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EFFECTS OF SEX AND AUTISM ON OXYTOCIN RECEPTORS IN THE SUBSTANTIA NIGRA OF THE HUMAN BRAIN

By

Kip Dooley

Capstone submitted in partial fulfillment of the requirements for graduation with

UNIVERSITY HONORS

With a major in
Human Biology
in the department of Biology

Approved:

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Capstone Mentor	**Departmental Honors Advisor**
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Abstract

Oxytocin, a hormone present in the mammalian brain, has been shown to be a vital component of social function in animals and may have a role in the social deficits associated with Autism Spectrum Disorder in humans. Based on previous studies from our lab, there are oxytocin receptors in the human substantia nigra, a basal ganglia structure in the midbrain that is important in both movement and reward pathways. The substantia nigra contains two subsections that are defined by the neurotransmitters they contain: the pars compacta, which is dopaminergic, and the pars reticulata, which is GABAergic. By localizing oxytocin receptors in either the pars compacta or pars reticulata, we can infer the role of that region as it relates to social function. We previously attempted to identify the pars compacta using immunohistochemistry for tyrosine hydroxylase, but the background signal was too high to reliably be used to delineate the boundaries, so we are trying a new approach. We used Nissl staining, which has been shown to reveal dopaminergic neurons in the substantia nigra and has been used to distinguish the pars compacta from the pars reticulata. Once identified, we used the borders of the pars compacta to quantify oxytocin receptors within the substantia nigra in a neuroanatomically informed way by overlaying microscope images of tissue (with the pars compacta outlined) with the receptor autoradiographs, which visualize oxytocin receptors in the substantia nigra. The tissue was acquired from four distinct groups: eight typically developing (TD) males, seven TD females, eight males with Autism Spectrum Disorder, and seven females with Autism Spectrum Disorder. We analyzed the oxytocin receptor binding to determine the effect of sex and autism on oxytocin receptor density in the pars compacta. Females with ASD exhibited significantly reduced OXTR density when compared to both males with ASD and TD females, which may be related to differences in expression of symptoms between males with ASD and females with ASD. Future

directions of this research are aimed at defining the role of the oxytocin system in individuals with Autism Spectrum Disorder and how it relates to the social deficits present in those individuals.

Acknowledgements

First, I would like to thank my research and capstone mentor, Dr. Sara Freeman for the support and guidance she has offered throughout both the capstone process and my time in her lab. I am very grateful to have had the opportunity to be a part of her lab and contribute to the amazing work she does. Her abundant knowledge and willingness to guide me through the writing process has been invaluable to my capstone project as well as my academic writing abilities, and for that I can not thank her enough.

I would also like to acknowledge Michelle Palumbo, who sliced and mounted the brain tissue used in this project.

Lastly, I want to thank my incredible support system in my friends and especially my family. My family, particularly my mom, has been incredibly supportive throughout this process and was very willing to listen and offer support in conversations about my capstone project.

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Introduction

Oxytocin (OT) has been shown to play an important role in social function in animals and is known to modulate social behavior (Carter et al., 2020). Because of its relationship with social functions, OT is implicated in the biology of Autism Spectrum Disorder (ASD), which is a developmental neuropsychiatric condition that is characterized in part by social deficits. OT is a neurotransmitter that acts in the brain by binding to the OT receptor (OXTR). Examining the site of action of OT can provide insight into its function as it relates to ASD in both males and females. Analyses of OXTR density differences between TD and ASD specimens may reveal some of the mechanisms of ASD and its associated social deficits.

ASD is a complicated condition that is a major area of ongoing research and is characterized by three classes of symptoms. These include difficulty interacting socially, deficits in both verbal and non-verbal communication, and repetitive or harmful behavior patterns (Beuker et al., 2012). ASD predominantly affects males, who are four times as likely to be diagnosed with ASD than females, which may be due to neuroanatomical differences or underdiagnosis in females (Fombonne, 2009). In order to gain a better understanding of ASD, many studies, including this one, focus on differences in brain chemistry and anatomy between typically developing brain tissue and brain tissue from individuals with ASD.

The substantia nigra (SN) is a small structure in the midbrain that contains two anatomically interwoven yet functionally distinct subunits, the dopaminergic pars compacta and the GABAergic pars reticulata. The pars compacta is involved in reward pathways and fine motor control while the pars reticulata has an inhibitory effect on downstream neural targets when stimulated (Caputi et al., 2013; see also Hodge & Butcher, 1980). Previously, Paval (2017) proposed that autistic behavior is partially a result of abnormalities in the dopaminergic system

housed in the midbrain. Previous work from our lab has identified dense OXTR binding in the SN of the human brain but has not been able to identify which of these two subunits the signal resides in. Knowing the specific location of the OXTR within the SN and whether they are present in one or both subdivisions influences how we interpret the function of OT in that area.

Other studies, such as Jacob et al. (2007), provide support for a connection between the OXTR gene and ASD in certain populations. Ribeiro et al. (2018) identified two polymorphisms at the OXTR gene that may play a role in the diagnosis of ASD and its associated behaviors. Over a decade of clinical research has provided evidence for symptom improvement in ASD after treatment with intranasal OT, although some studies have reported no changes (Guastella et al., 2015 see also; Anagnostou et al., 2012). However, for an OT treatment to be effective, the OXTR system must be working properly. Freeman et al. (2018) found evidence for dysregulated OXTR in the ASD brain. However, like the other studies mentioned previously, this study did not evaluate sex differences or study the SN. While these studies have suggested that dysfunctions and differences in both dopamine and OT circuits are potential contributing factors to ASD, neuroanatomical support for these hypotheses is lacking. Our study expands on these results with a targeted design that explores OXTR density as it relates to both the neuroanatomy and sex of the individual based on ASD diagnosis.

The most common technique used to investigate neural OXTR is receptor autoradiography. In this technique, brain tissue sections are incubated in a radioactively-labeled ligand that binds to the receptor of interest. Once the radioligand binds to the receptors in the tissue, it emits radiation, which is detected by radiosensitive film that darkens in the pattern and density of receptor binding. The OXTR radioligand binds selectively in rodents, but has been shown to exhibit cross-reactivity with AVPR1a receptors in primate brain tissue. To address this

cross-reactivity, a pharmacologically-informed, competitive binding protocol for receptor autoradiography that selectively distinguishes OXTR and AVPR1A distributions in primate brain tissue was used that yields macroscopic grayscale images of receptor locations and densities (Freeman et al., 2018).

This study focuses on the pars compacta and potential differences in OXTR density that may be associated with ASD. Experimental groups include: males with ASD, females with ASD, and matched neurotypical male and female control specimens. This experimental design allows us the ability to directly assess the effects of ASD and sex on OXTR binding in the SN. We expect that ASD individuals will have lower amounts of OXTR in the SN than the TD controls. Despite the male-bias in ASD diagnosis, a significant difference does not exist in the overall severity of symptoms between males and females diagnosed with ASD (Werling & Geschwind, 2014). However, differences exist in how these symptoms are presented. For example, females with ASD tend to internalize their symptoms resulting in anxiety, depression, and other emotional symptoms while males externalize behavior problems like aggressive behavior, hyperactivity, and increased restrictive behaviors (Werling & Geschwind, 2014). These sex differences in presentation of symptoms may be related to differences in OXTR density between males and females with ASD and their matched TD controls.

Materials and Methods

Specimens

Unfixed, frozen blocks of postmortem human brain tissue were provided by the University of Maryland Brain and Tissue Bank, which is a Brain and Tissue Repository of the NIH NeuroBioBank. The provided tissue includes 30 specimens and contains tissue from four

distinct groups: TD males (n=8), TD females (n=7), males with ASD (n=8), and females with ASD (n=7).

Tissue Preparations

The unfixed, frozen blocks of de-identified human brain tissue containing the SN were stored at -80℃, brought to -20℃, sectioned at 20 μm on a cryostat, and mounted to Fisher Superfrost-Plus slides. Slides were sealed in a box with the addition of a desiccant and were stored at -80℃ until they were removed for receptor autoradiography.

Competitive-binding receptor autoradiography was performed to selectively reveal OXTR as described in Freeman et al., 2018. Until recently, locations of OXTR and AVPR1a, a structurally-related receptor, were not able to be dependably mapped with the use of the commercially-available radioligands alone, specifically ^{125}I-ornithine vasotocin analog for OXTR (^{125}I-OVTA) and ^{125}I-linearized vasopressin antagonist for AVPR1a (^{125}I-LVA) (Perkin Elmer, Waltham, MA). Because of the structural similarities between OXTR and AVPR1a, there is pharmacological cross-reactivity in this system (Song & Albers, 2017). When used in primate brains, these radioligands are now known to bind to both receptors (Freeman et al., 2014). In order to avoid the receptor cross-reactivity, our lab developed the first dependable method for visualizing OXTR and AVPR1a in the primate brain through the use of a modified form of receptor autoradiography, where the brain tissue is co-incubated with the radioligand and a selective competitor compound that blocks one of the receptor subtypes to reveal binding only to the receptor of interest. This approach has proven to be valid in the use of postmortem brain tissue from monkeys (Freeman et al., 2014a; see also Freeman et al., 2014b) and humans (Freeman et al., 2017) to show, selectively, either OXTR or AVPR1a. In the current study, we used this method of competitive-binding receptor autoradiography to specifically locate OXTR

binding in the SN of the human brain. After the assay, the slides were exposed to Carestream BioMax MR film (Kodak, Rochester, NY, USA) for 10 days and then developed.

Visualization of Dopaminergic Neurons

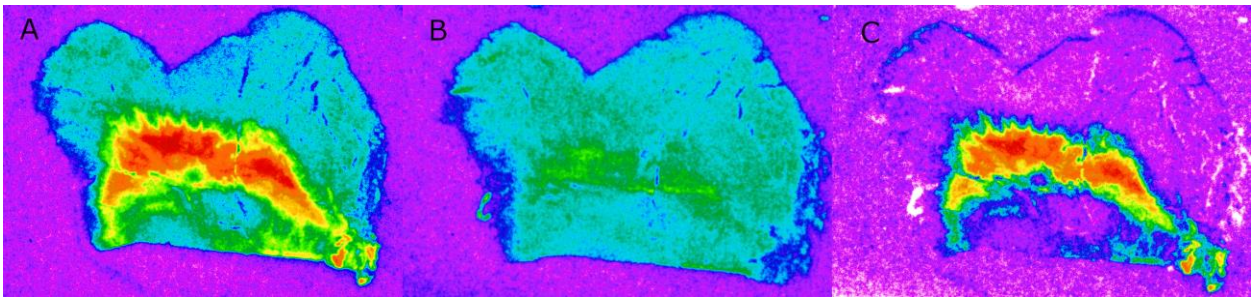
In order to identify the pars compacta, we initially attempted staining the tissue for tyrosine hydroxylase (TH) using immunohistochemistry (protocol adapted from Lonstein 2007) to visualize dopaminergic neurons. However, the background signal was too high to reliably use the TH stained sections in our analysis. Based on Domesick, Stinus, & Paskevich (1983), we know that dopaminergic neurons in the SN can be distinguished from GABAergic neurons when stained for Nissl substance using thionin. Fresh frozen 20 µm sections mounted to Fisher SuperFrost Plus slides were kept in 4% paraformaldehyde at 4°C for 1 week to fix the tissue. Slides were then dipped in deionized water and soaked twice in 50% chloroform and ethyl alcohol for 1.5 hours before being hydrated in descending concentrations of ethyl alcohol. The slides were dipped in 0.25% thionin then water before being dehydrated in ascending concentrations of ethyl alcohol and xylenes. Slides were coverslipped using CytoSeal 60 (Radnor, Wayne, PA, USA). The stained slides were examined using brightfield microscopy using a Keyence BZ-X800 (Keyence Corporation of America, Itasca, IL, USA) microscope. High resolution images of the Nissl-stained sections were used to anatomically determine the boundaries of the pars compacta and thus inform the analysis of the OXTR autoradiograms.

Quantification

Three representative sections of the SN from each specimen were quantified. For each section, images of radioligand binding remaining in the presence of the competitor were digitally subtracted from the corresponding image of total binding to yield an image that represented specific binding (Figure 1 A-C).

Figure 1: Total, nonspecific, and specific OXTR autoradiograms

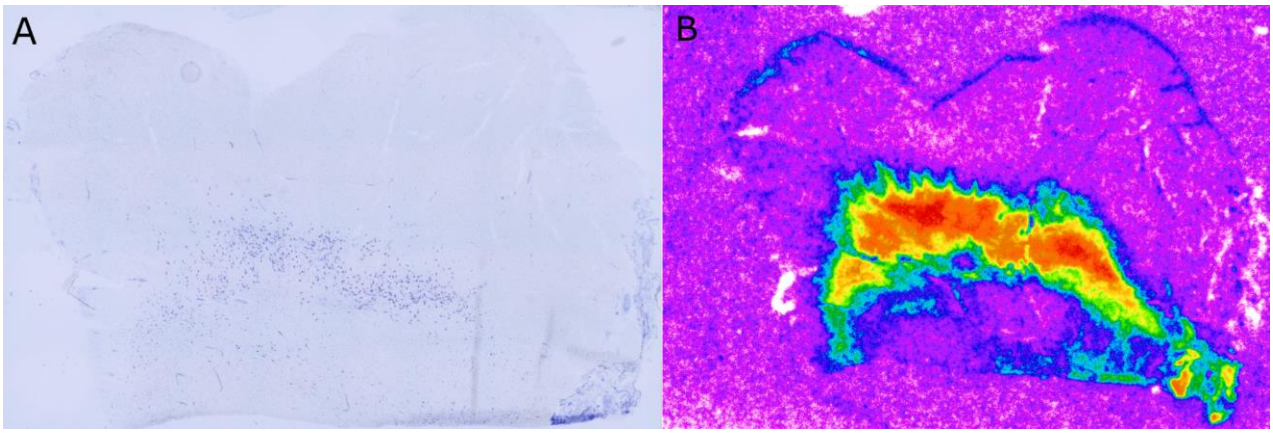
A. Autoradiogram showing total binding. **B.** Autoradiogram showing nonspecific binding. **C.** Autoradiogram showing OXTR specific binding (subtraction of **B** from **A**), which was used to measure OXTR binding densities.



Digital densitometry was performed on the specific OXTR autoradiograms using MCID Core to quantify the density of OXTR in the pars compacta. Images of Nissl stained tissue sections were placed side by side with the corresponding OXTR specific binding autoradiogram in order to accurately outline the pars compacta on the autoradiogram (Figure 2 A & B).

Figure 2: Nissl-stained section and specific binding autoradiogram

A. A Nissl-stained tissue section showing dark blue dopaminergic neurons of the pars compacta. **B.** The corresponding autoradiogram showing specific OXTR binding against background.



Statistical Analysis

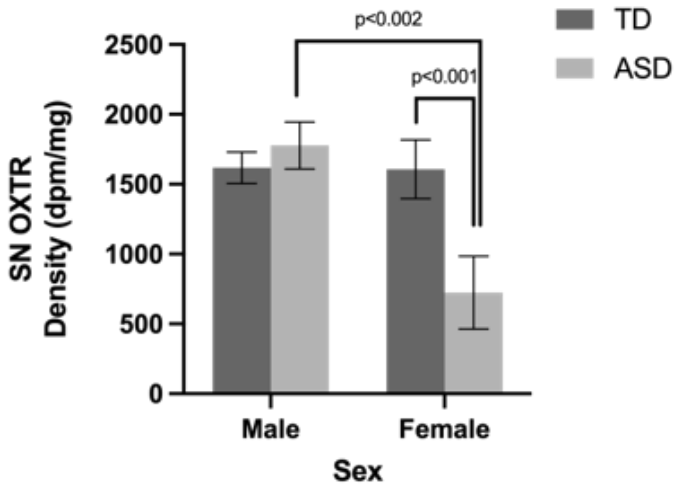
Statistical analyses and data visualization were performed in Graphpad Prism. A two-way ANOVA was used to determine whether there was a main effect of ASD or sex on OXTR density in the pars compacta and to identify a potential interaction effect between these two factors. Linear regressions were used to evaluate whether there was a correlation between OXTR binding and age and between OXTR binding and postmortem interval (PMI). An exploratory linear regression analysis on a subset of specimens was used to evaluate the potential relationship between OXTR density and ASD symptom severity by correlating OXTR binding with scores from the Autism Diagnostic Inventory-Revised (ADI-R) (Rutter, Lord, & LeCouteur, 2003). This psychiatric questionnaire is composed of three sections: Section A-Qualitative Abnormalities in Reciprocal Social Interaction, Section B (Verbal or Nonverbal)-Qualitative Abnormalities in Communication, and Section C-Restrictive, Repetitive, and Stereotyped Patterns of Behaviors. For each section, higher scores are indicative of greater impairment (Rutter & LeCouteur, 2003). For all tests, alpha was set to $p<0.05$.

Results

We found a main effect of sex ($F_{1, 23}=8.461$; $p<0.01$) and an interaction effect between sex and diagnosis ($F_{1, 23}=8.146$; $p<0.01$). The main effect of diagnosis trended toward, but did not reach, significance ($F_{1, 23}=3.926$; $p=0.0596$). A Šídák's post-hoc test for multiple comparisons revealed significant differences between females with ASD and males with ASD (adjusted

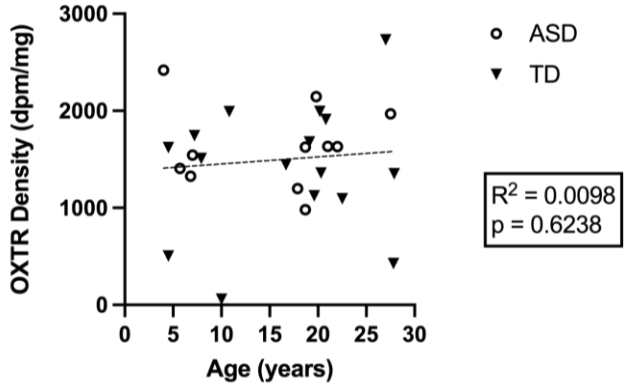
p=0.0012; Figure 3) and between females with ASD and TD control females (adjusted p=0.0093; Figure 3).

Figure 3: Effect of sex and ASD on OXTR density in the pars compacta of the SN



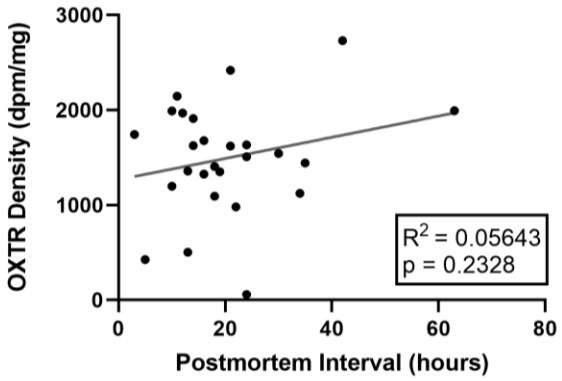
Because age has been found to impact OXTR binding in previous studies in human brain tissue (Freeman et al., 2018), we looked for an association between age and OXTR density in the pars compacta of the SN. We found no effect of age on OXTR density (Figure 4; $R^2 = 0.0098$, $p = 0.6238$).

Figure 4: Association between age and OXTR density in the SN



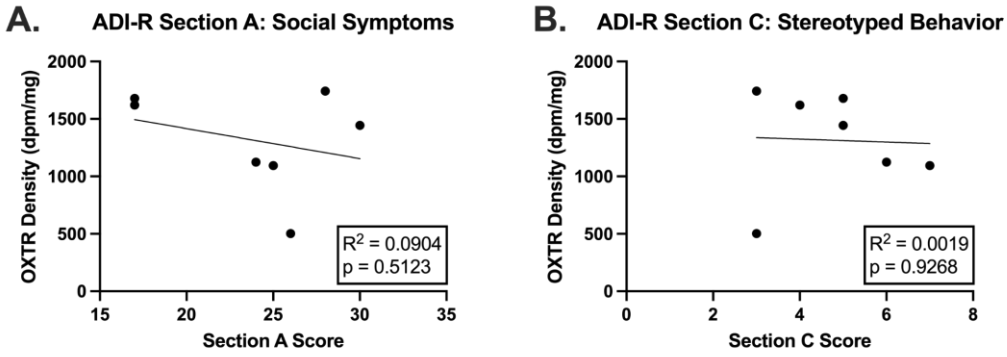
Because the brains of the donors in this study were removed at varying intervals after death, we looked for an association between postmortem interval (time between death and collection; PMI) and OXTR density. Although protein degradation after death is a concern that could reduce radioligand binding to our receptors of interest, we found no significant effect of PMI on OXTR density (Figure 5; R^2=0.0564; p=0.2328).

Figure 5: Association between postmortem interval and OXTR density in the SN



Expression of ASD symptoms can be evaluated using the ADI-R methods previously mentioned, which consist of three sections: A, B, and C. We looked for an association between severity of ASD symptoms, expressed in ADI-R score, and OXTR density. We found no significant effect of ADI-R score on sections A (Reciprocal Social Interaction) or C (stereotyped behavior) and OXTR density in the SN. Section B of the ADI-R test, which is not included in our study, evaluates verbal and nonverbal communication. Because OT has not been implicated in the early transition from nonverbal to verbal communication, we did not include it in this study.

Figure 6: Association between ASD symptom severity and OXTR density



Discussion

The current study is the first to evaluate whether OXTR differs in the SN of individuals with ASD compared to matched TD controls and whether sex differences exist in OXTR density. We found a main effect of sex and a significant interaction between sex and diagnosis. Females with ASD exhibited significantly reduced OXTR density when compared to both males with ASD and TD females, which may be related to difference in expression of symptoms between males with ASD and females with ASD.

Along with a male-bias, autism has been shown to produce unique symptoms in females compared to their male counterparts (Werling & Geschwind, 2014). As discussed previously, the severity of symptoms in males and females with ASD is not significantly different. However, females with ASD are more likely to internalize their symptoms, which is a stark contrast to the hyperactive and aggressive behavior typically seen in males with ASD (Werling & Geschwind, 2014). A recent study highlighting sex differences in ASD symptoms found that girls with ASD exhibit greater deviance from a sex-specific mean of standardized social scores when compared to boys with ASD (Lundström et al., 2019). These findings support the evaluation of ASD

symptoms and how those symptoms are presented from a sex-specific viewpoint that takes these differences into account. Our results provide a possible neurobiological explanation for the differences in presentation of symptoms between males and females, and may contribute to potential treatments of ASD in both males and females.

One method of treatment that has been explored is administration of intranasal OT to individuals with ASD. Bernaerts et al. (2020) found no significant improvements in 40 subjects that were administered nasal OT and that submitted follow-up questionnaires 24 hours, 4 weeks, and 1 year post-treatment. However, each of the 40 adult subjects involved in the study was male. Parker et al. (2017) found that intranasal oxytocin treatment led to improved social functions in children with ASD. 84% of the subjects in this study, though, were male, and the authors noted that their sample was not able to identify sex differences in response to treatment (Parker et al., 2017). Sex differences are emerging as important factors to consider in both the biology and treatment of ASD. Intranasal OT treatment, although promising in male children with ASD, should be further explored in females with ASD. Because of the reduced density of OXTR we found in females with ASD, potential treatment methods may differ in males and females with ASD.

Previous studies have found that age impacts OXTR binding in the human brain (Freeman et al., 2018). Among five brain regions investigated, Freeman et al. (2018) found that OXTR density was negatively correlated with age across all specimens, both TD and ASD. TD specimens also exhibited a peak of OXTR density in early life that was absent in ASD specimens (Freeman et al., 2018). Although an effect of age on OXTR density was found, that study did not include the SN. We looked for an association between age and OXTR density in the SN and found no significant association across all specimens. Our results support and expand on those

initial findings and indicate that the effect of age may be specific to certain regions of the brain, like the ventral pallidum.

In addition to a main effect of sex and an interaction between sex and diagnosis, we found multiple negative results worth noting. We found no association between PMI and OXTR density in the SN, as we hypothesized based on previous studies examining PMI and OXTR density. Our study supports the findings of Freeman et al. (2017) where effects of PMI were evaluated up to 33 hours post-death with no significant association. However, our specimens extend those findings and include PMIs above 60 hours in a novel region of interest in the SN while still exhibiting no significant association. We also found no effect of symptom severity on OXTR density in the SN.

Despite our results, it is important to acknowledge the limitations present in a subset of our statistical analysis. Although the sample size of our study is in line with other studies using postmortem brain tissue from clinical populations, the sample size of specimens that included Section A and Section C social scores (Figure 6) is limited. Because of this reduced sample size, further studies may be necessary to confirm a lack of correlation between ASD symptom severity and OXTR density in the SN.

Few studies examine sex-differences in ASD, and even fewer explore neuroanatomical differences between TD and ASD specimens. Our findings give insight into the known sex-differences in ASD symptom presentation and may provide a neurobiological basis for future exploration of ASD treatments involving OT and for future studies of ASD and how it relates to OT in the human brain. Single nucleotide polymorphisms (SNPs), or variations at a single nucleotide in a DNA sequence, present in the OXTR gene have been implicated in ASD (LoParo & Waldman, 2015). These DNA differences, in conjunction with our findings, support the need

for exploration of genetic mechanisms underlying ASD, which may be involved in the sex-differences in OXTR density between ASD females and ASD males, as well as differences between ASD females and TD females. Our lab plans to follow up this study by examining mRNA in the SN to determine whether the dopaminergic neurons of the pars compacta express OXTR.

Reflective Writing

Of my many experiences during my four years at Utah State University, tackling a research project in the lab of Dr. Freeman has been one of the most challenging and also one of the most rewarding. Despite experiencing multiple setbacks and readjustments, I was able to complete my capstone project and obtain exciting results. My capstone project is titled "Effects of Sex and Autism on Oxytocin Receptors in the Human Substantia Nigra" and is an intersection of my experiences in the classroom and lab with my career goal of becoming a physician.

I joined the Freeman lab at the beginning of my junior year at USU. Before joining Dr. Freeman's lab, I spent two years learning and practicing laboratory techniques as well as learning the importance of the research that takes place on campus and the impacts it has on life outside of the university. My work in the Freeman lab is a culmination of my classroom and course-associated lab experiences and is an appropriate capstone to my undergraduate education. Working on my capstone project has been a rare opportunity that has allowed me to apply a range of different skills including analytical thinking, hands-on laboratory techniques, written and verbal communication, design, and problem solving.

With my passion for medicine in mind, the topic of my capstone project is very relevant and timely. Because of Dr. Freeman's previous work, I was fortunate enough to be able to work with postmortem human brain tissue from clinical populations including those that had autism and those that were typically developing. Autism is a very prevalent yet mysterious condition that is an area of focus in many clinical studies. Although a reliable treatment for autism is not likely in the immediate future, our work is directly contributing to a growing field of knowledge surrounding autism. This study is the first to explore anatomical differences in the midbrain, and specifically the substantia nigra, of individuals with autism, and our results contribute to the understanding of the mechanisms underlying autism.

This project has helped me build a very strong relationship with my research mentor, Dr. Freeman, who is incredibly supportive of my education and future goals. One of the reasons this project has created such a meaningful relationship is the challenges and setbacks that we have faced throughout. Our initial plan was to automate the process in a way where we could write a macro, or script, in the program we used to produce our results and run images through the macro in batches, letting the computer do the work. However, that proved to be much more difficult than anticipated. After many brainstorming sessions and conference calls with software representatives, we decided that we were running out of time and that the only logical way forward was to do the analysis by hand, one image at a time. Before deciding to do the analysis manually, we had gotten so close so many times before finding a new problem that would halt the entire process. However, every time we hit a roadblock we were able to regroup and come up with another solution. Through this experience, Dr. Freeman also helped me understand that science is often very fluid, and the first approach is very unlikely to be the last as circumstances, methods, and ideas change.

Throughout my time in the Freeman lab I have presented research three times at USU at both the fall and spring research symposiums. When I arrived at the library for my first poster presentation, I was shocked by the magnitude of the event and by how many presenters there were. Every college in the university was represented and the research projects ranged from CRISPR-Cas9 gene editing to whether or not cats are friendlier than dogs. While I was aware of the research-oriented approach of Utah State, I did not understand that research was conducted to such an extent on campus. Getting to interact with dedicated researchers from so many disciplines offered a unique experience to engage with other motivated students, each working to make a difference in their respective field.

Although my capstone is not a hands-on service-oriented project, I believe that it has the potential to impact communities throughout the world. Autism is a very prevalent condition that is likely here to stay and is affecting more people every year. This project contributes to the understanding of the condition and provides insight into potential mechanisms that underlie the associated social deficits. Since little is currently understood of the neurobiology behind autism, every new finding presents opportunities to identify or create treatments that can alleviate some of the symptoms associated with the condition.

My honors capstone project has presented numerous challenges, but has led to tremendous growth both academically and personally. My capstone would not have been possible without the guidance and support of Dr. Freeman or collaboration with both students and professionals. Science is an interdisciplinary field where one person rarely has all the answers they need and where progress is not made alone. My research built on previous findings and I hope that others will look to my project as a starting point to further our collective knowledge and understanding of a condition that has affected nearly everyone in one way or another.

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