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Julianna Celeste March 15, 2023

Investigating natural variation in the oxytocin system of prairie voles: Do levels of oxytocin differ between two naturally occurring genotypes?

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An abstract of

a thesis submitted to the Faculty of Emory College of Arts and Sciences

of Emory University in partial fulfillment

of the requirements of the degree of

Bachelor of Sciences with Honors

Neuroscience and Behavioral Biology

2023

Abstract

Investigating natural variation in the oxytocin system of prairie voles: Do levels of oxytocin differ between two naturally occurring genotypes?

By Julianna Celeste

Importance: Oxytocin (OT) is a neuropeptide that modulates many aspects of social behavior. Previously, our lab has demonstrated natural variation in the oxytocin receptor (OXTR) levels of prairie voles, which are strongly associated with noncoding single-nucleotide polymorphisms in the oxytocin receptor gene (*Oxtr*). This difference in genetic background might also be associated with altered levels of OT. As OT-signaling is involved in the regulation of social behavior, understanding the genetic background of this system could lead to a better understanding of mental conditions characterized by social deficits

Objective: To determine if there is a significant difference in protein levels of oxytocin within the paraventricular nucleus (PVN) between voles with two different genotypes

Main outcome and Measure: Genotyping through PCR, immunohistochemistry, and imaging

Subjects: 14 voles, 5 females and 9 males between ages 50 and 113 days old.

Results: We did not observe significant differences in the number of OT-positive cells in the PVN between genotypes. We did find a significant difference in OT-cell count across bregma positions, indicating that the medial part of the PVN contains most OT-positive cells. Manual and automated cell count methods delivered similar results.

Conclusion and Relevance: While we hypothesized that variation in OXTR levels in prairie voles may impact OT levels, our analyses did not deliver evidence to support this possibility. We found that OT protein levels do not vary between the two genotypes. Therefore, we could not confirm

the differences that have been previously found in OT mRNA abundance between genotypes. A possible explanation is that differences in OT mRNA do not directly translate into differences in OT protein levels. In addition, it could be that confounding factors such as tissue isolation and/or processing could have affected experimental results. Overall, we did not observe any differences in OT levels between genotypes so it is unlikely that differences in OT levels are responsible for the differences in social strategies between the genotypes.

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Acknowledgements

I would like to acknowledge and give my warmest thanks to my advisor, Dr. Arjen Boender. He provided endless guidance and support while working on this project both in and out of the laboratory. I would also like to thank the Young Laboratory at the Emory National Primate Research Center for allowing me to complete my work and provide the necessary means to do so. I would also like to give a special thanks to my committee members, Dr. Larry Young and Dr. Aubrey Kelly, for being graciously flexible and for providing encouraging and brilliant comments and suggestions throughout this process.

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Introduction

Across the animal kingdom, social relationships are the foundation of societies. Ranging from reproductive behaviors to the formation of more complex social bonds such as biparental care, many social behaviors are controlled through the production and release of oxytocin (OT). This neuropeptide is an important modulator of and influences the formation of social behaviors. For example, OT is released during physical contact causing a positive sensation, giving it the nickname "the love hormone" (Wallum et al. 2017). Oxytocin is also known to be elevated during childbirth and lactation in women, facilitating the mother-infant bond (Olza et al. 2020). Oxytocin exerts its influence on social behavior through activation of its receptor (OXTR) and variations in OXTR levels are associated with differences in social behavior. Genetic variations in the human oxytocin receptor (OXTR) have been found to influence emotion recognition, and relationship quality but also are associated with autism spectrum disorder (ASD), underscoring the importance of OT signaling in the regulation of social behaviors (Liu et al. 2010; LoParo et al. 2017).

Not only is oxytocin important in humans, but there have been proven impacts on the social behavior of *Microtus ochrogaster*, a socially monogamous rodent also known as the prairie vole. In adolescent voles, differences in oxytocinergic signaling, induced by parental nurturing, has been shown to potentially modulate susceptibility to adverse experiences (Barret et al. 2015). When oxytocin receptors are genetically knocked out, voles showcase a decrease in helping behaviors and empathy (Kitano et al 2022). Ample studies of social bonding have been conducted in this species as prairie voles provide an interesting model akin to humans due to

similar social attachment styles. Socially monogamous, prairie voles form pair bonds, show empathy towards stressed partners, and exhibit biparental care (Johnson et al. 2015).

The main sites of oxytocin production are the paraventricular nucleus (PVN) and supraoptic nucleus of the hypothalamus (SON). Within these hypothalamic structures, two types of neurons, magnocellular and parvocellular, synthesize oxytocin (Liao et al. 2020). Differing electrophysiologically based on the necessary voltage for an action potential, these neurons contribute to the methods of OT release centrally and peripherally (Luther et al. 2000). Magnocellular neurons release OT centrally along with peripherally via axonal projections to the posterior pituitary into the bloodstream. In contrast, parvocellular cells are thought to release oxytocin only centrally, and in lower quantities (Lee et al 2009). Within this OT system, the genetic sequences that code for oxytocin and vasopressin have proven to be highly conserved across a multitude of species, ranging from nematodes to humans (Insel et al. 2000). Several studies have shown the presence of ancestral peptides with evolutionarily conserved functions relating to reproduction in invertebrates despite previously considered to be hormones specific to vertebrates. While invertebrates have a single OT-related peptide, a gene duplication in early vertebrates led to the evolution of two related peptides with diverging roles in mammals: OT and vasopressin (AVP) (Insel et al. 2000). The strong conservation of the OT system has been further demonstrated with correct expression in hypothalamic OT neurons of mice following genomic integration of isotocin, one of the OT homologs, of the teleost *Fugu rubripes*, commonly known as a blowfish (Venkatesh 1997).

The receptor for oxytocin (OXTR), however, is much more variable in its expression pattern, as strong diversity of OXTR expression can be observed within and across species. Most species previously studied have shown a unique pattern of OT receptor expression and regulation

with associated differences in behavioral responses (Insel et al. 2000). In King et al., variation in OXTR density in the nucleus accumbens (NAcc) of prairie voles was shown to be strongly associated with an intronic single nucleotide polymorphism (SNP), along with alterations in pair bond formations. This finding demonstrated that non-coding SNPs in the OXTR gene strongly predicts OXTR levels in a brain region-specific manner (King et al. 2016). Specifically, animals with genotype CC have high levels of OXTR in the NAcc, while the TT genotype have low levels of OXTR (Figure 1a).

Ongoing studies in the Young lab, utilizing single-cell sequencing experiments, have corroborated the strong association between this genetic variation and differences in OXTR levels. One intriguing finding that came from these recent experiments is that intronic genetic variation in the *Oxtr* gene is not only associated with differences in OXTR levels but with variations in OT expression itself (King et al. 2016). In this experiment, Sim1, a gene necessary for the development of neuronal cells in the PVN, was used to measure OT and AVP mRNA in PVN cells(Michaud et al. 1998) (Figure 1b). Once Sim1 was located, indicating the presence of PVN cells, OT and AVP cells were marked and the mRNA was quantified within both CC and TT genotype voles. The data showed equal amounts of Sim1 and AVP between both genotypes, but OT showed significantly higher levels within the CC genotype. This suggests that genetic variation in the *Oxtr* gene is not only associated with variation in OXTR levels, but also with altered OT levels. Since one of the hallmarks of OT is its consistent expression patterns across species, finding variation in OT levels is surprising, but potentially very interesting, considering the vital role that oxytocin plays in the regulation of social behavior.

To gain further insight into this potential difference in OT levels between OXTR genotypes, this thesis aims to validate if the observed differences in OT mRNA between two

vole genotypes, CC and TT for higher and lower expression respectively, can be observed at a protein level along with the implications that may hold.

Methods

Genotyping: Polymerase chain reaction was used to verify the genotype of each vole. Total DNA was isolated with the Qiagen Blood & Tissue Kit. Two PCRs (one for each allele) were executed with a polymerase that is sensitive to one-nucleotide differences. Both PCRs were executed with the following cycling conditions: 95℃ for 2 minutes for initial denaturation, then 34 cycles of 95℃ for 15 seconds for denaturation, 53℃ for 15 seconds for annealing, 72℃ for 30 seconds for elongation, and final elongation at 72℃ for 5 minutes. PCR products were then visualized on an agarose gel and the genotype was determined.

Tissue collection: Voles (male, female, n=16) were deeply anesthetized followed by transcardial perfusion with 0.1M phosphate-buffered saline (PBS, pH 7.4) containing 4% paraformaldehyde (PFA). The brains were extracted, post-fixed in 4% PFA, then saturated in PBS containing 30% sucrose, and stored at -80°C. PFA-fixed brains were cut into 40µm sections using a cryostat. Sections surrounding the paraventricular nucleus were then collected and stored in cryoprotectant buffer (PBS containing 25% polyethylene glycol and 25% glycerol) until further testing.

Immunohistochemistry: To visualize OT protein levels in the PVN immunohistochemistry was performed. After washing in PBS, sections were blocked in PBST (PBS with 0.05% Tween) supplemented with 5% BSA for 15 to 30 minutes. The sections were incubated in 1:10000 mouse OT antibody (MAB5296, Millipore) in PBST with 0.5% BSA at 4°C overnight. The following day, sections were washed in PBS before incubation in 1:400 anti-mouse 568 antibodies

(C755F84, Thomas Scientific) for 30 to 60 minutes at room temperature. Sections were then mounted in Fluoromount with DAPI, dried, and stored at 4°C.

Imaging: Immunofluorescent sections were imaged using a Keyence BZ-X800 microscope. At least six sections ranging from the whole anterior-posterior axis of the PVN were imaged per animal. Through imaging the PVN, the use of z-stacks at 20X magnification offers adequate resolution to quantify individual neurons. Images were categorized according to relative position along the anterior-posterior axis of the PVN based on the shape of the PVN. They were put into groups, or bins, of "anterior," "medial," and "posterior." ImageJ was then utilized to manually count OT-expressing cells while Keyence BZ-X800 Analyzer Hybrid Cell Count tool was also used to count cells and determine the average intensity of OT staining in each animal.

Statistics: The primary study objective was to determine a potential difference in OT protein levels between genotypes. All tests used a significance level of 0.05. Data was analyzed in R studio and visualized with the *ggplot2* package. Statistical tests utilized include ANOVA, Welch two sample t-tests, Shapiro-wilk tests, and Wilcoxon rank sum tests. An ANOVA test is used to analyze the difference between the means of multiple groups. The Welch two sample t-test compared the means of the cell count between the two genotypes. The Shapiro-Wilk test was used to test for normality of the computer cell count between genotypes, as well as fluorescent intensity between genotypes. If the data was not normally distributed, the Wilcoxon rank sum tests were used to compare the number and fluorescent intensity of OT-positive cells between genotypes.

Results

Immunohistochemistry to visualize OT-positive cells

After conducting immunohistochemistry to visualize OT-postive cells, we took a closer look to examine any differences in OT levels within subregions of the PVN. Based on appearance of the subregions and measured by relative bregma position, we divided the sections into three bins that run from the whole anterior-posterior axis of the PVN. The three bins are “anterior”, “medial”, and “posterior” respectively moving left to right, with examples of the relative shape underneath as seen in Figure 2. Once separated into the respective bins, cells were manually counted using the program Image J.

Quantification of OT-positive cell number

Figure 2 reports the cell count for each relative bregma position and split by genotype. We used ANOVA to test if the average number of OT-positive cells is different across the three PVN-bins; anterior, medial, and posterior. We did observe cell counts to differ across bins, with the medial bin containing the most cells ($P=0.004$, see Table 1). However, we did not find significant differences when comparing OT cell count between genotypes across the entire PVN (main effect), or when looking at the differences between genotypes within subregions (interaction effect).

To confirm the validity of our manual counting data, we turned to automated cell counting to quantify OT-cell number across the PVN and compare that to our manual count numbers, for which we used multiple t-tests to examine the different bins. This test showed no significant difference between the manual cell count and the Hybrid Cell Count computer

program numbers, with a p-value>0.05 for all bins (Table 2). From there, we ran a t-test looking at the automated cell count and genotype. The automated cell count confirmed results of the manual counting with respect to genotype across all bins (Figure 3, Table 3). A Shapiro test showed that the data was not normally distributed for the sections in the medial bin ($p<0.05$; Table 4), so a non-parametric Wilcoxon rank sum test was used. It showed no significant difference between genotypes in the number of OT-cells in the medial PVN (p-value>0.05; Table 5). Both the anterior and posterior bins showed normally distributed data with the shapiro test giving a p-value>0.05 (Table 4) so no further analysis was required. As a result of the data gathered, genotype differences do not significantly affect the number of OT-cells in the PVN, as visualized by immunohistochemistry.

Quantification of fluorescent intensity of OT-cells

While the number of OT-positive cells is not different between genotypes, it could be that OT levels within those cells are altered. To investigate this, we determined the fluorescent intensity of each OT-positive cell and compared these between genotypes for all of the PVN bins (Figure 5). The Welch two sample t-tests that were run for each bin showed no significant difference in fluorescent intensity between the genotypes ($p>0.05$; Table 6). Next, a Shapiro test was conducted (Table 7). For the medial bin, the intensity values were normally distributed, albeit that the Shapiro test was close to being significant ($p=0.06$; Table 7). To account for this, a Wilcoxon rank sum test was applied and returned a p-value greater than 0.05 (Table 8). This confirms that PVN OT levels are not significantly different between genotypes regardless of the relative bregma position of the sections.

Discussion

To our knowledge this is the only study looking at the difference in oxytocin protein levels in prairie voles of varying genotypes (CC and TT, Figure 3). Earlier, our lab found strong evidence for differences in *Oxtr* mRNA and associated protein levels depending on genetic background, and that this potentially impacts OT levels as well. Here, we found no evidence for a difference in CC compared to TT genotypes, as there were no significant differences in OT cell number or OT fluorescent intensity. We measured the fluorescent intensity of each cell to determine if there are differences in the levels PVN OT protein between CC and TT genotypes. While this showed no significant difference, this could be explained by a potential *ceiling effect*. It could be that very high levels of OT staining lead to saturated intensity levels, such that potential differences will not be registered. While care was taken to image OT-signal with appropriate exposure times, this could be one potential explanation for our results.

Another potential explanations for a lack of significance pertains to the OT mRNA itself. While we previously found differences in PVN *OT* mRNA between genotypes, it is possible that these differences are not translated to PVN OT protein levels, as we have observed in this study.

A third possibility is that the study on which this project was proposed could have had some limitations itself. This single-nucleus RNA-sequencing study used *Sim1* to identify PVN cells. These cells are known to produce OT and vasopressin (AVP). Within the previous study, cells that expressed *Sim1* included cells that expressed OT or AVP, indicating the that these cells were from the PVN. However, while *Sim1* expression was similar between genotypes, it is possible that there were differences in the amount of PVN tissue harvested between genotypes, which could have impacted the amount of OT mRNA.

A last possibility is another limitation that could have interfered with the data collected as well. While the Keyence BZ-X800 Hybrid Cell count program did show numbers similar to the manual count of cells within the PVN, the manual count could have been biased. As with any procedure that relies on human judgment, they are bound to contain a certain degree of error. The counting was conducted as unbiased as possible, and with collaboration to reach a consistent method. Some slides were more difficult to visualize and count, potentially leading to error. As mentioned, a consistent method was attempted as precisely and accurately throughout the entire process as possible.

Knowing the results of this study, there are ample future directions to further explore the enigmatic findings of differing OT levels in relation to varying OXTR density. In this study, the PVN was chosen in order to visualize the site of OT production. However, it is possible that the genotype associates with differences in OT levels in locations where it is released, such as the amygdala, ventromedial hypothalamus, brain stem, and nucleus accumbens, common areas that also contain OXTR (Lee et al. 2009). There is evidence that thyroid hormone is a physiological regulator of OT gene expression. In addition, the hormone stimulates the OT promoter activity through direct interaction with a component in the OT gene, a thyroid hormone-response element (Adan et al. 1992). Other studies have also implicated estrogen as a regulator of the OT gene promoter in humans (Richard et al. 1990). So, while we do not find differences in OT production, there may be alterations in OT release.

Furthermore, testing of the magnocellular and parvocellular neurons could be of interest. Based on the relative shape and grouping of bregma position, one could calculate the cell counts of the magnocellular and parvocellular neurons within the PVN. Differences in the OT protein levels within those neurons could show a relationship between the SNPs and those specific cells.

This could then link to specific behavioral aspects, as parvocellular neurons tend to release centrally, affecting fear responses in the amygdala or anxiety. In contrast, in magnocellular neurons, since they act more peripherally, they could have a greater influence on maternal bonding such as seen with lactation and parturition. While this study did not yield any significant results, it does open the avenue to look at other factors for what may cause higher levels of OT in voles with a CC genotype instead of a TT genotype

Tables and Figures

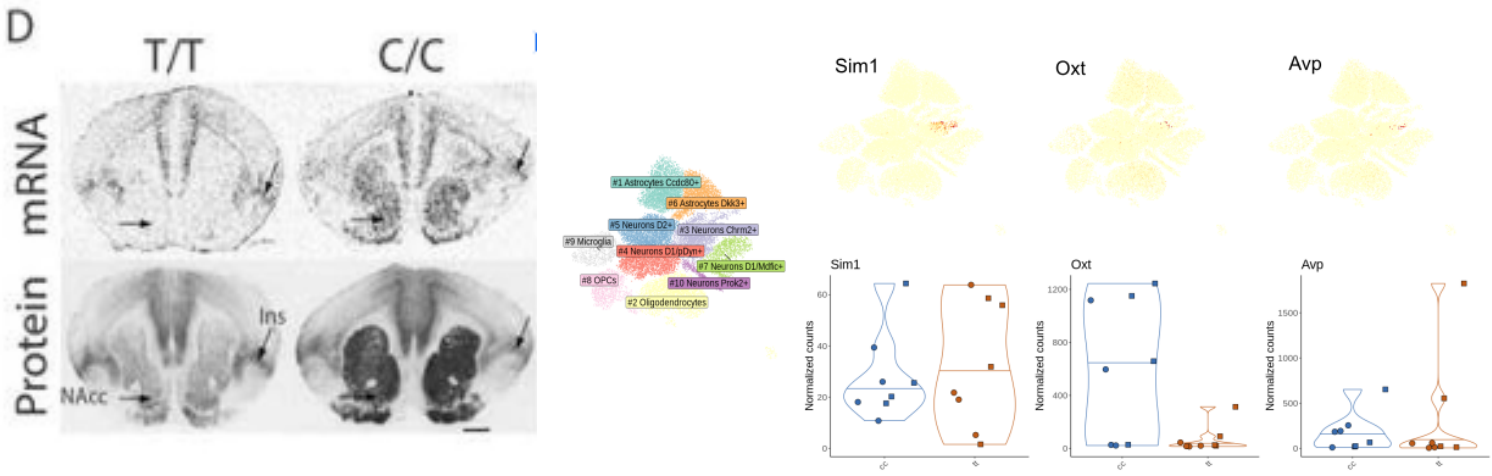

Figure 1: Variation of OXTR and OT mRNA. a. From King et al. 2016, shows individual variation in OXTR within prairie voles. Voles with the CC genotype show higher density of both OXTR mRNA and protein within the NAcc compared to voles with the TT genotype. b. Results from Sim1 experiment. Sim1 indicates presence of PVN cells, with OT and AVP expressing cells highlighted in red. Sim1 and AVP show equal levels between CC and TT genotypes, but OT mRNA is significantly higher in voles with the genotype CC compared to voles with the TT genotype.

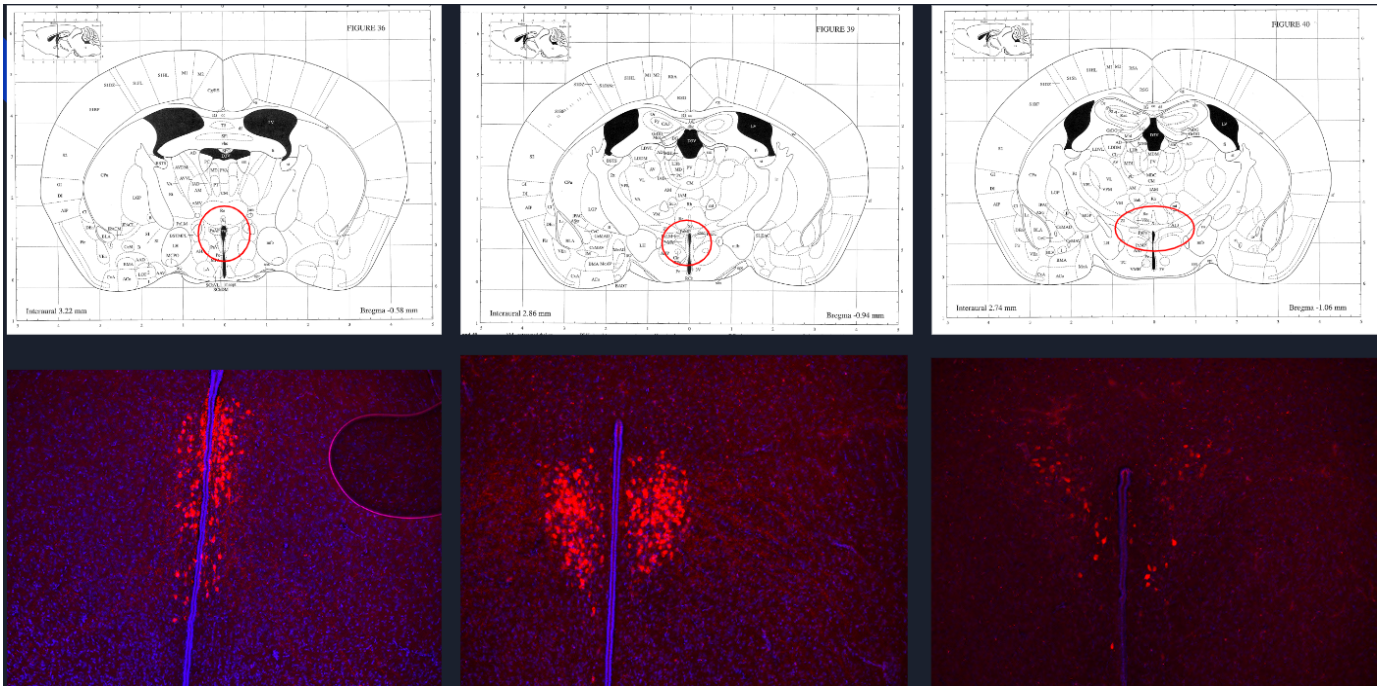

Figure 2: Example images of bregma position and shape. On the top, left to right, images show the relative bregma position of the bins “anterior,” “medial,” and “posterior.” On the bottom, example images of the visible shape of the PVN from immunohistochemistry data that correspond to the relative bregma position.

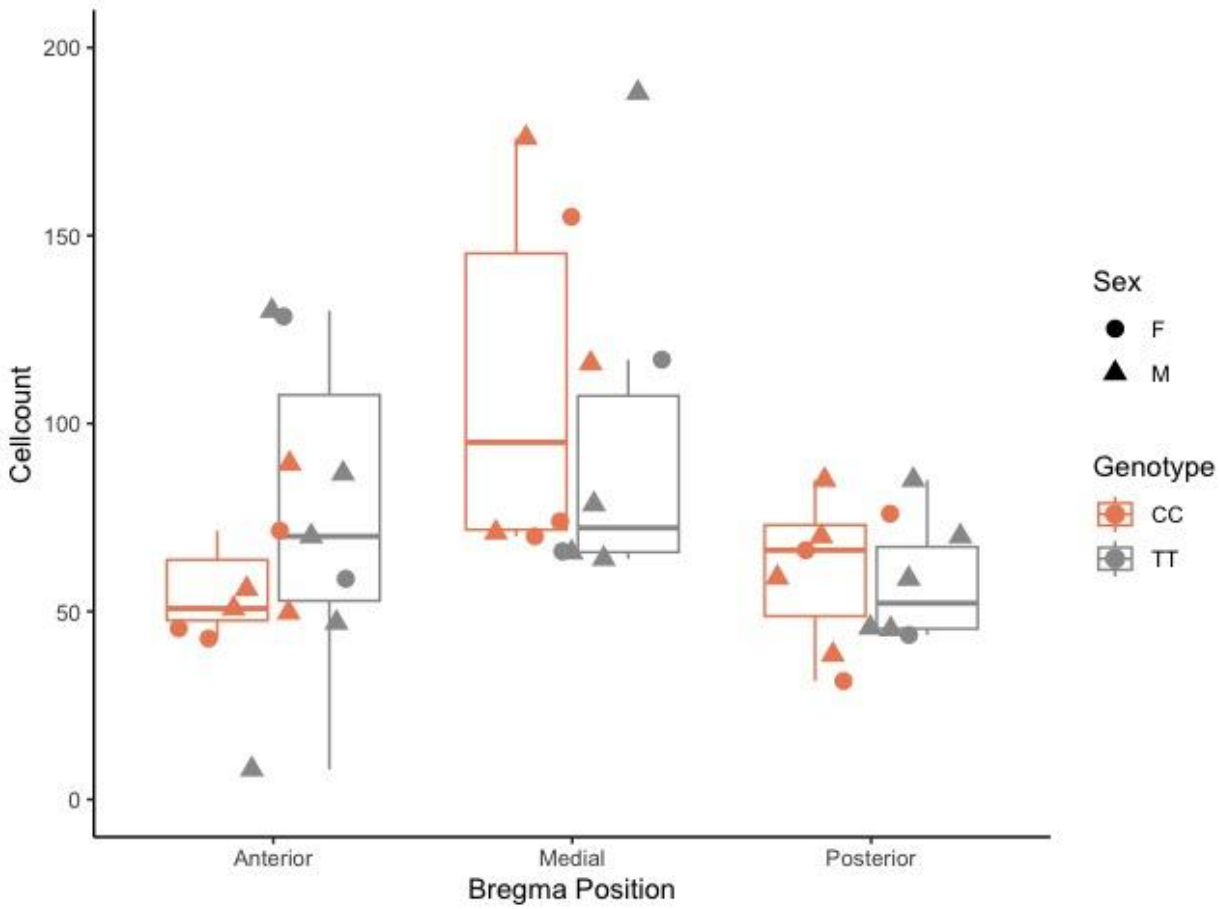

Figure 3: Cell count according to bregma position. This graph compares the cell count of each genotype according to relative bregma position. There appears to be a slightly higher count in the CC genotype of the medial group, with a $p<0.05$ (Table 1). The rest are not significantly different.

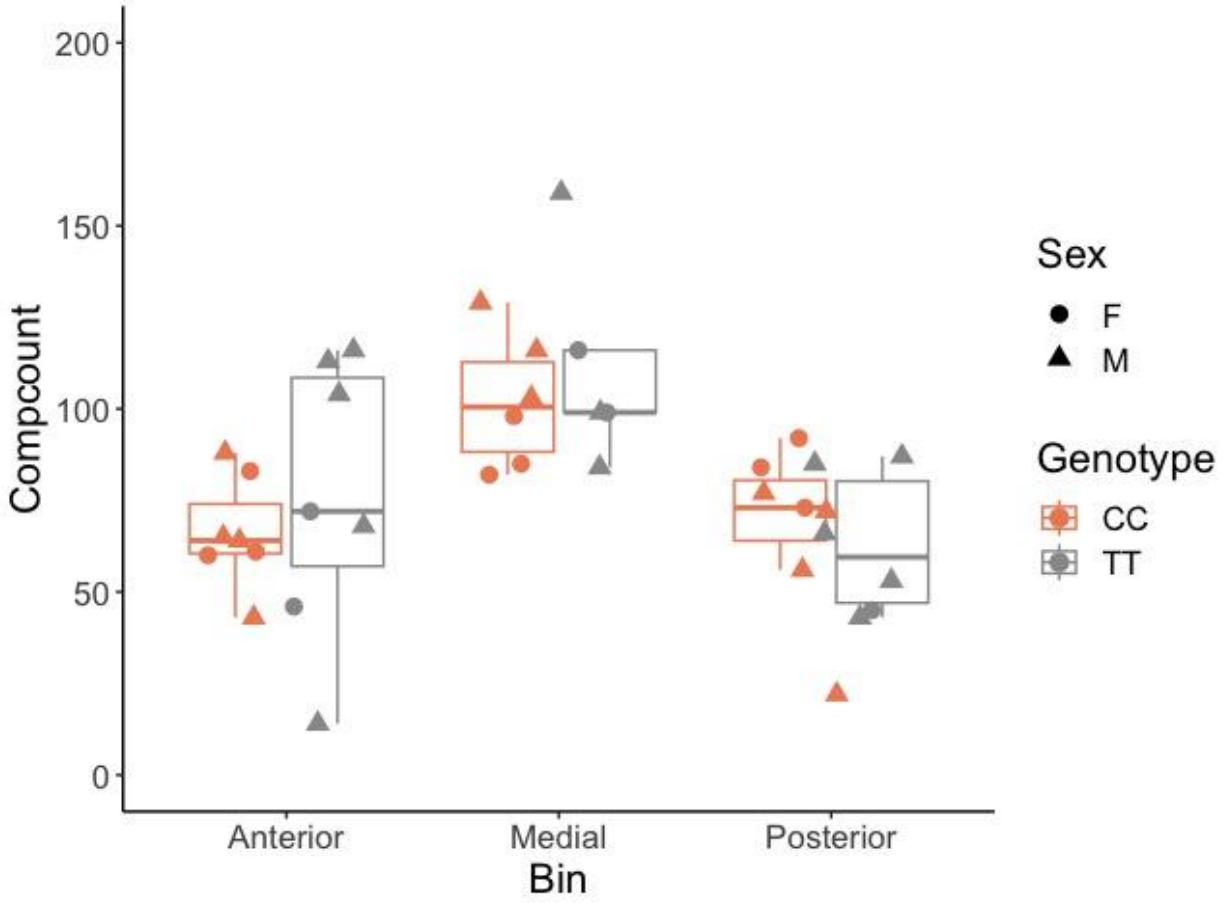

Figure 4: Computer cell count compared within bins. This graph reveals the cell count for sections in the Anterior, Medial, and Posterior group, split by genotype. The cell count was done using Keyence BZ-X800 Hybrid cell count computer program. There is no significant difference ($p>0.05$, Table 6).

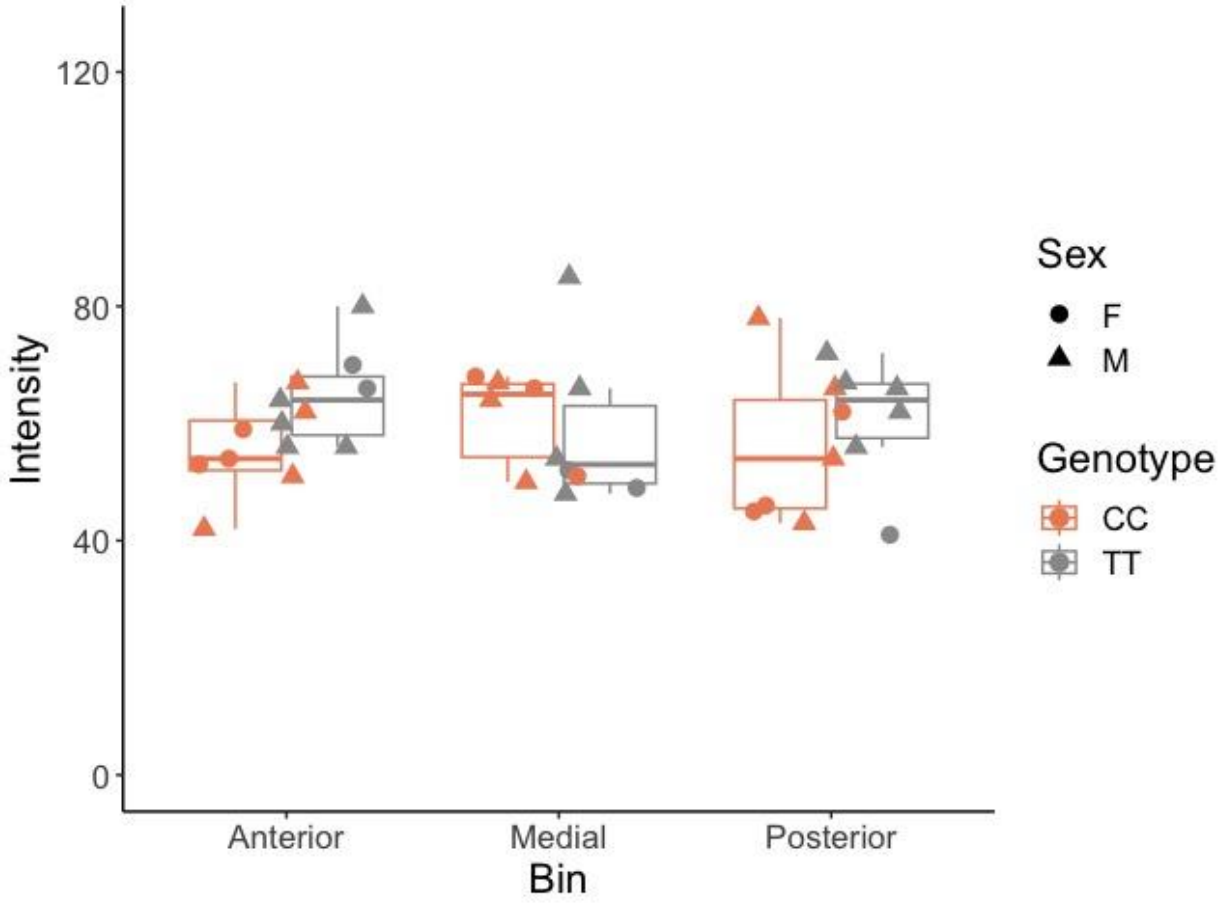

Figure 5: Intensity compared within bins. This graph shows the intensity of the fluorescence based on the genotype of each bin. Intensity of fluorescence on immunohistochemistry slides obtained through Keyence BZ-X800 Hybrid Cell count computer program. CCs show slightly higher mean, but not significant ($p>0.05$, Table 2-4)

Table 1: One-way ANOVA

Effect	Degrees of Freedom (numerator)	Degrees of Freedom (denominator)	F	p	ges
Genotype	1	9	0.194	0.670	0.009
Bin	2	18	7.460	0.004	0.327
Genotype:Bin	2	18	0.315	0.734	0.020

Table 2: Welch Two Sample t-test - Cell to Computer Count

	Mean Cell	Mean Computer	Difference	95% CI Lower	95% CI Upper	t	df	p-value
Anterior Bin Count	66.75	71.21	4.46	-28.41	14.48	-0.384	25.312	0.7044
Medial Bin Count	103.43	114.75	11.32	-46.59	23.96	-0.667	20.837	0.5118
Posterior Bin Count	59.60	65.77	6.17	-21.774	9.428	-0.818	23.355	0.4217

Table 3: Welch Two Sample t-test - Computer Count

	Mean TT	Mean CC	Difference	95% CI Lower	95% CI Upper	t	df	p-value
Anterior Bin Count	66.29	76.14	9.85	-45.446	25.732	-0.0641	7.8638	0.54
Medial Bin Count	127.33	102.167	25.66	-24.119	74.452	1.228	6.4556	0.2622
Posterior Bin Count	63.17	68	4.83	-21.174	30.84	0.4091	11	0.6904

Table 4: Shapiro-Wilk Normality Test - Computer Count

Computer Count by Genotype		
Anterior Bin	W	0.9621
	p-value	0.7572
Medial Bin	W	0.80849
	p-value	0.01172
Posterior Bin	W	0.9392
	p-value	0.4465

Table 5: Wilcoxon Rank Sum Test - Computer Count (Medial Bin)

Computer Count by Genotype (Medial Bin)	
W	12.5
p-value	0.4217

Table 6: Welch Two Sample t-test - Intensity

	Mean TT	Mean CC	Difference	95% CI Lower	95% CI Upper	t	df	p-value
Anterior Bin Intensity	65	55	10	-0.577	18.863	2.05	11.973	0.0629
Medial Bin Intensity	59	61	2	-17.53	13.53	-0.297	8	0.774
Posterior Bin Intensity	61	56	5	-10.281	19.043	0.657	11	0.524

Table 7: Shapiro-Wilk Normality Test - Intensity

Intensity by Genotype		
Anterior Bin	W	0.9916
	p-value	0.98396
Medial Bin	W	0.86962
	p-value	0.0646
Posterior Bin	W	0.9429
	p-value	0.4959

Table 8: Wilcoxon Rank Sum Test - Intensity (Medial Bin)

Intensity by Genotype (Medial Bin)	
W	22.5
p-value	0.5211

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