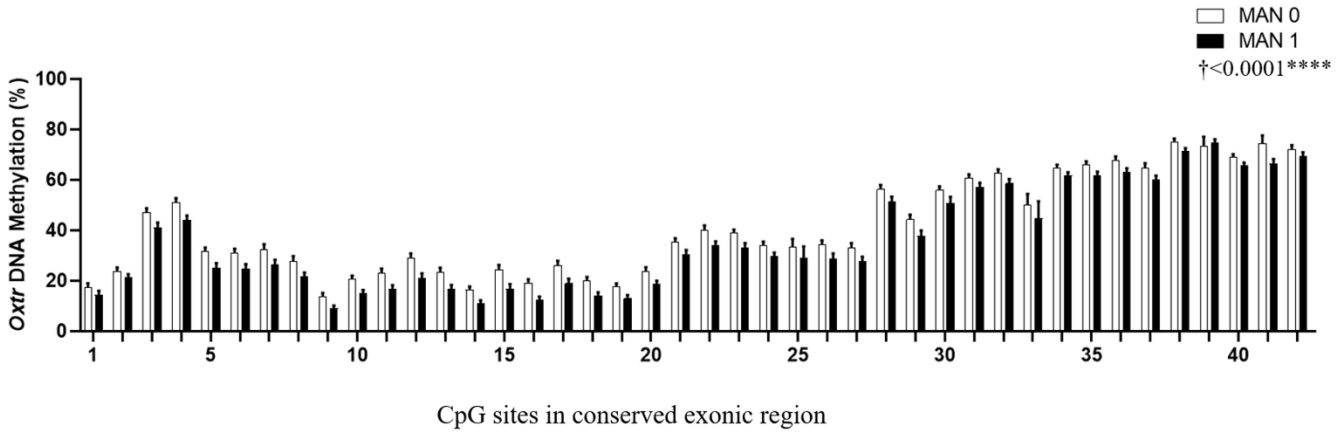


Figure 4A, 4B. Early life experience corresponds with changes in *Oxtr* DNA methylation. A) DNA methylation profiles of offspring of control group (MAN0) and offspring of parents manipulated to induce an increase in parental behavior (MAN1) across the conserved MT2 region in prairie voles. Overall, there is a significant difference between DNA methylation between MAN0 and MAN1 offspring, with decreased levels of DNA methylation in MAN1 offspring. CpG sites that have the greatest difference in DNA methylation between offspring that received greater amounts of parental care (MAN1) from control group (MAN0) include CpGs 14, -934_1, -934_2, -924, -901. (†2-way ANOVA, main effect of handling; error bars presented as mean +/- SEM, Sidak post-test by site, NAcc, PND 24 harvest; *p ≤ 0.05, **p ≤ 0.01, ***p ≤ 0.001, ****p ≤ 0.0001; MAN0 = 7 sibling pairs, MAN1 = 6 sibling pairs).
B) DNA methylation profiles of offspring of control group (MAN0) and offspring of parents manipulated to induce an increase in parental behavior (MAN1) across the conserved exonic region in prairie voles. Overall, there is a significant difference between DNA methylation between MAN0 and MAN1 offspring, with increased levels of DNA methylation in MAN0 offspring. No CpG sites survives multiple comparisons corrections. (†2-way ANOVA, main effect of handling; error bars presented as mean +/- SEM, Sidak post-test by site, NAcc, PND 24 harvest; MAN0 = 7 sibling pairs, MAN1 = 6 sibling pairs.)

C.

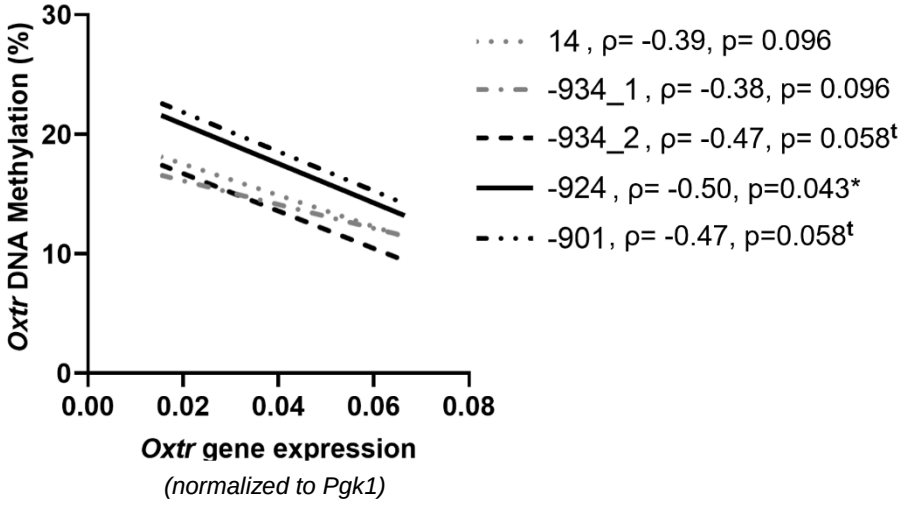
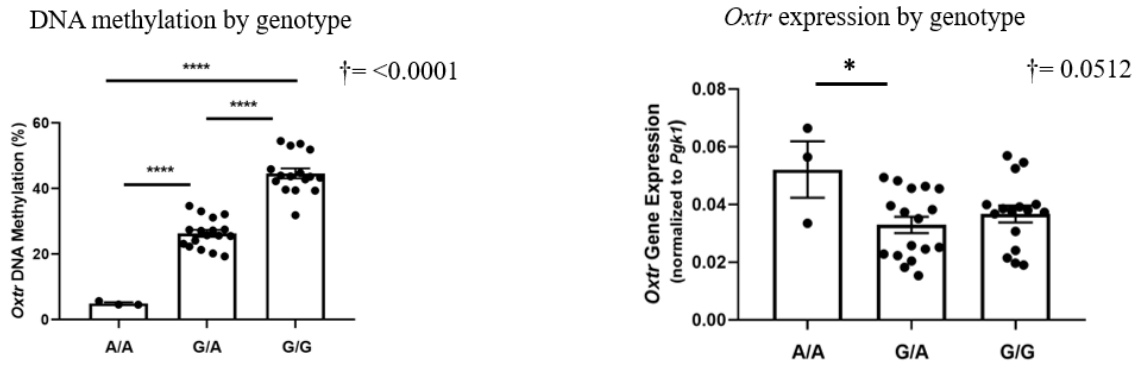


Figure 4C. DNA methylation in MT2 region influences *Oxtr* expression.
C) DNA methylation inversely correlates with *Oxtr* expression, revealed by correlating all significantly marked CpG sites between in MAN1 and MAN0 offspring. Of those sites, CpG, -934_2, -924, and -901 in the conserved MT2 region have a significant or strong trending inverse relationship between levels of DNA methylation and *Oxtr* expression. For visualization purposes, the linear regressions of these CpGs are highlighted in black. (MT2: CpG 14 ρ(26)= -0.390, *p*= 0. 0.096, CpG -934_1: ρ(26)= -0.382, *p*=0.096**;** CpG -934_2: ρ(26)= -0.473, *p*= 0.058; CpG -924: ρ(26)= -0.503, *p*=0.043, CpG -901: ρ(26)= -0.471, *p*=0.058); Holm-Sidak adjusted *p*-values; NAcc: PND24 harvest, sibling pairs = 13.)

A.

Polymorphic CpG 25



B.

Polymorphic CpG 33

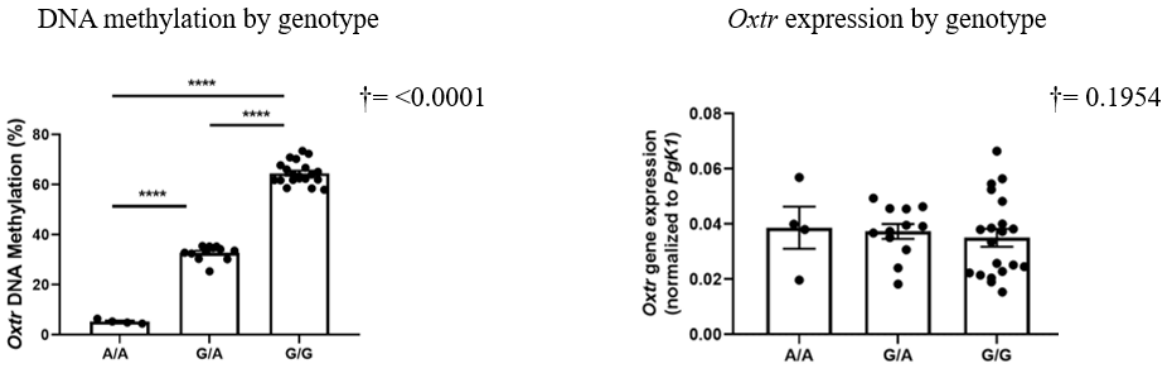


Figure 5. Exonic polymorphisms association with *Oxtr* expression.
A-B) CpG 25 and 33 in the exonic region contains a G to A single nucleotide polymorphism within our population. G/G represents animals with homozygous CpG alleles. G/A represents animals with a CpG allele and a CA allele. A/A represents animals with homozygous CA alleles. In both CpG 25 and CpG 33 polymorphisms, there is a significant difference in DNA methylation (†2-way ANOVA, main effect of genotype; error bars presented as mean +/- SEM, Sidak post-test by site, ****$p = \leq 0.0001$, NAcc, PND 24 harvest; n = 36).
A) Within our population of prairie voles, the CpG 25 polymorphism is associated with a strong trending difference in *Oxtr* expression by genotype, and a significant difference between A/A and G/A alleles (†2-way ANOVA, main effect of genotype; error bars presented as mean +/- SEM, Sidak post-test by site, *$p = \leq 0.05$, NAcc, PND 24 harvest; n = 36). B) There is no significant difference in *Oxtr* expression between CpG 33 polymorphic groups (†2-way ANOVA, main effect of genotype; error bars presented as mean +/- SEM, Sidak post-test by site, NAcc, PND 24 harvest; n = 36).

Table 1.

Correlation between *OXTR* DNA methylation and gene expression in the human brain across 22 CpG sites in the MT2 region

Human *OXTR* CpG site	Spearman's Rho	95% confidence interval	*P*-value (two tailed)
-1155	-	-	-
-1137	-	-	-
-1132	0.5000	-0.1627 to 0.8518	0.1818
-1126	-0.3000	-0.7711 to 0.3833	0.5455
-1122	0.5000	-0.1627 to 0.8518	0.1818
-1120	-	-	-
-1109	-0.5598	-0.8731 to 0.08078	0.0783
-1101	0.3000	-0.3833 to 0.7711	0.5455
-1098	-0.0939	-0.6685 to 0.5503	0.8000
-1085	0.07516	-0.5636 to 0.6577	0.8343
-1063	0.5798	-0.05121 to 0.8800	0.0727
-1057	-0.1753	-0.7117 to 0.4902	0.6364
-1051	0.3101	-0.3737 to 0.7756	0.3818
-1017	0.1000	-0.5463 to 0.6717	0.9091
-1002	0.5000	-0.1627 to 0.8518	0.1818
-990	-0.4000	-0.8134 to 0.2819	0.3636
-983	-0.3638	-0.7986 to 0.3205	0.2688
-959	-0.1284	-0.6872 to 0.5258	0.7108
-934	**-0.7818**	**-0.9429 to -0.3244**	**0.0064**
-924	**-0.6834**	**-0.9136 to -0.1214**	**0.0241**
-901	**-0.6560**	**-0.9050 to -0.07226**	**0.0323**
-860	-0.2187	-0.7332 to 0.4551	0.5148

In the MT2 region in humans, DNA methylation levels significantly contribute to *OXTR* expression in CpG sites -934, -924, and -901 (uncorrected *p*-values). Sites denoted by (-) display no methylation in the sample.

Table 2.

Correlation between *Oxtr* DNA methylation and gene expression in nucleus accumbens of prairie voles in 24 CpG sites in conserved MT2 region.

Prairie vole *Oxtr* CpG site	Spearman's Rho	95% confidence interval	*P*-value (two tailed)
1	-0.3332	-0.6100 to 0.01597	0.0541
2	-0.06066	-0.3996 to 0.2929	0.7333
3	-0.1665	-0.4903 to 0.1978	0.3545
4	-0.3133	-0.5885 to 0.02711	0.0628
5	**-0.3405**	**-0.6115 to 0.002061**	**0.0453**
6	-0.2166	-0.5288 to 0.1473	0.2260
7	-0.3010	-0.5870 to 0.05174	0.0836
8	-0.2976	-0.5808 to 0.04975	0.0825
9	-0.2798	-0.5678 to 0.06911	0.1035
10	-0.2723	-0.5585 to 0.07177	0.1081
11	-0.1674	-0.4821 to 0.1856	0.3365
12	**-0.4628**	**-0.6922 to -0.1485**	**0.0045**
13	-0.3261	-0.5978 to 0.01278	0.0522
14	**-0.3629**	**-0.6240 to -0.02898**	**0.0296**
15	-0.3269	-0.5984 to 0.01192	0.0517
16	-0.2798	-0.5640 to 0.06373	0.0984
17	**-0.5264**	**-0.7336 to -0.2297**	**0.0010**
18	**-0.3990**	**-0.6491 to -0.07103**	**0.0159**
-934_1	**-0.3436**	**-0.6103 to -0.006929**	**0.0402**
-934_2	**-0.4651**	**-0.6937 to -0.1514**	**0.0043**
-924	**-0.4788**	**-0.7027 to -0.1685**	**0.0031**
-901	**-0.4659**	**-0.6942 to -0.1523**	**0.0042**
23	**-0.4571**	**-0.6884 to -0.1415**	**0.0051**
24	**-0.3916**	**-0.6505 to -0.05121**	**0.0220**

In the conserved MT2 region in prairie voles, DNA methylation levels significantly contribute to *Oxtr* expression in CpG sites 5, 12, 14, 17, 18, -934_1, -934_2, -924, -901, 23, and 24 (uncorrected *p*-values).

Table 3

Correlation between *Oxtr* DNA methylation and gene expression in nucleus accumbens of prairie voles in 42 CpG sites in conserved exonic region.

Prairie vole *Oxtr* CpG site	**Spearman's Rho**	**95% confidence interval**	***P*-value (two tailed)**
1	-0.2835	-0.5668 to 0.05967	0.0938
2	**-0.3735**	**-0.6314 to -0.04117**	**0.0248**
3	-0.2570	-0.5471 to 0.08812	0.1302
4	**-0.4080**	**-0.6553 to -0.08173**	**0.0135**
5	-0.2569	-0.5470 to 0.08827	0.1304
6	-0.2785	-0.5631 to 0.06512	0.1000
7	-0.2965	-0.5763 to 0.04554	0.0791
8	-0.2461	-0.5388 to 0.09971	0.1480
9	-0.1951	-0.4997 to 0.1524	0.2541
10	-0.2108	-0.5119 to 0.1364	0.2171
11	-0.2466	-0.5392 to 0.09917	0.1471
12	-0.2649	-0.5529 to 0.07977	0.1185
13	-0.2402	-0.5343 to 0.1059	0.1583
14	-0.2344	-0.5300 to 0.1120	0.1689
15	-0.2584	-0.5481 to 0.08661	0.1280
16	-0.2398	-0.5341 to 0.1063	0.1590
17	-0.2340	-0.5297 to 0.1124	0.1696
18	-0.1990	-0.5027 to 0.1485	0.2447
19	-0.1887	-0.4947 to 0.1590	0.2704
20	**-0.3376**	**-0.6061 to -0.0001359**	**0.0440**
21	-0.2874	-0.5697 to 0.05548	0.0892
22	**-0.3902**	**-0.6430 to -0.06071**	**0.0186**
23	**-0.3942**	**-0.6458 to -0.06544**	**0.0174**
24	**-0.3709**	**-0.6296 to -0.03819**	**0.0259**
25	-0.2381	-0.5328 to 0.1081	0.1620
26	**-0.3820**	**-0.6474 to -0.03399**	**0.0282**
27	-0.2873	-0.5695 to 0.05564	0.0894
28	-0.3076	-0.5844 to 0.03337	0.0680
29	-0.2821	-0.5657 to 0.06122	0.0955
30	-0.2484	-0.5406 to 0.09727	0.1441
31	**-0.3426**	**-0.6096 to -0.005763**	**0.0408**

32	**-0.3593**	**-0.6215 to -0.02484**	**0.0314**
33	-0.1766	-0.4852 to 0.1711	0.3029
34	-0.2368	-0.5318 to 0.1094	0.1643
35	-0.2304	-0.5269 to 0.1161	0.1764
36	-0.3982	-0.6485 to -0.07012	0.0162
37	-0.1521	-0.4657 to 0.1954	0.3758
38	-0.1807	-0.4884 to 0.1670	0.2916
39	-0.2312	-0.5275 to 0.1153	0.1750
40	0.0007722	-0.3368 to 0.3382	0.9964
41	-0.004376	-0.3414 to 0.3336	0.9798
42	-0.1363	-0.4530 to 0.2109	0.4280

In the conserved exonic region in prairie voles, DNA methylation levels significantly contribute to *Oxtr* expression in CpG sites 2, 4, 20, 22, 23, 24, 26, 31, 32, 36 (uncorrected *p*-values).

Table 4.

Mean deviation *Oxtr* DNA methylation of 24 CpG sites in conserved MT2 region in prairie voles.

Prairie vole *Oxtr* CpG site	Mean Deviation
1	1.13%
2	1.23%
3	1.06%
4	1.29%
5	1.65%
6	1.16%
7	0.93%
8	1.13%
9	1.22%
10	1.44%
11	1.01%
12	1.57%
13	1.35%
14	2.29%
15	1.13%
16	1.31%
17	1.34%
18	1.23%
-934_1	1.61%
-934_2	1.50%
-924	1.73%
-901	1.55%
23	1.14%
24	1.20%

In measuring DNA methylation at CpG sites within the conserved MT2 region in prairie voles, all samples were amplified in triplicate and randomized for pyrosequencing to account for plate and run variability. Epigenotypes reported are an average of three replicates, and the percent each CpG site deviated from the mean (±) within our sample is listed in Table 4.

Table 5.

Mean deviation *Oxtr* DNA methylation of 42 CpG sites in conserved exonic region in prairie voles.

Prairie vole *Oxtr* CpG site	Mean deviation
1	1.39%
2	1.79%
3	1.44%
4	1.82%
5	1.72%
6	1.62%
7	1.72%
8	1.61%
9	1.15%
10	1.66%
11	1.82%
12	1.87%
13	1.61%
14	1.51%
15	1.75%
16	1.56%
17	1.88%
18	1.39%
19	1.56%
20	0.95%
21	0.95%
22	1.18%
23	1.08%
24	1.14%
25	0.99%
26	1.04%
27	1.36%
28	1.48%
29	1.70%
30	1.43%
31	1.41%
32	1.46%
33	1.34%

34	1.32%
35	1.51%
36	1.65%
37	1.87%
38	1.55%
39	1.63%
40	1.72%
41	1.82%
42	1.65%

In measuring DNA methylation at CpG sites within the conserved exonic region in prairie voles, all samples were amplified in triplicate and randomized for pyrosequencing to account for plate and run variability. Epigenotypes reported are an average of three replicates, and the percent each CpG site deviated from the mean (±) within our sample is listed in Table 5.

Table 6.

Primers for prairie vole DNA methylation

PCR Primer Name	Region of Intrest	PCR primers	PRC conditions	Pyrosequencing Primer Name	Pyrosequencing primers	CpG sites captured
KLW201_F TSL201_R	MT2_section 1	F-GTAGTTTTGTATTGTGGGAAAGT R-[biotin]CCAACAACCTCAAAACTCTACT	480 bp product, 50 ul PCR, 58.6°C annealing temperature, 45 cycles, 3.5 nM of $MgCl_2$	KLW201_S1	TTGGTTATAGTTTTTTTTTGTTTT	MT2: CpGs 1, 2
	MT2_section 2	↓		KLW201_S2	GTTATAGATTAGAGGATTGTAAAGA	MT2: CpGs 3, 4, 5, 6, 7, 8
	MT2_section 3			KLW201_S3	TGGAGTAGGTAGTTTTTTATTTTG	MT2: CpGs 9, 10, 11
	MT2_section 4			KLW201_S4	AGAGTTAGAGTATTTTTGAGGTTA	MT2: CpGs 12, 13, 14
	MT2_section 5			KLW201_S5	AGGTTTTTTTTAGGTGG	MT2: CpGs 15, 16
	MT2_section 6			KLW201_S6	GATGGTTAGTTAGTATTAGTG	MT2: CpGs 17, 18
	MT2_section 7			TSL201_S1	GAGGGAAGGTTTTGGAGTTTTTTATAT	MT2: CpGs -934_1, -934_2, -924
	MT2_section 8			TSL201_S2	AGGGATTGAAAAGTGA	MT2: CpGs -901, 23, 24
KLW301_F KLW301_R	Exonic_section 1	F-GAGGTGGAGGTGTTGTGTTTTATTTTGT R- [biotin]ACCAAACAACCCAACCATATC	375 bp product, 25 ul PCR, 58°C annealing temperature, 45 cycles, MinElute gel extraction	KLW301_S1	TTTTTTTTTATGAAGTATTTGAGTA	Exonic: CpGs 1, 2
KLW302_F KLW302_R	Exonic_section 2	F-AGGTGTTTTAGTAGTTGTTGTGGGATATT R-[biotin]CACCAAACAACCCAACCATAT	224 bp product, 25 ul PCR, 63°C annealing temperature, 45 cycles, 3.5nM of $MgCl_2$	KLW302_S2	GTTGTTGTGGGATATTATTTT	Exonic: CpGs 3, 4, 5, 6
	Exonic_section 3	↓		KLW302_S3	TTTGTAGGTGGTGGG	Exonic: CpGs 7, 8, 9, 10
	Exonic_section 4			KLW302_S4	GTTGTTTGGTTATTTGTTAGT	Exonic: CpGs 11, 12, 13, 14, 15, 16, 17, 18, 19
KLW305_F KLW305_R	Exonic_section 5	F-TATGGTTGGGTTGTTTGGTGGTTA R-[biotin]CATAAACCTTAAATCCCCAAAACTAAA	123 bp product, 25 ul PCR, 57°C annealing temperature, 45 cycles	KLW305_S5	GTTGTTTGGTGGTTAG	Exonic: CpGs 20, 21, 22, 23, 24, 25, 26
KLW306_F KLW306_R	Exonic_section 6	F-ATTTAGTTTTGGGGATTTAAGGTTTA R- [biotin]ACCAATACAATAATAAAAATCATCTTCACT	272 bp product, 25 ul PCR, 57°C annealing temperature, 45 cycles, 3.5nM of $MgCl_2$	KLW306_S6	GGGGATTTAAGGTTTATG	Exonic: CpGs 27, 28, 29, 30
	Exonic_section 7	↓		KLW306_S7	AGTTTTAAGATTTGGTAGAAT	Exonic: CpGs 31, 32, 33, 34, 35, 36
	Exonic_section 8			KLW306_S8	GAGGGGATTGAGGGA	Exonic: CpGs 37, 38, 39, 40, 41, 42

Table 7.

Primers for prairie vole polymorphism

PCR Primer Name	Region of Intrest	PCR primers	PRC conditions	Pyrosequencing Primer Name	Pyrosequencing primers
KLW325_F	Exonic CpG 25	F- TGTTCGCTTCCACCTACCTG	427 bp product, 25 ul PCR, 63°C annealing temperature, 45 cycles	KLW325_S1	TTTCTCACTGCGCGA
KLW325_R		R-[biotin]ATGAGCTTGACGCTACTGACTCG			
KLW333_F	Exonic CpG 33	F- GGACCCAAGGCCTATGTCA	209 bp product, 25 ul PCR, 58°C annealing temperature, 45 cycles	KLW333_S1	TCAAGATCTGGCAGAAC
KLW333_R		R-[biotin]ATGAGCTTGACGCTACTGACTCG			

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Chapter 3

Epigenetic Associations with Social Support in Adolescence Predict Early Adult Psychosocial Wellbeing

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Abstract

The oxytocin receptor (OXTR) directly regulates oxytocin's action on the cell and contributes to psychosocial functioning. Although the *OXTR* gene cannot change within individuals, intrapersonal variation in DNA methylation—an epigenetic modification that may influence behavioral phenotypes—is influenced by environmental factors. Previous research indicates that early human life experiences are associated with differences in methylation of the glucocorticoid receptor and ribosomal RNA gene promoters, but no research has investigated the relationship between supportive environments and *OXTR* DNA methylation in the MT2 region among human adolescents. In our study of 112 adolescents, greater perceived support at age 13 predicted less *OXTR* DNA methylation. Lower levels of *OXTR* DNA methylation predicted relative decreases in internalizing symptoms, less loneliness, and less stress in romantic relationships in these participants at age 24-25. Results suggest that supportive relationships during early adolescence predict less *OXTR* DNA methylation and that lower *OXTR* methylation predicts improving psychosocial functioning into early adulthood.

Introduction

Perceived support during adolescence, particularly from parents and peers, is considered among the most important predictors of psychosocial adjustment (Scholte, van Lieshout, & van Aken, 2001). Perceived parental and peer support relates to fewer internalizing symptoms, less loneliness, and fewer social problems (Kong & You, 2013; Scholte et al., 2001), as well as better adjustment and lower levels of depression and irritability as early adults (Aquilino & Supple, 2001; Rueger, Malecki, & Demaray, 2010). Research also indicates that the parent-adolescent relationship may provide a protocol for future relationships, influencing interpersonal expectations, strategies, and behavior.

Given the well-established importance of perceived support during adolescence, understanding the biological underpinnings by which experience of perceived support is linked to future psychosocial outcomes is crucial. The neuropeptide oxytocin is considered integral to social and psychological functioning, increasing social bonding (Bartz & Hollander, 2006; Baumgartner, Heinrichs, Vonlanthen, Fischbacher, & Fehr, 2008; Heinrichs & Domes, 2008) and decreasing susceptibility to depression, anxiety, loneliness, and maladaptive stress responses (Anderberg & Uvnäs-Moberg, 2000; Baumgartner et al., 2008; Carter, Grippo, Pournajafi-Nazarloo, Ruscio, & Porges, 2008; Cochran, Fallon, Hill, & Frazier, 2013; Neumann & Landgraf, 2012; Norman, Hawkley, Cole, Berntson, & Cacioppo, 2012; Slattery & Neumann, 2010; Yoshida et al., 2009; Young, Lim, Gingrich, & Insel, 2001). Most studies which focus on the link between oxytocin and psychosocial success have used administered oxytocin, rather than assaying the endogenous oxytocin system. Although administration studies are helpful in

learning about the *potential* effects of oxytocin, they may not yield fully accurate representations of how the endogenous oxytocin system *actually* functions.

The oxytocin receptor gene (*OXTR*) codes for a protein that receives and transmits the oxytocin signal and, thus, is the major regulator of oxytocin potential within the cell. Given that individual differences in oxytocin receptor density influence the oxytocin system (Walum et al., 2012), *OXTR* likely reflects variation in gene expression in the endogenous oxytocin system and, therefore, contributes to psychosocial functioning. Although the *OXTR* gene itself cannot change within each person, intrapersonal variation in DNA methylation, an epigenetic modification that may influence behavioral phenotypes, is possible. Elevated DNA methylation levels at a cluster of CpG sites in *OXTR* that includes sites -924 and -934 have previously been shown to impact gene transcription (Kusui et al., 2001). Because the oxytocin receptor is the gatekeeper of the oxytocin signal to the cell, *OXTR* DNA methylation has the potential to impact the readout of circulating oxytocin (Carter, 2014). Although previous research has examined the relationship between *OXTR* DNA methylation and depression, anxiety, social withdrawal, and stress response (Bell et al., 2014; Creswell et al., 2015; Jack, Connelly, & Morris, 2012; Lucht et al., 2009; Puglia, Lillard, Morris, & Connelly, 2015; Rodrigues, Saslow, Garcia, John, & Keltner, 2009; Ziegler et al., 2015), no research to date has studied the relationship between *OXTR* DNA methylation and relative changes in psychosocial functioning across time within the same individual and that doing so may provide insight into whether *OXTR* DNA methylation has the potential to influence psychosocial well-being.

Research has shown that blood can be used as a biomarker of the epigenetic state of the brain (Gregory et al., 2009) and that blood-derived DNA methylation levels can be used to identify individual differences in the brain's response to social perception (Jack et al., 2012; Puglia et al., 2015). This allows for the use of blood-derived DNA methylation as a biomarker for various psychiatric phenotypes (Costa et al., 2009; Dadds et al., 2014; Gregory et al., 2009; Guintivano et al., 2014; Reiner et al., 2015; Ziegler et al., 2015). These data suggest that the relationships we see in humans between *OXTR DNA* methylation as observed in the blood and a behavior/disorder may, in part, be due to the ability of the blood-based observations to predict oxytocin receptor availability in certain brain regions.

Due to the potential *effects* of *OXTR DNA* methylation, understanding factors that may be linked to its variability is critical. Yet, there is no existing research on the relationship between parental care and *OXTR* DNA methylation among human adolescents. Other research has indicated, however, that early life experiences of humans are associated with differences in methylation of the glucocorticoid receptor and ribosomal RNA gene promoters (Szyf & Bick, 2013). The present study explores both how relational exposures may be related to DNA methylation levels of *OXTR* and how DNA methylation levels predict psychosocial functioning over time. To do so, the present study uses uniquely rich longitudinal data from a community sample of 112 individuals followed prospectively across a 12-year span. Due to the extremely high developmental salience of friendship during adolescence (Collins & Laursen, 2004), the present study also looked at the relationship between perceived peer support and *OXTR* DNA methylation.

We hypothesize that the maternal/adolescent social environment influences *OXTR* DNA methylation variation. In this study we specifically investigated whether low levels of perceived maternal and peer support during early adolescence predict *OXTR* DNA methylation. We also hypothesize that variation in the level of DNA methylation of a regulatory region of the *OXTR* is associated with changes in psychosocial functioning from early adolescence to early adulthood. More specifically, we hypothesize whether higher levels of *OXTR* DNA methylation can predict the following behaviors at 24-25 years of age: (1) a relative increase in internalizing symptoms, (2) more loneliness, and (3) more stress in romantic relationships. Finally, we hypothesize whether *OXTR* DNA methylation will mediate the links between perceived support in early adolescence and psychosocial outcomes in early adulthood.

Method

Participants

This study was conducted as part of a larger longitudinal investigation of adolescent social development in familial and peer contexts. The final sample of 112 was a subsample of participants who had *OXTR DNA* methylation assessed as adults (from among 184 participants initially assessed at age 13, an attrition rate of less than 3%/year). The final sample included 46 males and 66 females and was racially/ethnically and socioeconomically diverse and representative of the community from which it was drawn: 64 adolescents 57% identified themselves as Caucasian, 33 (29%) as African American, 9 (8.04%) as of mixed race or ethnicity, and 3 (2.68%) as being from other minority groups. Adolescents' parents reported a median family income in the $40,000–$59,999 range at the initial assessment. Adolescents'

mothers (N=106) reported completing the following levels of education: less than high school (4.71%) high school or equivalent (17.93%), some college or technical training beyond high school (29.25%), associate's degree (5.66%), bachelor's degree (14.15%), some graduate work (9.43%), or post college degree (18.87%).

Adolescents were recruited from the seventh and eighth grades of a public middle school drawing from suburban and urban populations in the southeastern United States. Adolescents provided informed assent before each interview session, and parents and adult participants provided informed consent. Participants were assessed annually beginning at the age of 13 years (M=13.33, *SD* = 0.64). For the purposes of the present study, data were also drawn at ages 24 (M=24.65, *SD*=0.96) and 25 (M=25.69, *SD*=0.99). The standardized means of data at ages 24 and 25 (grand mean=25.2) were used. In early adulthood (ages 24-25), 168 (91.3%) of the original teens participated in at least one assessment. Individuals from the original study were recruited for an optional blood draw several years later as adults (M=28.31, *SD*=1.03). Out of the original 184 participants, 112 or nearly 61% (60.9%) consented to blood draws. We assume that *OXTR* DNA methylation is stable across adulthood and that our measure of methylation is representative of DNA methylation that has occurred by the time individuals reach their twenties.

Attrition Analyses

Attrition analyses examined missing data for each type of data available at baseline, and comparisons of the 112 participants from whom there was methylation data versus the remaining original participants for whom there was not methylation data indicated that those not continuing

were statistically indistinguishable from continuing participants on any baseline measure, suggesting that attrition was unlikely to have distorted any of the findings reported.

Procedure

Participants' data were covered under a confidentiality certificate issued by the U.S. Department of Health and Human Services, which protects information from subpoena by federal, state, and local courts. Adolescent/adult participants were paid for participation.

Measures

Blood collection, DNA isolation, and DNA methylation analysis (Age 28)

Individuals from the original parent study were recruited for an optional blood draw of which 112 individuals consented, and venipuncture was performed at the UVA Old Medical School Building (Charlottesville, V.A.). Eight and a half milliliters of whole blood was drawn into a PAXgene Blood DNA Tube (PreAnalytiX, Hombrechtikon Switzerland) and held at -20°C for short term storage (< 3 months) and held at -80°C for long term storage. Extraction of DNA was done using the PAXgene Blood DNA kit (PreAnalytiX, Hombrechtikon Switzerland) following manufacturer instructions. Two hundred nanograms of DNA, isolated from whole blood, was subject to bisulfite treatment (Kit MECOV50, Invitrogen, Carlsbad, CA), which allows for the detection of methylated cytosines by sequencing. Two ul of bisulfite converted DNA was used as a template for PCR using a Pyromark PCR kit (Qiagen, Valencia, CA) and 0.2 uM of primers was used for site -934 and -924 : TSL101F (5′-TTGAGTTTTGGATTTAGATAATTAAGGATT-3′) and TSL101R (5′-biotin-

AATAAAATACCTCCCACTCCTTATTCCTAA-3′). Samples were amplified in triplicate on three identical PCR machines (S1000 Thermal Cycler, Biorad, Hercules, C.A.). The following cycling conditions [Step 1: (95°C/15 min)/1 cycle, Step 2: (94°C/30 s, 56°C/30 s, 72°C/30 s)/50 cycles, Step 3: (74°C/10 min)/1 cycle, Step 4: 4°C hold] were used for amplification of fragments. Primers TSL101F and TSL101R amplifies a region on the coding strand of the *OXTR* gene that contains sites −934 and -924 (hg38, chr3: 8,769,044-8,769,160). Successful PCR amplification was confirmed using agarose gel electrophoresis for a representative of the sample and replicates, with the PCR product of TSL101F and TSL101R containing a single 116 base pair fragment. Standard controls of 0% and 100% methylated DNA, as well as a no DNA control and a positive control human standard were included for each PCR plate. All samples were amplified in triplicate and randomized for pyrosequencing to account for plate and run variability. Pyrosequencing was performed using primers TSL101S (5'-AGAAGTTATTTTATAATTTTT) for sites -934 and -924, and on a Pyromark Q24 using PyroMark Gold Q24 Reagents (Qiagen, Valencia, CA) per the manufacturer's protocol. Epigenotypes reported are an average of three replicates. On average, whole blood samples deviated from the mean by 1.49% for -934 and 1.86% for -924.

Perceived maternal and peer support (Age 13)

Adolescents' perceived maternal support was assessed at age 13 using the Children's Expectations of Social Behavior Questionnaire (Rudolph, Hammen, & Burge, 1995). This measure consists of 15 hypothetical vignettes in which teens are asked to imagine themselves interacting with their mothers (e.g., "You come home from school one day and you're feeling kind of sad because some of the kids at school were picking on you. You mention to your mom

what happened. What do you think she might do?"). For each vignette, teens were asked to indicate whether they expected an accepting maternal response (e.g., "She might talk it over with me so that I don't feel so bad"), an indifferent maternal response (e.g., "She might tell me that it's nothing to get upset about"), or a hostile maternal response (e.g., "She might ask me what I was doing that made the kids pick on me"). The scores were then summed, with higher scores representing teens' more positive expectations of maternal support. The internal consistency for valuing conformity was adequate (Cronbach's $\alpha = .67$).

Adolescents' perceived peer support was also assessed at age 13 using a parallel form of the same measure (Rudolph et al., 1995) in which items asked about imagined interactions with peers (e.g., "You're thinking about running for president of your class and you ask a friend to help you make up some posters to hang around school. What do you think she might say?"). The internal consistency for perceived maternal support was high (Cronbach's $\alpha = .76$). Perceived maternal support and perceived peer support were standardized and averaged to measure overall perceived support. Internal consistency for overall perceived support was good (Cronbach's $\alpha = .80$). We assume that this measure of perceived support represents a cumulative effect of social support throughout childhood and early adolescence.

Internalizing symptoms (Age 25)

Internalizing symptoms were assessed using the 39-item self-report internalizing symptoms subscale from the Adult Self Report (Achenbach & Rescorla, 2003). This subscale includes items concerning anxious/depressive symptoms, withdrawn symptoms, and somatic complaints. Items are scored on a 3-point scale ("Not true" to "Very true" or "Often True"), with higher

scores indicating greater internalizing symptoms. Sample items include "I am unhappy, sad, or depressed" (anxious/depressive symptoms), "I would rather be alone than with others" (withdrawn symptoms), and "I feel tired without good reason" (somatic complaints). Results were averaged across assessments at ages 24 and 25 to yield an overall measure of internalizing symptoms. The internal consistency for self-reported internalizing symptoms was high (Cronbach's α =.96).

Loneliness (Age 25)

Loneliness was assessed via the UCLA Loneliness Scale (Russel, Peplau, & Cutrona, 1980). This 20-item measure assesses loneliness using responses on a 4-point Likert-scale. Sample items include "I feel starved for company" and "No one really knows me well." Results were averaged across assessments at ages 24 and 25 to yield an overall measure of loneliness. The internal consistency for self-reported loneliness was high (Cronbach's α =.98).

Stress in romantic relationships (Age 25)

Stress in romantic relationships was assessed via the Romantic Life Satisfaction Scale (Hare & Miga, 2009). This five-item scale was created to measure satisfaction with the state of participants' current romantic life. The scale uses a 4-point Likert-scale and has a three-item romantic life stress subscale. Sample items include "The current state of my romantic life causes me a great deal of stress" and "I would like to make significant changes to the current circumstances of my romantic life." Results were averaged across assessments at ages 24 and 25 to yield an overall measure of stress in romantic relationships. The internal consistency for total stress in relationships was high (Cronbach's α =.83).

Baseline Depressive Symptoms (Age 13)

Adolescents reported the degree of their depressive symptoms using the Child Depression Inventory (Kovacs & Beck, 1977). This 27-item inventory is based on the Beck Depression Inventory and has been well-validated as a measure of depressive symptomatology linked to poor self-esteem, hopelessness, and negative cognitive attributions (Kovacs & Beck, 1977). Internal consistency for this measure was good (Cronbach's α =.85).

Baseline Close Friendship Competence (Age 13)

Adolescents completed a modified version of the Adolescent Self-Perception Profile (Harter, 1988) to assess their overall competence in forming and maintaining close friendships. Items in this subscale ask about individuals' abilities to form friendships in which they can share personal thoughts and secrets. Internal consistency for this subscale was adequate (Cronbach's α =.65).

Statistical Methods

For all primary analyses, SAS PROC CALIS (Version 9.4; SAS Institute, Cary, NC) was used to assess key relations in hierarchical regression models. Full-information maximum-likelihood methods were used to handle missing data. Conventional outlier analyses were performed in accordance with recommendations offered by Tabachnick and Fidell (1996) to account for multiple sources of methodological error or poor validity of operational definitions. variables containing outliers with scores greater than 3.5 standard deviations beyond the mean where removed (Tabachnick & Fidell, 1996).

Results

Means and standard deviations for all substantive variables examined and intercorrelations among primary variables are presented in Table 1. Initial analyses examined the role of sex and family income in early adolescence on the primary measures examined in the study. Most variables of substantive interest were not related to adolescent sex or income in the adolescent's family of origin.

Figure 1 shows the relationship between total perceived support and sites -924 and -934. Zero-order correlations never showed a link to site -934 and, therefore, this site is not discussed further. For the analyses below, the false discovery rate test (Glickman, Rao, & Schultz, 2014) was applied to control for the false positive rate from multiple tests.

Levels of DNA methylation can be influenced by racial origin (Heyn et al., 2013). Because our sample is a mixed-race sample, we examined the levels of DNA methylation in black participants vs. the levels of DNA methylation in white participants. There were no significant differences based on race in this sample ($F(1, 95)= 1.67$, $p= 0.20$). Even so, we tested models to control for race and results did not change. Levels of *OXTR* DNA methylation vary by sex (Puglia et al., 2015), therefore we examined the levels of DNA methylation in male participants vs. the levels of DNA methylation in female participants. There were no significant differences in the level of *OXTR* DNA methylation based on sex ($F(1, 110)= 0.13$, $p= 0.72$). We tested models to control for sex and, as expected, the models didn't change. There were also no

differences between white males and white females ($F(1,62)= 0.04$, $p= 0.83$) or between black males and black females ($F(1, 31)= 0.24$, $p= 0.63$). Therefore, we combined the data set since we found no difference between race and sex.

Perceived maternal and peer support during early adolescence predicts *OXTR* DNA methylation

Perceived maternal and peer support when adolescents were 13 was examined as a predictor of *OXTR* DNA methylation at site -924 in models that also accounted for sex and baseline family income. Results, presented in Table 2, indicated that more perceived support at 13 years old predicted less *OXTR* DNA methylation, after accounting for sex and baseline family income ($\beta= -0.24$, $p= 0.02$).

***OXTR* DNA methylation at site -924 predicts a relative increase in internalizing symptoms at 25 years old**

OXTR DNA methylation at site -924 was examined as a predictor of internalizing symptoms at 24-25 years old in models that also accounted for sex and baseline family income. Predictions also accounted for baseline depressive symptoms to allow for examination of the relative change in internalizing symptoms. Results, as seen in Table 3, indicated that *OXTR* DNA methylation predicted higher levels of future internalizing symptoms after accounting for sex r, baseline family income, and baseline depressive symptoms ($\beta = 0.29$, $p< 0.01$). Higher levels of *OXTR* DNA methylation results in relative increase in internalizing symptoms between early adolescence and young adulthood.

OXTR DNA methylation at site -924 predicts more loneliness at 25 years old

OXTR DNA methylation at site -924 was examined as a predictor of loneliness at 24-25 years old in models that also accounted for sex and baseline family income. Due to evidence that depressive symptoms in early adolescence predict a significant risk for social difficulty in early adulthood (Allen, Chango, Szwedo, & Schad, 2014), predictions also accounted for baseline depressive symptoms. Results, displayed in Table 4, indicated that *OXTR* DNA methylation positively predicted loneliness, after accounting for sex, baseline family income, and baseline depressive symptoms ($\beta = 0.29$, $p = 0.04$). This indicates that the relation of *OXTR* DNA methylation to adult loneliness existed above baseline psychological functioning, and that greater DNA methylation was linked to greater loneliness in young adulthood.

OXTR DNA methylation at site -924 predicts more stress in romantic relationships at 25 years old

OXTR DNA methylation at site -924 was examined as a predictor of stress in romantic relationships at 24-25 years old in models that also accounted for sex and baseline family income. Results, presented in Table 5, indicated that *OXTR* DNA methylation positively predicted stress in romantic relationships, after accounting for sex baseline family income, and baseline close friendship competence ($\beta = 0.24$, $p = 0.01$). This means that the relation of *OXTR* DNA methylation to adult stress in romantic relationships existed above baseline social functioning and that greater levels of *OXTR* DNA methylation was linked to more stress in romantic relationships.

OXTR DNA methylation at site -924 does not mediate the links between perceived support at 13 years old and psychosocial outcomes at 25 years old

We tested the significance of these hypothesized mediated paths using SAS PROC CALIS (Version 9.4; SAS Institute, Cary, NC). Effects of early adolescents' perceived support on young adults' internalizing symptoms, loneliness, and stress in romantic relationships was not mediated via *OXTR* DNA methylation at site -924.

Discussion

Results of this study provide evidence that supportive environments in early adolescence predict lower levels of *OXTR* DNA methylation and that lower levels of *OXTR* DNA methylation predict positive changes in psychological and interpersonal outcomes in early adulthood. This study builds from the growing recognition that oxytocin is integral to humans' psychosocial well-being (Carter, 2014) and that significant variation in DNA methylation of the oxytocin receptor gene may account for individual differences in functional outcomes (Kumsta, Hummel, Chen, & Heinrichs, 2015; Kusui et al., 2001).

The current study is the first to show the relationship between supportive environments and *OXTR* DNA methylation in humans, suggesting that self-reported perceived maternal and peer support during early adolescence has long-term implications for the epigenetic modification of *OXTR* at site -924. We used a measure of maternal and peer support as a measure of supportive environments, given that peers play an essential role in the psychological well-being of human adolescents (Collins & Laursen, 2004). Previous work provided evidence that early life

experiences of humans are related to other epigenetic modifications that influences behavioral phenotypes (Szyf & Bick, 2013). Recent research has found that physical and sexual abuse in childhood and adolescence predicts higher *OXTR* DNA methylation at a CpG site that is highly associated with site -924 (Gouin et al., 2017). It, however, looked at abuse retrospectively (at age 21), rather than at the ages in which the abuse occurred, only saw a relationship with female participants, and only examined the relationship between physical aspects of the parent-child relationship and *OXTR* DNA methylation. The present study is the first to investigate the emotional relationship between the parent-adolescent relationship—measured by adolescents' perceived support—and *OXTR* DNA methylation, specifically, and our study adds to the literature on gene-by-environment interactions with DNA methylation. It is also unique in looking at early adolescence, rather than early childhood, as a potentially important period for development in relation to DNA methylation.

The current study also extends previous research (e.g., MacDonald & MacDonald, 2010; Neumann & Landgraf, 2012; Slattery & Neumann, 2010) by showing that DNA methylation is linked to relative changes in psychosocial functioning over time, rather than just to contemporaneous assessments. This study is also the first to find that *OXTR* DNA methylation predicts self-reported stress in romantic relationships in early adulthood. This extends previous findings that *OXTR* DNA methylation predicts stress responses (Rodrigues et al., 2009; Taylor et al., 2006) and that it relates to negativity in romantic relationships (Ditzen et al., 2009). The finding of more internalizing symptoms and loneliness among young adults with more *OXTR* DNA methylation held even after accounting for depressive symptoms at age 13. The finding of more stress in romantic relationships among young adults with more *OXTR* DNA methylation

also held even after accounting for friendship competence at age 13. Accounting for baseline depressive symptoms in our analysis allowed for a measure of relative change in internalizing symptoms and worsening psychological functioning and accounting for baseline close friendship competence in our analysis was equivalent to a measure of worsening interpersonal functioning. This may indicate that DNA methylation observed in adulthood either was not present in early adolescence, or if it was, it did not yet have the relationships later observed.

These results provide compelling evidence to suggest that psychological and interpersonal functioning—thought to be influenced by the endogenous oxytocin system—may be governed by epigenetic processes and that this relationship may be predicted from tissue acquired through less invasive means. *OXTR* DNA methylation directly impacts gene expression such that increased levels of DNA methylation are associated with decreased gene transcription (Kusui et al., 2001). Therefore, lower levels of DNA methylation may increase ability to use available oxytocin, due to increased receptor expression. As predicted by this model, individuals in the current study with lower levels of *OXTR* DNA methylation showed higher levels of adaptive and positive psychosocial functioning than their counterparts with higher levels of *OXTR* DNA methylation.

A major advantage of our approach was that the reported epigenetic marker is a continuous variable directly relevant to gene function and capable of predicting individual variability without dichotomizing by allele groups. The design of this study also examined the endogenous OXTR system and its associations, rather than the associations with intranasally administered oxytocin. It is possible that variability that exists within groups when dichotomizing by allele

conceals the phenotypic variability related to the endogenous oxytocin system. Therefore, using *OXTR* DNA methylation as a putative predictor of endogenous oxytocin levels provides a biomarker now associated with psychological dysfunction and maladaptive interpersonal tendencies.

Several limitations to these findings warrant note. First, although this study addresses causal theories, its design only allows for potentially disconfirming, not directly supporting, such theories. Second, our measure of perceived support measures expected support, rather than perceived support during real situations. Although expected support in hypothetical situations presumably are based on previously received support, there is no way to be sure of this. Future research could test if there are differences in the relationship between perceived emotional vs. instrumental support received and *OXTR* DNA methylation. Future research could also investigate the relationship between support seeking and *OXTR* DNA methylation.

We do not yet know at what age *OXTR* DNA methylation occurs and stabilizes. Yet the tight linkage between adolescent experience and DNA methylation in animal models, together with findings that relative levels of methylation-linked pathologies emerged *after* adolescence in this sample, suggests adolescence as critical period during which methylation may be labile. Without definitive evidence, though, we can only hypothesize that the parental support precedes *OXTR* DNA methylation and that *OXTR* DNA methylation precedes our early adult outcomes.

Additionally, it is worth noting that the relationship between perceived support and *OXTR* DNA methylation was significant at site -924 but not at site -934. These two CpG sites are

correlated, and there is no evidence of a true functional difference between them. It is more likely that these two sites work together, although further research is warranted to investigate this. Finally, DNA methylation is highly tissue specific for some CpG sites (Ladd-Acosta et al., 2007), and when studying healthy humans, we are limited to assessments from peripheral tissue rather than the brain. However, recent studies have demonstrated that for some CpG sites, DNA methylation is correlated between tissues, and for CpG sites within *OXTR*, there is evidence for the maintenance of methylation levels between brain and blood (Walton et al., 2015), which suggests that our peripheral marker is likely to correlate with methylation levels in the brain. Despite these limitations, the current study provides insight both on how social support predicts epigenetic variability in the endogenous oxytocin system and how *OXTR* DNA methylation relates to psychological well-being and stress in relationships and is an important step to comprehend the relationships between environment, epigenetics, and psychosocial functioning.

Contribution of work: Coe-Odess wrote the manuscript and analyzed the data; Wroblewski designed and conducted the epigenetic portion of the research; Allen designed and contributed to the longitudinal investigation of adolescent social development in familial and peer contexts; and Connelly designed and contributed to the epigenetic portion of the research.

Figures

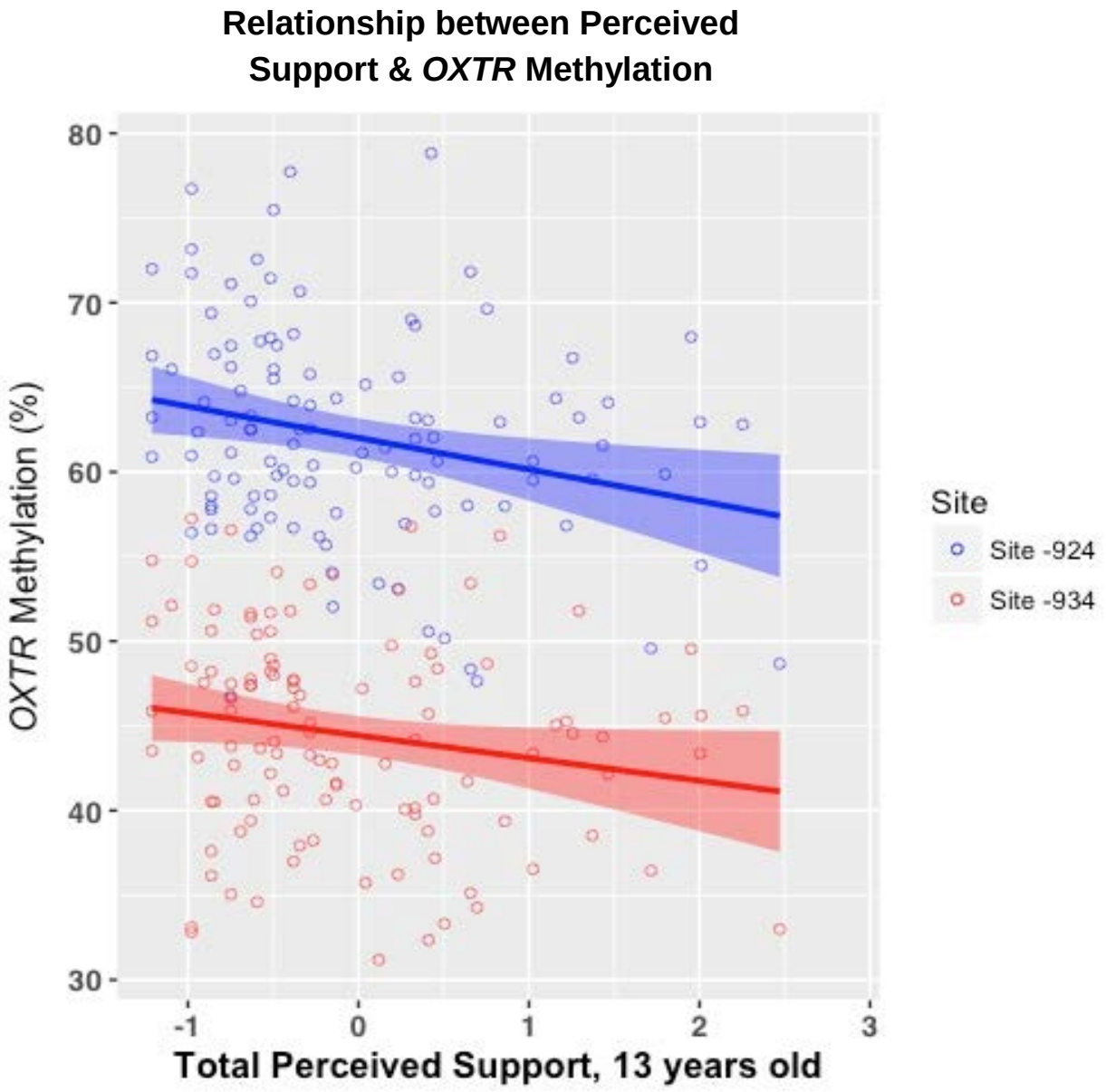

Figure 1. Total perceived support significantly negatively predicted *OXTR* DNA methylation at CpG site -924, $r(110) = -0.25$, $p < 0.01$. Total perceived support did not significantly negatively predicted *OXTR* DNA methylation at CpG site -934, $r(110) = -0.18$, $p = 0.052$.

Table 1

Intercorrelations Among Variables in the Primary Model

	M	*SD*	2	3	4	5	6	7
1. Total perceived support (Age 13)	0.00	0.85	0.06	0.14	-0.04	-0.25**	-0.13	-0.26***
2. Internalizing symptoms (Ages 24-25)	5.68	5.61	—	0.69***	0.47***	0.26**	0.05	0.13
3. Loneliness (Ages 24-25)	12.25	11.96		—	0.47***	0.17*	0.01	0.03
4. Total stress in romantic relationships (Ages 24-25)	5.89	2.00			—	0.23*	0.03	-0.04
5. *OXTR* DNA methylation at CpG site -924 (Ages 26-31)	62.09	6.39				—	0.03	0.09
6. Family income (age 13)	43,600	22,400					—	-0.11
7. Sex (male = 1, female = 2)	—	—						—

*p < .05. **p < .01. ***p < .001

Table 2 *Predicting OXTR DNA Methylation at Site -924 from Perceived Support in Early Adolescence (Age 13)*

OXTR DNA Methylation at Site -924 (Ages 26-31)			
Step and predictor	β	ΔR^2	Total R^2
Step 1			
Sex (1 = male, 2 = female)	-0.01		
Total family income (age 13)	0.03	0.01	0.01
Step 2			
Perceived maternal and peer support (age 13)	-0.24*	0.05	0.06

Note: The βs are from the final model.
*p = 0.019.

Table 3 *Predicting Internalizing Symptoms (Ages 24-25) From OXTR DNA Methylation at Site -924*

Internalizing Symptoms at Ages 24-25			
Step and predictor	β	ΔR^2	Total R^2
Step 1			
Sex (1 = male, 2 = female)	0.16		
Total family income (age 13)	0.08	0.02	0.02
Step 2			
Depressive symptoms (age 13)	0.25*	0.01	0.05
Step 3			
OXTR DNA Methylation at site -924 (ages 26-31)	0.29**	0.10	0.15

Note: The βs are from the final model.
*p = 0.010 **p = 0.003.

Table 4 *Predicting Loneliness (Ages 24-25) From OXTR DNA Methylation at Site -924*

	Loneliness at Ages 24-25		
Step and predictor	β	ΔR^2	Total R^2
Step 1			
Sex (1 = male, 2 = female)	-0.04		
Total family income (age 13)	0.06	0.00	0.00
Step 2			
Depression (age 13)	0.23*	0.03	0.03
Step 3			
OXTR DNA Methylation at site -924 (ages 26-31)	0.20**	0.03	0.06

Note: The βs are from the final model.
*p = 0.018. **=0.040.

Table 5
Predicting Stress in Romantic Relationships (Ages 24-25) From OXTR DNA Methylation at Site -924

	Stress in Romantic Relationships (Ages 24-25)		
Step and predictor	β	ΔR^2	Total R^2
Step 1			
Sex (1 = male, 2 = female)	0.07		
Total family income (age 13)	-0.05	0.00	0.00
Step 2			
Close friendship competence (age 13)	0.01	0.00	0.00
Step 3			
OXTR DNA Methylation at site -924 (ages 26-31)	0.25*	0.07	0.07

Note: The βs are from the final model.
*p= 0.012.

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Conclusion

This dissertation all began with a question, “What are the biological mechanisms driving social behavior?” and we had the goal of providing additional studies to help our understanding of this question. In observing the world around us, people noticed that there are differences in species typical social behaviors that varies between different types of animals. Yet, it is not just the differences in social behavior between different species, there are differences in social behavior within the same species. For example in humans, some people display lots of extroverted social behavior, whereas other people are more introverted. Interestingly, these differences of social behavior are not binary, but instead fall on a spectrum. This indicates that the biological mechanisms of social behavior are nuanced and can be fined tuned.

In investigating human social behavior, we are limited in the type of mechanistic studies that can be conducted, and scientists have turned to animal models to further our understanding of the biological mechanisms of social behavior. Prairie voles are a strong choice in an animal model of social behavior since they share many of human archetypes of social behavior, such as being socially monogamous, participating in biparental and alloparental care, and being highly affiliative. Importantly, many of these social traits are rare to find in other mammals, including commonly chosen animal models such as rats and mice. The shared social traits between humans and prairie voles are suggestive of a similar biological system shared between the two species, indicating that experimental manipulations that alter prairie vole social behavior may also influence human social behavior.

As scientists researched the mechanisms influencing social behavior, an overwhelming amount of evidence indicated that the oxytocin system plays a critical role in a variety of social perceptual and behavioral processes. Studies comparing the amount of oxytocin receptor present in regions of the brain associated with social behavior provided strong evidence that the amount of oxytocin receptors influenced the amount of displayed social behavior both between different species and within the same species. The association between differences in social behavior and oxytocin receptor density lead scientists to investigate the role of epigenetic regulation of the oxytocin receptor gene that could alter the amount of receptor produced and therefore, alter social behavior. Accordingly, if we want to gain a better understanding of the biological mechanisms of social behavior, we need to further investigate the epigenetic regulation of the oxytocin receptor gene.

In this dissertation, the epigenetic mark of interested is DNA methylation. I explored the epigenetic landscape of the conserved MT2 and exonic region in prairie voles and investigated how parental care corresponds with changes in DNA methylation across CpG sites at these regions. A pivotal *in vitro* study conducted by Kusui and colleagues provided evidence that DNA methylation in the MT2 region primarily regulated *OXTR* expression, but the select CpG sites within this region driving this response remained unknown. Additionally, in human research, DNA methylation in the MT2 and exonic region is associated with dysregulation of human social behavior and social perception. Information from these studies helped guide my hypothesis that DNA methylation in the conversed MT2 and exonic region in prairie voles may be associated with differences in oxytocin receptor gene expression. In both human and prairie vole brain tissue, I measured levels of DNA methylation across the entire MT2 region. The MT2 region

lays within a CpG island upstream to the transcription start site within the promoter region of the oxytocin receptor gene, typically these types of regions have low levels of DNA methylation, but when methylation does occur it tends to greatly impact gene expression. We determined that most CpG sites within the MT2 region in both humans and in prairie voles have low levels of DNA methylation, although there is a cluster of CpG sites that 1) have higher levels of DNA methylation, 2) are more variable in the level of DNA methylation between individuals, and 3) are conserved between humans and prairie voles. These CpG sites are -934, -924, and -901 in humans and -934_1, -934_2, -924, and _901 in prairie voles. I provide evidence that when DNA methylation occurs at these sites it corresponds with decreases in oxytocin receptor gene expression in the brain tissue of both humans and prairie voles. This evidence indicates that CpG sites -934, -924, and -901 may be more epigenetically sensitive to environmental factors and when methylation does occur at these sites, it corresponds to decreases in oxytocin receptor gene expression in both humans and the prairie voles.

An additional genomic region of interest was a segment within an exon. Previous studies in humans provide evidence that DNA methylation in this region corresponds with social dysfunction. Within a mouse model, DNA methylation in this region is associated with a decrease of *Oxtr* expression and is implicated in a decrease of maternal behavior and an increase of aggressive and anxiety behavior. To the best of our knowledge, I am the first to provide information of the epigenetic landscape of this region in our animal model, the prairie vole. I determined that there are higher levels of DNA methylation in this region, which is expected in exonic regions, and that there are 4 distinct clusters of correlated DNA methylation patterns. DNA methylation in select sites in the exonic region corresponded to differences in *Oxtr*

expression, CpG sites 2, 4, 20, 22, 23, 24, 26, 31, 32 and 36. Notably, the majority of CpG sites that when methylated, corresponded to decreases in gene expression are within cluster 3, suggesting that this region may be more sensitive to environmental factors associated with epigenetic changes and when methylated may affect *Oxtr* expression. Additionally, we determined that within our population of animals, there are two polymorphic CpG sites and one of these polymorphic sites (CpG 25) might be associated with differences in *Oxtr,* although this finding should be explored further with a larger sample size.

Epigenetic regulation of *OXTR* in humans is associated with several psychiatric disorders and social behaviors, yet the drivers of individual variability in the expression of this gene are unknown. Using the prairie vole as a model, I provided evidence that early life parental care is an environmental factor that corresponds with differences in DNA methylation and *Oxtr* expression. Previous work indicates that the amount of parental care received can alter the developmental trajectory of social behavior, although the mechanisms through which this occurs remained unknown. Therefore, to further investigate the biological mechanisms of social behavior, we used a handling paradigm that resulted in increased parental pup directed behavior and investigated the epigenetic effects of this behavior in offspring. While the handling paradigm was used in previous research studies, the length of time in which there was an increase of parental pup directed behavior remained unknown. The work in this dissertation expanded on our understanding of this handling paradigm by quantifying the amount of parental pup directed behavior at several time points. We determined that briefly handling the parents on the day their pups were born increased parental pup directed behavior on PND 1 through PND 7, compared to the control group that received no handling manipulation. However, by PND 8 there was no

significant difference in parental pup directed behavior and this pattern continued by PND 15. Therefore, we concluded that our handling manipulation results in a transient increase of parental care within the first week of their offspring's lives.

I provide evidence that prairie vole offspring which receive more parental care in early life corresponds to increases in *Oxtr* expression and overall lower levels of DNA methylation across the conserved MT2 and exonic region in the nucleus accumbens at PND 24, in comparison to offspring in our control group. Notability, only CpG sites 14, -934_1, -934_2, -924, and -901 within the MT2 region survived multiple comparison correction, indicating that these sites are more epigenetically sensitive to early life parental care. When I correlated DNA methylation of CpG sites 14, -934_1, -934_2, -924, and -901 within the MT2 to *Oxtr* expression, only sites -934_2, -924, and -901 resulted in significant or strongly trending correlations following multiple comparison corrections, with increase of DNA methylation corresponding to decreases in *Oxtr* expression. I provide evidence that out of the 24 CpG sites in the conserved MT2 region and the 42 CpG sites in conserved exonic region, only CpG sites -934_2, -924, and -901 are especially epigenetically sensitive to early life environment, and when methylated, are associated with decreases of *Oxtr* expression. These findings imply that in scientific research we can measure DNA methylation at the homologous CpG sites -934, -924, and -901 to gain a broader understanding of the endogenous oxytocin system.

In an additional experiment with a separate cohort, we performed the same parental handling manipulation to induce transient increases of parental care and, using autoradiography to quantify oxytocin receptor density, we determined that offspring which received more

maternal care early in life had more oxytocin receptors in the nucleus accumbens in young adulthood. Additionally, previous research established that offspring raised by high care parents develop into animals that spend more time socially investigating a novel animal, display more alloparental care to a novel pub, as well as display more pair-bonding behavior and more parental behavior when they produce their own offspring. Collectively, the research conducted in our lab and with our collaborators indicate that offspring receiving more parental care within the first week of life is associated with a decrease of DNA methylation across the conserved MT2 and exonic region in the NAcc, an increase of *Oxtr* expression in the NAcc, an increase of oxytocin receptor density in the NAcc, and an increase of species typical display of social behavior that extended in adulthood.

Additionally, our research provides evidence that just a select few CpG sites of the ones measured are particularly epigenetically sensitive to early life parental care and strongly correspond to *Oxtr* expression (CpG sites -934_2, -924, and -901). Strikingly, these CpG sites are homologous between prairie voles and humans and also display similar mechanistic characteristics. When we measured the levels of DNA methylation across the MT2 region in human brain tissue, the CpG sites -934, -924, and -901, when methylated significantly, corresponded with decreases of *OXTR* expression. This suggests that the environmental factors altering DNA methylation and oxytocin receptor gene expression may have similar effects in humans and our animal of model of social behavior, the prairie vole.

Accessing brain tissue in humans is challenging, and we wanted to explore if we could use a more accessible tissue to gather similar epigenetic information in the oxytocin receptor

gene. We turned to our animal model and measured DNA methylation in the homologous CpG sites -934_1, -934_2, -924, and -901 in both brain (NAcc) and whole blood tissue. We discovered that the levels of DNA methylation of these sites were highly correlated between brain and blood tissue, indicating that we can measure DNA methylation at the homologous CpG sites -934, -924, and -901 in peripheral blood tissue and can serve as a proxy for brain tissue in humans.

Finally, in the last section of this dissertation, we move from our animal model to humans, and examine the relationship of perceived social support, epigenetic marks, and emotional functions associated with social behavior. In young adolescences (age 13) we recorded the perceived maternal support, and examined the epigenetic and social developmental trajectories in adulthood within the same population of participants. We discovered that people who perceived receiving lower amounts of maternal care had higher levels of DNA methylation at CpG sites -934 and -924 in the MT2 region of *OXTR*. This was measured in whole blood tissue, and based on our findings in prairie voles, we infer that the levels of DNA methylation in whole blood corresponds to levels of DNA methylation of brain tissue. Evidence in our animal model also supports the hypothesis that people with higher levels of DNA methylation at CpG sites -934 and -924 would also have lower levels of oxytocin receptor density in regions of the brain associated with social behavior, indicating that the actions of endogenous oxytocin on social behavior would be restricted in people who have less receptors. Our findings of emotional functions associated social behavior in this study support this hypothesis, since people who perceived having less maternal care not only developed as adults with higher amounts of DNA methylation at these key CpG sites, they also developed as adults more likely to perceive having

feelings of loneliness, depression, anxiety, romantic stress, withdrawn symptoms, fatigue, and somatic pain.

In this dissertation we provided evidence in both humans and prairie voles that early life parental care can influence the biological mechanisms of social behavior through the epigenetic regulation of oxytocin receptor gene. We showed that homologous CpG sites -934, -924, and -901 in the MT2 region are epigenetically sensitive to early life environment, and that methylation at these sites strongly corresponds with decreases in oxytocin receptor gene expression in both human and prairie voles. Additionally, we provided evidence that the levels of DNA methylation of homologous sites -934, -924, and -901 in a brain region associated with social behavior (NAcc) corresponds to levels of DNA methylation in these sites in whole blood tissue. This indicates that we can use blood tissue as a proxy of DNA methylation levels within brain tissue at the CpG sites which are primarily regulating oxytocin receptor. Since brain tissue is difficult to obtain in humans, using whole blood as a proxy for brain DNA methylation levels opens up many areas of scientific inquiry in the study of social behaviors. There is a growing body of evidence indicating that the amount of care received in early life can epigenetically fine-tune systems associated with social behavior and stress, which in turn, can alter the developmental trajectory of social behavior and mental health in later development. As we gain a better understanding of how the environment can induce epigenetic changes in humans, we may be able to better design behavioral strategies and develop more targeted effective interventions to help improve quality of life.