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DNA Methylation Analysis of ECR3 of Oxytocin Receptor Gene

by:

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Undergraduate Honors Thesis under the direction of:

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Submitted to the LSU Honors College in partial fulfillment of the Upper Division Honors Program

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Louisiana State University
& Agricultural and Mechanical College
Baton Rouge, Louisiana

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Preface

One cannot fully understand science without *doing* science. Dr. Joomyeong Kim's lab is known for just that, doing "good science." During the summer of 2016 before I started my college career at Louisiana State University, I received an email from the LSU Department of Biological Sciences informing me that I am eligible to apply for a work-study program, in which as a science student, I could choose the option of working as an undergraduate assistant in a professor's research lab. While browsing through the online list of research labs in the Biology Department, I quickly became interested in Dr. Kim's lab with its genetic research on epigenetics and genomic imprinting. I met with Dr. Kim the first week of class my freshman year, asking if he had positions for any undergraduate assistants in his lab.

After asking me questions about my interests and academic goals, Dr. Kim kindly showed me around his lab, introduced me to the other undergraduate and graduate researchers, and embraced me into the Kim lab family. Besides being my lab instructor and genetics professor, Dr. Kim has been one of my most notable mentors throughout my four years at LSU. From advice on genetics, science, or general academics to some of the greatest life advice I have received as such a formative time in my life, Dr. Kim has proven to be one of the wisest, understanding, and trusted instructors a student could ask for.

During my first two years in his lab, I observed and assisted the graduate students with their projects, eventually learning the basic genetic protocols performed in Kim lab. As a Junior, I began to perform my own protocols during my year of an independent research course that prepared me for my thesis project. My time in the Kim lab has been one of my most fulfilling, challenging, and formative academic experiences, putting me out of my academic discomfort in

order to emerge a better student of science. Working in Dr. Kim's research lab has shown me the importance of fully understanding every detail, every mechanism behind a scientific concept in order to be able to fully comprehend its scope and apply it to a wider scientific understanding. I have learned how to fail and fail again gracefully, so that I can learn and value its necessity in order to succeed. I am forever grateful for the opportunity I have had during these past four years to apply the knowledge learned in the classroom and not only read about good science, but "*do* good science" as a mere undergraduate. The following pages are a summary of my project I have been building towards throughout my time in Dr. Kim's lab.

Abstract

Epigenetics is a rising field that reveals reversible modifications on the level of gene expression, rather than the exact genetic code. The field of epigenetics includes any process that alters the activity of a gene without altering the sequence. One of the most commonly studied epigenetic mechanisms is DNA methylation. This epigenetic modification involves the transfer of a methyl group to the C-5 position of a cytosine ring of DNA. In this study, DNA methylation is performed to analyze the differences in methylated regions of the Oxytocin Receptor (*Oxtr*) gene evolutionarily conserved region ECR3 in male versus female mice hypothalamus. This differential methylation of ECR3 will help to better understand how the chosen region affects Oxytocin receptor expression. The DNA methylation results are correlated to the activation or repression of the *Oxtr* gene. Analysis was conducted via combined bisulfite restriction analysis (COBRA), employing bisulfite conversion, bisulfite PCR, and restriction digestion with enzyme Hpy188I. The results of the study determined that all of the total samples of male and female DNA were digested, suggesting that all of the DNA samples for both male and females were methylated. The

methylation results suggest that the specifically chosen digested site on ECR3 either silences or does not play a significant role in the *Oxtr* gene. The results also suggest that there is no methylation difference based on sex in this region of the gene. Future studies need to be performed in order to determine which other site on ECR3 may affect the oxytocin receptor gene's expression. A substantial decrease in DNA methylation in the *Oxtr* gene has been shown to indicate the beginnings of social anxiety or disorder. A better understanding of the gene, and where its DNA methylation occurs in mice, can lead to a better understanding of the *OXTR* gene in humans, leading to future research in its correlation to sex-dependent bonding and social disorders, such as autism spectrum disorder (ASD).

Part One:

Background

Epigenetics

The original meaning of epigenetics stemmed from its basis in the studies of embryology, determining how a single fertilized egg can produce a variety of phenotypes. Before the 1950s, epigenetics was broadly used to study the full process of development from a zygote to a mature complex being. In the late 19th century, many embryologists began to debate about what was specifically responsible for the full development of an embryo. Many of those now referred to as "preformationists" argued that each embryo was created in completed but simply in miniature form and enlarged with development. However, on the opposite end of the debate were epigenesists, who wished to explain the gradual development of embryos in a complex plan. The original definition of "epigenesis" has now broadened with our increasing knowledge and has led to the field of epigenetics (Maienschein 2005). As the field expanded, the same original question remained, studying how one fertilized egg can result in multiple phenotypes. The term began to evolve to lay the groundwork for the study of genetics and the modern understanding that although all organisms possess the same basis of a genetic code and DNA sequence, genetic expressions can vary and can be inherited in offspring (Felsenfeld 2014).

In today's modern world, epigenetics is defined as "in addition to changes in genetic sequence," including any process that alters a gene's activity without altering its DNA sequence (Weinhold, 2006). These modifications occur naturally and can be transmitted to daughter cells, yet some have also been shown to be reversible. If an epigenetic modification does not form properly, it can severely alter an organism's functions and present major behavioral effects, which have encouraged scientists to thoroughly study each epigenetic mechanism in order to thoroughly understand its effects. Scientists have discovered numerous epigenetic processes including methylation, acetylation, phosphorylation, ubiquitylation, and sumolyation. With the clear

understanding that DNA is the primary carrier of genetic information and after clearly defining the science of epigenetics, DNA began to be studied on an epigenetic level.

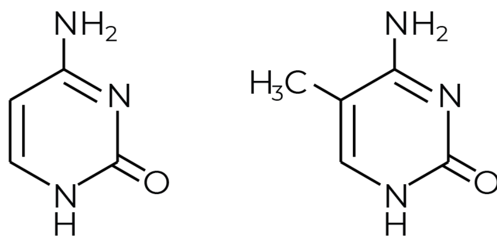
DNA Methylation

Although not understood until more recent times, DNA methylation was technically discovered in mammals as early as DNA was identified as genetic material with the Avery, Macleod, and McCarty experiment on bacterial transformation (Avery et al, 1944). However, the exact mechanism involving a modified cytosine was not discovered until 1948, when scientist Rollin Hotchkiss found a 5-methylcytosine (5mC) while studying a calf thymus using paper chromatography. Hotchkiss hypothesized that the 5mC had separated from cytosine in a manner similar to the mechanism of thymine (methyluracil) separating from uracil, however, he discovered that the modified cytosine actually already existed naturally in nature. Several subsequent studies revealed how DNA methylation is directly involved with gene expression, gene regulation, and cell differentiation (Moore et al, 2013). For example, in the early 1960s, X-chromosome inactivation was experimented on the DNA of the model organism *Mus musculus*. With the silenced X chromosome chosen at random, this experiment revealed that changes do not need to occur at a DNA sequence level for expressional changes to be shown. In 1975, scientists Holiday and Pugh proposed that the epigenetic mechanism DNA methylation could be responsible for gene inactivation, revealing DNA methylation to play one of the most significant roles in epigenetics (Holiday and Pugh, 1975).

Today, DNA methylation is the most highly studied epigenetic mechanism, directly modifying DNA chemically but not sequentially. DNA methylation regulates gene expression by turning off a gene's main function and silencing transcription semi-permanently. The methylation

can specifically control a gene's expression by inhibiting the binding of transcription factors that are responsible for activation or by recruiting proteins to enable gene repression. The process of *de novo* methylation and demethylation during development collaborate to develop a unique DNA methylation pattern and gene transcription. DNA methylation has been revealed to play a significant role in cellular processes such as X-chromosome inactivation, genomic imprinting, silencing of transposable elements, and embryonic development.

During DNA methylation, a methyl group from S-adenosyl-L-methionine (SAM) is covalently transferred by DNA methyltransferases (Dnmts) to the C-5 position of the cytosine ring of DNA. After DNA replication and cell division, DNA methylation is almost all erased, thus Dnmts are needed to restore the original methylation patterns. A family of Dnmts are involved in the methylation process. The key enzymes of DNA methylation include: Dnmt1, which maintains methylation patterns by copying pattern during replication, and Dnmt3a and Dnmt3b, which create *de novo* methylation patterns for DNA. The exact methylation location differs depending on the organism and its cell type. In mammals, the methylation can occur at any cytosine in the genome, but the methylation differs between somatic and embryonic stem cells. In mammalian, somatic cells, 98% of methylation takes place on CpG dinucleotide sites (a cytosine base near a guanine base, separated by a phosphate), except in genes' promoters. However, in mammalian embryonic stem cells, more than 25% of the methylation occurs at non-CpG sites that will be lost in mature tissues (Moore et al, 2013).



DNA methylation process.
(Figure from Zymo Research)

The highest levels of mammalian DNA methylation are located at CpG sites, with approximately 80% of CpG dinucleotides methylated. However, the overall mammalian genome is depleted of CpG sites due to the mutagenic potential of 5mCs that can deaminate to thymine, causing the conversion of CpG to TpG. This major mutability is shown over evolutionary time with the continual underrepresentation of CpG sites in the mammalian genome. Therefore, most of the methylation takes place at the remaining CpG sites spread throughout the body, with the exception of CpG islands that are unmethylated in order to maintain a richness in CpG sequences. CpG islands are DNA stretches of approximately 1000 base pairs that contain a high density of CpG sites normally located within a promoter region of a gene. During development in mammals, the methylation of cytosine (5mC) in these CpG islands results in long-term gene silencing, which is prevented by conserving the CpG islands in particular.

Expansion in DNA methylation studies have stemmed from its recent association with certain disorders and diseases, such as cancerous tumors. Studies have revealed that defects in DNA methylation are related to tumor development and cancer, which has led to an "epigenetic hallmark" of DNA hypo-methylation revealing signs of possible cancer (Jin and Robertson, 2011). All studied tumors exhibit DNA hypomethylation, while tumor suppressor genes display DNA hypermethylation. Furthermore, many scientists have begun to use this new understanding of DNA methylation to study the expression of certain genes, such as the Oxytocin Receptor Gene, the function of which is particularly important in the hypothalamus.

Oxytocin and the Oxytocin Receptor Gene

The peptide hormone and neuropeptide Oxytocin (OXT) plays a significant role for mammals' birthing, mother postpartum lactation, mother-infant bonding, stress regulation, and

social behaviors. OXT is synthesized in the hypothalamus, specifically by the *OXT* gene in magnocellular neurons in the paraventricular and supraoptic nuclei. The oxytocin is either transported directly to other regions of the brain involved in social and emotional behaviors or transported by axons to the pituitary lobe, where it is stored before it is released into the peripheral blood. Higher oxytocin levels have been shown to be linked to amplified maternal empathy, arousal, and response/ affection to the infant. Abnormal oxytocin levels can cause major changes in social behaviors, leading to social disorders such as autistic spectrum disorder (ASD), mood disorders, or dangerous increases in stress levels. Oxytocin treatments that administer calculated levels of oxytocin into the patient have been shown to reduce activation in the amygdala (the region of the brain responsible for processing emotions) and improve emotional behavior, stress levels, and mental health (Yoshida et al., 2009).

The body's oxytocin receptors are synthesized by the Oxytocin Receptor gene (abbreviated *OXTR* in humans and *Oxtr* in mice), which is located on human chromosome 3p25 and *Mus musculus chromosome* 6 and expressed in the brain and peripheral organs. The oxytocin receptor gene is 17 kilobases (kb) in length and contains 3 introns, which do not code for proteins although still considered part of the gene, and 4 exons, which do code for proteins. Exons 1 and 2 correspond to the 59-prime noncoding region, while exons 3 and 4 encode the amino acids of the Oxytocin receptor. The oxytocin receptor itself is comprised of 389-amino-acid G-protein coupled transmembrane receptor, which allows intracellular secondary messengers to activate for the oxytocin to travel along its pathways (Maud et al, 2018).

A new field of *Oxtr* epigenetics has emerged in order to study socio-emotional functions of humans by analyzing DNA methylation (DNAm) patterns, using *Mus musculus* as a model organism. While *OXT* has tight regional expressions, *OXTRs* are expressed throughout the brain

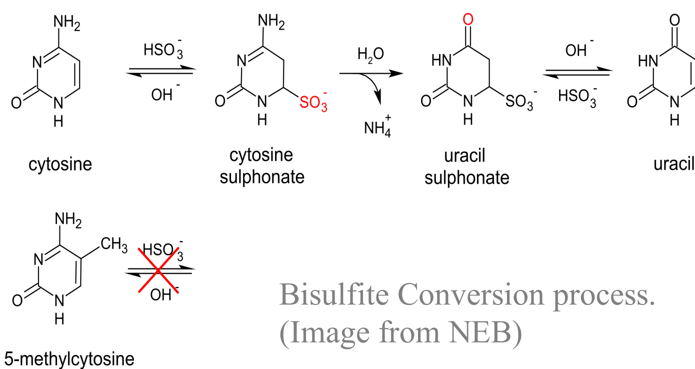
and body and can therefore, control the final rate-limiting step in oxytocin signal transmission (Maud et al, 2018). Methylation differences of Oxytocin receptor gene has been correlated to changes in the oxytocin receptor's expression in both humans and animals. Previous studies have shown that decreases in DNAm in the *Oxtr* gene can indicate the beginnings of social anxiety, stress, and autism in infants (Gould and Zingg, 2003).

In order to better understand the human *OXTR* gene, scientists study the *Oxtr* gene of *Mus musculus*, which shares 91% amino acid sequence identity with the human gene. Both genes contain the same number of exons and introns (4 and 3, respectively). Exons 1 and 2 contain the 5' untranslated region, exons 3 and 4 encode the receptor's amino acids, while intron 3 "interrupts the coding sequence after transmembrane domain 6" (Kubota et al, 1996). The gene's promoter region lacks a TATA box, but contains numerous interleukin and estrogen responsive elements (EREs) (Gruber et al, 2004). A recent study by Teruyama's group analyzed whether there are sex differences in the expression of *Oxtrs* and discovered that they are more often strongly involved in maternal behavior (Sharma et. al, 2019). The *Oxtr* gene includes all four evolutionarily conserved regions (ECRs) that have conserved their function in different genes across evolutionary time. Previous unpublished studies from Dr. Joomyeong Kim's lab has identified potential enhancers in the ECRs of *Oxtr* and specifically has identified that ECR3 could have transcription binding sites, hence why ECR3, which is located upstream of the promoter region, was chosen for this experiment.

Combined Bisulfite Restriction Analysis (COBRA)

In order to study DNA methylation, researchers can use the molecular biological technique COBRA, Combined Bisulfite Restriction Analysis, that specifically quantifies the methylation

levels at a given CpG site. This assay has been proven to provide great accuracy in its results and is applicable to large numbers of samples, unlike other existing techniques. To perform COBRA, a sodium bisulfite treatment is applied to genomic DNA in order to introduce methylation dependent sequence differences (Xiong and Laird, 1997). Unmethylated cytosine residues are converted to thymine, while methylated cytosines are protected and remain cytosines. The possible residue conversions can create new restriction enzyme sites or retain existing CG sites, such as CGCC. The bisulfite DNA is amplified by PCR. The primers do not contain CpG dinucleotides in order to not discriminate between DNA sequences based on the original methylation status. With these primers being used for PCR, the products will directly show the amount of DNA methylation in the original sequence. After PCR is complete, restriction enzyme digestion is performed (Xiong and Laird, 1997).



Bisulfite Conversion process. (Image from NEB)

Normally, restriction enzyme digestion is used to create compatible sticky ends of PCR products for DNA cloning. However, in respect to COBRA, restriction enzyme digestion is used for DNA methylation analysis. During restriction enzyme digestion, a DNA sequence is digested into smaller sequences by a specific restriction enzyme that normally recognizes 6-8 base pairs (bp) long. The restriction enzyme used in this experiment was Hpy188I. This enzyme originates from the Hpy188I gene from *Helicobacter pylori* 188. Hpy188I must be stored at 25°C at a pH of 7.4 to yield maximum results. Hpy188I cuts both strands of a DNA double helix at the restriction site 5'...TCN^GA...3', cutting between the N nucleotide and G, and 3'...AG^NC...5', cutting between the G and N nucleotide. The N nucleotide can vary depending on the sequence. For the

ECR3 sequence used in the experiment, the N is a C, meaning Hpy188I cuts at methylated C. This enzyme does Hpy188I is a type 2 enzyme, consisting of a simple structure of a restriction endonuclease and a methyltransferase. After cutting, one portion of DNA contains 3-4 nucleotides while the other portion contains 4-5 nucleotides, with each portion separated by 6-8 nucleotides (Xu et al., 2000). After restriction enzyme digestion, DNA methylation is quantified via gel electrophoresis, which separates DNA fragments from the PCR product according to its size, thus identifying which DNA sequences were digested or not (Xiong and Laird, 1997).

Part Two:

Experiment

1. Introduction

According to the most recent study taken by the Center for Disease Control and Prevention, roughly 1 out of 54 children are affected by autism spectrum disorder (ASD), with boys being affected four times as prevalent than girls (Maenner et. *al*, 2016). With its increasing prevalence, scientists have begun to further research potential causes for this disorder. Abnormal oxytocin (OXT) levels have been shown to correlate with abnormal social behaviors that can potentially lead to social disorders such as ASD (Yoshida et al., 2009). By studying the expression of the oxytocin receptor gene in mice, researchers can better understand what may affect its expression and how this gene can affect an organism's behavior.

With the rising field of epigenetics, scientists can study how a specific gene varies in expression and activity maintaining its underlying sequence (Weinhold, 2006). This variance in activity but not sequence is dependent on epigenetic modifications. The most commonly studied epigenetic modification is DNA methylation, involving the transfer of a methyl group to the 5' carbon position of cytosine, creating a 5'-methylcytosine. DNAm is correlated with the silencing of genes, usually in the maternally expressed thus by analyzing DNAm of a gene, a gene's activation or repression levels can be revealed (Moore et. al, 2013).

A previous study has shown that DNAm can be used to specifically study the Oxytocin receptor (*Oxtr*) gene of the model organism *Mus musculus* (house mouse), revealing that a decrease in DNAm of the *O*xtr gene can lead to of social disorders in infants (Gould and Zingg, 2003). Oxytocin (OXT) is a neuropeptide hormone that is important for mammals' birthing, maternal lactation, mother-infant bonding, stress regulation, and many social behaviors. OXT is synthesized in the hypothalamus by the OXT gene and received by the numerous oxytocin receptor genes throughout the body. Located on human chromosome 3p25 and *Mus musculus chromosome* 6, the

Oxtr gene in *Mus musculus* has 91% identical amino acids with the human gene, and thus, can be used as a model organism to better understand the gene (Maud et al, 2018).

The *Oxtr* gene includes the four known evolutionarily conserved regions (ECRs), which are located 5'-upstream of the gene that have remained relatively unchanged throughout evolution. In this experiment, ECR3 of *Oxtr* gene will be specifically studied in order to test this region for sex-dependent methylation differences that affect the expression of *Oxtr*.

DNA methylation analysis is performed to analyze the differences in methylated regions of *Oxtr* gene ECR3 in wildtype male versus wildtype female and post-delivery female mice hypothalamus. By studying the difference in methylation, the gene's activation can be better understood, and researchers can locate the origin of changes in oxytocin receptor gene's expression.

2. Methods

2.1 DNA isolation from *Mus musculus*

DNA was isolated from the hypothalamus of four separate *Mus musculus*— two separate wildtype males, a wildtype female, and a female one day after delivery. The DNA isolation protocol followed all protocols in the Guide for Care and Use of Laboratory Animals (National Research Council, 1996) and was approved by the Institutional Animal Care and Use Committees at UTHSC and Louisiana State University. The two wild type males were chosen in order to verify there is no fluctuations between different individuals. The wildtype female was chosen in order to compare sex related *Oxtr* gene activity, and the post-delivery female was chosen in order to study if there is an oxytocin change after a female uses a surplus of oxytocin during delivery. After DNA isolation, COBRA was performed.

2.2. Bisulfite Conversion

To analyze the methylation of each of the four DNA samples, the EZ DNA Methylation Kit (Zymo Research) was used in order to perform bisulfite conversion reactions. This kit involved a three-step reaction to convert cytosine into uracil. In order to do this, a CT Conversion Reagent was prepared by adding 750 μL water and 210 μL dilution buffer to a tube of CT Conversion reagent. After mixing at room temperature and shaking for 10 minutes, the CT conversion reagent is ready for use. For the 4 DNA sample studied in this experiment, a master mix (MM) of 172 μL water and 20 μL dilution buffer was made. 48 μL of MM and 2 μL of DNA was added to each tube. After incubating at 37 °C for 15 minutes, the protocol of the kit was followed, using the pre-made conversion reagent, a binding buffer, wash buffer, desulphonation buffer, and elution buffer to yield the final bisulfite conversion product. This bisulfite conversion will create a two-stranded sequence that is no longer complementary. The top strand will be targeted and amplified during the next PCR step.

2.3 Bisulfite- DNA PCR and Restriction Enzyme Digestion

After bisulfite conversion, bisulfite-DNA PCR is performed using the standard PCR conditions and ECR3 bis-a and bis-b forward and reverse primers. The PCR products then undergo restriction enzyme digestion using the enzyme *Hpy188I* to cut at methylated C in the ECR3 sequence of the *Oxtr* gene. A MasterMix(MM) of 75 μL water, 10 μL cutsmart, and 1 μL of *Hpy188I* was made. Four tubes were then prepared with18 μL of MM and 2 μL of each respective DNA. The single top strand from the bisulfite conversion becomes double stranded DNA that can now be digested by restriction enzymes.

2.4. Agarose Gel Electrophoresis and DNA quantification

After incubating overnight at 37°C, each tube's DNA solution is inserted into an agarose gel for gel electrophoresis to visualize the enzyme digestion results. Each DNA band in the gel image was quantified using ImageJ software in order to compare the DNA amounts in each sample.

3. Results

The gel electrophoresis imaging was analyzed in order to determine if digestion occurred in each sample, and if therefore, activation or repression of *Oxtr* gene occurred at each sample's ECR3 site. The gel image revealed that both male wildtype samples, the female wildtype sample, and the female one day post-delivery were all digested, shown with the double bands in each lane **(Fig. 1).** The restriction enzyme digestion with *Hpy188I* was replicated to ensure the results were accurate and both digestions revealed digestion in all four samples. From mere appearance of band intensity, sample 1 of wildtype male appeared to have the largest amount of DNA, followed by sample 3 of the wildtype female, sample 2 of wildtype male, and lastly, the female one day post-delivery **(Fig. 1).** All 4 lanes with each sample's unmethylated (top) and methylated (bottom) bands were quantified using ImageJ. The wildtype male in lane 1 showed 53.26% of unmethylated DNA and 46.74% of methylated DNA **(Fig. 2).** The wildtype male in lane 2 showed 49.59% of unmethylated DNA and 50.41% of methylated DNA **(Fig. 3).** The wildtype female in lane 3 showed 43.52% of unmethylated DNA and 56.48% of methylated DNA **(Fig. 4)**. The female one day post-delivery in lane 4 showed 45.11% of unmethylated DNA and 54.88% of methylated DNA **(Fig. 5)**.

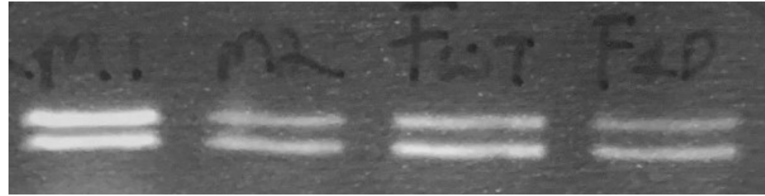


Fig. 1. Restriction Enzyme Digestion Products using Hpy188I on DNA samples of ECR3 of Oxtr gene hypothalamus. The samples from left to right are male wildtype 1, male wildtype 2, hypothalamus wildtype, and female one day post-delivery.

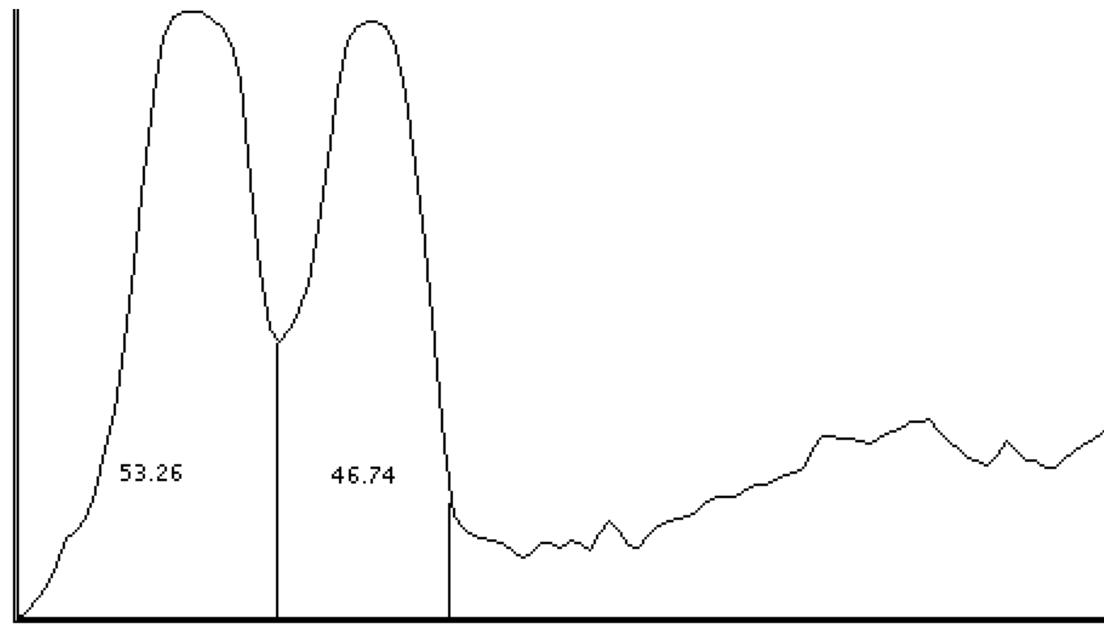


Fig. 2: ImageJ DNA quantification analysis of the Restriction Enzyme Digestion unmethylated (peak 1) and methylated (peak 2) products from the wildtype male 1 sample.

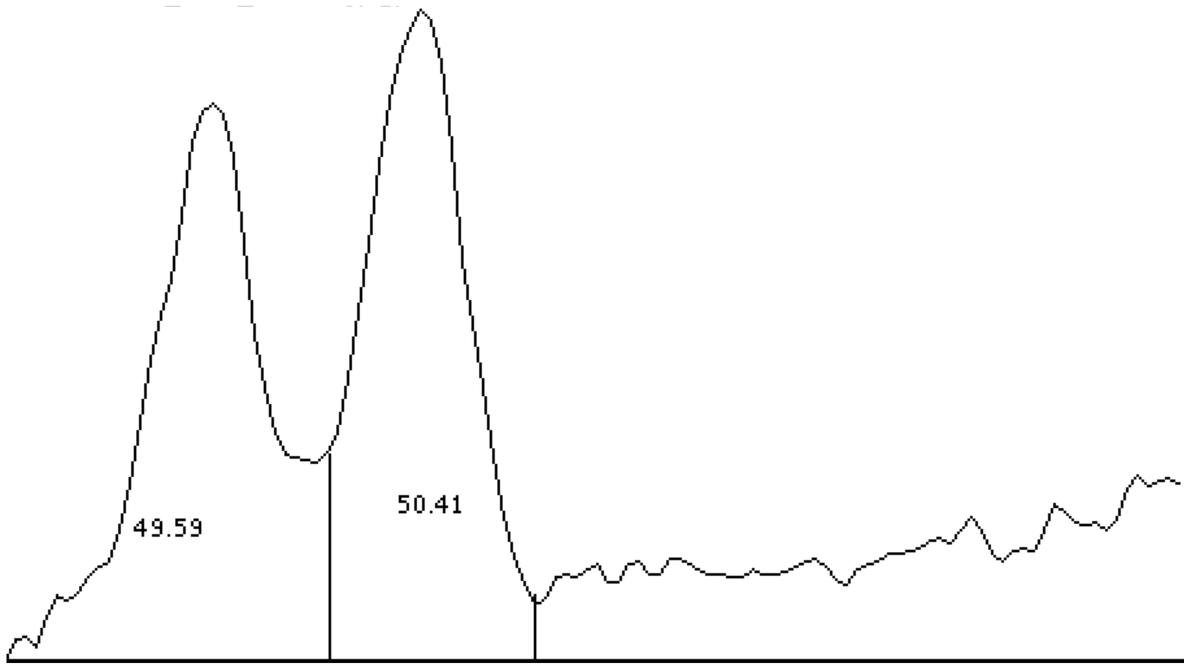


Fig. 3: ImageJ DNA quantification analysis of the Restriction Enzyme Digestion unmethylated (peak 1) and methylated (peak 2) products from the wildtype male 2 sample.

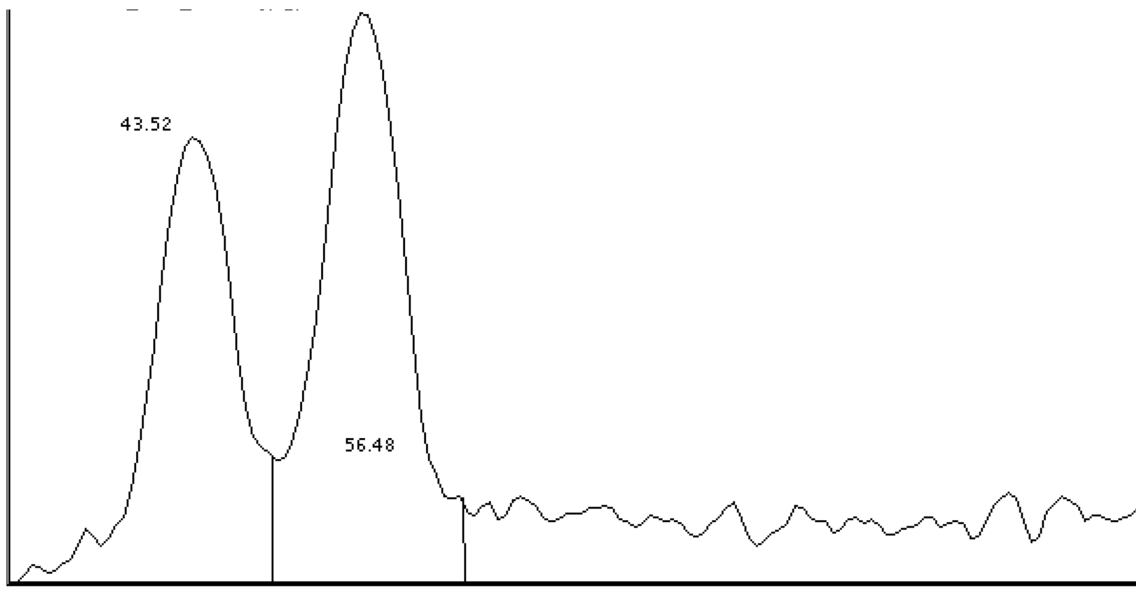


Fig. 4: ImageJ DNA quantification analysis of the Restriction Enzyme Digestion unmethylated (peak 1) and methylated (peak 2) products from the wildtype female sample.

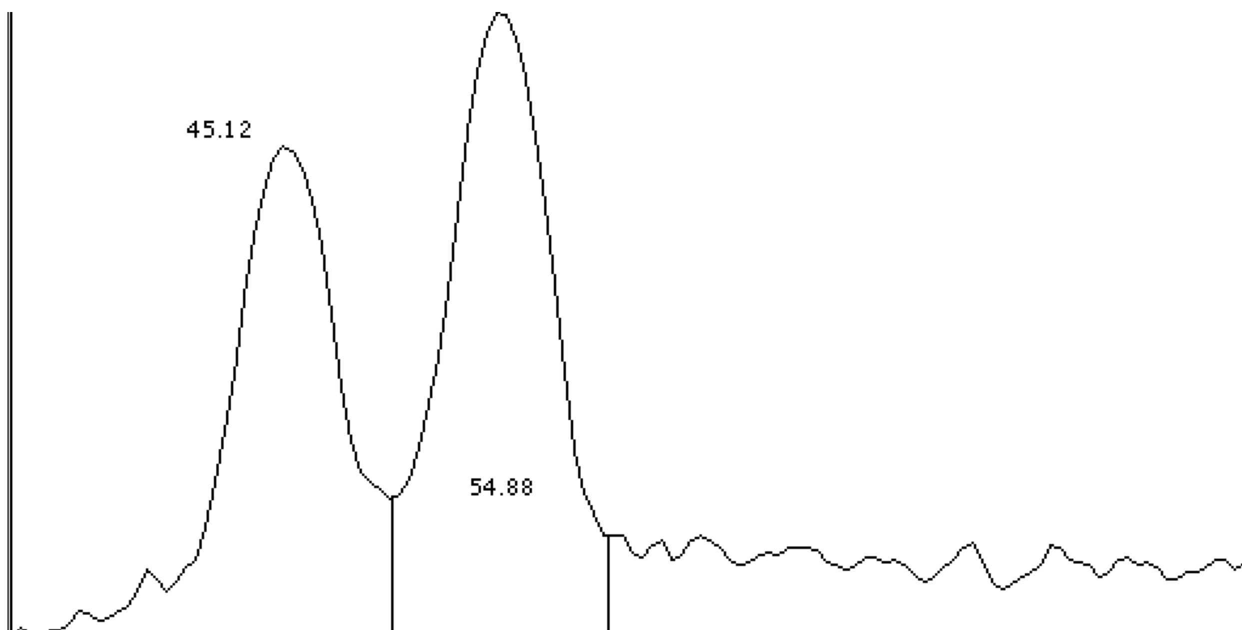


Fig. 5: ImageJ DNA quantification analysis of the Restriction Enzyme Digestion unmethylated (peak 1) and methylated (peak 2) products from the female one day post-delivery sample.

	WT M1	WT M2	WT Female	Female 1-day PD
Unmethylated DNA %	53.26%	49.59%	43.52%	45.12%
Methylated DNA %	46.74%	50.41%	56.48%	54.88%

Table summary of quantification results.

4. Discussion

The results suggest that all 4 samples were digested. The restriction enzyme digestion using *Hpy188I* normally cuts sequences as follows (with the red arrow indicating the cut site): 5'-TCN^GA-3', and 3'-AG^NC-5. In the original ECR3 sequence of the *Oxtr* gene used, the N nucleotide is a G, so *Hpy188I* digests the cut site 5'-TCGGA-3'. The enzyme cuts specifically at methylated CG sites, although restriction enzymes normally cannot cut at methylated sites. Since

all four samples showed 2 bands in the gel, the results suggest that all DNA samples were digested and hence, all are methylated.

In the original DNA sequence before bisulfite conversion, this site was 5'-CCGGA-3,' but after bisulfite conversion, the unmethylated C was converted to T, for the sequence to then become the recognized cut site 5'-TCGGA-3'. After bisulfite conversion, the newly converted double stranded DNA is no longer complementary, and only one strand will be targeted and amplified in PCR. In this case, the top strand is chosen because it has the recognized cut site 5'-TCGGA-3', while the complementary bottom strand of 5'-TCCGA-3' will either be converted to TTCGA or TTTGA, neither of which are a recognized cut site for *Hpy188I.*

Methylation correlates with the silencing or inactivation of genes, therefore, these methylated digestion results suggest that the specifically chosen site on ECR3 either silences or does not play a significant role in the *Oxtr* gene. The results also suggest that there is no overall methylation difference based on sex in this region of the gene. Since the results did show partial methylation, ECR3 may be functional in some cells and non-functional in others. The hypothalamus has around 30-50 neuron cells. The partial methylation in the results could either suggest that all of the different cells in the hypothalamus have the same partial methylation, or it could suggest that some hypothalamus cells have 100% methylation, while the other remaining could have 0%, then becoming an average methylation, which is more likely. However, since all different neuron cells have a different function, only a subset will have *Oxtr* and be partially methylated, while the cells that do not have *Oxtr* most likely will be completely methylated.

The wildtype male in lane 1 showed a greater percent of unmethylated DNA, suggesting greater activation of the oxytocin receptor's expression. The wildtype male in lane 2 showed almost identical methylated versus unmethylated amounts of DNA. However, when comparing the

unmethylated bands with the methylated bands, the two female samples show a significant increase in the DNA quantification of digested results due to methylation versus the quantification of the unmethylated fragments. This suggests that this ECR3 region of *Oxtr* has more of a silencing, insignificant effect on the expression of the oxytocin receptor. The post-delivery female sample in lane 4's increase in DNA quantification for methylation was slightly less than that of the wildtype female DNA, which could be related to post-delivery maternal mammals' greater use of oxytocin overall.

Since oxytocin is used for maternal infant bonding and for delivery, research suggests that females should have greater activation in their oxytocin receptors. Future research on another sequence of this gene or another site on the ECR3 must be performed in order to better explain if oxytocin receptor activation does indeed differ amongst males and females and between post-delivery females and virgin females.

Future studies need to be performed in order to determine which other site on ECR3 or which other sequence on *Oxtr* may affect the gene's expression. Previous studies have shown that a substantial decrease in DNA methylation in the *Oxtr* gene has been shown to indicate the beginnings of social anxiety or disorders (Yoshida et al., 2009). Therefore, if research can pinpoint which site on which sequence is correlated to the methylation changes in this gene, there will be a better understanding of some of the potential causes and correlations of oxytocin related social disorders such as ASD or sex-dependent bonding.

Acknowledgments

I would like to thank all of those who have generously helped me complete this project. First and foremost, I would like to thank my advisor, Dr. Joomyeong Kim. He took me into his lab four years ago and has followed me along all of my trials and tribulations with my experiments. Dr. Kim has guided me every step of the way, and without his help, I would not have been able to complete this project. I would also like to thank the members of the Kim lab, Subash Ghimire and Haley Williams, whose help and support was also equally as significant for me to understand my work every step of the way. I would also like to thank the members of my committee Dr. Leaf Boswell and Dr. Kevin Bongiorni for kindly take time out of their schedules to be a part of my defense. All of the aforementioned people's help was crucial for the completion of my honors degree, and I am forever appreciative.

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