A Molecular Mechanism of *Oxtr* Gene Regulation Through a Single CpG Site

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Abstract

The parent-offspring dyad is critical in preparing offspring for their future environment (Yoshihara et al 2018; Esposito et al, 2019). This relationship encompasses a variety of actions including proximity of parent to offspring, engagement, and thermoregulation (Mateo, 2014). Variation in early parental care can modulate offspring developmental outcomes through epigenetic tuning of genes involved in social behavior (Perkeybile et al, 2019; Krol et al, 2019). The oxytocin signaling pathway has been extensively studied in relation to its effect on social behavior through epigenetic changes of the oxytocin receptor gene (*OXTR*), acquired through variable parental care. However, the mechanism of change in expression has yet to be fully deduced. This study seeks to provide functional evidence that environmentally induced DNA methylation of key CpG sites within *Oxtr* results in a transcriptional response in the monogamous prairie vole. We provide preliminary evidence that by creating a germline knockout of a single key CpG site from a CpG rich region (MT2) within *Oxtr*, the epigenetic state of the gene is altered and results in a change in *Oxtr* RNA expression.

Introduction

Oxytocin-Oxytocin Receptor

The oxytocin signaling pathway modulates numerous physiological functions including uterine contractions, milk letdown, and social behavior (Gimpl, & Fahrenholz, 2001). While many of this pathways' functions are activated through changes in endocrine, proinflammatory, and immune responses, the activation and effect this pathway has on social behaviors on a longitudinal scale remain unknown (Wirobski et al, 2021; Carter et al, 2020). Oxytocin is dependent on binding to its receptor, OXTR, to conduct intracellular signaling in several regions of the brain and periphery (Jurek & Neumann, 2018). OXTR, a G-protein coupled receptor, is encoded by its gene, *OXTR*. In both the human and prairie vole (*Microtus ochrogaster*), *Oxtr* contains four exons, two of which are translated to form the protein (Danoff et al, 2021). In 2001, Kusui and colleagues profiled the promoter region of *OXTR*, which begins approximately 2860 base pairs (bp) upstream of the transcription start site (TSS) and extends through the end of exon 1 and start of intron 1 to approximately 1342 bp downstream of TSS. Kusui defined a CpG rich region within the promoter, denoted as the MT2 region, to be a key regulatory region in DNA methylation dependent regulation of *OXTR* transcription (Kusui et al, 2001). Using the prairie vole as an animal model, our lab has provided further evidence for the role of the MT2 region in methylation-dependent regulation of *Oxtr* (Gregory et al, 2009; Perkeybile et al, 2019; Danoff et al, 2019). Specifically, we have shown that environmental factors, such as parental care during early development, impact *de novo* methylation on CpG sites within MT2 to drive a change in *Oxtr* RNA expression (Perkeybile et al, 2019).

Use of the prairie vole as an animal model

Our lab focuses on the socially monogamous prairie vole as an animal model to study the effects of environmental factors on *Oxtr* DNA methylation and resulting RNA expression. While previous research has used the mouse or rat model to study *Oxtr* regulation and expression (Pisansky et al. 2017; Ayers et al. 2016; Beery et al, 2016), we use the prairie vole as our translational model due to their similarity to humans in oxytocin-dependent social constructs. An example of this is their parental care, wherein both parents rear the litter, taking cooperative responsibility to huddle, groom, nurse, etc. until weaning (Tabbaa et al, 2016). In addition to this, prairie voles pair bond as adults, a highly social behavior not typically found in other rodents (Aragona & Wang, 2004). Even more, the *Oxtr* sequence is highly conserved in prairie vole when compared to the human *OXTR* sequence, specifically the MT2 region in which CpG sites (-934_2, -934_1, -924) are biologically conserved (Danoff et al, 2021).This is not the case in other rodent models such as mice and rats.

Effects of environmental factors on *Oxtr* DNA methylation

Our lab has studied the effects of early life experience, specifically, parenting, on DNA methylation of *Oxtr* in the socially monogamous prairie vole. Our results found that CpG sites within the MT2 region of *Oxtr* are protected from *de novo* methylation as a result of exposure to high care parenting early in life (Perkeybile et al. 2019). This reduction of *de novo* DNA methylation within this regulatory region, when compared to offspring that received lower levels of parental care, has a direct correlation with an increase in *Oxtr* RNA expression. This finding suggests that CpG sites within MT2 may impact *Oxtr* RNA expression through epigenetic state change. In addition to this, our lab has identified that

an increase in DNA methylation within MT2 is negatively associated with *Oxtr* RNA expression (Perkeybile et al, 2019). Within MT2, there are a subset of CpG sites; -924, and -934_1 and -934_2 that are more sensitive to epigenetic change from environmental factors when compared to 20 other sites within MT2 (Danoff et al, 2021). This finding suggests that these three CpG sites, which are homologous to CpG sites within MT2 of *OXTR* in humans, are most related to *Oxtr* expression and potentially contribute to the largest portion of methylation-dependent regulation on *Oxtr*. Furthermore, in humans DNA methylation of these sites are correlated with DNA methylation in peripheral tissue in humans, including saliva, blood, and PBMCs (Krol et al, 2019), suggesting a global functional role in *OXTR* regulation.

Here, we show that by genetically removing CpG site -934_2 of *Oxtr* in the prairie vole using CRISPR-Cas9 gene editing techniques, we have altered the epigenetic state of the MT2 region, evidenced by the hypermethylation of adjacent CpG sites. This change in epigenetic state results in a reduction in *Oxtr* RNA expression. These findings provide further evidence for the importance of DNA methylation in regulating the expression of *Oxtr* early in life.

Methods

Animal Model

The prairie vole (*Microtus ochrogaster*) was used as the animal model for this study. Animals used in both DNA methylation and RNA expression analysis were descended from the offspring of the iGONAD surgical technique outlined below. Heterozygote breeding pairs (n = 8) were housed in polycarbonate hamster cages and given food and water ad libitum. Two cotton nestlets were provided for enrichment and nesting material. Subjects in this study were euthanized within 24hours postnatally (P0) or 7 days post-birth (P7) via isoflurane anesthesia followed by cervical dislocation. Brains were collected and flash frozen on dry ice before being transferred to a -80℃ freezer for storage.

iGONAD Surgical Technique

The iGONAD surgical technique was previously described in the mouse model (Takahashi et al, 2015). A guide RNA (gRNA) sequence targeted to the site of interest (-934_2) was designed using CRISPOR.net. CRISPR-Cas9 reagents were designed and sourced from Integrated DNA technologies (IDT, Coralville, IA). To induce estrus, the virgin female prairie vole was paired with a male for 24 hours before a cage divider was placed between them for a further 48-72 hours. The divider was removed and the breeding pair was monitored until copulation occurred. Surgery was conducted approximately 14-16 hours post-copulation in order to modify the single cell zygote. The pregnant female was induced into anesthesia using 3-4% isoflurane and maintained at approximately 3% isoflurane throughout the surgery. Ketoprofen (25mg/kg) was administered as an anti-inflammatory to treat any subsequent pain. The animal was

placed on a warming mat set at 37℃ to maintain thermal stability. Hair was removed from the dorsal skin and cleaned using a combination of betadine and saline. A two centimeter incision was made on the midline and the abdominal wall was cut. The ovary-oviduct was located and then exposed, clamped in place on a sterile Kimwipe using an aortic clamp. CRISPR-Cas9 reagents (1.5µl) were collected using a glass pipette and administered to the infundibulum of the oviduct. The entire oviduct was electroporated at 50V for 8 pulses with a 1-second interim delay, as previously described (Gurumurthy et al, 2019; Ohtsuka et al, 2018). This process was repeated on the opposing side before the animal was sutured closed and monitored for 7 days for post-anesthesia recovery. The animal was weighed at 14 days post-surgery to determine pregnancy. Tissue derived from the offspring of the surgery was taken to identify any mutations created and genotyped via Sanger sequencing. One male, denoted CR101B, was identified with the Δ-934_2 mutation and crossed to a wildtype female. The offspring were genotyped to confirm germline transmission of the putative mutation and used to establish the heterozygote breeding colony.

Tissue Collection, DNA & RNA extraction

Brain tissue was collected at postnatal day 0 (P0) or 7 (P7). Subjects were anesthetized using 5% isoflurane followed by cervical dislocation. Brains were extracted, flash frozen on dry ice, and transferred to a -80C freezer for storage until processing. Tissue was gross dissected using a -20℃ Cryostat to isolate the forebrain. DNA and RNA extraction was conducted using Qiagen AllPrep DNA/RNA Mini Kit (Qiagen, Valencia, CA) following manufacturer instructions.

DNA methylation and Pyrosequencing

Extracted DNA (10ng/µl) underwent bisulfite conversion using Invitrogen Methyl Code Bisulfite Conversion Kit (MECOV50, Invitrogen, Carlsbad, CA). Following column isolation of bisulfite-converted DNA, the samples were used as a template for PCR. PCR was conducted using Qiagen Pyromark kit (Valencia,CA) under conditions previously described by our lab (Danoff et al. 2021). Samples were run in triplicate on three separate machines to account for variability. Three DNA controls were used for each PCR plate: 0% methylated DNA, 100% methylated DNA, and a negative control. Agarose (1.5%) gel electrophoresis confirmed a single PCR product at the predicted molecular weight (375bp). Pyrosequencing was conducted on the Qiagen Pyromark Q24 machine and performed per manufacturer's instructions. Epigenotypes of three CpG sites, -934_1, -934_2, and -924 are reported as an average over the individual triplicate reactions. Allele Specific Methylation was determined using the same processes as above. The sequencing primers used for the WT allele were; 5' – TTTTTGGTTTTAGAAATAGTTTAGGGT – 3' and for the Mut4 allele; 5' – TTTTTGGTTTTAGAAATAAGGGT – 3'.

Real time quantitative PCR

Extracted RNA (33.33ng/µl) underwent a reverse transcriptase reaction to obtain cDNA using BioRad iSCRIPT cDNA synthesis kit (Hercules, CA). Real-time quantitative PCR (RT-qPCR) was performed on the CFX96 System (BioRAD) using SYBR Green (Applied Biosystems). RT-qPCR was conducted on all samples for both *Oxtr* and *Gapdh*.

Reactions were run in triplicate with the mean and standard deviation calculated. *Gapdh* was used as a normalizing gene based on previous literature showing its stability across development in the mouse brain.

Quantification of Parental Care

Heterozygote breeding pairs were observed for pup directed behavior during P0-P3 for three consecutive litters, as previously described (Perkeybile et al. 2013). The breeders were monitored for four maternal and paternal observation sessions per litter at 20 minutes per session. Total contact times for pup-directed behavior including licking and grooming, nursing (active versus passive), huddling etc. were recorded. The behaviors recorded were determined based on findings from Stone and Bales (Stone and Bales, 2010), and recorded using the software behaviortracker.com. To rank breeding pairs into categories of high, medium, or low care parental groups, thresholds of pup-directed behavior were taken as previously described by our lab (Perkeybile et al. 2013). The total time of pup-directed contact was summed across all observations individually for mother and father. The average pup-directed behavior was calculated for each individual parent and for the breeding pair as a dyad. The scores were then ranked, with the bottom quartile breeding pairs being defined as low care versus the upper quartile defined as high care.

Results

Confirmation of removal of CpG site -934_2

Δ-934_2 pups were generated using iGONAD surgical technique. iGONAD utilizes the CRISPR-Cas9 system by injecting CRISPR reagents, including a Cas9 nickase and gene specific gRNA, into the oviduct of a pregnant female vole, 14 hours post-copulation. The entire oviduct was electroporated to allow the reagents access the single cell zygotes, removing the need for microinjections and a recipient female prairie vole. Confirmation of the existence of mutation in the offspring was confirmed via DNA isolation followed by Sanger sequencing. The Δ-934_2 mutation was characterized by the loss of the guanine in the CpG site -934_2, no longer allowing the cytosine to be methylated. Along with the removal of the guanine, three additional nucleotides were deleted **(Fig 1)**. DNA methylation analysis was conducted on F2-generated heterozygotes and homozygotes, and their wildtype siblings as control.

Hypermethylation of adjacent sites to -934_2

DNA methylation of CpG sites -934_1, -934_2, and -924 of offspring derived from Δ-934_2 /WT heterozygous breeding pairs was conducted. Offspring displayed three different genotypes: WT/WT, WT/Δ-934_2, and Δ-934_2/Δ-934_2 . At site -934_2, average DNA methylation of WT/WT offspring (n = 11) was approximately 8%, a value typically seen in our wildtype colony **(Fig 2A)**. In contrast, Δ-934_2 homozygotes (n = 3) demonstrated approximately 1% methylation at -934_2, the 1% taken as negligible machine background. In the Δ-934_2 heterozygote (n = 12), DNA methylation averaged to approximately 4%, an unsurprising finding given the halving of alleles containing CpG

site -934_2. At sites adjacent to CpG site -934_2, we find that at both -934_1 and -924 hypermethylation occurs in both the Δ-934_2 heterozygote and Δ-934_2 homozygote when compared to the wildtype siblings **(Fig 2B & C).**

Hypermethylation of Adjacent CpG sites occurs on the mutant allele

Based on our finding of hypermethylation of adjacent sites to -934_2 in both the Δ-934_2 homozygote and Δ-934_2 heterozygote, we next sought to determine if this hypermethylation was occurring in a bi-allelic fashion or if it was allele-specific. Pyrosequencing primers that would specifically target the WT or Δ-934_2 allele were designed. Using this method, we were able to decipher the hypermethylated allele in the Δ-934_2 heterozygote. Our findings provided preliminary evidence that hypermethylation occurs on the mutant allele with the wildtype allele retaining the typical epigenetic state found in wildtype siblings **(Fig 3).**

***Oxtr* RNA expression is reduced in mutant lines**

Our methylation findings suggest a shift in epigenetic state within the MT2 region of *Oxtr*. Based on this, we sought to determine if this shift results in a change in the RNA expression levels of *Oxtr* in the WT/WT homozygotes, Δ-934_2 heterozygotes, and Δ-934_2 homozygotes. In figure 3, we show *Oxtr* RNA expression by genotype, providing preliminary evidence that Oxtr expression is reduced in lines containing the Δ-934_2 allele (WT/Δ-934_2, Δ-934_2 /Δ-934_2) **(Fig 4).**

Parental Care across the colony is variable but stable

Previously, our lab has shown that variability in parental care received by offspring can impact DNA methylation within the MT2 region of *Oxtr*. As all offspring used in this study are derived from parents that are heterozygous for the Δ-934_2 allele, we sought to determine if parental care is the cause for hypermethylation/reduced expression rather than the mutation itself. We quantified parental care across the colony and find that parental care is normally distributed into high, medium, and low care parents indicating no effect of the heterozygous mutation on parenting behavior **(Fig 5).**

Discussion

Oxtr is a critical regulator of social behaviors in monogamous prairie vole. This study provides further evidence of the importance of DNA methylation in regulating the epigenetic state of *Oxtr* and its expression. We show that by removing one of the key CpG sites from the MT2 region, local hypermethylation occurs. This hypermethylation is specific to the mutant allele and in impacts the level of *Oxtr* RNA expression such that mutants display reduced expression when compared to their wildtype counterparts.

Evidence has shown the interplay between the environment and the epigenetic regulation of *Oxtr*. In the prairie vole, we have shown that parental care impacts *de novo* methylation of key CpG sites within MT2 (-934_1, -934_2, -924) and results in a change in *Oxtr* RNA expression (Perkeybile et al, 2019) such that higher parental care leads to lower methylation and higher expression. Similarily, in humans, our lab has shown that maternal engagement early in life impacts DNA methylation levels within the MT2 region of *OXTR* (Krol et al, 2019) such that high engagement is associated with decreased methylation

across time.These studies underscore the sensitivity of *Oxtr* to environmental factors through DNA methylation and resulting RNA expression. Interestingly, researchers have proposed manipulation of oxytocin levels as treatment for those with neuropsychological disorders, including ASD, obsessive compulsive disorder, and borderline personality disorder (Yamasue et al, 2020). Intranasal oxytocin administration initially provided preliminary hope of mediating symptoms associated with these disorders (Huang et al, 2020), however, upon further discovery, adverse effects on the expression of *OXTR* have been identified with this type of treatment. This provides unsurprising evidence of the complexity of the oxytocin system and suggests the need to further characterize the impact of environmentally induced variability on this pathway, both on an organismal and cellular level. This study provides preliminary data on the importance of understanding the regulation of *Oxtr* on a genetic scale, as disruption can lead to complex epigenetic effects.

As detailed, the regulation of *Oxtr* is critical in multiple pathways, including pair bonding, maternal care of offspring, and social behaviors. Previous research has looked to understand the functional mechanism of this pathway by genetically deleting *Oxtr* in the hopes of observing a clear phenotypic effect (Pobbe et al, 2011). Recently, Manoli and colleagues created a germline knockout of *Oxtr* in the prairie vole and assessed downstream phenotypic changes including: changes to individual paternal and materal care of offspring, the ability to form a strong pair bond, and the ability to keep the pups alive to weaning age. In this model, no significant changes to behavior were observed, suggesting that *Oxtr* is not required for this set of behaviors (Berendzen et al, 2022). This

study is one of many that have found variable results from knocking out *Oxtr*. In the mouse, a study found that Oxtr knockout dams were more likely to abandon their pups after birth (Rich et al, 2014), another showed that germline versus conditional knockout of *Oxtr* resulted in an increase in male aggression (Dhakar et al, 2012). Given this, it is crucial to understand the mechanism by which this gene is regulated, both on a genetic and epigenetic level, before we can truly understand its impact on social processes. Rather than genetically removing a gene, we have induced a small subtle knockout of a CpG site that has shown to regulate *Oxtr* expression based on environmental factors through changes to DNA methylation. This process can provide information on the influence epigenetic change within this region has on social behaviors and may provide a more attuned outlook on the oxytocin signaling pathway's role in social behaviors.

Caveats and Future Directions

While this study shows evidence that removal of CpG site -934_2 results in a molecular change in the state of *Oxtr* and an early impact on RNA expression, we have not yet shown a behavioral consequence of this mutation. In addition to this, further quantification of the environment the subjects are being exposed to prior to tissue collection and analysis is necessary. To expand this study from molecular to behavioral evidence, we will conduct the following experiments:

1. *Establish that deletion of Oxtr CpG site -934_2 blunts environmentally induced change in Oxtr gene expression and leads to changes in juvenile behavior.* Given our evidence of hypermethylation and reduced RNA expression in both Δ-934_2 homozygotes and Δ-934_2 heterozygotes early in life, we hypothesize that regardless of the parental care received by the mutant lines (high care versus low care) the RNA expression will remain

lower when compared to wildtype siblings, suggesting that we have created a mutant vole line that is no longer pliable to environmental influence on DNA methylation change. To test this, we will conduct a cross-fostering experiment. Cross-fostering will occur by first characterization of a natural high care parental group and low care parental group. P0 pups (WT/WT, WT/Δ-934_2, Δ-934_2 /Δ-934_2 siblings) will be taken from the surrogate parents and placed in the cage with either high or low parental care parents. Baseline DNA methylation and RNA expression of *Oxtr* will be quantified by taking a P0 pup prior to receiving care. DNA and RNA will be isolated from P20 pups and quantified for changes in *Oxtr* RNA expression and percentage methylation given their genotype and level of care received from the foster parents.

2. *Establish the behavioral consequences of CpG site -934_2 removal*. To determine if the epigenetic and RNA expression changes we are observing early in life persist through to adulthood and result in a phenotypic change with regard to social behavior, we will conduct a series of behavioral analyses on P24 to P60 animals. These analyses will include alloparental testing at P24, in which the test animals will be exposed to a P0 stranger pup. Typically, the juvenile will conduct behaviors emulative of parental behavior including licking, grooming, and huddling. These behaviors will be recorded to determine the level of the juvenile's alloparental abilities. We hypothesize that the Δ-934_2 homozygotes will show reduced pro-social behaviors towards the novel pup due to the decrease in *Oxtr* RNA expression. At P60, we will conduct partner preference tests across all three genotypes. This test involves partnering the test animal with a novel opposing sex animal for approximately 24 hours to initiate a pair bond. After this time the partner and a stranger animal will be tethered into separate cages with access by the test animal

to each cage through a tunnel. Typically, the test animal will spend a short period of time investigating both cages before huddling next to the partner animal. If we have disrupted the homeostatic epigenetic state of *Oxtr* by removing CpG site -934_2 we hypothesize a shift in the ability to form and maintain pair bonds.

3. Examine the changes in chromatin structure of mutant lines. Our preliminary results suggest a shift of the DNA methylation state of the MT2 region within *Oxtr*. We have yet to determine if this shift is occurring locally or if it persists throughout the gene and has subsequent effects on the chromatin landscape of the gene. To investigate this, we will analyze DNA methylation within Exon 3 of *Oxtr,* which has previously shown to be correlated with methylation within the MT2 region. In addition to this, we will conduct a Chromatin Immunoprecipitation (ChIP) assay along *Oxtr,* to investigate if there is any change to active/repressive chromatin marks in the Δ-934_2 homozygotes compared to their WT/WT siblings. Should our RNA expression results persist through to adulthood, we would hypothesize a shift in chromatin structure to a more inactive state in the mutant lines compared to the wildtype.

This study demostrates the importance of key CpG sites in the methylation dependent regulation of *Oxtr*. Furthermore, we have shown that by removing the ability to methylate single CpG site within the MT2 region (-934_2), we can shift the local epigenetic state to a hypermethylated state which results in reduced expression. Our goal moving forward is to determine if this shift in the epigenetic state of *Oxtr* persists through to adulthood, resulting in a phenotype such as decreased social behavior and reduced parental care of offspring. In addition, we seek to determine if this mutant line can be rescued by

environmental factors such as exposure to high parental care and propose that the removal of site -934_2 may create a mutant line that is no longer pliable to environmental factors.

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Figures

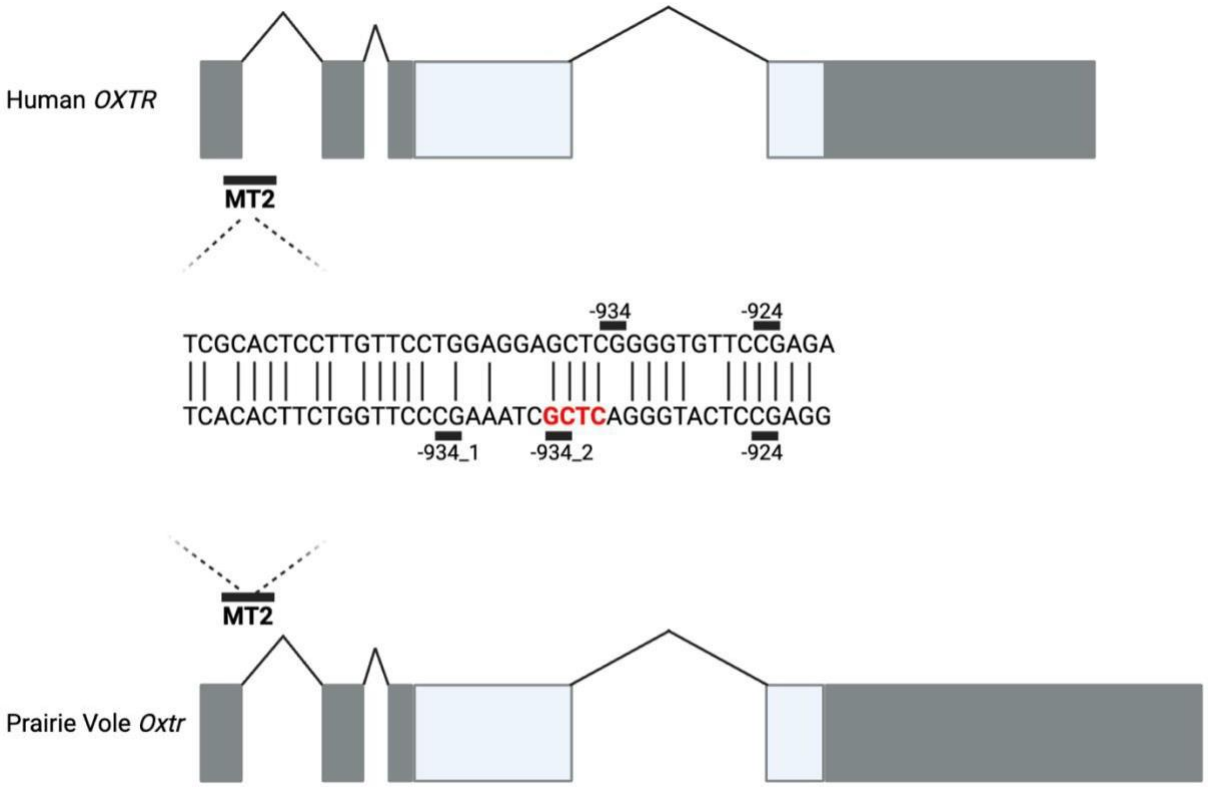

Figure 1. Gene Schematic of Human *OXTR* and Prairie Vole *Oxtr*. Exons are denoted in boxes; grey boxes are untranslated, blue boxes are translated to the protein. Introns are marked by black solid lines. The MT2 region is located at the 3' end of Exon 1 and persists into Intron 1. Alignment of conserved human and prairie vole sequence from the MT2 region is shown using a vertical line. The CpG sites of interest are marked and the sequence removed using the CRISPR-Cas9 reagents are highlighted in red.

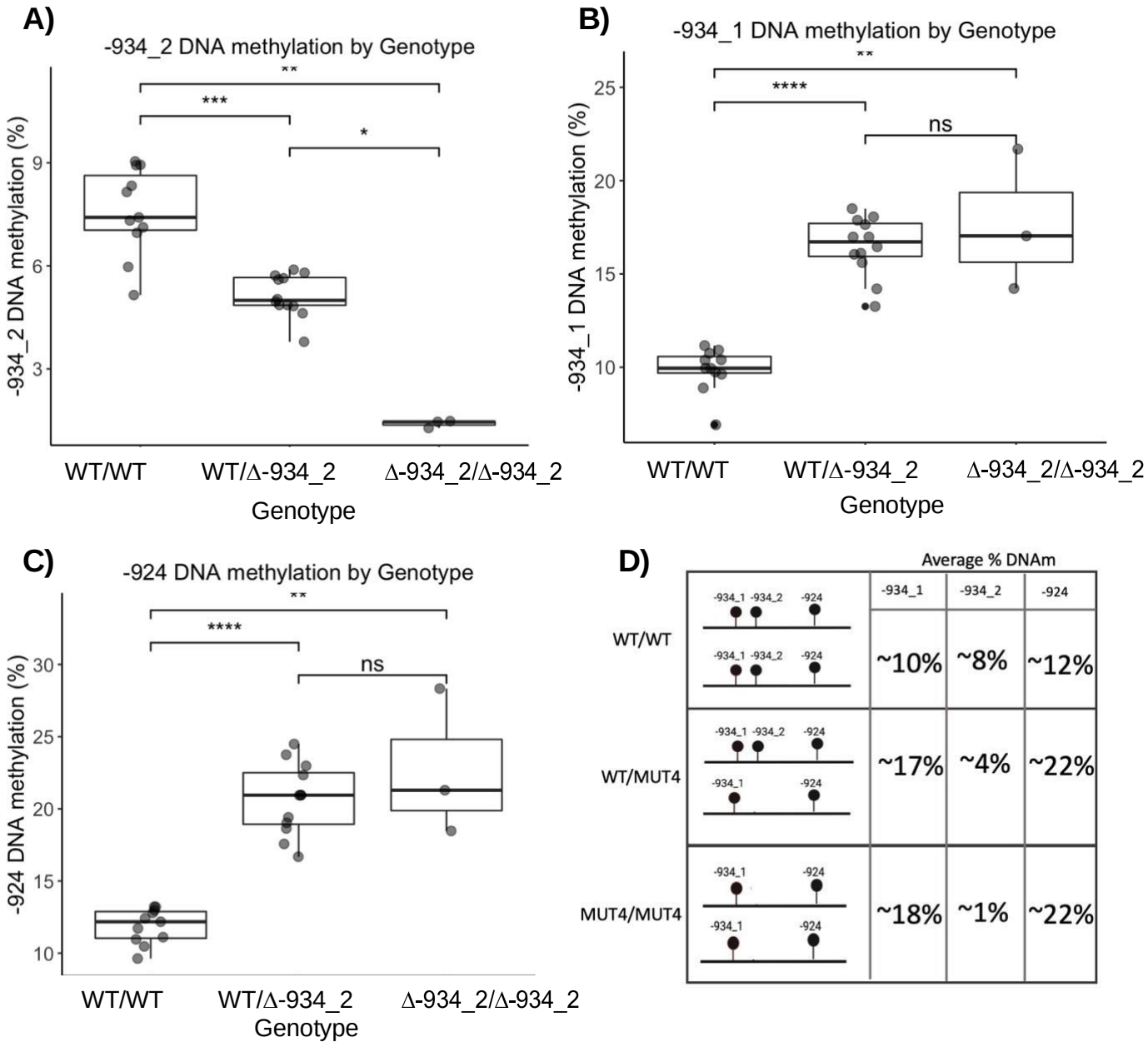

Figure 2. Removal of CpG site -934_2 results in hypermethylation of adjacent CpG sites. **A)** DNA methylation of site -934_2 by genotype. **A)** Methylation of CpG site -934_2 in the WT/WT offspring is approximately 8%, which is typically seen in our wildtype colony. The WT/Δ-934_2 offspring methylation, only containing one copy of site -934_2, is reduced to approximately 4%. The Δ-934_2 /Δ-934_2 subjects are methylated at >1%. **B)** DNA methylation of site -934_1 by genotype. Methylation of WT/WT remains typical at adjacent site -934_1 at between 10-12%. The WT/Δ-934_2 and Δ-934_2 /Δ-934_2 have an increased DNA methylation percentage to approximately 20%, providing preliminary data that hypermethylation has occurred. **C)** DNA methylation of site -924 by genotype.

WT/WT methylation remains consistent at approximately 12%. The WT/Δ-934_2 and Δ-934_2 /Δ-934_2 have an increased DNA methylation percentage to a range of 20-22%. **D)** Summary table of DNA methylation at each site by genotype. Schematic representation of the location of CpG sites by genotype with corresponding DNA methylation percentages. (WT/WT: n = 11, WT/Mut4: n = 12, Mut4/Mut4: n = 3, Welch's t-test, $p < 0.001$).

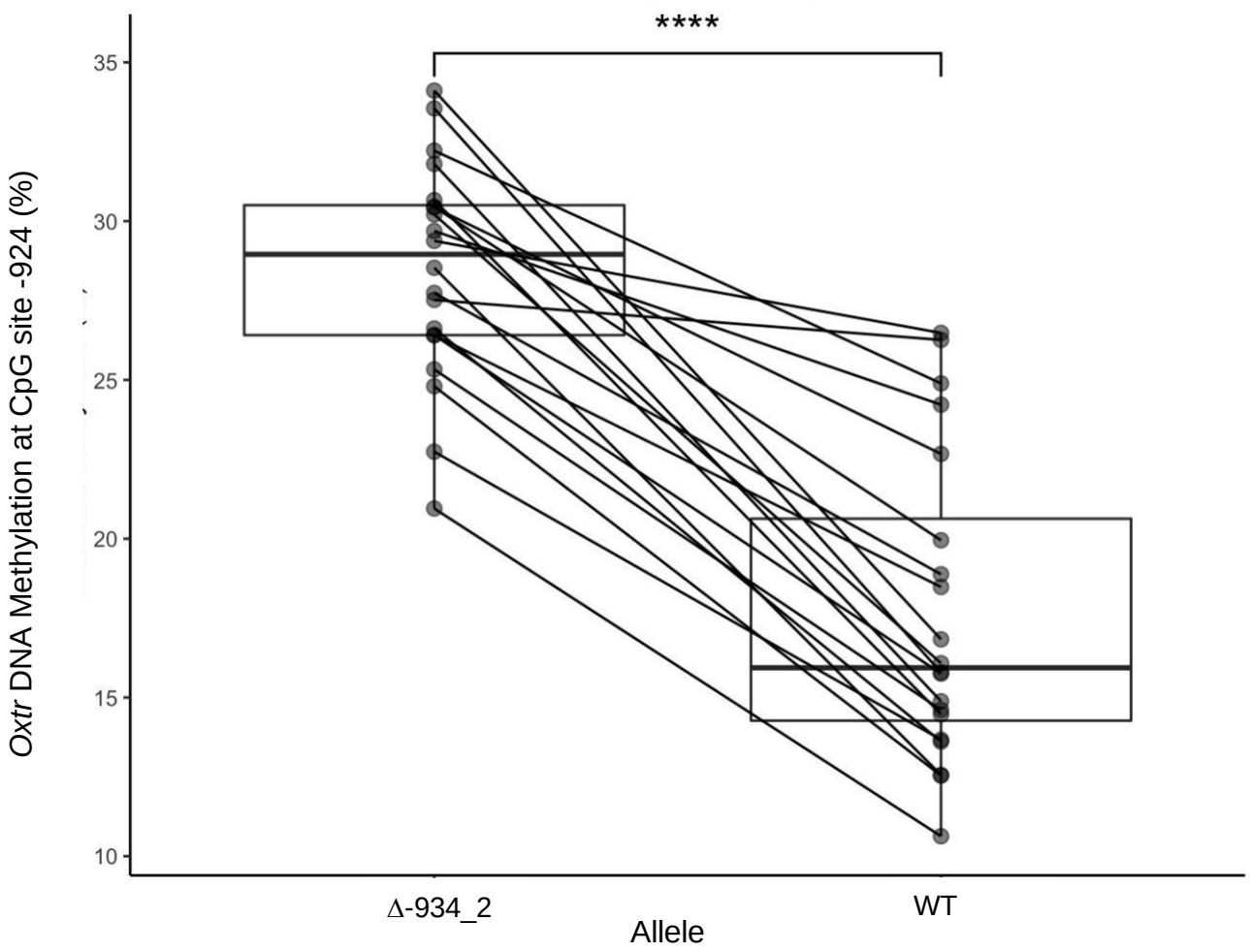

Figure 3. Hypermethylation of CpG site -924 occurs on the mutant allele in the heterozygote. Allele specific methylation identifies hypermethylation occurs at CpG sites adjacent to Δ-934_2. DNA methylation at site -924 was quantified in heterozygote offspring, Mut (Δ-934_2) and WT (wildtype alleles) are shown per animal (n = 21). Lines are drawn to connect alleles measured in same animal. ****Paired Sample t-test ($p < 0.001$) indicates significant difference in methylation state of the alleles.

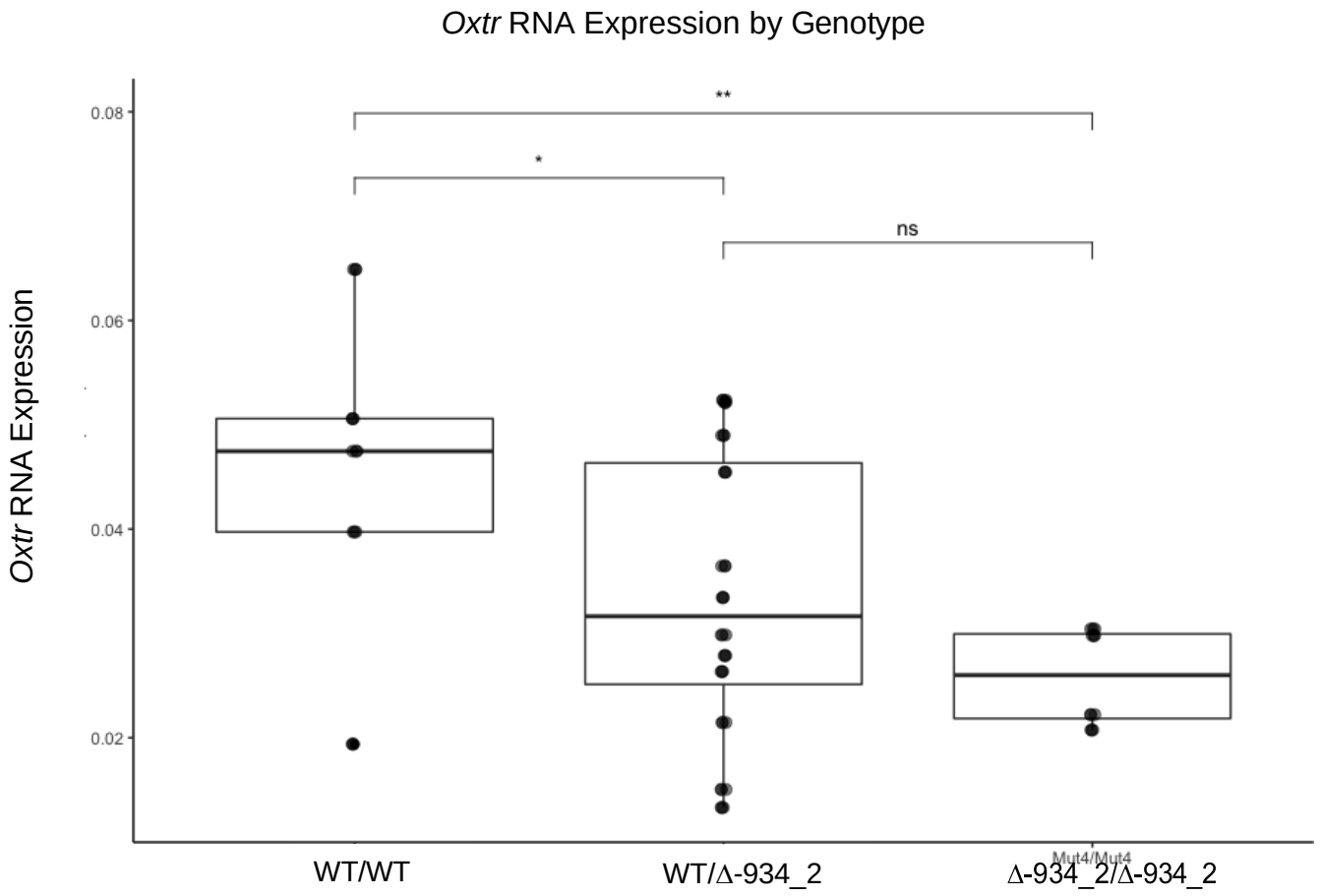

Figure 4. Creation of the Δ-934_2 mutation within the MT2 region of *Oxtr* results in a decrease in *Oxtr* RNA expression. Heterozygote(WT/Δ-934_2) and homozygote (Δ-934_2/Δ-934_2) siblings demonstrate decreased expression when compared to WT/WT siblings. *Oxtr* gene expression was determined by normalization of *Oxtr* Cq values to *Gapdh* Cq values. (WT/WT: n = 5, WT/Mut4: n = 16, Mut4/Mut4: n = 5. Paired t-test, $p < 0.01$)

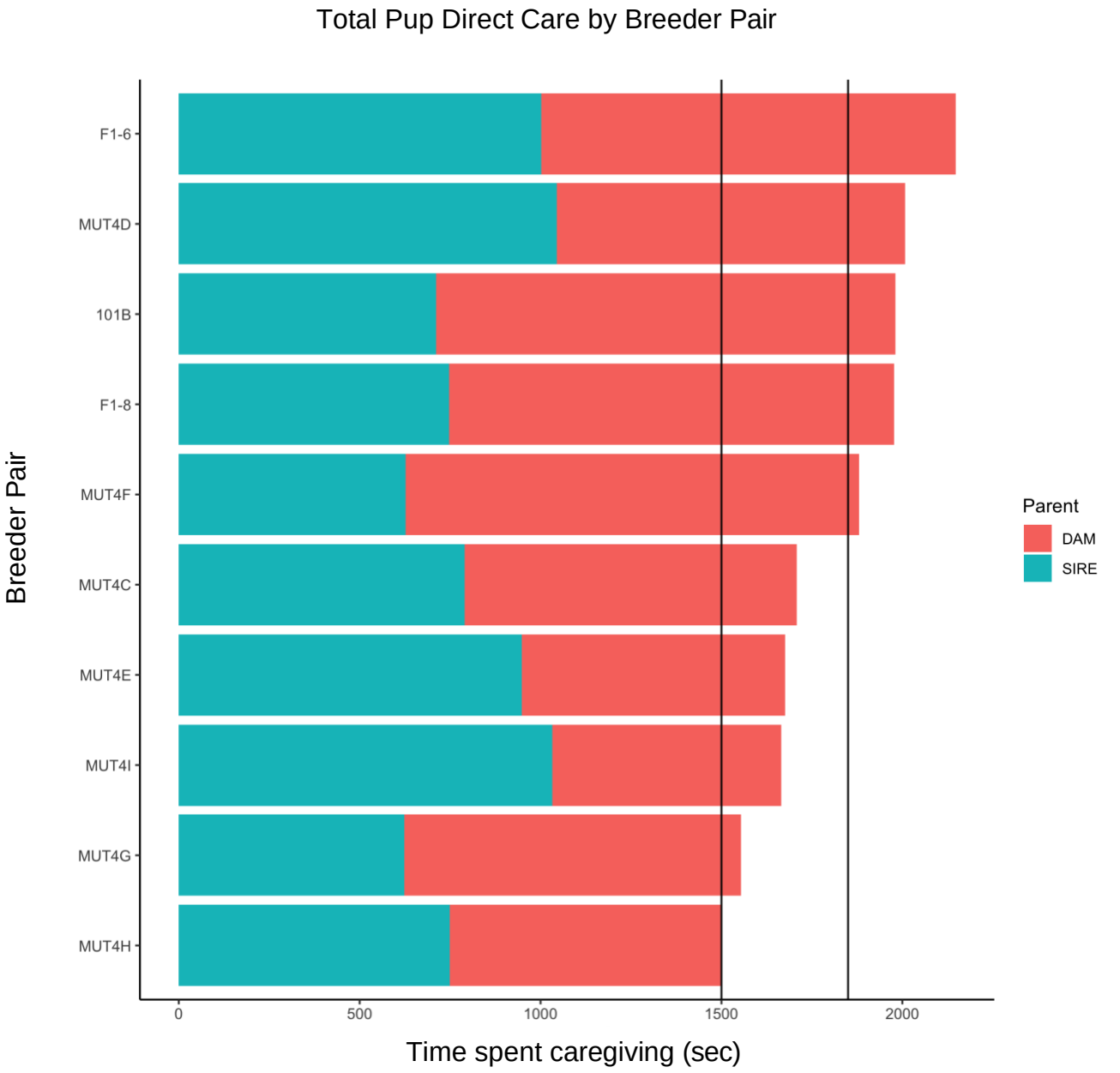

Figure 4. Representation of Parental Care Quantification across CRISPR Colony. Quantification parental care given to pups during first three days (P0 to P3). Parental care is split by Sire (Blue) and Dam (Red). Overall Parental care is ranked from lowest to highest. Solid vertical lines denote the cut-off for low parental care (>1500 seconds of parental care received by pups) versus high care (<1850 seconds of parental care received by pups). All breeding pairs listed are WT/Δ-934_2 heterozygote pairs, providing preliminary data that there is no effect of the heterozygous mutation on parenting behavior (n = 10).