COMPARING THE DENSITY OF BETA-ADRENERGIC RECEPTORS IN THE PREFRONTAL CORTEX OF MALE AND FEMALE C57BL/6 MICE

by

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Abstract

Norepinephrine, a hormone and neurotransmitter, is primarily produced in a brain region called the locus coeruleus and is directly projected to the prefrontal cortex and other areas of the brain. This production and projection of norepinephrine are heavily involved in attention regulation. Norepinephrine regulates attention similarly in all mammals, but there are known sex differences in the noradrenergic system, such as differences in locus coeruleus volume and size. Sex differences found in the locus coeruleus may affect norepinephrine transmission to the prefrontal cortex and its regulation of attention behaviors. When norepinephrine reaches the prefrontal cortex, it can bind to alpha or beta-adrenergic receptors. This study compared the density of beta-1 adrenergic receptors in prefrontal cortices of male and female C57 black six mice. The hypothesis of this study was that female mice have a lower density of beta-1 adrenergic receptors in the prefrontal cortex than males, because they have a higher potential for norepinephrine release. To test this hypothesis, an RNAscope assay was used to detect beta-1 adrenergic receptor RNA in the tissue of the prefrontal cortex of both male and female mice, and the relative density of these receptors was quantified using ImageJ software. I found that males have more beta-1 adrenergic receptor RNA on average than females. These findings established the relative density of beta-1 adrenergic receptors within the prefrontal cortex in both sexes and provided a better understanding of attention regulation in the prefrontal cortex and its impact on a variety of psychiatric and neurodegenerative disorders.

Introduction

The noradrenergic system refers to the production of norepinephrine (NE) in the locus coeruleus (LC) and its projection to the prefrontal cortex (PFC) and other areas of the brain (Berridge et al., 2003; Figure 1). Because NE is projected to many different areas of the brain, the noradrenergic system is involved in a diversity of behaviors such as attention, arousal, learning, stress, and memory (Stone et al., 2011). Any differences in these behaviors may be attributed to differences in NE transmission, signaling, and integration in the brain (Xing et al., 2016; Fornito and Bullmore, 2015; Marien, Colpaert, and Rosenquist, 2004). However, disruptions in these processes have been linked to various psychiatric and neurodegenerative disorders such as attention-deficit/hyperactivity disorder (ADHD), autism spectrum disorder (ASD), schizophrenia, Alzheimer's disease, Parkinson's disease, and more (Cash et al., 1986; Nicholas et al., 1993). For the purposes of this thesis, I would like to focus on how the noradrenergic system regulates attention in the PFC, which has implications to many of these disorders, using ADHD as an example.

ADHD, also known as attention-deficit/hyperactivity disorder, is a behavioral disorder that has variable diagnostic rates worldwide, ranging from about 1% to 20% among children and 4% to 5% in adults (Faraone et al., 2003; Polanczyk et al., 2007; Wilens et al., 2009). ADHD is commonly characterized by symptoms of inattention, impulsivity, and hyperactivity (Faraone et al., 2003). If these symptoms are left untreated, they may persist into adolescence and adulthood, impacting an individual's way of life (Faraone et al., 2003).

ADHD is hypothesized to be regulated by the noradrenergic system (Biederman et al., 1999). Inattention and hyperarousal are often the result of altered LC firing patterns (Howells,

Stein, and Russell, 2012). LC neurons involved in attention regulation transition between tonic and phasic modes of activity, where an overly tonic LC means that the individual surveys more stimuli in their environment as compared to an overly phasic LC (Aston-Jones et al., 2000). LC activity is associated with NE release at target regions such as the PFC, and the binding of NE to noradrenergic receptors is involved in the regulation of attention (Xing et al., 2016).

NE regulates attention similarly in all organisms, but there are still sex differences in how this behavior is being regulated. More specifically, there are sex differences in the LC-NE system and likely its regulation of attention, but the extent of these sex differences is currently unknown. In healthcare, sex is an important factor for doctors to consider when they are treating patients, because differences in male and female physiology may lead to different responses to medications (Campesi, Fois, and Franconi, 2012). For this reason, understanding sex differences is important for the treatment of ADHD and nearly all other disorders. Because the noradrenergic system is heavily involved in attention, any sex differences in this system may result in differences in ADHD behavior as well as other cognitive and executive functions (Gaub and Carlson, 1997; Xing et al., 2016). ADHD is more commonly diagnosed in males than in females by approximately a 3:1 ratio, likely due to underlying sex differences in the noradrenergic system (Gaub and Carlson, 1997; Mulvey et al., 2018). There are also sex differences in levels of hyperactivity and inattention in children who have ADHD, with girls showing lower levels in both of these behaviors than boys (Gaub and Carlson, 1997). This means that if there are sex differences in ADHD behavior, there could also be sex differences in the neural mechanisms underlying that behavior.

Sex differences in the LC indicate that there may also be differences in the way that NE is being transmitted to the PFC (Pinos et al., 2001; Xing et al., 2016). NE acts on alpha and beta-adrenergic receptors, which are mostly found in the PFC (Barr et al., 2001; Figure 2). A previous study showed that NE binding to beta-adrenergic receptors has more of an effect on attention-related behaviors in rats than NE binding to alpha-adrenergic receptors, and therefore this thesis will investigate the role of beta-adrenergic receptors in the PFC. Because of previously found sex differences in attention-related behavior and the role of beta-adrenergic receptors in regulating this behavior, there may be sex differences in the density of beta-adrenergic receptors as well. More specifically, the hypothesis of this thesis was that female mice have a lower density of beta-1 adrenergic receptors in the PFC than males, because they have a higher potential for NE release.

In this thesis, I quantified beta-1 adrenergic receptors in three different subregions of the PFC that were posterior to, but still included: the anterior cingulate cortex (ACC), the secondary motor cortex (M2), and the primary motor cortex (M1) in male and female mice. The posterior sections of these regions were chosen because this study was a trial that aimed to establish parameters for the beta-1 adrenergic receptor that can be applied to a larger sample. The overall goal of this thesis was to compare the density of beta-adrenergic receptors in the PFC of male and female mice and to quantify any sex differences. This goal was only one specific part of a larger study regarding the extent of sex differences in the noradrengeric system and how these differences influence attention-related behaviors. The findings of this thesis provided valuable information about specific NE receptors that regulate attention. Targeting these receptors allowed

a better understanding of sex differences in the noradrenergic system, which could contribute to treating disorders, like ADHD, more effectively in both sexes.

Literature Review

Norepinephrine (NE), also known as noradrenaline, is a catecholamine hormone and neurotransmitter involved in attention regulation (Saboory et al., 2020). It is primarily produced in the LC, a brain region that directly projects NE throughout the cortex, with particularly strong innervation targeted to the PFC (Robertson et al., 2009; Figure 1). The PFC also innervates the LC in return, and has an excitatory influence on LC activity (Jodo et al., 1998). In addition to attention, the PFC is involved in regulating a variety of other executive behaviors, such as working memory and decision making (Benarroch, 2009; Xing et al., 2016). The region's functions are greatly mediated by NE, dopamine, and serotonin (Cerpa, Marchand, and Coutureau, 2019). Noradrenergic and dopaminergic receptor density on PFC cells may affect its regulation of executive and cognitive behaviors (Xing et al., 2016). These receptors are involved in excitation and inhibition of neurons in the PFC, and disrupting the balance between excitation and inhibition is associated with a variety of psychiatric disorders (Xing et al., 2016).

Known Sex Differences in Attention-Related Behavior in Rodents

While the PFC regulates similar behaviors in all organisms, there are still differences in how these behaviors are regulated in males and females (Roesch-Ely, 2009). For example, Bayless and colleagues found sex differences in attentional processes in adult Long-Evans rats performing the 5-choice serial reaction time task (Bayless et al., 2012). In this study, there were sex differences in errors of vigilance, which is defined as the ability to continuously allocate attentional resources to detect rare events, and inhibitory control, which is the ability to refrain

from making a premature or inappropriate response (Bayless et al., 2012). Both vigilance and inhibitory control are considered attentional processes, and are likely regulated by NE in the PFC (Bayless et al., 2012; Robbins, 2002). This suggests that there could be sex differences in the noradrenergic system as a whole. The goal of this project was to determine what these differences are, specifically in regards to NE receptor concentration in the PFC. Most studies that have investigated sex differences in the noradrenergic system have used animal models, notably rats. Because brain organization is conserved across all mammals, the sex differences found in rodent brains will likely be echoed in human brains.

Known Sex Differences in the Locus Coeruleus of Rodents and Humans

Previous studies have found differences, specifically in the LC, between male and female Wistar rats (Pinos et al., 2001). For example, in some rat strains, females have more LC cells and communication routes (Pinos et al., 2001; Bangasser et al., 2011). One study discovered that at 3 months, female Wistar rat pups already show a significantly larger LC volume than male rat pups (Guillamón et al., 1988). In a different study, researchers found that LC dendrites in female Sprague-Dawley rats have a more complex pattern of branching, which shows that the LC of these female rats may be structured to receive and process more information compared to their male counterparts (Bangasser et al., 2011). In humans, there are differences in the total number of LC cells between men and women (Busch et al., 1997; Ohm et al., 1997). In two separate studies involving degradation of the locus coeruleus in patients with aging and Alzheimer's disease, Busch and colleagues were able to find a baseline number of LC cells from healthy adult males and females. The average number of LC cells in men is approximately 15,731 cells, whereas the average number of LC cells in women is approximately 18,307 cells (Busch et al.,

1997; Ohm et al., 1997). This means that similarly to female rats, women have a more dense LC than men, because they have more LC cells on average.

These differences are just a few examples of sex differences in the LC in both rats and humans. Understanding sex differences in the noradrenergic system as a whole is crucial for understanding differences in vulnerability to disorders caused by dysfunction in the noradrenergic regulation of the PFC (Bangasser et al., 2011). Many diseases are more commonly diagnosed in one sex over the other, like ADHD, which is more common in males than in females (Mulvey et al., 2018). The sex differences found in the LC may affect NE transmission to the PFC, and its regulation of attention behaviors. For example, female rats have more LC cells than male rats, and if there are more LC cells producing NE and releasing it in the PFC, then this sex difference in NE production and its projection to the PFC may be underlying the observed sex differences in attention behavior (Pinos et al., 2001).

Alpha and Beta-Adrenergic Receptors

NE can have a variety of effects in the PFC depending on the receptor it binds to. NE is primarily targeted to alpha (subtypes 1 and 2) and beta (subtypes 1, 2, and 3) adrenergic receptors (Barr et al., 2001; Figure 2). These receptors are found all throughout the PFC and have a role in helping individuals maintain attention (Nicholas et al., 1993; Barr et al., 2001). Alpha and beta receptors are coupled to guanine nucleotide regulatory proteins, also known as G proteins, and generally have opposite effects (Kobilka et al., 1988). Alpha-adrenergic receptors generally couple to G_i proteins, which are inhibitory and therefore have inhibitory effects (Kobilka et al., 1988). More specifically, alpha-1 adrenergic receptors can decrease activity in the

cell that they're acting on, whereas alpha-2 adrenergic receptors can increase cellular activity (Dinh et al., 2009; Simson and Weiss, 1987).

In contrast, beta-adrenergic receptors generally couple to G_s proteins, which are stimulatory (Kobilka et al., 1988). More specifically, beta-1 and beta-2 adrenergic receptors increase cellular activity, whereas beta-3 adrenergic receptors may decrease cellular activity, but this is still unknown (Engelhardt et al., 2001; Johnson, 2006; Hayward, Mueller, and Hasser, 2004). Beta-1 adrenergic receptors are more highly concentrated in the PFC as compared to other receptor subtypes (Rainbow et al., 1983; Ramos and Arnsten, 2007). Although NE has a lower affinity for beta-adrenergic receptors as compared to alpha-adrenergic receptors, beta-adrenergic receptors may be more involved in regulating attention-related behaviors, such as extinction learning (Ramos and Arnsten, 2007; André et al., 2015).

Alpha and Beta-Adrenergic Antagonist Drugs

Propranolol, a non-selective beta-adrenergic receptor antagonist drug, is widely used to treat a variety of diseases including ADHD, and those related to anxiety, stress, and schizophrenia (Ananth and Lin, 1986; Fitzgerald, 2015). In contrast, prazosin is a drug that blocks alpha-1 adrenergic receptors and is primarily used to treat symptoms of posttraumatic stress disorder (PTSD), hypertension, and heart failure (Fitzgerald, 2015; Koola et al., 2013; Colucci 1982). A previous study found that propranolol administration leads to decreased performance in a decision making task. In the same study, prazosin had less of an effect on performance. This data suggests that NE binding to beta-adrenergic receptors has more of an effect on attention-related behaviors than NE binding to alpha-adrenergic receptors. Therefore,

this study will focus on beta as opposed to alpha-adrenergic receptors for insights on how the noradrenergic system regulates attention differently in male and female mice.

Methods

Mouse Brain Collection and Brain Tissue Preparation

Mice were terminally anesthetized with Ketamine/Xylazine and the brains were extracted. Within 2 to 3 minutes of decapitation, 1 male and 1 female brain from C57 black six (C57BL/6) mice were removed and immediately frozen for 20 seconds in -80°C isopentane. The brains were then wrapped in aluminum foil, sealed in a zippered plastic bag, and stored at -80°C.

The brains (bregma A/P +0.5 to +2.5) were cut into 16 μm thick sections. These sections were dry-mounted directly onto Superfrost Plus slides. The slides were then stored at -80°C until the RNAScope process.

RNAScope Preparation

On the day of staining, the oven was set to 40°C. A wash buffer was diluted by mixing 10 mL of wash buffer and 490 mL of milli-Q H_2O. The wash buffer was equilibrated to room temperature in a water bath for about 10-20 minutes at 40°C before dilution. All tools in the area were cleaned thoroughly with RNAse Away. Filter paper was placed into a humidifying box with a thin layer of milli-Q H_2O on top. The Multiplex Fl v2 Amp 1, 2, 3, and 4, and HRP C1, 2, and 3, were equilibrated to 40°C in a water bath. The probes used in RNAScope were prepared by warming in a water bath at 40°C for 10 minutes, and then cooling to room temperature.

RNAScope Procedure

3 frozen slides (positive control, negative control, and the beta-1 adrenoceptor probe) were taken from -80°C, placed into a dunking slide holder, and submerged in ice cold formalin for 15 minutes to keep the tissue from degrading over time. After 15 minutes, the slides were rinsed twice with 0.1M phosphate buffer (PB), and placed on a paper towel to absorb excess liquid between each rinse. The slides were then dehydrated with 50% ethanol alcohol (EtOH) for 5 minutes at room temperature. This process was followed by additional dehydrations using 70% EtOH and 2 rounds of 100% EtOH, each for 5 minutes at room temperature. The slides were allowed to air dry after this dehydration process. Select sections of the tissue were then outlined with a barrier pen and allowed to dry for approximately 1 minute.

2 drops of hydrogen peroxide (H_2O_2) were added to cover each section of tissue entirely. The tissue was incubated at room temperature in a humidified box for 10 minutes. The remaining H_2O_2 was decanted onto a paper towel and the slides were washed twice with milli-Q H_2O at room temperature. 5 drops of protease III were added to permeate the tissue and the tissue was left to settle for 15 minutes at room temperature. After 15 minutes, the remaining protease III was decanted onto a paper towel. The slides were then washed twice with 0.1M PB at room temperature. Following this wash, the probes were added to each of the sections to target the RNA, and the sections were incubated for 2 hours at 40°C in the oven.

After 2 hours of incubation, the remaining probes were decanted onto a paper towel. The slides were washed twice in the wash buffer at room temperature. The excess liquid was decanted after 2 minutes. 4 drops of Multiplex Fl v2 Amp 1 were added to cover the sections, and the slides were incubated for 30 minutes at 40°C in the oven. After 30 minutes, Amp1 was

decanted and the slides were washed twice in the wash buffer. Then, any remaining wash buffer was decanted. This process was repeated for Multiplex Fl v2 Amp2, and Multiplex Fl v2 Amp3. The purpose of the addition of Amp1, 2, and 3 was to strengthen the probe signals.

HRP-conjugated signals were developed by adding approximately 4 drops of Multiplex Fl v2 HRP-C1 to cover each section. The slides were incubated in the oven at 40°C for 15 minutes. The slides were decanted and washed twice in the wash buffer at room temperature for two minutes. Diluted Amp4 with green fluorescent dye was added to each section, and the slides were then incubated in the oven at 40°C for 30 minutes. After incubation, the decanting process was repeated. Approximately 4 drops of Multiplex Fl v2 HRP blocker was added to cover each section, and the slides were incubated in the oven at 40°C for 15 minutes. Following incubation, the decanting process was repeated. This process to develop HRP-conjugated signals was repeated for Multiplex Lf v2 HRP-C2 , and Multiplex Lf v2 HRP-C3. A coverslip was then added to the slides using a mounting medium, and they were stored in the fridge in the dark.

ImageJ Analysis

Images of the ACC, M2 and M1 regions of the stained brain tissue were taken using a Zeiss Epi-Fluorescence microscope at 10x zoom, and ImageJ was used to analyze how much RNA staining there is in these subregions of both the male and female prefrontal cortices (Figure 3). Prior to analysis, each colored RNAscope image was converted to an 8-bit image. Then, the brightness and contrast of each image was adjusted to help visualize and prep the regions of interest for analysis. A threshold was set to target the regions of interest, making these regions white against a black background (Figure 4). The Analyze Particles function on ImageJ was used to obtain a count of how many particles are on the image.

A particle size range of 6.00 to 18.00 pixels was used to omit background noise in the analysis. This range was obtained prior to the analysis by measuring the size (in pixels at 300% zoom) of 50 individual particles of beta-1 adrenergic receptor RNA on a colored image of the stained tissue. The smallest and largest sizes across all the particles provided a range that was then expanded to +/- 2 standard deviations. Each particle counted represented 1 beta-1 adrenergic receptor RNA. The total particle count was averaged across each PFC region, and this average was the relative density of beta-1 adrenergic receptor RNA. Other measurements included the total area of the regions of interest ($pixels^2$) and the average size of the regions of interest (pixels). The figures outlining the results of this analysis were made using Prism software.

COVID-19 Impact Statement

Originally, I had planned to collect data from a sample of 4 male and 4 female Long-Evans rats and perform a thorough analysis of this data. However, this was no longer possible due to the COVID-19 pandemic, because I was restricted from entering the lab and troubleshooting RNAscope. Therefore, I analyzed preexisting pilot images of 1 male and 1 female brain from C57 black six mice for this thesis. The limited data set is a shortcoming of this thesis, and in the future, more data would be obtained from a larger sample.

Results

I analyzed pilot RNAscope images of 1 male and 1 female brain from C57 black six mice, looking at the ACC, M2, and M1 regions of the PFC. Due to symmetry in the brain, I was able to collect two data points for each sex and subregion, one for each hemisphere, and

averaged the results that represented a quantification of beta-1 adrenergic receptor RNA. This analysis established parameters for future use in a larger scale study with a greater sample size.

Sex and Subregion Differences in Beta-1 Adrenoceptor RNA

I found an overall sex difference in beta-1 aderenrgic receptor RNA across the regions of the PFC that were assessed (Figure 5). This means that there are differences in the density of beta-1 adrenergic receptor RNA between males and females in the ACC, M2, and M1 regions of the PFC. After quantifying an average of beta-1 adrenergic RNA puncta across two hemispheres per animal, females had fewer RNA puncta compared to males. More specifically, in the ACC, males had 300.5 RNA puncta on average whereas females had 147 RNA puncta on average; in the M2 region, males had 184 RNA puncta on average and females had 142 RNA puncta on average, and in the M1 region, males had 181.5 RNA puncta on average and females had 86 RNA puncta on average (Table 1). Overall, males had more beta-1 adrenergic receptor RNA compared to females across all of the PFC subregions assessed, but this was most apparent in the ACC (Figure 5).

In addition, I also found an overall subregion difference in beta-1 adrenergic receptor RNA across all the regions of the PFC that were looked at (Figure 5). This indicates that the differences in average beta-1 adrenergic receptor RNA puncta between males and females was observed among all of the subregions of the PFC that were looked at, and was not specific to only one subregion.

Discussion

In this thesis, I investigated if there were sex differences in the density of beta-1 adrenergic receptors in three different subregions of the PFC in mice. Overall, males had more beta-1 adrenergic receptor RNA puncta than females. If there is more beta-1 adrenergic receptor RNA puncta in males, they would have a higher beta-1 adrenergic receptor density. This result was consistent with my hypothesis that females have a lower density of beta-1 adrenergic receptors in the PFC than males.

Based on this result, NE may be more likely to bind to a beta-1 adrenergic receptor compared to an alpha-adrenergic receptor in the male brain because there is a greater quantity of them. The lower density of beta-adrenergic receptors in the female brain suggests that perhaps, NE is more likely to bind to alpha-adrenergic receptors as opposed to beta-adrenergic receptors in the female brain. This finding is also consistent with previous data that showed that males are more affected by propranolol, a beta-blocker, compared to females. This makes sense because there are more beta-1 adrenergic receptors, so it shows that beta-1 adrenergic receptors may have a larger role in attention regulation in males. In addition, the male brain may have more potential for excitation than the female brain, because beta-adrenergic receptors generally couple to G_s proteins, which are stimulatory and can increase activity in the cell (Kobilka et al., 1988). This may relate to the Yerkes-Dodson relationship in the LC-NE system, which suggests that optimal performance on tasks is associated with an intermediate level of arousal (Aston-Jones and Cohen, 2005). In other words, the male brain may need more excitation to reach the optimal level of arousal, which is the reason why there are different densities of beta-1 adrenergic receptors in the male and female brain.

I also found a subregion difference in beta-1 adrenergic receptor RNA across the three regions of the PFC that were looked at. The subregions of the PFC that were investigated in this study included the ACC, M2, and M1 regions. This sex difference of beta-1 adrenergic receptor RNA density was most apparent in the ACC. As a whole, the ACC is involved in higher-order cognitive processes like emotion, impulse control and decision-making, whereas M2 and M1 are regions that are more involved in movement (Bush, Luu, and Posner, 2000; Cao et al., 2015). Therefore, the differences in the density of beta-1 adrenergic receptors may be related to the differences in function of these regions. This finding is also consistent with a theory that there is increased NE receptor density in brain regions that are closer to the midline (Cash et al., 1986; Palacios and Kuhar, 1980). Based on this theory, the ACC would have more receptors because the ACC is closer to the midline than the M2 and M1 regions are. Males and females also exhibit differences in behaviors that are regulated by the ACC, and these findings suggest that perhaps these differences in behavior may also be attributed to differences in the density of beta-1 adrenergic receptors (Xing et al., 2016).

My results indicate that there could be a sex difference in the relative density of beta-1 adrenergic receptors in the PFC of mice, and this may be a contributor to the differences in attention regulation in males and females. The next step is to further investigate these differences by obtaining more data and running statistical analyses. However, a future direction for this study, and this topic, could be to investigate if this sex difference in the density of beta-1 adrenergic receptors is present in other areas of the brain, because NE is projected throughout the cortex (Robertson et al., 2009). The cortex is responsible for a variety of behaviors, like memory, emotion, hormonal regulation, processing and interpreting sensory information and planning

motor tasks, and beta-adrenergic receptors have a role in many of these behaviors (Sansom and Livesey, 2009; Cahill et al., 1994). Therefore, it would be interesting to see if this sex difference is present in other regions of the brain that are primarily involved in functions other than attention regulation.

Another future direction for this study could be to examine the specific cell type that the beta-adrenergic receptors are on. In the PFC, there are both excitatory and inhibitory interneurons (Ährlund-Richter et al., 2019). There are also pyramidal cells, which are the most common type of neuron in the cortex, and the main source of excitatory synapses (Elston et al., 2011). When NE binds to beta-adrenergic receptors on these cells, cellular activity may increase and this increase may affect activity in the PFC and the noradrenergic system as a whole (Kobilka et al., 1988; Nicholas et al., 1993). However, the effect that occurs in the PFC is dependent on the cell type that the beta-adrenergic receptors are acting on. For example, if a cell normally engages in inhibitory activity, activated beta-adrenergic receptors would increase this inhibition. On the other hand, if a cell is normally excitatory, activated beta-adrenergic receptors would increase this excitation. Examining whether there's a sex difference in beta-adrenergic receptor density on interneurons versus pyramidial cells would provide a better understanding of its effects in the NE system in the PFC and the behaviors it regulates in both sexes.

The findings of this study allowed the relative density of the beta-1 adrenergic receptor within the PFC to be established in both sexes and these trends in density could be expected in a larger study. These findings may also contribute to a better understanding of attention regulation in the PFC that can impact a variety of psychiatric disorders and neurodegenerative disorders. While this study provides new insights on sex differences in the neural mechanisms of attention

regulation, further research is needed to figure out the extent of sex differences in the noradrenergic system.

Limitations

One limitation of this study included small sample size. All of the images taken were from one male mouse brain and one female mouse brain. This largely affected the data that I was able to collect, and in the future, data would be obtained from a larger sample size. More data would allow me to run reliable analyses with high statistical power. Another limitation included the data collection method using ImageJ. While a size range was set to eliminate background noise, the threshold on ImageJ was not able to pick up all of the RNA particles on the image. This was dependent on how bright the stain was for each RNA particle. However, this should have not had a large effect on the results of this study.

Acknowledgements

Throughout this process, I have received so much support and would like to thank everyone who was involved in helping me complete my thesis.

I would like to first thank the Principal Investigator of the lab and my committee chair, Dr. Elena Vazey, as well as committee member Dr. Heather Richardson, for providing me with useful insight and feedback. I would also like to thank my mentor, Emma Dauster, for helping me through everything.

Appendix

Abbreviations:

ADHD (attention-deficit/hyperactivity disorder)

ACC (anterior cingulate cortex)

A/P (anterior/posterior)

EtOH (ethanol)

HRP (horseradish peroxidase)

LC (locus coeruleus)

M1 (primary motor cortex)

M2 (secondary motor cortex)

NE (norepinephrine)

PB (phosphate buffer)

PFC (prefrontal cortex)

RNA (ribonucleic acid)

Figures:

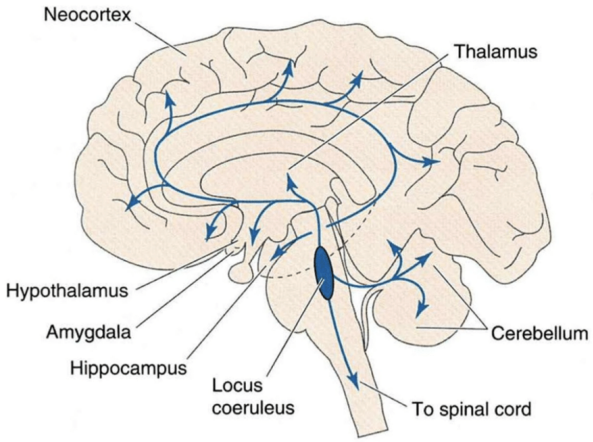

Figure 1. The noradrenergic system. This figure shows an overview of the noradrenergic system. The blue arrows indicate where in the brain norepinephrine is projected to after its production in the locus coeruleus. Image adapted from Lin, H. and Vartanian, O. (2018). *Projections of the locus coeruleus-norepinephrine (LC-NE) system.* [Figure]. ResearchGate. https://www.researchgate.net/profile/Hause-Lin/publication/325625804/figure/fig1/AS:634881906335756@1528379050789/The-Locus-Coeruleus-Norepinephrine-LC-NE-System.png

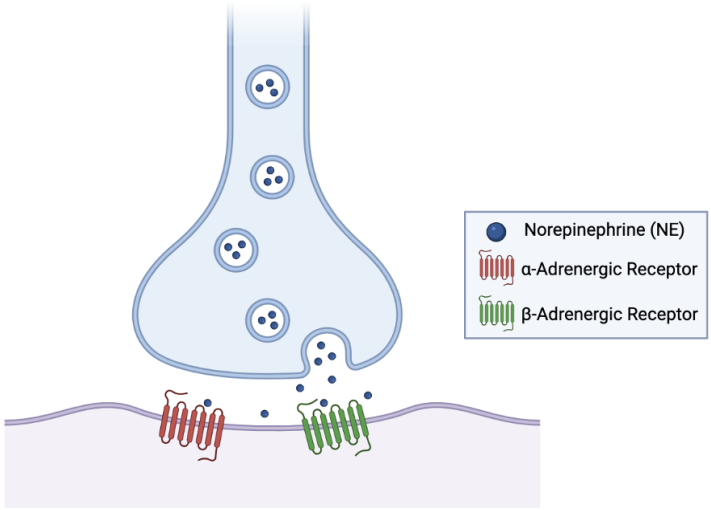

Figure 2. Alpha- and beta-adrenergic receptors in the prefrontal cortex. This figure illustrates norepinephrine being released from a presynaptic neuron and binding to either an alpha-adrenergic receptor or a beta-adrenergic receptor on a postsynaptic neuron in the prefrontal cortex. Alpha-adrenergic receptors (shown in red on the left) generally couple to G_i proteins, which are inhibitory and can decrease activity in the cell, whereas beta-adrenergic receptors (shown in green on the right) generally couple to G_s proteins, which are stimulatory and can increase activity in the cell.

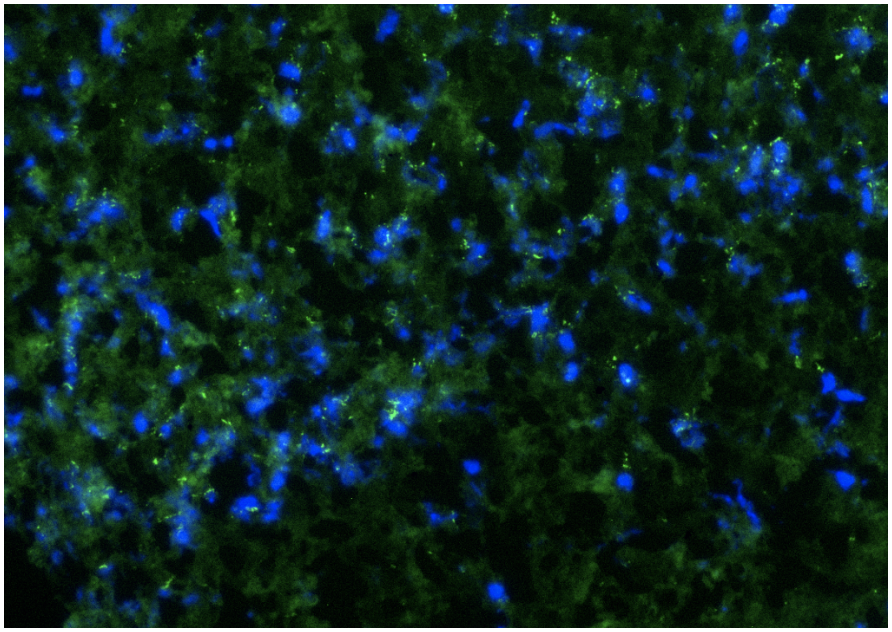

Figure 3. RNAscope assay of anterior cingulate cortex in male C57 black six mouse. This image shows a close-up of a coronal section of the ACC in a male C57 black six mouse after the RNAscope in situ hybridization assay has been performed. RNA for the beta-1 adrenergic receptor is stained green, and the cell nuclei is stained blue. The bright green particles that are more prominent on the image represent beta-1 adrenergic receptor RNA.

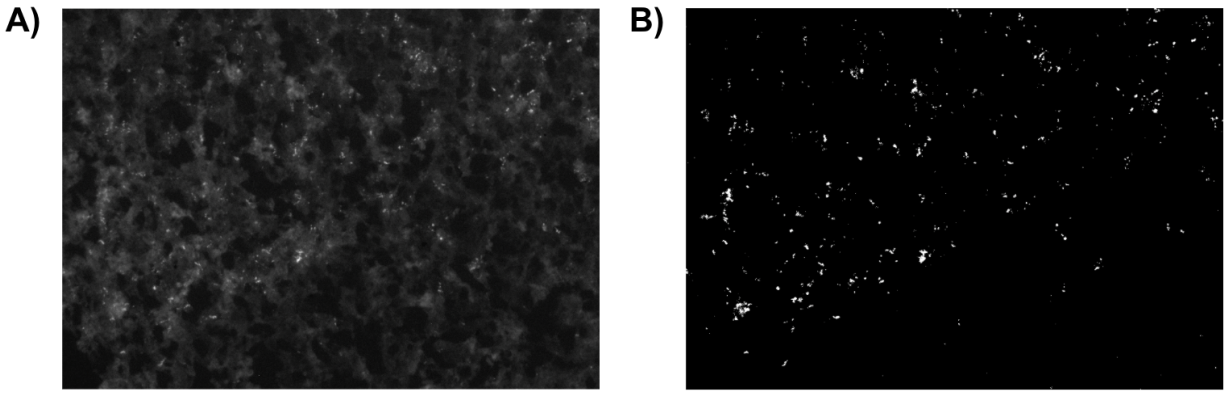

Figure 4. ImageJ analysis of RNAscope assay of anterior cingulate cortex in the left hemisphere of a male C57 black six mouse. This image shows two separate images of the same RNAscope assay after they have been edited in the ImageJ software. (A) This image shows the staining for only the beta-1 adrenergic receptor RNA. The brightness and contrast of this image has been adjusted for visual clarity. (B) This image is the same image from Figure 3A, but a threshold has been set to target the regions of interest for quantitative analysis. The regions of interest are shown in white and each particle on the image represents RNA for the beta-1 adrenergic receptor.

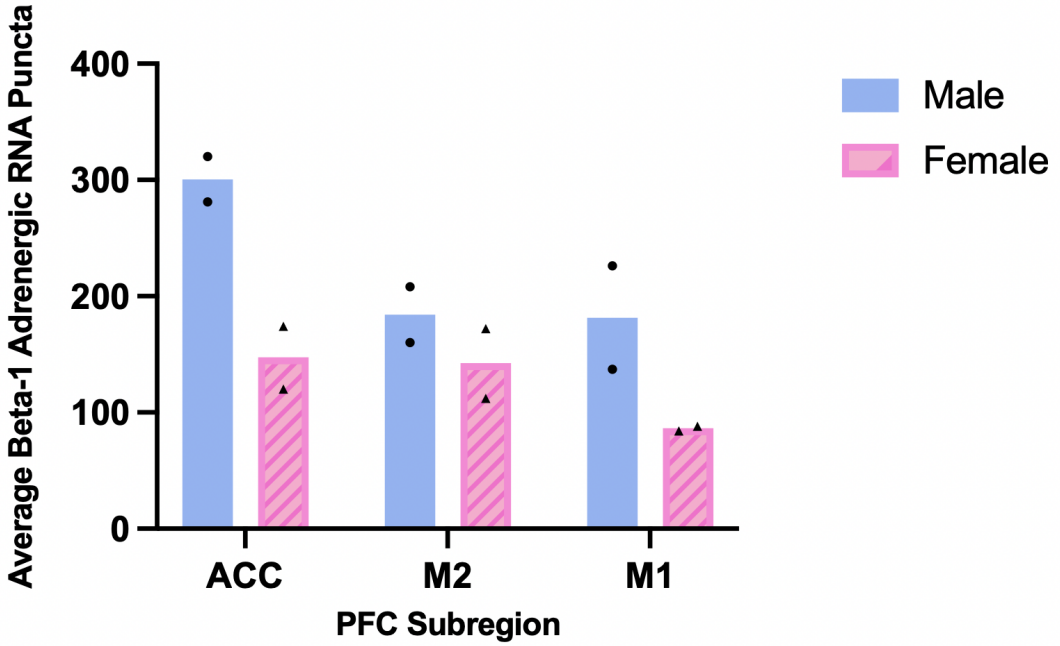

Figure 5. Average beta-1 adrenergic receptor RNA puncta in 3 PFC subregions of male and female C57 black six mice. I obtained an average count of beta-1 adrenergic receptor RNA for 3 different subregions of the PFC from a male and female mouse: the ACC, M2, and M1 regions. These results were an average count of 2 sites per subregion per animal. The average count of beta-1 adrenergic receptor RNA puncta for the male mouse is shown in blue and the individual data points are shown as circles. The average count of beta-1 adrenergic receptor RNA puncta for the female mouse is shown in pink with diagonal stripes, and the individual data points are shown as triangles.

Tables:

	Male	Female
ACC, Left Hemisphere	320	120
ACC, Right Hemisphere	281	174
ACC Mean	300.5	147
M2, Left Hemisphere	160	112
M2, Right Hemisphere	208	172
M2 Mean	184	142
M1, Left Hemisphere	137	88
M1, Right Hemisphere	226	84
M1 Mean	181.5	86

Table 1. Count of beta-1 adrenergic receptor RNA puncta for male and female C57 black six mice in 3 PFC subregions: the ACC, M2, and M1 regions. The first two numbers for each PFC subregion are a count of beta-1 adrenergic receptor RNA from images of the left and right hemispheres for that particular subregion and were used to calculate the mean.

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