NORADRENERGIC REGULATION OF DECISION-MAKING IN FEMALE AND MALE RATS

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NORADRENERGIC REGULATION OF DECISION-MAKING IN FEMALE AND MALE RATS

A Dissertation Presented

by

Emma Sophia Dauster

Submitted to the Graduate School of the
University of Massachusetts Amherst in partial fulfillment
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Neuroscience and Behavior Graduate Program
NORADRENERGIC REGULATION OF DECISION-MAKING IN FEMALE AND MALE RATS

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Emma Sophia Dauster

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Elena Vazey and Emma Dauster conceived the research conducted during Emma’s Ph.D. Emma planned, constructed, conducted, analyzed, and presented each experiment, but would not have been able to complete this much work in this period of time without the help of hardworking undergraduate and high school research assistants. Cam Donahue, Kara Conlan, and Ben Earle consistently tested, fed, and cared for the rats that provided all of the data outlined in this Ph.D. Those three researchers along with Judy Luu and Harish Ganesh asked thoughtful questions that propelled the research forward. Each of those researchers contributed to brain tissue preparation and/or analysis. Then Emma put it all together and constructed a narrative with Elena to tell the story of the research findings. Mariana Pereira, Heather Richardson, Stephanie Padilla, and Elena Vazey provided revisions to the dissertation document. The 2AFC task schematic was created by Emma Dauster with inspiration from a figure by Mark Presker.
ABSTRACT

NORADRENERGIC REGULATION OF DECISION-MAKING IN FEMALE AND MALE RATS

MAY 2023

EMMA SOPHIA DAUSTER, B.S. CASE WESTERN RESERVE UNIVERSITY

Ph.D. UNIVERSITY OF MASSACHUSETTS AMHERST

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Decision-making is regulated by many associated brain regions, including the locus coeruleus (LC) and the prefrontal cortex (PFC). Disruptions in decision-making are a key feature of many disorders including attention-deficit/hyperactivity disorder which is disproportionately diagnosed in one sex over another for reasons unknown. LC or its primary neurotransmitter norepinephrine (NE) have been implicated in the etiology or treatment of disrupted decision-making. Understanding the relationship among LC, PFC, and decision-making across sexes may provide insight into the basic neurobiology of cognition and disorders that lead to disrupted decision making.

There are sex differences in LC anatomy, however studies investigating sex differences in LC-PFC regulation of decision-making are limited. This dissertation aims to investigate key substrates in the LC-PFC system underlying decision-making in females and males. As similar sex differences have been observed in behavior and anatomy of human and rodent LCs, the following experiments were conducted in Long-Evans rats:

Chapter 2 objective: Determine the decision-making effect of increased LC activity, with additional study of impact on motivation and reward consumption as control behaviors. This was tested by administering excitatory chemogenetics into the LCs before performance of the two-alternative forced choice (2AFC) decision-making task. Results indicated that females were more sensitive to increases to LC activity than males.
Chapter 3 objective: Assess behavioral effects of NE activity. This was tested by administering atomoxetine systemically before 2AFC performance. Results indicated that males were more sensitive to increases to NE availability than females.

This chapter further assessed the influence of adrenergic receptors on decision-making. This was tested by administering propranolol systemically before 2AFC performance. Results indicated that both females and males were similarly sensitive to decreases to NE receptor availability.

Chapter 4 objective: Compare NE projections to the PFC. This was tested by comparing density of NE projections to the PFC, density of NE release sites in the PFC, and cell-type distribution of RNA coding for NE receptors in the PFC. Results indicated that NE projections and release sites in the PFC were similar in females and males. However, β1 receptor RNA had higher expression on glutamatergic neurons in females, and β2 in males.
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CHAPTER 1

INTRODUCTION

1.1 Exploration of sex differences in behavior

Many neuropsychiatric disorders are disproportionately diagnosed more in one sex over the other. For example, attention-deficit/hyperactivity disorder (ADHD) is up to 16 times more frequently diagnosed in males than females [Kessler et al., 2006; Cantwell, 1996; Novik et al., 2006]. Additionally, females have about double the diagnosis rate of post-traumatic stress disorder and anxiety disorders [Valentino and Bangasser, 2016]. Some of these behaviors may be related as well, since arousal-related symptoms characterize all of the aforementioned behaviors as well as depression and Alzheimer’s Disease, which are also associated with stress and diagnosed more frequently in females than males [Slavich and Sacher, 2019; Valentino and Bangasser, 2016]. Despite these known sex differences, females are underrepresented in biological research [Beery and Zucker, 2011; Woitowich et al., 2020]. By neglecting a large portion of the population, researchers are missing critical sex differences across anatomy and behavior. For example, there are limited studies investigating the behavior and anatomy that may be associated with decision-making across sexes. There is currently a gap in research about sex differences in the neural regulation of most biological functions including decision-making.

1.2 The LC-NE system as a regulator of decision-making behavior

The locus coeruleus-norepinephrine (LC-NE) system plays a critical role in regulating decision-making behavior. Previous studies from the Vazey lab found that tonic activation of LC activity impacted task engagement, accuracy, and distractibility in a decision-making task, driving inattentive behavior in the two-alternative forced choice (2AFC) decision-making task. Additional studies have concluded that phasic activity is involved in maintaining focused attention, while tonic
activity is more involved in behavioral flexibility [Aston-Jones and Cohen, 2005]. In both cases, the LC is involved in regulating key facets of decision-making behavior.

In previous studies that recorded LC activity during performance of decision-making tasks, it was concluded that the LC is highly plastic and will tune activity to the rewarded target even in reversal tasks where a previously unrewarded target suddenly becomes rewarded [Aston-Jones and Cohen, 2005]. The LC response to such changes occurred after the cue and before behavior reflected an understanding of the change, suggesting that the LC is driving behavioral response rather than these observations being categorized as a coincidental downstream effect [Aston-Jones and Cohen, 2005]. Further, this trend is correlated with task performance, so when animals were not attending to the task, their LCs were not displaying these strong patterns [Aston-Jones and Cohen, 2005].

However, decision-making requires a balance of LC activity. Too much or too little LC-NE activity can lead to decreased performance in decision-making tasks [Arnsten 2006; Aston-Jones and Cohen, 2005]. Thus, it has been proposed that there is an optimal amount of LC activity and NE release for optimal behavioral performance, a relationship known as Yerkes-Dodson [Gamo et al., 2010; Aston-Jones and Cohen, 2005; Arnsten, 2011]. Deviations from that optimal NE signaling can lead to decreased performance in decision-making behavior, as observed in different behavioral and pathological states e.g., ADHD [Gamo et al., 2010].

1.3 The 2AFC task as an assessment of decision-making behavior

Therefore, decision-making behavior can be probed in rodents by the two-alternative forced choice task (2AFC), which uses cued lever presses to gauge an individual’s focused attention [Shea-Brown et al., 2008; Kilpatrick et al., 2019]. Many other tasks can also assess attention, decision-making, or cognitive flexibility, like the attentional set shifting task [Newman et al., 2008; Cain et al., 2011; Bradshaw et al., 2016]. Additional factors involved in decision-making, such as arousal, impulsivity, and motivation can be assessed in behavioral tests including the five-choice serial reaction time task, the go/no-go task, and the progressive ratio task [Newman et al., 2008; Cain et
al., 2011; Bradshaw et al., 2016; Higgins et al., 2020]. However, the 2AFC task allows for a wider variety of responses that provides a better window into the decision-making process and trade-offs. In this task, a rat self-initiates each trial, is presented with a stimulus cue dictating which lever will be rewarded, and leaves the initiation port to press either lever or neither lever.

Further, the LC-NE system can be manipulated during 2AFC testing to assess its role in decision-making behavior in females compared to males. There are several ways that this can be accomplished, including chemogenetics and optogenetics. Optogenetics stimulate phasic activity, while chemogenetics stimulate tonic activity [Goutaudier et al., 2019]. Previous studies have concluded that phasic activity is involved in maintaining focused attention, while tonic activity is more involved in behavioral flexibility [Aston-Jones and Cohen, 2005]. Thus, a simpler task like the sustained attention, signal detection tasks or go/no-go paradigm might be better suited for optogenetic LC manipulation, while the 2AFC task is a good task to introduce chemogenetic manipulations.

2AFC testing can be preceded by a surgery targeted to the LC to virally introduce the chemogenetic tool: designer receptors exclusively activated by designer drugs (DREADDs). DREADDs come in a variety of options, including excitatory and inhibitory, with a variety of activating ligands including clozapine-N-oxide (CNO). The Gq-coupled DREADDs can reversibly activate cells within a known temporal window and return to baseline behavior outside of that window when the effect wears off. Additionally, neither the DREADDs nor CNO are naturally occurring in the body, so they are designed to bind to each other with minimal off-target effects.

To assess the influence of the LC-NE system on decision-making behavior, Gq-coupled DREADDs can specifically target LC cells for activation during performance of the 2AFC task. This cell-type specificity can be accomplished using the PRSx8 promoter, activated by the transcription factor Phox2 found in LC cells [Hwang et al., 2001; Vazey and Aston-Jones, 2014]. Just before 2AFC testing, DREADDs, and thus LC cells, can be reversibly activated by intraperitoneal injection of
the ligand CNO. This manipulation allows for decision-making data collection upon increased LC activity. Since decision-making is such a complex behavior, this LC-DREADD manipulation was also implemented before motivation probing tasks such as the progressive ratio task and general reward consumption measures (Chapter 2).

1.4 NE pharmacology

However, LC activity is not necessarily synonymous with NE activity. To assess the behavioral effects of NE activity, the downstream consequences of NE signaling regarding decision-making behavior and its underlying driving factors can be tested using pharmacology. Rats can receive atomoxetine (ATM) injections systemically before performance of the 2AFC task as a NE reuptake inhibitor tool that increases the available NE [Gamo et al., 2010; Aston-Jones and Cohen, 2005; Arnsten, 2011; Bymaster et al, 2002]. ATM has been previously found to dose-dependently improve performance in measures of cognitive flexibility and impulsive behavior [Newman et al., 2008; Cain et al., 2011; Bradshaw et al., 2016; Higgins et al., 2020].

However, NE can have a variety of cellular outcomes depending on the receptor to which it binds. For example, when NE binds to β receptors, it generally increases neuronal activity [Saboory et al., 2020; Liu et al., 2014]. However, previous investigations have found different effects of different β receptors [Ramos et al., 2005; Ramos et al., 2008]. In those studies, β1 receptors were more inhibitory and β2 receptors were more excitatory [Ramos et al., 2005; Ramos et al., 2008]. Therefore, the behavioral influence of the receptors to which NE binds should be assessed using pharmacological tools such as the β adrenergic receptor blocker propranolol intraperitoneally injected before performance of tasks including the 2AFC, progressive ratio, and sucrose consumption task (Chapter 3). Limited studies exist comparing the effects of these drugs in females and males [Chernoff et al., 2021].

1.5 Anatomical sex differences in the LC-NE system
Decision-making and arousal are in part regulated by a dense cluster of cells in the dorsal pons of the brainstem and a major source of norepinephrine (NE) in the brain: the locus coeruleus (LC) [Berridge and Waterhouse, 2003; Aston-Jones and Bloom, 1981; Grant et al., 1988; Herve-Minvielle and Sara, 1995; Rasmussen et al., 1986]. Sex differences have been observed in the LC of humans and rats [Bangasser et al., 2011; Bangasser et al., 2013; Pinos et al., 2001; Busch et al., 1997; Ohm et al., 1997]. More cells have been found in the LC in women compared to men [Busch et al., 1997; Ohm et al., 1997]. This finding has been similarly observed in rats, along with denser dendritic arborizations in females compared to males, indicating an increased potential for output and increased ability to incorporate inputs [Bangasser et al., 2011; Bangasser et al., 2013; Pinos et al., 2001].

Additional sex differences have been found in cortical inputs to the LC [Sun et al., 2020]. Female mice have increased inputs to the LC from the midbrain and hindbrain compared to males, while males have increased inputs to the LC from the cerebrum [Sun et al., 2020]. Females receive a greater proportion of LC inputs from the pons; thus, they may be influenced to a greater extent by LC autoinhibition than males. This sex difference in LC innervation may also drive sex differences in LC-regulated behavior regardless of sex differences in the LC itself. These sex differences in LC and NE signaling may contribute to sex differences in behavior. However, outputs from the LC to the cortex have not yet been investigated.

Previous investigations of β adrenergic receptors revealed potential sex differences as well. These publications focused on male data, and when female data was introduced, it appeared that β receptors produced different and potentially opposite effects [Ramos et al., 2005; Ramos et al., 2008]. Therefore, studies designed to assess the influence of female and male β receptors in the PFC are needed.

1.6 Noradrenergic signaling to the PFC regulates decision-making behavior
One of the main pathways that the LC regulates decision-making behavior through in both humans and rodents is the direct projections of NE to the cortex [Morilak et al., 2005; Aston-Jones and Cohen, 2005; Anden et al., 1966; Fuxe et al., 1968; Olson and Fuxe, 1971; Sachs et al., 1973; Berridge and Waterhouse, 2003]. Of note, the LC specifically innervates the prefrontal cortex (PFC), which is a key regulator of higher order cognitive functioning [Chandler et al., 2014; McGaughy et al., 2008; Rich et al., 2018; Arnsten, 2006].

In males, LC neurons fire at a higher discharge rate into the PFC than other brain regions such as the motor cortex [Chandler et al., 2014]. However, it is unknown how female discharge rates in the PFC compare. The extent of anatomical sex differences in NE circulation is currently unknown. Therefore, sex differences in LC projections to the PFC, NE release sites in the PFC, and/or NE receptors in the PFC may contribute to behavioral sex differences. The present experiments aim to fill the knowledge gap linking sex differences in decision-making and sex differences in LC-NE circuitry.

This can be accomplished using immunohistochemical methods that assess NE projections to the PFC in females and males with antibodies targeting a conversion factor required to create NE called dopamine-β-hydroxylase. Using advanced imaging technology such as StereoInvestigator software, NE release sites in the PFC can be quantified in both sexes. These tools are ideal for assessment of NE regulation of decision-making behavior because the brain tissue of animals that performed the 2AFC decision-making task can be assessed using these measures for direct analysis of anatomy and behavior in the same individual.

Then, the other end of the synapse can be assessed as well. Of β adrenergic receptors in the PFC, β1 and β2 receptors are the most prevalent [Ramos et al., 2007]. However, NE can bind to β adrenergic receptors and enhance either excitatory synaptic transmission or GABAergic inhibition [Waterhouse et al., 1982; Huang et al., 1996; Ji et al., 2008]. Therefore, RNAscope technology was used to label RNA coding for likely glutamatergic and GABAergic cells overlaid with β1 and β2
receptor RNA to see how the proportion of glutamatergic cells labeled with β receptors, GABA-ergic cells labeled with β receptors, and other cells labeled with β receptors compare in female and male PFCs (Chapter 4). This tool was ideal for preliminary analyses of sex comparisons in β adrenergic receptors that had never been previously investigated. By assessing pure anatomy, the results provide a jumping point for future more complicated and time-consuming behavioral assessments. Additionally, assessing RNA that codes for receptors provides information as to which cells are capable of expressing those receptors without the confound of assessing receptor expression and capturing a snapshot in time that may or may not be representative.

1.7 Objectives

There is a knowledge gap in which the extent and functional consequences of sex differences in the LC-NE system are largely unknown. However, the LC-NE system acts throughout the cortex, so this dissertation narrowed the focus to outline sex differences in LC-NE signaling that may impact decision-making specifically. The overarching hypothesis was that females are more resilient to manipulations to the LC-NE system than males with regard to decision-making because their anatomy is better suited for higher LC-NE levels. Each chapter of this research independently contributed to the field by determining the role of increased LC activity in female and male decision-making (Chapter 2), determining the role of noradrenergic signaling in female and male decision-making (Chapter 3), and identifying substrates of NE-PFC innervation across female and male rats (Chapter 4).

The following studies investigated sex differences in noradrenergic regulation of decision-making through a set of functional, pharmacological, and anatomical experiments that compared female and male brains and behavior to begin to probe the root of sex differences in LC-NE signaling to the PFC. These experiments went beyond the question of if there are sex differences in behavior to expose how sex differences in behavior can arise.
Chapter 2 assessed the role of increased LC activity in female and male decision-making. The hypothesis was that female rats can manage a higher amount of LC activity than males before it impacts their decision-making performance. Based on that hypothesis, it was predicted that the same increase in LC activity across females and males would decrease decision-making performance in males more than females because females are better suited for higher LC activity. This was tested by administering excitatory DREADDs into the LCs of female and male rats before performance of the 2AFC task.

Chapter 3 assessed the role of NE signaling in female and male decision-making. The hypothesis was that males are more sensitive to increases in NE than females with regard to decision-making performance. Based on that hypothesis, it was predicted that the same increase in NE across females and males would decrease decision-making performance in males more than females because females are better suited for higher LC activity. This was tested by administering the NE reuptake inhibitor, atomoxetine, to female and male rats before performance of the 2AFC task. Chapter 3 further assessed the influence of adrenergic receptors on female and male decision-making. The hypothesis was that females are more sensitive to decreased availability of β adrenergic receptors than males with regard to decision-making performance. Based on that hypothesis, it was predicted that the same decrease in β adrenergic receptor availability across females and males would decrease decision-making performance in females more than males because females are better suited for higher LC activity. This was tested by administering the β adrenergic receptor antagonist, propranolol, to female and male rats before performance of the 2AFC task.

Chapter 4 assessed substrates of NE-PFC innervation across female and male rats. The hypothesis was that a more fortified neural infrastructure for LC-NE function in the female brain compared to the male brain not only exists in the brainstem, but also extends to NE projections and/or their binding sites in prefrontal target regions. Based on that hypothesis, it was predicted that there are more axons and/or β adrenergic receptor RNA in the female brain compared to the male brain. This was tested by staining axons and release sites in the female and male PFC for the compound that
turns dopamine into NE: dopamine-β-hydroxylase. This was also tested by staining RNA that codes for β adrenergic receptors and excitatory and inhibitory cells in the PFC of female and male rats.

The extent and functional consequences of sex differences in the LC-NE system are largely unknown. Inputs to the LC have been mapped in females and males previously, but projections from the LC, especially to regions of interest in the regulation of decision-making behaviors like the PFC, have not been mapped in females and males. The following research expanded this foundation by assessing LC-PFC anatomy and the behavioral consequences of manipulating the LC-NE system in both females and males. The research below outlined downstream outputs from the LC to the PFC in female and male Long-Evans rats.

The findings of the proposed research increased our understanding of sex differences in LC-NE circuitry to the frontal cortex and associated behaviors. After conducting these experiments, the comparative role in females and males of vital components of LC activity and projections, NE availability, and adrenergic receptor distribution in regulating decision-making is now known.
Chapter 2

THE ROLE OF INCREASED LC ACTIVITY IN FEMALE AND MALE DECISION-MAKING

2.1 Abstract

Sex differences have been observed in human attention and decision-making deficits such as attention-deficit/hyperactivity disorder [Kessler et al., 2006; Cantwell, 1996; Novik et al., 2006]. The present study aimed to investigate the relationship between locus coeruleus (LC) activity and decision-making behavior across sexes by comparing the performance of female and male Long-Evans rats in a 2AFC (two alternative forced choice) decision-making task before and after artificial increases to LC activity. Task engagement, inattention, and accuracy were measured before and after targeted chemogenetic manipulation of LC cells in both females and males. Results revealed sex differences in LC-regulated decision-making behavior. Decreased accuracy and task engagement in females compared to males from the same LC manipulation indicates that females were more sensitive to these manipulations than males. Taken together, these results suggest that sex differences in LC anatomy contribute to sex differences in decision-making behavior.

2.2 Background

LC-norepinephrine (NE) cells projecting throughout the cerebral cortex play a critical role in regulating arousal and decision-making [Berridge and Waterhouse, 2003; Aston-Jones and Bloom, 1981; Grant et al., 1988; Herve-Minvielle and Sara, 1995; Rasmussen et al., 1986]. They act in a Yerkes-Dodson relationship, where both overactivity and underactivity lead to decreased performance [Gamo et al., 2010; Aston-Jones and Cohen, 2005; Arnsten, 2011]. This means that maintaining an optimal level of LC activity is key to achieving focused attention on cognitive tasks. However, anatomical and behavioral sex differences suggest that the optimal level of LC activity
may vary across sexes [Bangasser et al., 2011; Bangasser et al., 2013; Pinos et al., 2001; Busch et al., 1997; Ohm et al., 1997].

Traditionally, LC activity and decision-making behavior have been investigated in males, but not females. Previous studies have investigated the role of LC-NE on decision-making behavior using chemogenetic tools, but neglecting sex comparisons [Cope et al., 2019; Perez et al., 2022]. Decision-making behavior, as probed by the two-alternative forced choice task (2AFC), can be assessed through cued lever press [Shea-Brown et al., 2008; Kilpatrick et al., 2019]. In previous 2AFC studies that involved LC recordings, it was concluded that the LC drives behavioral responses [Aston-Jones and Cohen, 2005]. Further, this trend is correlated with task performance, so when animals were not attending to the task, their LCs were not displaying as strong patterns [Aston-Jones and Cohen, 2005]. Additionally, previous work from the Vazey lab in male rodents showed that chemogenetic activation of tonic LC activity impacted 2AFC task engagement, accuracy, and distractibility, driving inattentive behavior. However, this inattentive behavioral effect has not been evaluated in females. Therefore, sex comparisons in LC regulation of 2AFC performance are yet to be investigated.

The chemogenetic method, designer receptors exclusively activated by designer drugs (DREADDs), can be used to reversibly excite overall activity specifically in the LC, targeted by the PRSx8 promoter, to identify causal relationships. The PRSx8 promoter is specifically activated by the transcription factor Phox2 found in LC cells [Hwang et al., 2001; Vazey and Aston-Jones, 2014]. Previous work from the Vazey lab in male rodents showed that this kind of chemogenetic activation of tonic LC activity impacted task engagement, accuracy, and distractibility in a decision-making task, driving inattentive behavior. However, this inattentive behavioral effect has not been evaluated in females.

2.2.1 Present study
The objective of the present study was to determine the behavioral effect of increased LC activity in females, specifically regarding decision-making, with additional study of impact on motivation and reward consumption as control behaviors. A secondary goal was to determine whether sex differences exist in the effects of LC activation on the behaviors under investigation. The hypothesis was that female rats can manage a higher amount of LC activity than males before it impacts their decision-making performance. Based on that hypothesis, it was predicted that the same increase in LC activity across females and males would decrease decision-making performance in males more than females because females are better suited for higher LC activity. This was tested by administering excitatory DREADDs into the LCs of female and male rats before performance of the 2AFC task. Since females have more LC cells, they may activate downstream targets to a greater extent than males at baseline. Therefore, artificially increasing LC activity would overstimulate the male system, while it would stimulate the female system within the range that their anatomy is setup for.

To probe the impact of changes in LC activity on decision-making behavior, female and male Long Evans rats were tested in the 2AFC task with chemogenetic LC activation. To test if the results from the 2AFC task were primarily driven by changes to motivation or overall reward consumption, rats were tested in the progressive ratio test and a sucrose consumption test respectively. Before testing, each rat underwent a craniotomy to infuse a PRSx8 promoted adeno-associated virus (DREADDs or mCherry) specifically into their LC cells followed by an intraperitoneal injection of the DREADD-activating ligand, clozapine-N-oxide (CNO), or vehicle on testing days.

2.3 Methods

2.3.1 Animals

Female (weight 175-200 g, n=43) and male (weight 275-300 g, n=28) adult wild type Long-Evans rats (Charles River Laboratories, Wilmington, Massachusetts, USA) were kept in temperature- (68-78° F) and humidity-controlled (30-70%) conditions under a 12 h reverse light/dark cycle (lights off at 9am). Training, handling, and tests were all performed during the dark phase of the cycle.
Rats were consistently trained and fed at the same time within one-hour variation every day to avoid circadian effects on feeding or arousal. All animals were individually housed. Of the original 73 rats that underwent craniotomy surgery, 41 met criteria for inclusion in final analysis based on histologically confirmed virus expression. All experimental protocols were approved by the UMass Amherst Institutional Animal Care and Use Committee, and all procedures were carried out in compliance with the policies and regulations of the guidelines approved by the National Institutes of Health *Guide for the Care and Use of Laboratory Animals*.

<table>
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<th>Total</th>
<th>Locomotor</th>
<th>2AFC</th>
<th>Sucrose</th>
<th>PR</th>
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<td>21</td>
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<tr>
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<tr>
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<table>
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<th>2AFC</th>
<th>Sucrose</th>
<th>PR</th>
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<tbody>
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</tr>
<tr>
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<td>0</td>
</tr>
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<td>10</td>
<td>4</td>
<td>7*</td>
<td>7*</td>
</tr>
</tbody>
</table>

**Table 2.1.** Total number of animals included in LC experiments and final analysis. All animals received a craniotomy surgery upon arrival at the lab, but only a portion of those surgeries were successful and included in analysis. Further, some of the control viruses were contaminated with DREADDs, leading to a higher number of DREADD rats after histological confirmation than were intended at time of surgery (*

### 2.3.2 Experimental design

The overall experimental design is shown in the timelines below. All animals received either PRSx8-hM3Dq or PRSx8-mCherry viral infusions to the LC (Figure 2.3). One group of animals underwent the 2AFC test (Figure 2.4) and locomotor test (Section 2.3.8; female mCherry n=9, male mCherry n=2, female DREADD n=6, male DREADD n=4) and a second group underwent the PR test (Figure 2.5), sucrose test (Section 2.3.11), and locomotor test (female DREADD n=7, male DREADD n=7). After surgery, rats were mildly food restricted for training in the 2AFC task. Upon completion of 2AFC testing, each rat performed a locomotor test. After completing those tests, rats were perfused 90 minutes after ligand injection and their brains were collected for
immunohistochemical analysis to confirm viral expression in LC and that the chemogenetic manipulations influenced the LC.

2AFC Timeline

Figure 2.1. Example 2AFC training schedule. All rats were given a one-week adjustment period after arriving at our animal facility before surgery. After surgery, they recovered for two weeks. 2AFC training (green) took on average five weeks for rats to progress through each stage to the final 2AFC task. Once performance on the final task was stable and above criterion, testing began. During the testing period, rats were brought to the behavior room, allowed to acclimate for 30 minutes, injected with the scheduled drug, and left for about 30 minutes before the session began. After the 40-minute test period, each rat was removed from the operant box, weighed, lavaged if applicable, and returned to their home cage where they received free access to water. Then they were all returned to the animal facility until the same time the next day.

To probe the effects of increased LC activity on motivation and reward consumption, a separate group of animals were tested in progressive ratio (PR), sucrose consumption, and locomotion. These rats all received either PRSx8-hM3Dq or PRSx8-mCherry infusions to the LC. Of note, some animals received a variation of that testing schedule in which the order of testing was reversed. Upon completion of each of these experiments, all rats were perfused and their brains were collected for post-hoc verification of viral expression.

PR and Sucrose Test Timeline
Figure 2.2. Example PR and sucrose test training schedule. All rats were given a one-week adjustment period after arriving at our animal facility before surgery. After surgery, they recovered for two weeks. Some started with locomotor testing, while others started with sucrose testing. Sucrose testing took on average two weeks for rats to complete. During the testing period, rats were brought to the behavior room, allowed to acclimate for 30 minutes, injected with the scheduled dose of CNO or vehicle, and left for about 30 minutes for activation. After the 60-minute test period, each rat was removed from the operant box, weighed, lavaged if applicable, and returned to their home cage where they received free access to water. Then they were all returned to the animal facility until the same time the next day. Some then completed PR training for about two weeks and began testing once the criterion was reached. During the testing period, rats were brought to the behavior room, allowed to acclimate for 30 minutes, injected with the scheduled drug, and left for about 30 minutes for activation. After the 60-minute test period, each rat was removed from the operant box, weighed, lavaged if applicable, and returned to their home cage where they received free access to water. After completion of the PR testing schedule, they completed sucrose testing as described above. For some animals, the order of testing was reversed.

2.3.3 Food restriction
Rats were mildly food restricted (12g female daily rations, 20 g male daily rations, LabDiet iso-pro 3000 irradiated rodent diet catalog #5P76) beginning on the first day of 2AFC training. Rats were weighed every 2-3 days to ensure that >80% of their projected free-fed body weight was maintained [Pahl, 1969]. Animals received ad libitum water access except during the 40-60 min training/testing period, during which rats earned liquid rewards.

2.3.4 Viral manipulation
Custom viral vectors were obtained from the University of Pennsylvania Vector Core. Rats (female n=25, male n=16 included in final analysis) were bilaterally infused into the LC with either an adeno-associated viral vector containing the PRSx8 promoter driving HA tagged hM3Dq DREADD (AAV9-PRSx8-HA-hM3Dq) or control vector with PRSx8 promotor driving only a reporter protein (AAV9-PRSx8-mCherry) [Hwang et al., 2001; Roth, 2016; Vazey and Aston-Jones, 2014]. Of the rats that performed the 2AFC and locomotor tests, half received the hM3Dq DREADD virus (female n=13, male n=11) and half received the control virus (female n=12, male n=5). Follow up data in the progressive ratio and sucrose consumption tests exclusively report within subject findings from DREADD rats due to technical difficulties (female n=7, male n=7). DREADDs provide reversible activation of the Gq signaling cascade in LC cells [Roth, 2016]. The PRSx8 promoter ensured specific expression of the DREADDs only on Phox2 expressing cells,
which restricts expression to LC-NE cells within the infusion area. The control rats were infused with AAV9 PRSx8-mCherry virus that specifically targeted LC neurons and expressed mCherry rather than the DREADD. This marker revealed the transduced cells in post-hoc immunohistochemical analysis but did not alter LC cell activity. The membrane-bound DREADDs utilized here have a haemagglutinin (HA) tag that was immunohistochemically targeted (Figure 2.6, magenta). The overlap confirms that the virus was expressed in the LC.

2.3.5 Surgery

All surgeries were performed under continuous vaporized inhaled isoflurane (around 2% induction and around 0.5% maintenance at a flow rate around 0.5 liters per minute). Immediately prior to surgery, NSAID analgesia (0.1 mg/100g of metacam was injected subcutaneously), and antibiotics (0.1 ml cefazolin injected intramuscularly) were administered. Surgeries were performed on a small animal Kopf stereotaxic frame (California, USA). Viral vectors were delivered bilaterally to the LC by pressure injection using glass pipettes (picospritzer, tip size 25-35 microns) lowered to the following coordinates on a flat skull: Anterior-Posterior (AP) -9.5 mm from Bregma, Medial-Lateral (ML) +/-1.3 mm from midline, and Dorsal-Ventral (DV) evenly distributed between -6.6- and -6.3-mm ventral from the skull surface for females, and AP -9.8 mm posterior from Bregma, ML +/-1.3 mm from midline, and DV -7.4- -6.8 mm for males. The virus was evenly distributed across DV of the LC, starting at the ventral end and moving dorsal over about three administration sites, depositing ~1 µl total virus per side, then left to diffuse through the tissue for 20 minutes before the pipette was pulled dorsally out of the brain. Given the small size of the LC (~1,600 cells), a PRSx8 promoter was used to limit the DREADD expression to the LC. Sterile sutures were used to seal the incision site and antibacterial ointment and numbing cream was applied to the incision site every day until completely healed.
Figure 2.3. Selective chemogenetic control of LC cells in female and male Long-Evans rats. A) Schematic of infusion site (females: +/-1.3 mm M/L, -9.5 mm A/P, and -6.6/-6.3 mm D/V; males: +/-1.3 mm M/L, -9.8 mm A/P, and -7.4/-6.8 mm D/V; Paxinos and Watson, 2007). B) DREADD transcription took place exclusively after interaction between the PRSx8 viral promoter and Phox2 transcription factor, creating cell selectivity. The receptor remained inactive until the activating ligand CNO was systemically injected and bound to the hM3Dq receptor.

2.3.6 Drugs

Experimental groups were given the selective ligand clozapine-N-oxide (CNO; NIMH Chemical Synthesis Supply) to activate DREADDS during behavioral testing [Armbruster et al., 2007; Roth, 2016]. CNO was diluted in a vehicle of 0.9% sterile sodium chloride and 1% dimethyl sulfoxide to 0.1 mg/ml, 1 mg/ml, and 10 mg/ml, delivered at 1 ml/kg intraperitoneally.

2.3.7 LC manipulation

The LC was manipulated for each behavior test using intraperitoneal (IP) injection of 1 ml/kg CNO. In the present study, three doses of CNO: low (0.1 mg/kg), medium (1 mg/kg), and high (10 mg/kg) were used on different test days to produce a dose response curve. To avoid a general order effect, CNO doses were administered in a Latin square design (example below in Table 2.2), with each dose three days apart from each other to minimize residual effects from the last CNO injection. Each CNO injection was paired with a preceding vehicle injection (0.9% sodium chloride and 1%
dimethyl sulfoxide) to control for the effects of injection and solvent. Behavioral data was also collected the day after each dose to ensure that the rat’s behavioral performance returned to baseline.

Table 2.2. Latin Square injection schedule for LC experiments. Three examples of the DREADD activation schedule applied to each rat. A vehicle control injection day preceded each CNO injection test day, with at least three baseline task performance days in between each test day. CNO concentrations were administered in different sequences for each rat to remove any time or learning confound.

2.3.8 Locomotor testing

Spontaneous locomotion was evaluated in each rat for 1 hour. Each rat was placed in an empty 11in x 11in x 13in chamber following CNO or vehicle injection (IP) and monitored via camera for total distance traveled (m/30min). These empty chambers were novel to the rats. In a between-subjects design, DREADD (female n=14, male n=13) and control (female n=12, male n=5) rats received either a CNO or vehicle injection before introduction to the testing chamber. A camera mounted above the chamber captured the entire chamber. Overall rat movement was tracked and binned in 30-minute increments using AnyMaze software (Stoelting, USA). This test followed a similar timeline to progressive ratio testing in which the rat receives an injection, waits about 30 minutes, is introduced to the testing chamber, and is monitored for the next hour.

2.3.9 Two-alternative forced choice (2AFC) task

2.3.9.1 Apparatus

All behavioral testing was performed in Med Associates (Vermont, USA) operant chamber equipped with a central reward well with a recessed panel of LED cue lights and infrared (IR) entry beam, as well as two laterally located levers (one on each side of the reward well), and a house
light by the ceiling. Each operant chamber was 23.5 inches wide, 22 inches tall, and 16 inches deep, and was situated within a sound-attenuating chamber.

2.3.9.2 Training

Rats (female n=15, male n=6) initiated trials by breaking the IR beam in front of the LED cue panel. After maintaining the beam break for a variable hold (200-700ms), a red or green LED cue light illuminated (50% probability) to indicate which lever would be rewarded. Light-lever associations remained constant throughout training and testing (red LED indicated that the left lever would be rewarded, and green LED indicated the right lever would be rewarded). The LED cue remained illuminated for up to 5s or until the rat left the IR beam. Pressing the correct lever indicated by the LED cue elicited a 5KHz tone (100ms) and 13.7% liquid sucrose reward (0.16ml female, 0.24ml male), followed by a 5s inter-trial interval (ITI) in which the house light was illuminated. The reward amount was adjusted for males and females to reduce satiation and maintain consistent baseline task engagement throughout the 40-minute task. Pressing the incorrect lever elicited a 10s timeout, cued by the house light, before a new trial could be initiated. Trials in which there was no lever press response within 5s of the LED cue onset were counted as omissions and led to a 5s ITI. Extinguishing the house light signaled to the rats that a new trial could be initiated.

On the first day of training, rats started with a fixed-ratio 1 schedule with every press on each lever rewarded (one lever press resulted in one reward). After the rat pressed each lever at least 50 times within a session, the rat trained on the second phase to break the IR beam across the central reward port to produce the reward. The rat trained on this phase for one day. The next phase taught the rat to lever press in response to a cue light, and to establish specific cue-lever associations (red-left, and green-right). During this stage the cue light remained illuminated until the rat completed their response, and cues repeated-on-error. The rat trained on this phase until they achieved more than 165 correct responses within a session for two consecutive days. Then they advanced to the 2AFC phase where cue lights extinguished upon leaving the IR beam and they no longer received repeat-
on-error cues. Final 2AFC sessions were limited to 40min or a maximum of 251 trials, whichever came first. There was no punishment for reduced participation. As measures of focused attention in this task, each rat’s data was collected and analyzed in three measures: 1) total trials initiated, 2) percent completed correct responses, and 3) percent exploratory trials (premature withdrawal from IR beam before the cues were presented). Total initiated trials show the raw number of trials that each rat self-initiated during the testing session(s) to assess task engagement. Percent completed correct trials assess accuracy and are calculated from the number of correct responses divided by the number of correct plus incorrect responses. Percent exploratory trials was calculated by the portion of initiated trials that were not completed with a lever press (but rather the rat left the initiation port to explore the chamber), to assess focused task completion. Rats were trained to perform consistently at or above 75% accuracy in the 2AFC task before testing.
Figure 2.4. Two-alternative forced choice task schematic. A rat displayed focused accuracy if it self-initiated a trial, held the nose in the initiation/reward port for a variable time, attended to a stimulus cue, and pressed the indicated lever. The light turned on and a tone played indicating that a reward has been dispensed and a new trial could not be initiated for the next five seconds. The rat then returned to the initiation/reward port for a 13.7% sucrose-water reward. Alternatively, the rat could perform an incorrect trial when it pressed the opposite lever from that which was indicated by the cue light. The rat could not initiate another trial for 10 seconds after an error. In another alternative outcome, the rat performed an exploratory omitted trial when the rat initiated a trial but did not wait for a cue and did not press either lever for five seconds.

2.3.10 Progressive ratio (PR) test

To determine whether LC activation impacted motivation for sucrose rewards, animals were assessed in a progressive ratio test. Rats were trained on FR-1 for sucrose rewards as above, after meeting criterion (>50 presses per day, two consecutive days) an inactive lever was introduced to the chamber, and animals again had to meet a criterion of >50 rewarded presses per day for two consecutive days. Animals were then trained on a FR-3 schedule to criterion before performing the progressive ratio test with the following schedule 1, 2, 4, 9, 12, 15, 20, 25, 32, 40, 50, 62, 77, 95, 118, 145, 178, 219, 268, 328, 402, 492, 603, 737, 901, 1102, 1347, 1647, 2012, based on Richardson and Roberts, 1996. Animals received vehicle or 10 mg/kg CNO injections in a repeated-measures, counterbalanced design with three days of PR training in between. PR sessions terminated after 60 minutes or if rats failed to press the lever for 5 minutes. The highest response ratio completed for each session was recorded.

Figure 2.5. Progressive ratio task schematic. A rat displayed motivated behavior if it pressed the lever until a 13.7% sucrose-water reward was dispensed. To achieve the first reward, the rat pressed the left lever once and returned to the center port for reward. To achieve subsequent rewards within the same testing session, the rat pressed the left lever incrementally more times before receiving a reward. At any point in time, the rat could stop pressing the lever or start again until the hour was complete.
2.3.11 Sucrose drinking test

To determine whether LC activation impacted consumption of freely available sucrose rewards, a 60-minute sucrose test was performed. Each rat was brought to the behavioral testing room and allowed to recover from transport. Rats received an injection of either vehicle or 10 mg/kg CNO in a repeated-measures, counterbalanced design with three days in between injections. Thirty minutes after the injection, their home cage water bottle was removed and replaced with an identical bottle filled with the 13.7% sucrose solution in a one-bottle-one-choice paradigm. They were left to drink freely from that bottle for 60 minutes, to match the progressive ratio test time. The content of the bottle was measured by volume and by weighing the bottle before and after each testing session.

2.3.12 Estrus lavage

Female rats were tested for estrus cycle effects on 2AFC task performance across at least one full estrous cycle, with sample taken immediately after daily 2AFC sessions using vaginal smears. Only data from actively cycling rats was analyzed.

2.3.13 Perfusion and brain retrieval

Female (n=9) and male (n=8) rats that had undergone behavioral testing were injected with CNO 50-140 minutes before perfusion to activate DREADD cells during fixation for later quantification. After CNO injections, each rat received at least 1.5 ml/kg ketamine, xylazine, and saline solution (97.5 mg/kg ketamine and 15 mg/kg xylazine) 20-80 minutes before perfusion (e.g.: A rat that weighed 510 g received 0.77 ml of the solution; Henry Schein Animal Health from USA). Once unresponsive to toe pinch and righting reflex was lost, each rat was perfused transcardially with 200 ml 0.9% saline, followed by 400 ml 10% formalin (VWR International, USA). The brains were immediately removed and stored in 10% formalin overnight. The next day, the brains were transferred to a 20% sucrose-azide solution until it was fully absorbed, as observed by the brains sinking to the bottom of a brain jar. The tissue was then submerged in -80° C isopentane, mounted on a cryostat (Leica CM3050S, Germany) at -20° C, and serially sliced from Bregma -11.16 mm
A/P to Bregma -8.52 mm A/P reference brain coordinates from the Paxinos and Watson Rat Brain Atlas, to collect 40 micron thick serial coronal sections, (160 microns between adjacent sections within a series) of the entire LC [Paxinos and Watson, 2007]. This tissue was stored in phosphate buffered saline (PBS)-azide until fluorescent staining.

2.3.14 Immunohistochemistry

DREADD and mCherry viruses were labeled with fluorescent antibodies, along with the LC cells themselves, characterized as a dense cluster of large TH positive cells ventrolateral to the fourth ventricle in the dorsal pons of the brainstem. In tissue analysis performed after behavior was complete, the brain tissue was stained with antibodies that amplified and added fluorescent tags to the viruses previously infused into the LC to produce double labeled sections: mouse monoclonal anti-TH primary antibodies to identify LC (Immunostar, USA), donkey anti-mouse Alexa Fluor 488 secondary antibodies (Jackson ImmunoResearch Laboratories, USA), rabbit polyclonal anti-DsRed primary antibodies to identify mCherry expression (Takara, USA), rabbit monoclonal anti-HA primary antibodies to identify DREADD expression (Cell Signaling Technology, USA), and donkey anti-rabbit Alexa Fluor 594 secondary antibodies (Jackson ImmunoResearch Laboratories, West Grove, PA, USA). With this stain, the tissue was microscopically assessed for virus expression in part or all the LC of each hemisphere of each rat.

LC sections were washed three times for five minutes in PBS solution on an orbital shaker at 100 rpm. Then the tissue was washed three times for five minutes in PBS with 0.2% Triton (PBST) and then incubated for 60 min in a blocking solution of PBST and 3% Normal Donkey Serum. The tissue incubated in blocking solution and 1:1000 primary antibody (Mouse anti-TH and Rabbit Rnti-HA or Rabbit Rnti-DsRed) at room temperature overnight. The next day, tissue was washed three times in five-minute increments in PBST and then incubated in blocking solution with 1:500 secondary antibodies (Donkey anti-mouse and anti-rabbit) for three hours. Next, tissue was washed two times for five minutes in PBST and finally one five-minute wash in 0.1 M phosphate buffer
solution. The tissue was wet mounted in 0.1 M PB with citifluor mounting solution and coverslipped on Superfrost Plus slides (Fisher Scientific, Pittsburgh, PA, USA) in the dark to avoid bleaching the fluorescent label.

Active LC cells were also labeled with a DAB-Ni solution for c-Fos reactivity. LC sections were washed three times for five minutes in PBS solution on an orbital shaker at 100 rpm, then incubated in 1% hydrogen peroxide for 10 minutes and incubated for 60 minutes in a blocking solution of PBST and 3% Normal Donkey Serum. The tissue incubated in blocking solution and 1:15,000 primary antibody (Rabbit anti-c-Fos; lot no. 2672548; Millipore Corp. Bedford, MA, USA) overnight at 4°C. The next day, sections were washed three times for five minutes in PBST solution on an orbital shaker at 100 rpm in room temperature, then incubated for 2.5 hours in a 1:500 dilution of biotinylated secondary antibody (Donkey anti-rabbit; lot no. 122849; Jackson ImmunoResearch. West Grove, PA, USA). Next, the sections were washed three times for five minutes in PBST solution and incubated at room temperature for 2 hours in a 1:500 dilution of avidin-biotin complex (ABC Vectastain, Burlingame, CA, USA), followed by 0.2 mg/ml DAB and 6 mg/ml Nickel ammonium sulfate (Fisher Scientific, Pittsburgh, PA, USA). The tissue was wet mounted in 0.1 M PB on Superfrost Plus slides and left to dry overnight. Tissue then went through dehydration with ethanol and defatting with xylene and completed NISSL staining before cover-slipping with DPX mounting solution (Electron Microscopy Sciences, Hatfield, PA, USA). Figure 2.7 shows one anterior LC, one medial LC, and one posterior LC section labeled for c-Fos from a single rat. After brightfield imaging on a Zeiss Axioimager, c-Fos puncta within LC (as identified by neutral red counterstain) were thresholded and manually counted before averaging across six data points (two hemispheres from three sections) for each rat.

The Zeiss Axioimager M2 epi-fluorescent microscope picked up any Alexa-Flour 488 and 594 fluorescently labeled cells and the MBF StereoInvestigator software was used to image the cells. Three images (anterior to posterior) were taken at 10x magnification in both channels across each
LC bilaterally of each animal, then artificially colored magenta for DREADDS and mCherry and green for tyrosine hydroxylase (LC; Figure 2.6). ImageJ was used to quantify the fluorescence in each image. LC cells were manually counted. In addition, the fluorescent LC area was divided by the overall LC area to produce a proportional analysis. Inclusion criterion for successful transduction of LC and study inclusion was >50% LC co-stained for virus expression.

2.3.15 Statistical analyses

All data were baseline corrected and presented as mean +/- SEM unless noted otherwise. Normally distributed data were analyzed by mixed model repeated measures two-way analysis of variance (ANOVA) for drug dose and sex, followed by post hoc Dunnett’s or Sidak’s test. Kolmogorov-Smirnov test was used to assess normality in all data sets. Statistics and graph composition was undertaken in Matlab and/or GraphPad Prism version 7 for Windows. α≤0.05 was accepted. Outliers (one female DREADD and one male DREADD rat from PR analysis) were removed based on ROUT test, Q=1.

2.4 Results

2.4.1 LC cells were specifically targeted by PRSx8 vectors

After completion of behavioral tests outlined above, all brains were immunohistochemically analyzed for accurate placement of LC-hM3Dq DREADDs or LC-mCherry virus bilaterally. All rats included in final analysis had 78.62 +/-18.88% coverage across both hemispheres (female n=26, male n=18). Figure 2.6 shows highly localized virus expression (magenta) in LC (green). The virus was clustered dorsally in this example. These analyses support majority coverage of the LC by virus after surgery, as shown by overlap between LC cells and virus stain.
Figure 2.6. Immunohistochemical confirmation of virus expression location in the brain. Each panel shows DREADD virus coverage of the LC in the right hemisphere of the same rat. White arrow shows the fourth ventricle for reference. A) Anti-tyrosine hydroxylase antibody stained with 488 fluorescence. B) Anti-HA antibody stained with 594 fluorescence. C) The merged image of A and B.

2.4.2 DREADD manipulations elicited c-Fos expression in LC

Female (n=9) and male (n=8) rats were tested for expression of immediate early gene, c-Fos, in response to CNO activation of LC-hM3Dq DREADDs in a between subjects design (females vs males). LC cells labeled for c-Fos activity were readily identifiable and quantified manually.

Figure 2.7. Immunohistochemical confirmation of DREADD activation in the LC. Each panel shows c-Fos stain in black on the left hemisphere of a DREADD-infused rat’s LC (outlined in panel B). Black arrow (panel A) shows example c-Fos cell, white arrow (panel A) shows example background neutral red stained cell. c-Fos activity was assessed from three sections across LC at approximately A) Bregma -9.36 mm (anterior) B) Bregma -9.72 mm (medial) C) Bregma -10.2 mm (posterior).

Analysis of c-Fos expression confirmed that there was no significant difference between female and male LC activation after CNO administration to LC-DREADD animals (Figure 2.8a;
t_{(1, 4)}=0.4754, p=0.6953, Welch’s t-test). One limitation of this study was the lack of significant correlation between c-Fos activation and DREADD coverage across the LC (Figure 2.8b; Female slope=0.1197+/-0.3616, Pearson r=0.1634, p=0.7571; Male slope=0.5402+/-1.183, Pearson r=0.2227, p=0.6715).

Figure 2.8. There was a comparable number of c-Fos labeled cells across sexes. A) When data from each animal was averaged across both LCs and anterior to posterior cross sections, c-Fos quantification suggested no statistically significant sex differences in cell activity in the DREADD animals upon CNO administration. B) In the same animals, c-Fos was not significantly correlated with DREADD coverage across the LC.

2.4.3 Reproductive hormones did not change 2AFC performance

To determine whether variations in indicators of basal hormonal state influenced performance on the 2AFC outside of LC manipulation, female (n=10) rat performance was evaluated across estrous stage (based on post-performance estrous status, Miller and Takahashi, 2013) and male (n=15) performance was evaluated across the day (based on circadian changes in testosterone, Nyby et al., 2008). Female performance was evaluated across four days of the estrous cycle (diestrus, proestrus, estrus, and metestrus) tracked by vaginal lavage, because estradiol peaks during the proestrus phase (Miller and Takahashi, 2013). This window into hormonal fluctuations suggests that what was probed in these assessments had no influence on any facet of behavior tested here: ITI impulsivity (Figure 2.9a; F_{(3,12)}=0.2022, p=0.9772), goal-directed performance (Figure 2.9b; F_{(3,12)}=3.023, p=0.388), task engagement (Figure 2.9c; F_{(3,12)}=1.12, p=0.7721), focused task completion (Figure
2.9d; \( F_{(3,12)}=1.15, \ p=0.7649 \), hyperactivity (Figure 2.9e; \( F_{(3,12)}=2.117, \ p=0.5486 \)), and exploratory behavior (Figure 2.9f; \( F_{(3,12)}=0.2989, \ p=0.9602 \)) were all statistically unchanged by cycle (One-way ANOVA).

Male performance was also sampled between circadian states, as male testosterone tends to peak early in the active cycle with daily troughs occurring about 5 hours later (Nyby et al., 2008). The same measures were sampled in males as in females. In males, impulsivity (Figure 2.9g; \( t(12.84)=0.2207, \ p=0.8288 \)), goal-directed performance (Figure 2.9h; \( t(10.08)=0.9675, \ p=0.3559 \)), task engagement (Figure 2.9i; \( t(11.25)=0.7573, \ p=0.4644 \)), focused task completion (Figure 2.9j; \( t(12.68)=0.4583, \ p=0.6545 \)), hyperactivity (Figure 2.9k; \( t(12.73)=1.543, \ p=0.1474 \)), and exploratory behavior (\( t(12.67)=0.03498, \ p=0.9726 \)) were all statistically unchanged by active cycle time (Welch’s t-test).

There was no significant variability in basal performance of 2AFC metrics within females or males across expected cycles of reproductive hormone fluctuation, specifically across the estrous cycle for females or circadian time points for males (Figure 2.9). These findings support 2AFC performance consistency across regular variations in basal hormonal state, as shown by a lack of change in any 2AFC measures.
Figure 2.9. Performance on the 2AFC task was consistent across phases of the reproductive cycle. Females (n=10) performed consistently across the estrous cycle, assessed by (A) percent of ITI lever presses out of total lever presses across a testing session, (B) percent of correct responses out of total completed trials (C) total number of trials initiated (D) percent of trials in which a rat neglected to respond to a received cue out of total trials initiated (E) percent of trials in which a rat leaves the initiation port before a cue has been presented and presses either lever out of total trials initiated (F) percent of trials in which a rat leaves the initiation port without receiving a cue or pressing a lever out of total trials initiated. Number of lever presses during the inter-trial interval as a percent of total lever presses throughout a session, (B) number of rewarded trials as a percent of total completed trials, (C) total number of trials initiated throughout a session, (D) the number of neglected trials as a percent of total trials, (E) number of rushed trials as a percent of total trials, and (F) number of exploratory trials as a percent of total trials. Male daily performance at 10am-11:30am or 1-2.5 hours after lights off (n=8) and 1:30pm or 4.5 hours after lights off (n=7) was also assessed by the same measures (G-L).

2.4.4 Locomotion was unaffected by increased LC activity
A subset of animals (female DREADD+CNO n=6, male DREADD+CNO n=7, female controls n=16, male controls n=8) was tested for locomotor activity in a novel chamber in response to CNO. Overall distance travelled was unaffected by these manipulations. There was no correlation between locomotion or any testing group assessed here (Figure 2.10; Pearson r=0.2473, p=0.4909). These findings rule out locomotor impairment as a cause for any 2AFC performance changes, as shown by consistent locomotor trends across LC activating manipulations and controls.

Figure 2.10. CNO injection and DREADD activation did not significantly impact rat movement. In this sample of the rats that performed the behavioral tasks above, each rat was injected with CNO or vehicle and placed in an open chamber and left to move freely for the next hour, similar to the progressive ratio timeline. Their total cumulative movement was recorded and averaged across 30-minute increments above. Rats that received the DREADD infusion and CNO injection are in dark colors (triangle and square). Rats that received the mCherry infusion and received CNO or vehicle injections along with rats that received the DREADD infusion and received vehicle injections are in light colors (upside down triangle and diamond). Females are pink triangles and males are blue squares.

2.4.5 Females, but not males, decreased task engagement and accuracy with increased LC activity

To determine the impact of chemogenetically upregulating LC activity on decision making activity, female (n=6) and male (n=4) rats with LC-hM3Dq DREADDs were tested in the 2AFC task. Sex differences were observed in task engagement and accuracy measures after LC activation. Female task engagement, as measured by total number of trials initiated during the testing session, decreased compared to baseline performance (Figure 2.11a). A significant main effect of sex on task engagement confirmed that females reduced the number of trials they initiated with increased LC activity (Figure 2.11a; F(1,39)=10.5, p=0.0024, Two-way ANOVA). In contrast, males showed
no notable change in performance (Figure 2.11a). Females also trended toward more exploratory performance, as measured by portion of initiated trials that were abandoned before receiving a cue, than males in the 2AFC task (Figure 2.11b; $F_{(1,40)}=2.647$, $p=0.1116$, Two-way ANOVA). Finally, female accuracy, as measured by percent correct responses out of all correct and incorrect trials, decreased after administration of the highest dose of CNO but not significantly (Figure 2.11c; $F_{(1,40)}=1.782$, $p=0.1895$, Two-way ANOVA). Performance returned to baseline the next day, confirming that this was a reversible effect induced by CNO injection acting on LC-hM3Dq (Figure 2.11). Males showed no significant change in performance (Figure 2.11). These findings suggest that females were more sensitive to CNO administration activating LC cells than males, as shown by decrease in trials initiated. However, there was considerable variability in individual female responses to the present manipulation, so some of these effects seem to be driven by sensitive individuals rather than all females.
Figure 2.11. Increased LC activity decreased female performance in the 2AFC task, while males’ performance remained unaffected. Data presented from the day of each dose CNO injection and from the day after the highest dose of CNO. Lighter lines represent individual data points, while darker lines represent the average performance. A) There was a significant main effect of sex in task engagement, where females decreased performance upon increased LC activity but not males. $F(1,39)=10.5$, $p=0.0024$, Two-way ANOVA. B) Females trended more exploratory than males upon increased activity. $F(1,40)=2.647$, $p=0.1116$, Two-way ANOVA. C) The high dose of CNO, and greatest increase in LC activity reduced task accuracy in females but not significantly and not in males. $F(1,40)=1.782$, $p=0.1895$, Two-way ANOVA.

2.4.6 CNO alone in mCherry controls did not impact performance on the 2AFC task.
To determine the effects of CNO itself on 2AFC performance, a group of female (n=9) and male (n=2) control rats expressing LC-mCherry virus also went through the same testing paradigm. No sex differences were observed in these control animals. Female and male task engagement (Figure 2.12a; $F_{(4,45)}=0.08948$, $p=0.9853$, Two-way ANOVA), exploratory trials (Figure 2.12b; $F_{(4,45)}=0.6775$, $p=0.6112$, Two-way ANOVA), and accuracy (Figure 2.12c; $F_{(4,45)}=0.0711$, $p=0.9905$, Two-way ANOVA) were unchanged by all doses of CNO. These findings support that previous 2AFC performance impairment was related to CNO activation of the DREADD rather than CNO administration.
Figure 2.12. Control rats with mCherry viral infusions did not significantly alter their performance when administered any dose of CNO. Lighter lines represent individual data points, while darker lines represent the average performance. A) The accuracy, as measured by percent of correct responses out of all completed trials, did not significantly change for females or males administered three doses of CNO. B) The task engagement, as measured by total number of trials initiated in a testing session did not significantly change in females or males administered three doses of CNO. C) The task disengagement, as measured by percent of exploratory trials, did not significantly change in females or males administered three doses of CNO. For ease of comparison with the DREADD rats, all the control data is plotted with the same axis ranges as the DREADD data.

2.4.7 LC activation did not significantly impact motivation for sucrose rewards but decreased sucrose consumption
To determine if the behavior observed in 2AFC task performance was driven by underlying sucrose consumption or motivational factors, a group of female (n=7) and male (n=8) rats were tested in the PR task and the sucrose consumption task. Motivation to achieve reward, as measured by progressive ratio break points, was unaffected by increased LC activity in both female and male rats. The last number of lever presses completed before reaching a breakpoint and becoming disengaged in the task was not statistically significantly different between females and males (Figure 2.13a; F(1,10)=4.361, p=0.0608, Two-way ANOVA) or between vehicle and CNO injections (Figure 2.13a; F(1,10)=1.004, p=0.3400, Two-way ANOVA).

Despite no main effect of DREADD activation there was a main sex effect in the sucrose test, as females generally consumed more sucrose per body weight when freely-available compared to males (Figure 2.13b; F(1,13)=5.752, p=0.0322, Two-way ANOVA). This sex difference was a mild effect. To determine whether it was related to LC activation, the relationship to c-Fos activity was assessed. Greater LC cell activation, as detected by c-Fos stain, did not change motivation and consumption in females or males (Figure 2.13c; Female slope=-1.703+/-1.137, Pearson r=-0.6542, p=0.2311; Male slope=0.5009+/-0.3314, Pearson r=0.5601, p=0.1910; Figure 2.13d; Female slope=-3.593+/-3.115, Pearson r=-0.3997, p=0.2865; Male slope=5.39+/-2.825, Pearson r=0.6491, p=0.1147). These findings suggest that 2AFC performance impairment was independent of motivation or consumption.
Figure 2.13. Increased LC activity had no effect on motivation in females or males, but decreased male consumption. Lighter lines represent individual data points, while darker lines represent the average performance. A) Female and male motivation were unaffected by increased LC activity. B) Females consumed more sucrose than males, as calculated by ml consumed as a percent of body weight (ml/g*100). This effect was driven by LC manipulation, which further decreased male consumption. $F_{(1,13)}=5.752$, $p=0.0322$, Two-way ANOVA C) Female c-Fos activation trended negatively but was not correlated with performance in the progressive ratio task. Male c-Fos activation trended positively but was not correlated with performance in the progressive ratio task. D) Female c-Fos activation trended negatively but was not correlated with sucrose consumption. Male c-Fos activation trended positively but was not correlated with sucrose consumption. Stats: $^\wedge p \leq 0.05$.

2.5 Discussion

Overall, LC upregulation with DREADDS decreased female task engagement in a decision-making task but did not affect male performance. These results were not driven by sex differences in LC activation on locomotion, motivation, or sucrose consumption. These results suggest that female decision-making behavior was more sensitive than male to the LC manipulations performed here.

2.5.1 2AFC

Female decision-making behavior was disrupted to a greater extent than male behavior by artificial increases to LC activity. It has been proposed that there is an optimal amount of LC activity and NE release for optimal behavioral performance, a relationship known as Yerkes-Dodson [Gamo et al., 2010; Aston-Jones and Cohen, 2005; Arnsten, 2011]. Going above that activity level could lead to hyperarousal and inability to focus on a task, while going below that activity level could lead to hypoarousal and inability to attend to a task. The 2AFC findings reported here complement this idea and build on it to establish a different optimal LC-NE level for females and males. While female performance decreased upon LC-DREADD manipulation, male performance was largely unchanged, suggesting that females and males may have different thresholds for LC activation before behavior is impacted.
Figure 2.14. Proposed Yerkes-Dodson relationship with regard to LC activity. Females (pink triangle) and males (blue square) both perform optimally in the 2AFC decision-making task at baseline. However, females may be farther along the curve than males. This would yield decreased performance for females compared to males when pushed farther along the curve by artificial increases to LC activity.

Previous literature reported that the LC regulates arousal and decision-making, as determined through behavioral tests including lever pressing tasks based on visual cues in monkeys, reinforced by a consumed reward [Ivanova et al., 1997; Berridge and Waterhouse, 2003]. The present study discussed lever pressing tasks based on visual cues in rodents, concluding that manipulations to the LC significantly impacted performance. Although the manipulations were different, in previous studies monkey LC activity patterns were not manipulated with chemogenetics, the findings agreed that the LC played a critical role in decision-making behavior [Ivanova et al., 1997; Berridge and Waterhouse, 2003]. Further, previous studies reported that pharmacologically increasing LC activity decreased task performance in monkey [Ivanova et al., 1997; Berridge and Waterhouse, 2003]. The female rat results reported here agreed with those findings.

Previous work from the Vazey lab in male rodents suggested that chemogenetically increasing tonic LC activity dose-dependently reduced task engagement and accuracy while increasing distractibility in a decision-making task, thus driving inattentive behavior. In the investigations reported here, male performance of the same task was not significantly altered by the same manipulations, but female performance was. There could be several reasons for differences in the male data across studies. The present study altered test parameters such as the reward volume. In addition, these analyses differed as well. The data in the present study were analyzed using more specific sub-categories of behavioral output that allowed significant effects that had been
previously obscured by competing results in slightly different behaviors to come to light. For example, an omitted trial could have been the result of a rat never leaving the initiation port, leaving early before receiving a cue, or leaving after receiving a cue and never pressing a lever. In the present study, those behaviors were separately assessed to find that they each had different trends that could not be observed when they were combined as in previous work from the lab.

2.5.2 Viral expression and activation

Each of the animals included in final analysis expressed either LC-hM3Dq DREADDs or LC-mCherry in the bulk of LC unilaterally or bilaterally. This is consistent with previous studies that similarly achieved a high level of LC coverage with DREADDs [Perez et al., 2022; Fortress et al., 2015]. Were the DREADDs expressed in a lower percentage of the LC, the behavioral results would have been driven by a smaller portion of LC cells, which might lead to less significant behavioral differences. The portion of the LC covered by DREADDs trended positively with number of c-Fos activated cells, and that measure trended negatively with female motivation and sucrose consumption, and positively with male motivation and sucrose consumption. Some researchers suggest that the dorsal LC projects preferentially to the cortex, while the ventral LC projects primarily down the spine [Chandler et al., 2019; Hirschberg et al., 2017; Loughlin et al., 1986]. But in this case, there is not a bias (data not shown), so viral coverage of the LC was consistent and likely to impact all brain activity. Since the animals included in this study all had virus coverage across more than half of the LC, activation larger than 50%, and no difference between female and male viral targeting, it is likely that the above manipulations affected the intended targets.

Because the animals included in final analysis were all required to meet this 50% LC coverage threshold, many data points were removed from the original sample size. This resulted in a very small sample size and a lower power than the study would ideally have. Due to that shortcoming, the results of this chapter must be taken with a grain of salt. It is possible that an increased sample size would reveal no sex differences or even an increased sensitivity to the present manipulations
in males compared to females. Future directions include repeating this investigation with more animals.

It was also a shortcoming of this study that the mCherry rats that underwent c-Fos analysis were contaminated with DREADDs, so the only c-Fos data available compared female and male DREADD rats rather than mCherry vs DREADD. Therefore, it could not be concluded from this dataset whether DREADDs successfully increased LC activity, as evidenced by c-Fos analysis. However, that would be consistent with their intended use to be activated by CNO and lead to active cells [Roth, 2016]. It also would be consistent with previous studies that used c-Fos expression as a validation tool for LC-hM3Dq virus activity [Gompf et al., 2015]. While females had a slightly higher number of c-Fos activated cells, there was no statistical difference between female and male c-Fos activation in the present study [Figure 2.8a]. Similarly, c-Fos indication of cellular activation by DREADDs changed rodent behavior in certain previous studies where DREADD activation of LC cells led to a decrease in latency to reach a platform by about 30% on later trials [Rorabaugh et al., 2017]. Future investigations should include the comparative c-Fos results of DEADD and mCherry animals or at least comparison animals decapitated at a similar time of day.

The DREADD activating ligand, CNO, had no effect on control animals in 2AFC, locomotion, and motivational measures. The present results were representative of LC manipulations rather than CNO or surgery effects because the control animals underwent the same manipulations without DREADDs and had no statistically significant change in performance. This is also consistent with previous studies that assessed the behavioral effects of CNO administration [Tran et al., 2020]. While CNO can have off-target effects in Long-Evans rats, it largely doesn’t affect behavior, except for a reduced acoustic startle reflex [Tran et al., 2020; MacLaren et al., 2016]. To validate that CNO was not having off-target behavioral effects relevant to this study, all rats were injected with a vehicle and CNO on separate 2AFC testing days. The control rats performed comparably across
those testing days, even when exposed to auditory stimuli during the 2AFC task, suggesting that CNO itself was not responsible for behavioral effects observed in DREADD rats.

There were no sex differences in c-Fos activation. The relationship between c-Fos activation and behavioral performance in the progressive ratio task and sucrose consumption did not lead to any significant sex differences in overall results from those assessments.

2.5.3 Reproductive hormones

Vaginal smears were taken after 2AFC testing, which occurred at different times of day for different rats, all of which were on a reverse light/dark cycle. Therefore, these smears had high likelihood of covering the entire estrous cycle. However, each smear was individually assessed to determine the stage of the estrous cycle that each rat was in every day based on cellular morphology. Rats whose lavaged cells could not be identified as each of the four estrous stages for a complete cycle were excluded from analysis. By assessing decision-making performance across each stage of the rodent estrous cycle, this study assessed performance across varying estrous status [Miller and Takahashi, 2014]. Several, if not all, of the reproductive hormones associated with different estrous stages have been previously associated with altered cognitive performance [Broestl et al., 2018]. Some previous studies reported that rodents in estrous cycle stages with high estrogen were associated with increased neural network activation and decreased cognitive performance, while rodents in stages with high progesterone were associated with decreased neural network activation and increased cognitive performance [Broestl et al., 2018]. Therefore, monitoring cognitive performance across estrous stages provides a window into the effects of estrous status on decision-making performance. In this study, estrous status had no observable effect on 2AFC performance in females and males were also unchanged throughout the day. This is consistent with previous studies that used the same vaginal lavage measure after rodent performance of decision-making tasks, but inconsistent with studies that report altered cognitive performance across the estrous stages [Georgiou et al., 2018; Broestl et al., 2018]. This inconsistency could be related to differences in task. Decision-making
tasks like the 2AFC might be influenced differently by estrous status compared to memory-centered tasks like those performed in previous literature, due to lower dependence on the hippocampus or different activation of prefrontal cortex subregions [Broestl et al., 2018]. However, hormones are important factors in cognitive performance. To fully investigate the role of reproductive hormones in future studies, investigations of cognitive performance could be assessed in gonadectomized animals.

2.5.4 Locomotion

Locomotion was unaffected by LC-hM3Dq DREADD activation with CNO. Previous studies reported that CNO activating DREADDs had no effect on locomotor activity, which was consistent with these findings [Fortress et al., 2015; Tran et al., 2020]. Therefore, our findings are consistent with previous literature concluding that LC-hM3Dq DREADDs alone do not lead to changes in locomotor activity, despite their potential effects when used in conjunction with genetic models. This conclusion removes a locomotor confound from the present study, in which rats were required to move from an initiation port to press a lever and then back to the reward port to complete the 2AFC task. As movement was unaffected by DREADD manipulations in the locomotor task, those manipulations were not likely to affect the rat’s ability to complete the 2AFC task. Therefore, the change in performance between trials with and without DREADD activation in the 2AFC task was not likely to be the result of altered motion, but rather cognition. Had DREADD manipulations altered locomotor performance, it could have indicated that the rat’s time spent moving to initiate a new trial and to press a lever were affected.

2.5.5 Motivation and sucrose consumption

The sex differences in 2AFC performance after LC activation were not driven by sex differences in motivation. There was no significant difference in PR breakpoint, which was inconsistent with previous literature [Grimm et al., 2022]. However, similar performance was observed across vehicle and CNO test days for females, so the LC manipulation did not significantly alter
motivation to complete a behavioral task. This validation suggests that any changes in performance of the 2AFC task upon LC manipulation were not the effect of lower motivation, but rather an altered ability to perform a decision-making task. Previous studies have pointed to the LC as a critical brain region in making cost-benefit analyses such as the progressive ratio task [Varazzani et al., 2015]. They suggested that LC neurons are activated upon exerting more effort for the same reward [Varazzani et al., 2015]. However, those studies were conducted in monkeys that were required to press down harder on the lever rather than press the lever more times, so the specific context of the effort required in a task is likely an important factor in LC involvement and behavioral outcome [Varazzani et al., 2015].

Similarly, the sex differences in 2AFC performance were not driven by sex differences in sucrose consumption. Female rodents have been found to consume more sucrose than males per body weight at baseline consistently in this study and in previous literature [Grimm et al., 2022]. And LC-NE manipulations did not alter sucrose consumption in this study or previous studies [Cao et al., 2018]. Again, vehicle and CNO performance remained similar in this task, suggesting that any changes in performance of the 2AFC task upon LC manipulation were not the effect of lower sucrose consumption, but rather an altered ability to perform a decision-making task.

2.6 Conclusion

Based on these findings, we now know that there are sex differences in LC-regulated decision-making behavior. Specifically, increased LC function decreases female task engagement, unrelated to effects on motivation, sucrose consumption, and locomotion. These findings suggest that females are more sensitive to increased LC activity in regulating cognitive engagement compared to males. This would indicate that females should be included in all LC-NE research and investigated as a unique test group from males, as well as considered for unique interventions upon decreased task performance.

2.7 Future studies
Having determined that there are sex differences in LC regulation of decision-making behavior, future studies should investigate the role of adrenergic signaling in producing those sex differences. One possible mechanism of this sex difference is the co-release of additional neurotransmitters by the LC. While the LC is the main cortical source of NE, it also has more subtle dopaminergic, galanin, glutamate, or neuropeptide Y activity in some brain regions [Kempadoo et al., 2016]. Therefore, it is possible that the manipulations to LC activity discussed here had many downstream effects through the activity of several different neurotransmitters. Future studies should elucidate how an increase in available NE and other involved neurotransmitters in decision-making centers of the brain like the prefrontal cortex affect female and male performance of the 2AFC task. One way to determine this relationship would be through blocking the synaptic clearance of these neurotransmitters after release in the prefrontal cortex, thus determining the role of increased neurotransmitter availability on behavior. Similar studies have been conducted in monkeys, but they did not investigate sex differences, and used a slightly different decision-making task [Gamo et al., 2010; Callahan et al., 2010; Dela Pena et al., 2019].

The functional impact of LC upregulation can also have a variety of downstream effects depending on the receptor to which it binds, including α1, α2a, α2b, β1, etc. NE receptors [Ramos et al., 2007]. Understanding how NE signaling through specific adrenergic receptors impacts sex differences in NE regulation of decision-making behavior in the 2AFC would make strides towards filling the knowledge gap of mechanism by which this regulation occurs. Therefore, the next chapter will address the effects of atomoxetine and propranolol on female and male rodent 2AFC performance.
Chapter 3

THE ROLE OF NORADRENERGIC SIGNALING IN FEMALE AND MALE DECISION-MAKING

3.1 Abstract

Norepinephrine (NE) is a key chemical regulator of decision-making behavior, acting on a variety of adrenergic receptors in the brain. This chemical is mainly circulated through the brain from a cluster of cells called the locus coeruleus (LC), which has been linked to different regulation of decision-making behavior in females and males. The present study aimed to determine if the sex differences in LC regulated decision-making behavior are NE dependent. To investigate this question, female and male Long-Evans rats were tested in a decision-making task before and after pharmacologically increasing the amount of NE available in the brain. Further experiments investigated the role of β adrenergic receptors by pharmacologically blocking them before task completion. Task engagement, inattention, and accuracy were measured. Decreased performance in males compared to females suggests that there are sex differences in NE-regulated decision-making behavior. Further, β adrenergic receptors were found to be involved in NE regulation of decision-making behavior, but not in the sex differences observed therein. These findings suggest that NE may be involved in producing sex differences in decision-making behavior, and that β adrenergic receptors play a role in regulating decision-making behavior in both females and males.

3.2 Background

Decision-making and arousal are regulated by many regions, including a dense cluster of cells in the dorsal pons of the brainstem called the LC, which provides most of the NE acting in the rest of the brain [Berridge and Waterhouse, 2003; Aston-Jones and Bloom, 1981; Grant et al., 1988; Herve-Minvielle and Sara, 1995; Rasmussen et al., 1986]. NE release can produce a variety of cellular outcomes depending on the receptor to which it binds. NE can bind to several different
adrenergic receptors (adrenoceptors), including β1 and β2 adrenoceptors [Ramos et al., 2007]. When NE binds to β receptors, it generally increases neuronal activity, although through different mechanisms [Saboory et al., 2020; Liu et al., 2014]. β adrenoceptors are Gs coupled. While other adrenoceptors, such as α2 receptors, have high affinity for NE, β adrenoceptors play a valuable role in times of increased NE circulation [Aoki et al., 1994; Aoki et al., 1997; Zerbi et al., 2019]. Therefore, NE binding to each adrenoceptor may play a critical role in regulating decision-making behavior. The present study investigated the role of NE in decision-making using two pharmacological manipulations: 1) increasing synaptic NE levels and 2) blocking the impact of NE on β adrenoceptors.

The pharmaceutical agent, atomoxetine (ATM) was used to increase NE levels in previous publications [Newman et al., 2008; Cain et al., 2011; Bradshaw et al., 2016; Higgins et al., 2020]. ATM is a NE reuptake inhibitor that increases NE in the synaptic cleft [Bymaster et al, 2002]. Previous studies found that low doses of ATM increased cognitive flexibility, while moderate and high doses of ATM decreased performance in male rats completing attentional set shifting tasks [Newman et al., 2008; Cain et al., 2011; Bradshaw et al., 2016]. In these previous studies, rats were trained to dig through media to reach a buried food reward in terra cotta pots outfitted with different textures, digging media, and odors. The rats were trained to associate the food reward with one of the textures, media, or odors and attend to those factors to reach criterion quickly.

Additional previous tests of ATM on cognitive performance in male rats used the five-choice serial reaction time task to test impulsive action [Higgins et al., 2020]. In the five-choice serial reaction time task, rats were placed in a testing arena with five light stimuli with five associated nose-poke ports and reward trays. The rats received food pellet rewards upon entry of the nose-poke port associated with the randomly illuminated light. Between each new trial, there was a 10 s inter-trial interval during which no nose-pokes were rewarded. Rats who received an injection of ATM before performing this task generally reduced nose-pokes during the unrewarded inter-trial interval,
suggesting that ATM decreases impulsive action to increase task performance [Higgins et al., 2020]. However, rats that had been performing close to optimally before ATM injection decreased performance afterward [Higgins et al., 2020]. These findings lend support to a relationship known as Yerkes-Dodson, in which optimal performance can shift to sub-optimal if under- or over-stimulated by something like ATM which increases NE levels [Gamo et al., 2010; Aston-Jones and Cohen, 2005; Arnsten, 2011].

Further tests using the Go/No-Go task measured the effect of ATM on response inhibition by offering rats rewards for both action and inaction. Each rat was tested in an operant chamber similar to those described in Chapter 2 of this dissertation with levers on either side of a food reward port containing stimulus lights. One light indicated that a lever should be pressed to achieve the reward and the other light indicated that no lever should be pressed to achieve the reward. After ATM injection, the rats reduced the number of incorrect trials in which a lever was pressed for a no-go stimulus [Higgins et al., 2020]. Therefore, ATM improved the rats’ response inhibition in this task, particularly at high doses.

Previous experiments in male rats, but not females, have also tested the effect of ATM in a task outlined below in this chapter, the Progressive Ratio (PR) task. Each trial required a rat to press the lever more times than the last trial before receiving the food reward. In this measure of motivation, male rats decreased performance more as ATM dose increased [Higgins et al., 2020]. While previous studies suggest that ATM may act as a cognitive enhancer, they do not support ATM for improvements in motivation.

The present study also assessed the role of β adrenoceptors independently in regulating decision-making behavior using the pharmacological agent propranolol, a non-selective β1, β2, and β3 adrenoceptor blocker. Previous studies have assessed these effects in male rats’ performance of a decision-making and impulsivity test called the delay discounting task and found that propranolol increases preference for delayed large reward over immediate small rewards [Schwager et al.,
Other relevant studies in sign tracking and goal tracking assessed male rats’ performance exclusively and found that propranolol impaired the rats’ sign tracking, with no effect on goal tracking [Pasquariello et al., 2017]. Thus, previous investigations suggest that propranolol can impair decision making-behavior in male rats.

However, limited female data exists regarding the effect of atomoxetine or propranolol on decision-making behavior. One recent study compared the effects of ATM on a gambling task in females and males and found an improvement in performance across both sexes, but additional research in this area is needed [Chernoff et al., 2021]. Further, comparative adrenergic receptor studies in females and males are missing.

### 3.2.1 Present study

In the present study, pharmacological manipulations of increased NE availability using ATM and restricted NE receptor binding with propranolol were used to assess behavioral effects of NE activity in female and male rats. These drugs were systemically injected into both females and males to determine the behavioral effects of adrenergic signaling on decision-making, using the two-alternative forced choice task (2AFC). The role of adrenergic signaling in motivation was compared, using the progressive ratio task. Finally, their role in reward consumption was compared in an assessment removed from focused attention. Together, these experiments investigated the specific downstream consequences of NE signaling regarding decision-making behavior and its underlying driving factors.

The present study compared females and males in the above investigations, to fill the previous gap in female and male comparative studies. Previous studies have found more cells and denser dendritic arborizations in the LC of females compared to males, suggesting that they may have an increased potential for output and increased ability to incorporate inputs [Bangasser et al., 2011; Bangasser et al., 2013; Pinos et al., 2001; Busch et al., 1997; Ohm et al., 1997]. That would mean that females have more fortified neural infrastructure for LC-NE function.
Therefore, the hypothesis was that males are more sensitive to increases in NE than females with regard to decision-making performance. Based on that hypothesis, it was predicted that the same increase in NE across females and males would decrease decision-making performance in males more than females because females are better suited for higher LC activity. This chapter further assessed the influence of adrenergic receptors on female and male decision-making. The hypothesis was that females are more sensitive to decreased availability of β adrenergic receptors than males with regard to decision-making performance. Based on that hypothesis, it was predicted that the same decrease in β adrenergic receptor availability across females and males would decrease decision-making performance in females more than males because females are better suited for higher LC activity.

3.3 Methods

3.3.1 Animals

Female (n=30, weighing 175-200 g) and male (n=24, weighing 275-300 g) adult wild type Long-Evans rats (Charles River Laboratories, Wilmington, Massachusetts, USA) were kept in temperature- (68-78°F) and humidity-controlled (30-70%) conditions under a 12 h reverse light/dark cycle (lights off at 9am). Training, handling, and tests were all performed during the dark phase of the cycle. Rats were consistently trained and fed at the same time within one-hour variation every day to avoid circadian effects on feeding or arousal. All animals were individually housed. All experimental protocols were approved by the UMass Amherst Institutional Animal Care and Use Committee, and all procedures were carried out in compliance with the policies and regulations of the guidelines approved by the National Institutes of Health *Guide for the Care and Use of Laboratory Animals*. 
Table 3.1 Total number of animals included in NE experiments and final analysis. All animals received at least one of the three pharmacological injections intraperitoneally (ATM and propranolol). For a single animal, each injection and behavioral test occurred on an independent day, at least three days after the previous injection.

3.3.2 Experimental design

The overall experimental design is shown in the timelines below [Figure 3.1]. All animals received either PRSx8-hM3Dq or PRSx8-mCherry viral infusions to the LC for unrelated testing in a separate experiment [Chapter 2]. These viral infusions were inert during the present testing. One group of animals underwent the 2AFC test (female n=23, male n=15) and a second group underwent the progressive ratio (PR) test and sucrose test (female n=7, male n=9). After surgery, rats were mildly food restricted, never below 80% body weight, for training in the 2AFC task [Figure 3.3]. Once they reached the final criterion for 2AFC training, they began testing with pharmacological manipulations before performance of the 2AFC task. Upon completion of 2AFC testing, female rats received vaginal lavage for estrous tracking and each rat performed a locomotor test [results outlined in Chapter 2]. After completing those tests, rats were perfused, and the brains collected.

<table>
<thead>
<tr>
<th></th>
<th>Total</th>
<th>2AFC</th>
<th>Sucrose</th>
<th>PR</th>
</tr>
</thead>
<tbody>
<tr>
<td>ATM</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Female</td>
<td>30</td>
<td>23</td>
<td>7</td>
<td>5</td>
</tr>
<tr>
<td>Male</td>
<td>24</td>
<td>15</td>
<td>9</td>
<td>7</td>
</tr>
<tr>
<td>Propranolol</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Female</td>
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<td>7</td>
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<tr>
<td>Male</td>
<td>24</td>
<td>15</td>
<td>9</td>
<td>7</td>
</tr>
</tbody>
</table>
Figure 3.1. Example 2AFC training schedule. All rats were given a two-week adjustment period after arriving at our animal facility before surgery. After surgery, they recovered for two weeks. 2AFC training (green) took on average five weeks for rats to progress through each stage to the final 2AFC task. Once performance on the final task was stable and above criterion, testing began. During the testing period, rats were brought to the behavior room, allowed to acclimate for 30 minutes, injected with the scheduled drug, and left for about 30 minutes before the session began. After the 40-minute test period, each rat was removed from the operant box, weighed, lavaged if applicable, and returned to their home cage where they received free access to water. Then they were all returned to the animal facility until the same time the next day.

To investigate how NE signaling affects motivation and reward consumption, animals were tested in PR and sucrose consumption [Figure 3.2]. Each rat was trained and tested on the PR test, which determined how motivated the rats were to achieve the sucrose reward solution [Figure 3.4]. Following PR testing, a sucrose test determined how much reward rats would consume across an extended testing period of one hour when given free access to the reward and whether this was affected by pharmacological manipulation. Of note, some animals received a variation of that testing schedule in which the order of testing was reversed. Upon completion of each of these experiments, all rats were perfused.

**PR and Sucrose Test Timeline**

Figure 3.2. Example PR and sucrose test training schedule. All rats were given a one-week adjustment period after arriving at the UMass animal facility before surgery. After surgery, they recovered for two weeks. Some started with locomotor testing, while others started with sucrose testing. Sucrose testing took on average two weeks for rats to complete. During the testing period, rats were brought to the behavior room, allowed to acclimate for 30 minutes, injected with the scheduled dose of ATM, propranolol, or vehicle, and left for about 30 minutes for activation. After the 60-minute test period, each rat was removed from the operant box, weighed, lavaged if applicable, and returned to their home cage where they received free access to water. Then they were all returned to the animal facility until the same time the next day. Some then completed PR training for about two weeks and began testing once the criterion was reached. During the testing period, rats were brought to the behavior room, allowed to acclimate for 30 minutes, injected with the scheduled drug, and left for about 30 minutes for activation. After the 60-minute test period, each rat was removed from the operant box, weighed, lavaged if applicable, and returned to their home cage where they received free access to water. After completion of the PR testing schedule, they completed sucrose testing as described above. For some animals, the order of testing was reversed. All animals also performed a locomotor test.
3.3.3 Food restriction

Rats were mildly food restricted (12g female daily rations, 20g male daily rations, LabDiet iso-pro 3000 irradiated rodent diet catalog #5P76) beginning on the first day of 2AFC training. Rats were weighed every 2-3 days to ensure that >80% of their projected free-fed body weight was maintained [Pahl, 1969]. Animals received *ad libitum* water access except during the 40-60min training/testing period, during which rats earned liquid rewards.

3.3.4 Pharmacological manipulation

In the present study, three doses (low 0.1 mg/kg, medium 0.3 mg/kg, and high 1.0 mg/kg doses) of the NE reuptake inhibitor, ATM, were used on different test days to produce a dose response curve. To avoid a general order effect, ATM doses were administered in a Latin square design (example below in Table 3.2), with each dose more than three days of stable drug-free performance apart from each other to minimize residual effects from the last injection. On a separate testing day prior to ATM testing, each animal received a sterile saline injection to control for the effects of injection. Behavioral data was also collected the day after each dose to ensure that the rat’s behavioral performance returned to baseline. All drugs were administered systemically via IP injection 20-30min before testing. All animals received one vehicle injection and three doses of ATM (low 0.1mg/kg, mid 0.3mg/kg, high 1mg/kg; TCI America, Portland, Oregon, USA). ATM dilutions were dissolved in sterile saline. These doses were previously used in similar rat experiments [Newman et al., 2008; Hosking et al., 2015; Higgins et al., 2021; Chernoff et al., 2021].

<table>
<thead>
<tr>
<th>Rat ID</th>
<th>Injection #1</th>
<th>Injection #2</th>
<th>Injection #3</th>
<th>Injection #4</th>
</tr>
</thead>
<tbody>
<tr>
<td>ESD-17-002</td>
<td>vehicle</td>
<td>0.1 mg/kg ATM</td>
<td>0.3 mg/kg ATM</td>
<td>1 mg/kg ATM</td>
</tr>
<tr>
<td>ESD-17-004</td>
<td>vehicle</td>
<td>1 mg/kg ATM</td>
<td>0.1 mg/kg ATM</td>
<td>0.3 mg/kg ATM</td>
</tr>
<tr>
<td>ESD-17-006</td>
<td>vehicle</td>
<td>0.3 mg/kg ATM</td>
<td>1 mg/kg ATM</td>
<td>0.1 mg/kg ATM</td>
</tr>
</tbody>
</table>
Table 3.2. Latin Square injection schedule for NE experiments. A vehicle control injection day preceded the first ATM injection test day, with at least three baseline task performance days in between each test day. ATM concentrations were administered in different sequences for each rat to remove any time or learning confound.

In addition to the ATM injections, on separate days each rat was injected with one dose of the active isomer of the β adrenoceptor blocker, propranolol (10 mg/kg). This dose was previously used in similar rat experiments [Schwager et al., 2014; Pasquariello et al., 2018].

3.3.5 Two-alternative forced choice (2AFC) task

3.3.5.1 Apparatus

All behavioral testing was performed in Med Associates (Vermont, USA) operant chambers equipped with a central reward well with a recessed panel of LED cue lights and infrared (IR) entry beam, as well as two laterally located levers (one on each side of the reward well), and a house light by the ceiling. Each operant chamber was 23.5 inches wide, 22 inches tall, and 16 inches deep, and was situated within a sound-attenuating chamber.

3.3.5.2 Training

Rats (female n=23, male n=15) initiated trials by breaking the IR beam in front of the LED cue panel. After maintaining the beam break for a variable hold (200-700ms), a red or green LED cue light illuminated (50% probability) to indicate which lever would be rewarded. Light-lever associations remained constant throughout training and testing (red LED indicated that the left lever would be rewarded, and green LED indicated the right lever would be rewarded). The LED cue remained illuminated for up to 5s or until the rat left the IR beam. Pressing the correct lever indicated by the LED cue elicited a 5KHz tone (100ms) and 13.7% liquid sucrose reward (0.16ml female, 0.24ml male), followed by a 5s inter-trial interval (ITI) in which the house light was illuminated. The reward amount was adjusted for males and females to reduce satiation and maintain consistent baseline task engagement throughout the 40-minute task. Pressing the incorrect lever elicited a 10s timeout, cued by the house light, before a new trial could be initiated. Trials in
which there was no lever press response within 5s of the LED cue onset were counted as omissions and led to a 5s ITI. Extinguishing the house light signaled to the rats that a new trial could be initiated.

On the first day of training, rats started with a fixed-ratio 1 schedule with both levers rewarded (one lever press resulted in one reward). After the rat pressed each lever at least 50 times within a session, the rat trained on the second phase to break the IR beam across the central reward port to produce the reward. The rat trained on this phase for one day. The next phase taught the rat to lever press in response to a cue light, and to establish specific cue-lever associations (green-left, and red-right). The rat trained on this phase until they achieved more than 165 correct responses within a session for two consecutive days. Then they advanced to the 2AFC phase to learn that they needed to respond promptly and after an inter-trial interval had passed, they would be required to start a new trial. This phase continued until they achieved more than 165 correct trials for one day. Then they performed the 2AFC task, with sessions limited to 40 minutes. Final 2AFC sessions terminated after 40min or a maximum of 251 trials, whichever came first.

In the final task, each trial was self-initiated by the rat breaking the IR beam in the center port. Correct trials, in which the rat pressed the cued lever, were rewarded with sucrose solution at the central reward port [Figure 3.3]. There was no punishment for incorrect responses, in which the rat pressed the non-cued lever, or reduced participation. In those cases, the rat received the same five second timeout as for rewarded trials. As measures of focused attention in this task, each rat’s data was collected and analyzed in three measures: total trials initiated, percent correct responses, and percent exploratory trials. Total initiated trials show the raw number of trials that each rat self-initiated during the testing session(s) to assess task engagement. Percent completed correct trials show calculated from the number of correct responses divided by the number of correct and incorrect responses, to assess accuracy. Percent exploratory trials was calculated by the portion of initiated trials that were not completed with a lever press (but rather the rat left the initiation port
to explore the chamber prior to cue delivery), to assess focused task completion. Rats were trained to perform consistently at or above 75% accuracy in the 2AFC task before testing.

**2AFC task**

![Figure 3.3](image)

**Figure 3.3.** Two-alternative forced choice task schematic. A rat displayed focused accuracy if it self-initiated a trial, held the nose in the initiation/reward port for a variable time, attended to a stimulus cue, and pressed the indicated lever. The light turned on and a tone played indicating that a reward has been dispensed and a new trial could not be initiated for the next five seconds. The rat then returned to the initiation/reward port for a 13.7% sucrose-water reward. Alternatively, the rat could perform an incorrect trial when it pressed the opposite lever from that which was indicated by the cue light. The rat could not initiate another trial for 10 seconds after an error. In another alternative outcome, the rat performed an exploratory omitted trial when the rat initiated a trial but did not wait for a cue and did not press either lever for five seconds.

**3.3.6 Progressive ratio (PR) test**

To determine whether pharmacological manipulation impacted motivation for sucrose rewards we assessed females (n=5) and males (n=7) on a PR test [Figure 3.4]. Rats were trained on FR-1 for sucrose rewards as above, after meeting criterion (>50 presses per day, two consecutive days) an inactive lever was introduced to the chamber, and animals again had to meet a criterion of >50 rewarded presses per day for two consecutive days. Animals were then trained on a FR-3 schedule to criterion before performing the PR test with the following schedule 1, 2, 4, 9, 12, 15, 20, 25, 32, 40, 50, 62, 77, 95, 118, 145, 178, 219, 268, 328, 402, 492, 603, 737, 901, 1102, 1347, 1647, 2012,
based on [Richardson and Roberts, 1996]. Animals received vehicle, ATM, or propranolol injections in a repeated measures and counterbalanced design with three days of PR training in between. PR sessions terminated after 60 minutes or if rats failed to press the lever for 5 minutes. The highest response ratio completed for each session was recorded.

### Progressive Ratio

![Progressive Ratio Diagram](image)

**Figure 3.4.** Progressive ratio task schematic. A rat displayed motivated behavior if it pressed the lever until a 13.7% sucrose-water reward was dispensed. To achieve the first reward, the rat pressed the left lever once and returned to the center port for reward. To achieve subsequent rewards within the same testing session, the rat pressed the left lever incrementally more times before receiving a reward. At any point in time, the rat could stop pressing the lever or start again until the hour was complete.

### 3.3.7 Sucrose drinking test

To determine whether pharmacological manipulation impacted consumption of freely available sucrose rewards, a 60-minute sucrose test was performed. Each rat was brought to the behavioral testing room and allowed to recover from transport. Rats received an injection of either vehicle, ATM, or propranolol, in a repeated-measures, counterbalanced design with three days in between injections. Thirty minutes after the injection, their home cage water bottle was removed and replaced with an identical bottle filled with the 13.7% sucrose solution in a one-bottle-one-choice paradigm. They were left to drink freely from that bottle for 60 minutes, to match the PR test time. The content of the bottle was measured by volume and by weighing the bottle before and after each testing session.

### 3.3.8 Statistical analyses
All data were baseline corrected and presented as mean +/- SEM unless noted otherwise. Normally distributed data were analyzed by mixed model repeated measures two-way analysis of variance (ANOVA) for drug dose and sex, followed by post hoc Dunnett’s or Sidak’s test. Kolmogorov-Smirnov test was used to assess normality in all data sets. Statistics and graph composition was undertaken in Matlab and/or GraphPad Prism version 7 for Windows. The accepted α<=0.05. Outliers were removed based on ROUT test, Q=1, one female and one male rat from PR analysis.

3.4 Results

3.4.1 Males, but not females, decreased task engagement and accuracy with increased NE availability

To determine the impact of pharmacologically increasing NE availability on decision-making behavior, female (n=23) and male (n=15) rats were tested in the 2AFC task after IP injection of ATM. Sex differences were observed in task engagement (total trials initiated), disengagement (exploratory trials), and accuracy (correct trials) measures after ATM administration. Male task engagement, as measured by total number of trials initiated during the testing session, decreased with increasing ATM dose (Figure 3.5a Interaction between sex and dose: F(3,108)=9.604, p<0.0001, two-way ANOVA). Male task engagement at the high dose of ATM was significantly lower than the vehicle (Figure 3.5a; p<0.0001, Sidak’s test). Male task engagement at the high dose of ATM was also significantly lower than the female high ATM dose Figure 3.5a; p<0.0001, Sidak’s test). In contrast, females showed no significant change in performance (Figure 3.5a). Female and male exploratory performance, as measured by portion of initiated trials that they disengaged from before receiving a cue, significantly increased with ATM, as observed in a main effect of dose (Figure 3.5b; F(3,108)=4.225, p=0.0072, two-way ANOVA). This effect was driven by the high ATM dose, which made rats significantly more exploratory than the vehicle (Figure 3.5b; p=0.0230, Tukey’s test) and the low dose of ATM (Figure 3.5b; p=0.0206, Tukey’s test). Accuracy, as measured by percent correct responses out of all correct and incorrect trials, also significantly decreased after
administration of the highest dose of ATM in males but not females (Figure 3.5c; p=0.0213, Sidak’s test), observed in a significant main effect of sex (Figure 3.5c; $F_{(1,36)}=7.48$, $p=0.0096$, two-way ANOVA). All three measures showed the greatest behavioral response at the highest dose of ATM and a return to baseline performance the next day. These findings support 2AFC performance impairment in males but not females after ATM administration, as shown by decrease in trials initiated and decrease in accuracy despite no sex difference in exploratory responses.
Figure 3.5. Males significantly decreased 2AFC performance upon increases to available NE. Lighter lines represent individual data points, while darker lines represent the average performance. A) Males decreased task engagement in the 2AFC task most significantly at the highest dose of ATM, but not females. $F(3,108)=9.604$, $p<0.0001$, two-way ANOVA B) All rats were significantly more exploratory as ATM dose increased; an effect driven by males at the high dose. $F(3,108)=4.225$, $p=0.0072$, two-way ANOVA C) Males decreased accuracy in the 2AFC task, particularly at the highest dose of ATM, but not females. $F(1,36)=7.48$, $p=0.0096$, two-way ANOVA. Stats: ^ $p\leq0.05$, ~~~~$p<0.0001$, * represents sex effects, ^ represents significantly different from vehicle (1 mg/kg).
3.4.2 Higher NE availability decreased motivation for sucrose rewards and sucrose consumption in males but not females

To determine if the behavior observed in 2AFC task performance above was driven by underlying motivational factors, a group of female (n=5) and male (n=7) rats were tested in the PR task and the sucrose consumption task. Motivation to achieve reward, as measured by the last number of lever presses completed before reaching a breakpoint and becoming disengaged in the PR task, was significantly decreased by increased NE availability (Figure 3.6a; F(1,10)=6.166, p=0.0324, two-way ANOVA, p=0.0495, Sidak’s test). In addition to this drug effect, there was a main sex effect with females at lower breakpoints than males overall (Figure 3.6a; F(1,10)=8.934, p=0.0136, two-way ANOVA).

Motivation to consume sucrose reward was also significantly decreased in males (n=9) after ATM (Figure 3.6b; p=0.0208, Sidak’s test), while female consumption (n=7) did not change. A main effect of sex confirmed that, as described in Chapter 2, females consumed more free sucrose than males, despite being less willing to work for sucrose rewards (Figure 3.6b; F(1,14)=8.168, p=0.0126, two-way ANOVA). While baseline sex differences may play a role, the ATM 2AFC results may also have been related to decreased interest in reward and motivation in males upon drug administration. These findings support motivation and consumption impairment in males but not females after ATM administration, as shown by decrease in PR breakpoint and decrease in volume of reward consumed.
Figure 3.6. Males decreased motivation and interest in reward upon ATM administration. Lighter lines represent individual data points, while darker lines represent the average performance. A) Males decreased motivation for reward upon ATM administration, but not females. $F_{(1,10)}=6.166$, $p=0.0324$, two-way ANOVA, $F_{(1,10)}=8.934$, $p=0.0136$, two-way ANOVA B) Males decreased sucrose consumption upon ATM administration, but not females. $F_{(1,14)}=8.168$, $p=0.0126$, two-way ANOVA. Stats: ^ p≤0.05.

3.4.3 β adrenoceptors impaired decision-making performance significantly more than vehicle in both sexes

To determine the impact of pharmacologically blocking β NE adrenoceptors on decision-making behavior, female (n=7) and male (n=15) rats were tested in the 2AFC task after IP injections of propranolol. Across all three measures, propranolol decreased 2AFC performance, indicating that optimal performance from both females and males required β adrenoceptors (Figure 3.7). Blocking β adrenoceptors decreased task engagement significantly more than vehicle injections, as observed by a main treatment effect of propranolol (Figure 3.7a; $F_{(1,20)}=20.46$, $p=0.0002$, two-way ANOVA).
Propranolol also increased disengagement, as observed in a main treatment effect of propranolol on exploratory trials (Figure 3.7b; $F_{(1,20)}=7.836$, $p=0.0111$, two-way ANOVA). Blocking β adrenoceptors also decreased accuracy in the 2AFC task more than vehicle injections, as observed by a main treatment effect of propranolol (Figure 3.7c; $F_{(1,20)}=6.993$, $p=0.0156$, two-way ANOVA). These data suggest that β adrenoceptors are involved in the 2AFC decision-making tested here. These findings support 2AFC performance impairment in both sexes after ATM administration, as shown by decrease in trials initiated, increase in exploratory responses, and decrease in accuracy.
**Figure 3.7.** β adrenoceptors decreased performance of the 2AFC task in both sexes. Lighter lines represent individual data points, while darker lines represent the average performance. A) Propranolol decreased task engagement. $F_{(1,20)}=20.46$, $p=0.0002$, two-way ANOVA B) Propranolol increased exploratory behavior. $F_{(1,20)}=7.836$, $p=0.0111$, two-way ANOVA C) Propranolol decreased accuracy. $F_{(1,20)}=6.993$, $p=0.0156$, two-way ANOVA.

### 3.4.4 Decreased β adrenoceptor activity altered sucrose consumption but not motivation for sucrose reward in both females and males

Based on 2AFC outcomes, the next experiment assessed the role of β adrenoceptor antagonist propranolol in motivated behavior and sucrose reward consumption to identify potential underlying drivers of decision-making performance in female ($n=5$) and male ($n=7$) rats. Propranolol had no significant effect on motivation to achieve a reward as measured by progressive ratio testing (Figure 3.8a). Females showed lower motivation than males overall, as observed in a main sex effect (Figure 3.8a; $F_{(1,20)}=11.48$, $p=0.0029$, two-way ANOVA).

However, in testing free sucrose intake both females ($n=7$) and males ($n=9$) decreased reward consumption upon propranolol administration. Blocking β adrenoceptors significantly decreased sucrose consumption compared to vehicle with a main treatment effect (Figure 3.8b; $F_{(1,14)}=18.66$, $p<0.0001$, two-way ANOVA) supported by post-hoc analyses for females (Figure 3.8b; $p=0.0064$, Sidak’s test) and males (Figure 3.8b; $p=0.0001$, Sidak’s test). There was also a main sex effect, as males had decreased sucrose consumption compared to females at baseline (Figure 3.8b; $F_{(1,14)}=11.7$, $p=0.0041$, two-way ANOVA). Therefore, the 2AFC results could have been impacted by decreased interest in reward. These findings support 2AFC performance impairment driven by decreased consumption but not motivation after propranolol administration, as shown by decrease in volume of reward consumed by both sexes.
3.5 Discussion

The hypothesis for this chapter was that males are more sensitive to increases in NE than females with regard to decision-making performance. Overall, artificially increasing NE availability with ATM disrupted male task engagement, disengagement, and accuracy in a decision-making task more than females. These changes in decision-making were likely influenced by sex differences in motivation and sucrose consumption after ATM. These results supported the prediction that the same increase in NE across females and males would decrease decision-making performance in males more than females because females are better suited for higher LC activity.
The hypothesis for further investigations in this chapter was that females are more sensitive to decreased availability of β adrenergic receptors than males with regard to decision-making performance. However, results showed that artificially blocking NE receptors with propranolol disrupted decision-making performance and sucrose consumption, but not in a sex-dependent manner. These results suggest that male decision-making behavior was more sensitive than female to the NE manipulations performed here, but not the adrenoceptor manipulations.

3.5.1 Atomoxetine

Male decision-making behavior in the 2AFC task was disrupted to a greater extent than female behavior by artificial increases to NE availability. Previous literature reported that NE availability regulates arousal, impulsivity, motivation, and decision-making in rodents, as determined through behavioral tests including attentional set shifting, the five-choice serial reaction time task, the go/no-go task, and the progressive ratio task [Newman et al., 2008; Cain et al., 2011; Bradshaw et al., 2016; Higgins et al., 2020]. The present study investigated the 2AFC decision-making task in rodents, concluding that manipulations to NE availability significantly impacted performance. Although the manipulations were different, in some previous studies rodents were digging rather than lever pressing, the findings agreed that NE availability played a critical role in decision-making behavior [Newman et al., 2008; Cain et al., 2011; Bradshaw et al., 2016].

The present results also agreed with previous studies that found greater behavioral detriments at moderate and high doses of ATM, further supporting the inverted U function of NE [Newman et al., 2008; Cain et al., 2011; Bradshaw et al., 2016; Higgins et al., 2020]. Further, when female and male performance was compared, the results of the present study suggested that males were more sensitive to ATM manipulations than females. While there was no significant sex difference due to ATM administration in a previous study using the gambling task, a more straight forward decision-making task like the 2AFC task, motivation test, and sucrose consumption test resulted in significant sex differences [Chernoff et al., 2021].
Like the 2AFC results, males in the present study were significantly more sensitive to ATM manipulations in measures of motivation and sucrose consumption compared to females. These findings support previous progressive ratio results in males, finding decreased performance with high doses of ATM, and add female data [Higgins et al., 2020]. The data further suggest that the 2AFC results may have been driven by underlying sex differences in motivation and interest in the reward.

If females had more drastically decreased performance of the 2AFC task upon increased NE availability, it might suggest that females are already operating under greater NE activity and the same increase in NE availability would push females out of their optimal LC-NE range faster than males. In this case, males would have to build up to female levels before getting pushed out of the optimal range so it would take greater pharmacological manipulation to decrease their decision-making performance. Alternatively, if there were no sex differences in 2AFC performance, it might suggest that other neurotransmitters aside from NE could be driving the sex differences observed in Chapter 2. For example, while the LC is the main cortical source of NE, it also has more subtle dopaminergic, galanin, glutamate, or neuropeptide Y activity in some brain regions [Kempadoo et al., 2016].

The present study concluded that there were sex differences in decision-making behavior when NE became artificially more available. The data supported the prediction that the same increase in NE across females and males would decrease decision-making performance in males more than females because females are better suited for higher LC activity. The next question those results lead to is what anatomical feature(s) underly that sex difference. The findings of the present study might be influenced by a variety of factors including different receptor levels. Greater NE availability could lead to more NE binding to more receptors and/or to different, lower affinity receptors, which could produce different behaviors.

3.5.2 Propranolol
The behavioral effects of some of those adrenoceptors were tested in this chapter. When β adrenoceptors were artificially blocked by propranolol, both female and male decision-making behavior in the 2AFC task was disrupted. Changes in decision-making behavior were likely driven by propranolol driven changes in reward consumption but not motivation to seek reward. This effect replicates a trend observed in previous male rat studies using the delay discounting task and adds female data to the conversation [Schwager et al., 2014]. In the previous study, propranolol increased male preference for delayed large over immediate small rewards [Schwager et al., 2014]. This is considered an improvement in delayed decision-making. However, previous studies report no change in reward consumption, possibly due to the complexity of previous tasks in comparison to the present sucrose consumption measure [Schwager et al., 2014]. Perhaps the sucrose consumption in previous studies was decreased or increased based on desire to perform or fatigue from performing the decision-making component of the task.

Adrenoceptors have been shown to regulate decision-making in rodents, as determined through behavioral tests including delayed discounting and sign vs. goal tracking [Schwager et al., 2014; Pasquariello et al., 2017]. The present study tested the 2AFC task in rodents, concluding that manipulations to β adrenoceptors significantly impacted performance. Although the manipulations were different, in previous studies reward sizes varied throughout the task or the reward itself was flavored food pellets, the findings agreed that adrenoceptors played a critical role in decision-making behavior and took the field one step further to suggest that β adrenoceptors may play a critical role in regulating decision-making behavior [Schwager et al., 2014; Pasquariello et al., 2017]. However, different β adrenoceptors play different roles in regulating behavior. β adrenoceptors are found on glutamatergic pyramidal cells and GABAergic interneurons, stimulating adenylyl cyclase and cyclic adenosine monophosphate-dependent protein kinase (PKA) via the Gs pathway [Aoki et al., 1997; Liu et al., 2014]. Once PKA is activated, many different cellular responses and behaviors can follow. For example, the β1 and β2 adrenoceptors can suppress
cortical circuits or inhibit working memory in the PFC, potentially leading to inattention or reduced hyperactive behavior [Aoki et al., 1997; Liu et al., 2014]. Considering those activities, the results reported here could be interpreted as increased inattention in decision-making tasks via blocking β adrenoceptors, because they decreased task engagement and increased task disengagement. This effect was observed in females and males, suggesting that the prediction that the same decrease in β adrenergic receptor availability across females and males would decrease decision-making performance in females more than males because females are better suited for higher LC activity was not supported. These findings have clinical implications for individuals whose baseline decision-making leans toward hyperarousal, such as those diagnosed with attention-deficit/hyperactivity disorder. Blocking these adrenoceptors could bring them to a more productive level of NE activity.

3.6 Conclusion

This study confirms that the same manipulations of NE signaling can drive differences in decision-making behavior between males and females. Specifically, the results of ATM administration before 2AFC performance impacted males but not females, showing the opposite trend from previous investigations that impacted females more than males by chemogenetically increasing LC activity before 2AFC performance [Chapter 2]. Rather than females decreasing performance, males drove a decrease in performance of this decision-making task, especially with the strongest manipulation. Additionally, sucrose consumption and motivation were impacted by ATM in both females and males. In contrast, the propranolol manipulation that blocked β adrenoceptors decreased performance in both sexes. Sucrose reward consumption may have driven the decision-making disruption from blocking β adrenoceptors, while motivated behavior was not a likely driver. These results indicate that females should be included in all LC-NE research and investigated as a unique test group from males, as well as considered for unique interventions upon decreased task performance.
Figure 3.9. Summary schematic. A) At baseline and upon vehicle administration, NE binds to β adrenoceptors on post-synaptic cells. This sets a Gs-coupled cascade in motion and produces average behavior in both females and males. B) ATM administration maintains NE in the synaptic cleft for longer periods of time, so there is greater chance that NE binds to more adrenoceptors. In females, this does not change behavior. However, in males, this decreases decision-making behavior. C) Propranolol administration blocks β adrenoceptors so NE cannot bind to them. In both females and males, this decreases performance in the 2AFC decision-making task.

3.7 Future studies

Having determined that there are sex differences in NE regulation of decision-making behavior, future studies should investigate the density of LC projections to decision-making centers of the brain, like the PFC, to determine the extent of adrenergic signaling in producing those sex differences. Due to the common misconception that findings from investigations of males exclusively can be applied to females, it is important to establish that sex differences in NE regulated decision-making behavior exist. But if we do not understand how these sex differences
came to be, then we cannot establish interventions to return both females and males to optimal
decision-making abilities. One possible mechanism of this sex difference is different density of
LC-PFC projections in females and males. While the LC maintains direct projections to the PFC,
there could be different strength of projections in female and male brains based on previous studies
that have found denser projections from the cortex to the LC in males and denser LC
collateralizations in females [Sun et al., 2020; Bangasser et al., 2011; Bangasser et al., 2013; Pinos
et al., 2001]. Therefore, it is possible that the manipulations to LC activity discussed here were
influenced by underlying sex differences in brain region connectivity. Future studies should
determine the comparative strength of female and male LC-PFC projections. One way to determine
this relationship would be through tissue stains and quantification of PFC axons originating in the
LC. Similar studies have been conducted in other neural circuits, but they did not investigate this
circuit [Sun et al., 2020].

The functional impact of sex differences in LC-PFC projection strength can also have a variety of
downstream effects depending on the density of PFC adrenoceptors. Just as it is important to
understand sex differences in LC-PFC projection density, it is important to understand sex
differences in PFC adrenoceptor density. Understanding how NE signaling through specific
adrenergic receptors impacts sex differences in NE regulation of decision-making behavior in the
2AFC would make strides towards filling the knowledge gap of mechanism by which this
regulation occurs. These investigations may also add context in which to better understand the
propranolol results from the present chapter. Blocking β adrenergic receptors was the only LC-NE
manipulation reported here that produced similar decision-making results in females and males. It
is possible that the use of a non-selective β receptor antagonist such as propranolol obscured further
sex differences in receptor influence on decision-making behavior. Previous studies have reported
opposing effects of β1 and β2 receptors, as well as conflicting behavioral results upon addition of
females to an otherwise male data set [Ramos et al., 2005; Ramos et al., 2008]. Therefore, future
studies should investigate these receptors independently in females and males to identify their role in the brain and behavior across sexes. The next chapter will address the density of LC-PFC projections and PFC adrenoceptors in female and male rats.
Chapter 4

SUBSTRATES OF NE-PFC INNERVATION ACROSS FEMALE AND MALE RATS

4.1 Abstract

Sex differences are known to exist in the neural anatomy of the locus coeruleus (LC) norepinephrine (NE) system, from cortical inputs to cell number and dendritic arborization. LC-NE sex differences may have behavioral relevance, leading to sex differences in decision-making and other noradrenergic mediated functions. This study aimed to broaden the extent of sex differences investigated by comparing NE projections to a region known as a main regulator of decision-making behavior, the prefrontal cortex (PFC), in female and male Long-Evans rats. A three-pronged approach was taken to address this topic: 1) compare density of NE projections to the PFC across females and males, 2) compare density of NE release sites in the PFC across females and males, and 3) compare cell-type distribution of RNA coding for NE receptors in the PFC across females and males. The present study found no significant sex differences in NE projections or release sites. However, β1 receptor RNA showed a differential expression pattern in females with higher expression than males in glutamatergic projection neurons and lower expression in non-glutamatergic PFC cells. Further, β2 receptor RNA revealed the opposite relationship, with higher expression in male glutamatergic projection neurons compared to females. This suggests a potential role of β receptors that may underlie the sex differences observed in NE-PFC regulated decision-making across prior Chapters.

4.2 Background

As noted in prior Chapters, the LC in the dorsal pons of the brainstem and the PFC are both key regulators of decision-making. The LC has been described as a center for arousal regulation and a major neuromodulator for cognitive processes [Berridge and Waterhouse, 2003; Aston-Jones and Bloom, 1981; Grant et al., 1988; Herve-Minvielle and Sara, 1995; Rasmussen et al., 1986]. The
PFC is critical for higher order cognitive functioning including decision-making [Rich et al., 2018; Arnsten, 2006]. These two regions are linked, as the LC releases NE and neuromodulatory peptides in the PFC via direct projections [Chandler et al., 2014; McGaughy et al., 2008]. The LC is the main source of NE to the PFC [Moore and Bloom, 1979; Berridge and Waterhouse, 2003; Aston-Jones and Cohen, 2005; Robertson et al., 2013]. The connections between the LC-NE system and the PFC are well understood in males, but that understanding is not fully extended to females. Due to their respective roles in decision making, the present study assessed NE regulation of the PFC from an anatomical perspective. The anatomical relationship of LC-PFC projections across males and females was explored to investigate potential mechanisms that may underlie the sex differences observed in motivated behavior including decision-making behavior in Chapters 2 and 3.

Although most research is not sex inclusive, several comparative studies including previous chapters of this dissertation have highlighted sex differences in behavior and anatomy. Additional sex differences have been independently observed in decision-making behaviors and the LC-NE system. This Chapter focuses on NE regulation of decision-making through LC projections to, and NE targets within, the PFC [Bangasser et al., 2011; Bangasser et al., 2013; Pinos et al., 2001; Busch et al., 1997; Ohm et al., 1997]. Previous studies have described sex differences in LC size, dendritic arborizations, and inputs, but LC projection outputs have not previously been compared in females and males.

Once NE is released in the PFC, it must bind to an adrenergic receptor (adrenoceptor) to take effect. To gain an understanding of the effect NE has on the PFC, RNA coding for different adrenoceptor subtypes that NE could bind to in the PFC was investigated. Previous unpublished results from the Vazey lab suggested that β adrenoceptors were most influential in performance of the decision-making behavior tested here. However, there is more than one subtype of β receptor [Ramos et al., 2007]. As β1 and β2 receptors are most prevalent in the PFC and there is limited evidence for β3 receptors, this chapter focused on β1 and β2 adrenoceptor RNA in the PFC [Ramos et al., 2007].
The two main cortical subtypes, β1 and β2, can be distinguished by expression of *adrb1* or *adrb2* RNA by the post-synaptic cell.

Different β receptors yield different cellular and behavioral results [Ramos et al., 2005; Ramos et al., 2008]. β1 receptors have been found to be more inhibitory and β2 receptors have been found to be more excitatory [Ramos et al., 2005; Ramos et al., 2008]. Previous investigations of β adrenergic receptors revealed potential sex differences as well. These publications focused on male data, and when female data was introduced, it appeared that β receptors produced potentially opposite effects [Ramos et al., 2005; Ramos et al., 2008]. However, the role of β receptors in the PFC has not been specifically compared in females and males.

Within post-synaptic targets, NE acts as a neuromodulator via multiple G-protein coupled receptors. Adrenoreceptors are expressed on glutamatergic projection neurons, interneurons, and non-neuronal cells such as astrocytes and microglia, creating additional complexities in post-synaptic impacts [Waterhouse et al., 1982; Huang et al., 1996; Ji et al., 2008] (Figure 4.1). The response of a brain region to NE is related to which receptor it binds to in the cells of that region. β receptors investigated in this Chapter are generally Gs-coupled [Xing et al., 2016].

![Figure 4.1. Adrenoceptor activity schematic. β adrenoceptors can have excitatory or inhibitory effects in the PFC by binding to different postsynaptic targets. Via the Gs pathway, β adrenoceptors stimulate adenylyl cyclase, which converts adenosine triphosphate into cyclic adenosine monophosphate (cAMP) [Aoki et al., 1997; Liu et al., 2014]. Then, cAMP-dependent protein kinase (PKA) phosphorylates calcium channels to let more calcium into the cell, leading to overall excitation. Alternatively, β adrenoceptors can interact with GABA activity in a variety of ways, some of which result in overall inhibition [Waterhouse et al., 1982; Zsiros and Maccaferri, 2008].](image)
In the present investigation, tissue was labeled not only for β noradrenergic receptors, but also target cell phenotype based on \textit{vGlut1} (excitatory neuron) or \textit{vGat} (inhibitory neuron) RNA expression. Further, \textit{vGlut1} cells are more likely to be pyramidal projection neurons and \textit{vGat} cells are more likely to be local interneurons [Warren et al., 2019]. Cells that are neither are more likely to be glial cells like astrocytes.

The present study compared the NE-PFC pathway in females and males via three avenues: 1) NE projections to the PFC, 2) NE release sites in the PFC, and 3) NE receptors in the PFC. Differences in any of these measures may contribute to behavioral sex differences observed in Chapters 2 and 3 of this dissertation.

A previous study in mice found sex differences in cortical inputs to the LC [Sun et al., 2020]. However, LC-NE inputs to the PFC have not been outlined in females and males. Therefore, in the first avenue of investigation this chapter anterogradely traced NE axons in the PFC of females and males to fill that gap in comparative anatomy.

However, synaptic NE release sites exist along the axon and their quantification cannot necessarily be predicted purely by axon density [Aoki et al., 1997]. Therefore, in the second avenue of investigation, this chapter further quantified NE boutons in the PFC.

NE released from those boutons into the PFC could produce a variety of cellular effects depending on the receptors and cells on which it acts [Waterhouse et al., 1982; Huang et al., 1996; Ji et al., 2008]. Therefore, in the third avenue, this chapter quantified the distribution of β adrenoceptors on different cell-types in females and males.

The present experiments aim to fill the knowledge gap linking sex differences in decision-making and sex differences in NE circuitry. The extent of anatomical sex differences in LC-NE of females compared to males is currently unknown.

\textbf{4.2.1 Present study}
This chapter assesses signaling from NE projections in the PFC of females and males. Axons in the PFC that are capable of NE release, likely from the LC, were investigated. Quantifying the density of these projections will aid in our understanding of sex differences in decision-making. Next, this chapter assesses the NE release sites along those axons in the PFC of females compared to males. Even if axon density differs between sexes in the PFC, it remains important to identify whether release sites will be equally dense in both sexes. Finally, this chapter assesses β adrenoceptor RNA in the PFC of females and males to address whether differences in adrenergic receptor distribution may underpin some behavioral sex differences in prior Chapters.

The present study assessed females and males in the above investigations, to fill the previous gap in female and male comparative studies. Previous studies have found more cells and denser dendritic arborizations in the LC of females compared to males, suggesting that they may have an increased potential for output and increased ability to incorporate inputs [Bangasser et al., 2011; Bangasser et al., 2013; Pinos et al., 2001; Busch et al., 1997; Ohm et al., 1997]. This would mean that females have more fortified neural infrastructure for LC-NE function. The hypothesis of this chapter is that a more fortified neural infrastructure for LC-NE function in the female brain compared to the male brain not only exists in the brainstem, but also extends to NE projections and/or their binding sites in prefrontal target regions. Thus, predicted outcomes are more axons and/or receptor RNA in the female brain compared to the male brain.

4.3 Methods

The schematic below shows the overall methodological approach for the current Chapter. Brains were collected from female and male Long-Evans rats and processed for immunohistochemistry to identify noradrenergic axons from the LC and their bouton-en-passant for select PFC regions for analysis by approaches 1 and 2 (outlined above). In a separate set of animals, brains were processed for in situ hybridization using RNAscope to identify β adrenoreceptor expression in select PFC regions for approach 3.
Figure 4.2. Methods overview schematic. Different rats were assessed for different experiments. For axon and bouton analysis, eight females and eight males were perfused, labeled with antibody tags, and microscopically imaged and analyzed. For RNA analysis, four different females and four different males were fresh frozen, labeled with fluorescent RNA tags, and microscopically imaged and analyzed.

4.3.1 Animals

Female (n=12, weighing 175-200 g) and male (n=12, weighing 275-300 g) adult wild type Long-Evans rats (Charles River Laboratories, Wilmington, Massachusetts, USA) were kept in temperature- (68-78°F) and humidity-controlled (30-70%) conditions under a 12 h reverse light/dark cycle (lights off at 9am). Animals included in approaches 1 and 2 were individually housed, while those included in approach 3 were pair housed. All experimental protocols were approved by the UMass Amherst Institutional Animal Care and Use Committee, and all procedures were carried out in compliance with the policies and regulations of the guidelines approved by the National Institutes of Health Guide for the Care and Use of Laboratory Animals.

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Table 4.1. Total number of animals included in anatomical experiments and final analysis.

4.3.2 Axon and bouton analysis

4.3.2.1 Perfusion and brain retrieval

Female (n=8) and male (n=8) rats that had undergone behavioral testing were injected with at least 1.5 mg/ml ketamine, xylazine, and saline solution (97.5 mg/kg ketamine and 15 mg/kg xylazine) 20-80 minutes before perfusion (e.g.: A rat that weighed 510 g received 0.77 ml of the solution; Henry Schein Animal Health from USA). Once unresponsive to toe pinch and righting reflex was lost, each rat was perfused transcardially with 200 ml 0.9% saline, followed by 400 ml 10% formalin (VWR International, USA). The brains were immediately removed and stored in 10% formalin overnight. The next day, the brains were transferred to a 20% sucrose-azide solution until it was fully absorbed, as observed by the brains sinking to the bottom of a brain jar. The tissue was then submerged in -80°C isopentane, mounted on a cryostat (Leica CM3050S, Germany) at -20°C, and serially sliced from Bregma -11.16 mm A/P to Bregma -8.52 mm A/P reference brain coordinates from the Paxinos and Watson Rat Brain Atlas, to collect 40 micron thick serial coronal sections, (160 microns between adjacent sections within a series) of the entire LC [Paxinos and Watson, 2007]. This tissue was stored in phosphate buffered saline (PBS)-azide until chromagen staining.

4.3.2.2 Staining and imaging noradrenergic axons in female and male PFCs

Dopamine-β-hydroxylase (DBH) is a conversion factor required to make NE [Kaufman and Friedman, 1965]. By quantifying the DBH axons in the PFC, this experiment quantified the axons capable of NE release in the PFC, differentiating them from other monoaminergic projections in the region. Projections to the PFC were isolated with DBH using immunohistochemically labeled tissue with anti-DBH antibodies. DBH was labeled with DAB stain across the PFC (Bregma + 2.6-3.4). NE cells were labeled with a DAB-Ni solution for DBH reactivity. The brain tissue was stained with primary antibodies that bound to DBH (lot no. MAB308, Millipore Corp, Bedford,
MA, USA) and biotinylated secondary antibodies for later detection (lot no. 122849, Jackson ImmunoResearch Laboratories, West Grove, PA, USA). With this stain, the tissue was microscopically assessed for DBH in the PFC of each hemisphere of each rat. Specifically, PFC sections were washed three times for five minutes in PBS solution on an orbital shaker at 100 rpm. Then the tissue was incubated for ten minutes in a H$_2$O$_2$, methanol, and RO H$_2$O solution. The tissue was then washed three times for five minutes in PBS with 0.2% Triton (PBST) and then incubated for 60 min in a blocking solution of PBST and 3% Normal Donkey Serum. The tissue incubated in blocking solution and 1:15,000 primary antibody at -4°C for two days on an orbital shaker. The next day, tissue was washed three times in five-minute increments in PBST and then incubated in blocking solution with 1:150 secondary antibody for 2.5 hours. Next, tissue was washed three times for five minutes in PBST and incubated for 2 hours in a 1:500 dilution of avidin-biotin complex (ABC Vectastain, Burlingame, CA, USA), followed by two washes for five minutes each in PBST. After one last wash for five minutes in Tris buffer, the tissue incubated for ten minutes in 0.2 mg/ml DAB and 6 mg/ml Nickel ammonium sulfate (Fisher Scientific, Pittsburgh, PA, USA). The tissue was wet mounted in 0.1 M PB on Superfrost Plus slides and left to dry overnight. Tissue then went through dehydration in 50%, 70%, 100%, and 100% ethanol for 10 minutes at room temperature and defatting with xylene and completed NISSL counter staining before cover-slipping with DPX mounting solution (Electron Microscopy Sciences, Hatfield, PA, USA).

The Zeiss Axioimager M2 microscope with brightfield imaging was used to visualize DAB-Ni stained DBH labeled axons and the MBF StereoInvestigator software was used to collect images at 10x magnification. The following subregions of PFC were evaluated: Secondary motor cortex (M2), anterior section of the anterior cingulate cortex (ACC), Prelimbic PFC, ventromedial PFC, medial orbitofrontal cortex (OFC), and lateral OFC [Figure 4.3]. Each subregion of the PFC was imaged separately (Figure 4.3).
Figure 4.3. PFC subregions assessed. Secondary motor cortex (M2) in red, anterior section of the anterior cingulate cortex (ACC) in purple, Prelimbic in blue, ventromedial PFC in green, medial orbitofrontal cortex (OFC) in yellow, and lateral OFC in orange. A) Bregma +3.4 mm, B) Bregma +3 mm, C) Bregma + 2.6 mm A/P.

Next, ImageJ image processing software was utilized to make the image binary, which makes the black axons stand out against a white background (Figure 4.4b). By adjusting the threshold of this image, any cells in the background were removed to assess only axons. Next, the same image was analyzed again to gather information on what region of the image was covered by the tissue (Figure 4.4c). Some brain regions sampled were on the outer edge of the brain, so the images occasionally included empty space where the tissue ended. There were also instances where the tissue was torn during immunohistochemical processing (Figure 4.4c). If the tissue were simply quantified using the black area on an axon thresholded image without taking this empty space into consideration, it would have confounded results. Therefore, for each image, the area that contained tissue was outlined and called quantified area. Then, the quantification of black area in each axon image was divided by the quantification of black area in the corresponding tissue image. This allowed for
creation of a numerical representation of the percent of tissue area covered in DBH axons for each subregion of the PFC.

Figure 4.4. Tissue processing for axon density measurement. A) Tissue was immunohistochemically stained. B) Using imageJ image processing software, the image was thresholded to highlight the axons and remove background cells. C) Using imageJ, any regions of the image that were not brain tissue were outlined so that tears or edges didn’t influence the axon density representation.

A total of four subregions of the PFC were analyzed bilaterally in each animal (Figure 4.3). Thus, the axon experiments analyzed the anatomy of 16 female and 16 male Long-Evans rat brain hemispheres. A power analysis using G*Power software revealed that 13 female and 13 male data points would yield sex comparison results with 0.81 power. Additionally, each data point represented one animal. The average of three sections of brain tissue from anterior to posterior and two hemispheres totaling six data points went into each animal’s data point.

4.3.2.3 Analysis of noradrenergic release sites along axons in female and male PFCs

The DBH axon release sites in the PFC were quantified using the same DBH DAB immunohistochemistry stained tissue and additional StereoInvestigator analysis (Figure 4.5). StereoInvestigator software was used to input the relevant parameters of the samples so that the software could calculate estimates of total boutons based on the samples counted. Counting frame size was set to x (width)=25, y (height)=25. Grid size was set to x=250 μm, y=250 μm. This way, only the release sites in a representative sample of the tissue were counted rather than the entire tissue section. Three samples of 40 micron sections were analyzed, each 8 sections apart from the last (as recommended by MBF Bioscience; average Gundersen’s coefficient of error=0.06133).
Additionally, the tissue thickness was measured once for every three sections analyzed. The region of interest was outlined based on the Paxinos and Watson Rat Brain Atlas, for each subregion of the PFC on each section of tissue. Then, a grid was overlaid onto the live image of each sample section and the number of boutons in a small square shown in red and green was manually counted once per each section of that overlaid grid (Figure 4.5). While counting boutons, the 40 microns of tissue was surveyed dorsal to ventral. A bouton was considered to be any location where the axon bulged out in a dark circle, indicating an accumulation of NE ready for release. The grid was set at 10x magnification and boutons assessed at 40x magnification. The same number of images from the same number of animals and the same brain regions were assessed in bouton analysis as in the previous axon analysis.
Figure 4.5. StereoInvestigator was used to count boutons. First, a virtual grid was placed over the tissue for manual count of each bouton in a sample (the green and red box) at each cell of the grid (sampling sites in the top right corner). Then those counts were used to estimate the overall population of the entire image. Each blue dot represented one bouton marked. The red and green scale bar on the left of the tissue shows depth through the image. As release sites were counted, the microscope focus was accordingly adjusted to count the sites at different depths of the tissue.

4.3.3 RNA analysis

4.3.3.1 Brain retrieval and freezing

Female (n=4) and male (n=4) rats were injected with at least 1.5 mg/ml ketamine, xylazine, and saline solution (97.5 mg/kg ketamine and 15 mg/kg xylazine) 20-80 minutes before decapitation (e.g.: A rat that weighed 510 g received 0.77 ml of the solution; Henry Schein Animal Health from USA). Once unresponsive to toe pinch and righting reflex was lost, each rat had their spine lethally severed. The head was then removed and the brain extracted for analysis. RNAscope brains were fresh frozen at -80° C wrapped in foil. The tissue was then dry mounted on a cryostat at -15° C, and serially sliced in 16 micron sections to collect coronal slices of the PFC. This tissue was stored at -80° C on SuperFrost+ slides until RNAscope staining.

Some groups have successfully performed similar RNAscope stains in slices as thick as 40 microns [Grabinski et al., 2015], but to optimize results the tissue used in the present study was sliced in 16 micron sections [Padilla et al., 2019; Rubio et al., 2015; Wang et al., 2012; Warren et al., 2019].

4.3.3.2 Labeling of β adrenoceptor RNA in female and male PFCs

Using RNAscope (ACD Bio) multiplex RNA labeling, β adrenoceptor RNA, vGat RNA, and vGlut1 RNA were targeted and made fluorescent as indications of probable cells with NE receptors, inhibitory interneurons, and excitatory pyramidal cells in the same tissue (Figure 4.6). Two rounds of RNAscope labeling were completed for each subregion and each sex. One round included probes for adrb2 RNA, vGat RNA, and vGlut1 RNA, while the other round included probes for adrb1 RNA and vGlut1 RNA. This multiplex approach was utilized to label each of these qualities in the same cell and quantify the cell type that β adrenoceptors dominated. This allowed for estimation of
the relative influence of β adrenoceptors on excitatory and inhibitory PFC activity in females and males.

The RNA probes in this study were from the RNAscope Multiplex Fluorescent Reagent Kit (Advanced Cell Diagnostics). Slides with PFC sections were dehydrated in 50%, 70%, 100%, and 100% ethanol for 10 minutes at room temperature. They air dried and then a barrier was drawn around the tissue using a hydrophobic pen. The tissue was then incubated in a humidified box with hydrogen peroxide inside the tissue barrier for 10 minutes and was washed twice with distilled water. The tissue was treated with protease pretreatment III (Advanced Cell Diagnostics) for 15 minutes, then the tissue was rinsed twice in distilled water. Next, the tissue was incubated with the RNA probes (or a positive ubiquitin probe vehicle or negative dapB probe vehicle) at 40° C for 120 minutes, then washed twice in wash buffer and incubated at room temperature for 2 minutes. Then, the tissue was incubated in preamplifier and amplifier probes at 40° C for 30 minutes each: Amp1, Amp2, Amp3 with wash buffer washes in between. Next, the horseradish peroxidase (HRP) C1 was added to the tissue and left to incubate for 15 minutes at 40° C, then washed in wash buffer and added Amp4 dye and incubated at 40° C for 30 minutes. Finally, the tissue was washed in wash buffer, incubated in HRP blocker at 40° C for 15 minutes, washed in wash buffer, and cover-slipped in mounting medium. The tissue was stored in the dark to avoid bleaching the fluorescent labels.
**Figure 4.6.** Two representative cells stained using RNAscope technology. Blue = nucleus, green = adrb2 (or adrb1 in other images), magenta = vGlut1, and yellow = vGat. A) Neuron that NE binds to. Filled in arrow indicates a cell, as identified by blue DAPI stain, with green fluorescence labeling a β adrenoceptor and far red fluorescence artificially colored magenta labeling RNA commonly found in glutamatergic cells. This cell is likely to be a neuron that NE binds to. B) Interneuron that NE does not bind to. Hollow arrow indicates a cell, as identified by blue DAPI stain, with red fluorescence artificially colored yellow to stand apart from the other colors, labeling RNA commonly found in GABAergic cells. This cell is likely to be an interneuron that NE does not bind to.

PFC tissue was imaged on a Zeiss Axioimager M2 epi-fluorescent microscope at 20x magnification for green, blue, red, and far red fluorescent signals. The amount of RNA labeling was manually assessed by counting discrete fluorescent puncta in cells of female and male tissue. Additionally, MBF Stereoinvestigator and ImageJ software were utilized to assess the amount of RNA label compared to cell label.

A total of six subregions of the PFC were analyzed bilaterally in each animal (Figure 4.3). RNA data was also split across four different RNA markers per animal. The RNA data included four animals per sex and two hemispheres per animal.

### 4.3.4 Statistical analyses

All data were presented as mean +/- SEM unless noted otherwise. Normally distributed data were analyzed by mixed model repeated measures two-way analysis of variance (ANOVA) for PFC subregion and sex, followed by post hoc Dunnett’s or Sidak’s test. Kolmogorov-Smirnov test was used to assess normality in all data sets. Statistics and graph composition was undertaken in Matlab and/or GraphPad Prism version 7 for Windows. The accepted \(\alpha\leq 0.05\).

### 4.4 Results

#### 4.4.1 Females had similar NE axon innervation of the PFC compared to males

To identify potential anatomical underpinnings for sex differences in NE-PFC regulated behavior identified in Chapters 2 and 3 of this dissertation, female (n=8) and male (n=8) brains were stained for NE projections in the PFC. There were no sex differences in the tissue (Figure 4.7).
4.4.2 Females had similar NE bouton density in the PFC compared to males

In quantitative analysis, females and males yielded similar results (Figure 4.8a; $F_{(1,14)}=3.151$, $p=0.0976$, two-way ANOVA, n=8 females, n=8 males). Additionally, NE release sites in the PFC were evaluated. There were no sex differences in bouton density across the four PFC subregions analyzed with comparable results across females and males (Figure 4.8b). Neither of the quantitative assessments yielded statistically significant results. Together, these results suggest that females may have slightly more NE projections, but are statistically similar to males with a similar density of release sites in the PFC.
Females trended toward more NE innervation of the PFC, but not NE release, than males. A) Females trended toward denser noradrenergic capable axons in the PFC than males overall. B) There were no significant sex differences in the number of NE release sites in the PFC.

4.4.3 RNA reveals sex differences in the PFC

The following assessment continued to trace the path of NE through the PFC by quantifying a selection of adrenoceptor RNA in the PFC of females (n=4) compared to males (n=4). Overall, there were significantly more cells labeled with β2 adrenoceptor RNA than β1 across PFC regions (Figures 4.9a and 4.11a; F(3,72)=36.44, p<0.0001, two-way ANOVA, n=4 females, n=4 males).

4.4.3.1 β2 RNA reveals limited sex differences in the PFC

There was a main effect of sex on the average number of cells labeled with β2 RNA (Figure 4.9a; F(1,36)=25.13, p<0.0001, two-way ANOVA, n=4 females, n=4 males). Females had more β2 RNA labeled cells than males, particularly in the ACC (Figure 4.9a; p=0.0455, Sidak’s test, n=4 females, n=4 males). Among cells stained with β2 RNA, there was no clear cell type dominance, although males had a larger proportion of β2 RNA on glutamatergic cells than females, as observed in a main effect of sex (Figure 4.9b; F(1,36)=6.222, p=0.0173, two-way ANOVA, n=4 females, n=4 males). β2 RNA was generally split evenly between GABAergic and glutamatergic cells, with some additional labeling on other cell types (Figure 4.9b-d). Therefore, β2 RNA was relatively evenly distributed across cell types within the PFC (Figure 4.10).
Figure 4.9. Cells labeled with β2 adrenoeceptor RNA were not significantly sexually differentiated in the PFC, with the exception of the ACC. Each data point on these graphs represents the average of data from one image of the left hemisphere and one image of the right hemisphere. Percents were out of all labeled cells in that image. A) Females had more cells with β2 RNA than males. This result was driven by the ACC. \( F_{(1,36)}=25.13, p<0.0001, \) two-way ANOVA B) There were no significant sex differences in glutamatergic cells labeled with β2 RNA. \( F_{(1,36)}=6.222, p=0.0173, \) two-way ANOVA C) There were no significant sex differences in GABAergic cells labeled with β2 RNA. D) There were no significant sex differences in non-glutamatergic, non-GABAergic cells labeled with β2 RNA.
**Figure 4.10.** Representative images of β2 RNAscope data. Blue = nucleus, green = adrb2, magenta = vGlut1, and yellow = vGat. Star = glutamatergic cells with β2 RNA, diamond = GABA-ergic cells with β2 RNA, hourglass = other cells with β2 RNA. A) Female tissue. B) Male tissue.

### 4.4.3.2 β1 RNA shows sex differences in PFC distribution

β1 RNA, on the other hand, was almost entirely clustered on glutamatergic cells (Figure 4.11b). Previous studies have claimed that β1 RNA is much less commonly found on GABAergic neurons in the PFC than β2 RNA, so it was not co-labeled in the present experiment [Hertz et al., 2010]. A larger proportion of β1 RNA labeled cells were glutamatergic in females compared to males, as observed in a main effect of sex (Figure 4.11b; F(1,36)=47.52, p<0.0001, two-way ANOVA, n=4 females, n=4 males). This sex effect was driven by the ACC (Figure 4.11b; p=0.0143, Sidak’s test, n=4 females, n=4 males), ventromedial PFC (Figure 4.11b; p=0.0014, Sidak’s test, n=4 females, n=4 males), and the medial OFC (Figure 4.11b; p=0.0125, Sidak’s test, n=4 females, n=4 males).

In contrast, males had a larger proportion of β1 RNA labeled non-glutamatergic cells than females (Figure 4.11c; F(1,36)=47.52, p<0.0001, two-way ANOVA, n=4 females, n=4 males). The sex difference was particularly prominent in medial subregions of the PFC including the ACC (Figure 4.11c; p=0.0143, Sidak’s test, n=4 females, n=4 males), the ventromedial PFC (Figure 4.11c; p=0.0014, Sidak’s test, n=4 females, n=4 males), and the medial OFC (Figure 4.11c; p=0.0125, Sidak’s test, n=4 females, n=4 males).

Previous studies that did not include females have identified β1 receptors present on non-neuronal cells including astrocytes [Hertz et al., 2010]. The results of the present study support that finding. In addition, the current findings show that non-glutamatergic cells (including interneurons and astrocytes) labeled with β1 RNA are present in very low numbers in females (Figure 4.11c). These results were not driven by sex differences in the total number of cells labeled with β1 RNA (Figure 4.11a). Overall, while a majority of β1 RNA labeled cells were glutamatergic in both females and males, the proportion of non-glutamatergic cells labeled with β1 RNA was higher in males than females (Figure 4.12).
Figure 4.11. β1 adrenoceptor RNA distribution across cell-type was sexually differentiated. Each data point on these graphs represents the average of data from one image of the left hemisphere and one image of the right hemisphere. Percents were out of all labeled cells in that image. A) Females and males had a similar number of β1 RNA labeled cells. B) Females had more β1 RNA labeled glutamatergic cells than males. This effect was driven by the ACC, PFCvm, and OFCm. F(1,36)=47.52, p<0.0001, two-way ANOVA C) Males had more β1 RNA labeled non-glutamatergic, non-GABAergic cells than females. F(1,36)=47.52, p<0.0001, two-way ANOVA.
4.5 Discussion

The present study compared the NE-PFC pathway in females and males via three avenues: 1) NE projections to the PFC, 2) NE release sites in the PFC, and 3) NE receptors in the PFC. The hypothesis for this chapter was that a more fortified neural infrastructure for LC-NE function in the female brain compared to the male brain not only exists in the brainstem, but also extends to NE projections and/or their binding sites in prefrontal target regions. Thus, predicted outcomes were more axons and/or more adrenoceptor RNA labeled on excitatory cells in the female brain compared to the male brain. Overall, significant sex differences were found in the distribution of NE receptors across different cell-types in the PFC, and not in NE-PFC projections. These results may contribute to an explanation for sex differences observed in decision-making behavior from earlier chapters of this dissertation.

4.5.1 NE projections to the PFC of females vs males

Figure 4.12. Representative images of β1 RNAscope data. Blue = nucleus, green = adrb1, and magenta = vGlut1. Star = glutamatergic cells with β1 RNA, hourglass = other cells with β1 RNA. A) Female tissue. B) Male tissue.
No significant differences in adrenergic axonal density in the PFC were found between females and males (Figure 4.8a). Previous studies found increases in the complexity of LC dendrites in females compared to males [Bangasser et al., 2011; Bangasser et al., 2013; Pinos et al., 2001]. One previous study investigated sex differences in inputs to the LC, while the present investigations filled the gap of projections from NE sources [Sun et al., 2020]. The present results did not find overt sex differences in noradrenergic axon density in the PFC (Figure 4.8a). This suggests that anatomical sex differences in NE axonal density within the PFC are unlikely to be the substrate for sex differences in decision-making behavior.

4.5.2 NE release sites in the PFC of females and males

LC axons do not solely release NE from axon terminals, but rather have boutons en passant release sites along their axons (Figure 4.5). Through NE bouton quantification in the PFC, a similar number of release sites were identified in females compared to males (Figure 4.8b). Had there been denser NE release sites in females compared to males, it would suggest that females have greater NE innervation of the PFC than males, which would be a likely source of sex differences in decision-making behavior. However, the results are more in line with no sex differences in NE axons or boutons in the PFC. This suggests that anatomical sex differences in axonal density or release sites within the PFC are unlikely to be the substrate for differences in NE mediated behaviors between female and male rats. It could be implied that sex differences observed in previous chapters of this dissertation are not related to density of projections and NE release, but rather what happens after NE has been released, which is why the experiments in this chapter complement each other.

4.5.3 Adrenoceptor RNA in the PFC of females vs males

Males had more β2 adrenoceptor RNA on glutamatergic cells than females (Figure 4.9b). In contrast, females had more β1 adrenoceptor RNA on glutamatergic cells, while males had more β1 adrenoceptor RNA on other non-neuronal cells (Figure 4.11b-c). This sex difference in cell-type labeled adrenoceptor RNA may underly the lack of sex differences in propranolol data reported in
Chapter 3. Since propranolol antagonizes both β1 and β2 receptors, potential sex differences in their activity may have been obscured. A selective β1 antagonist might have resulted in decreased PFC excitation in females more than males, while a selective β2 antagonist might have resulted in decreased PFC excitation in males more than females (see 4.7 Future Directions). The results from blocking both of those β receptors revealed decreased performance of the 2AFC decision-making task in both females and males, but potentially via different β receptors.

While the overall number of cells labeled with β1 adrenoceptor RNA was similar between sexes, female β1 adrenoceptor RNA in the PFC was biased toward projection neurons and male β1 adrenoceptor RNA in the PFC was biased toward other cells. Previous studies reported definitive presence of β1 adrenoceptor RNA on astrocytes, but females were not mentioned in their investigations [Hertz et al., 2010]. The male RNAscope results reported in this chapter agree with those findings and the female results uncover additional sex differences in PFC adrenoceptor RNA. These results could have implications for sexually differentiated astrocyte, interneuron, or other neuronal subtype activity in the PFC. Previous studies report the presence of excitatory adrenoceptors on astrocytes in the PFC [Pittolo et al., 2022; Domin et al., 2014]. Once the astrocyte has been activated, it can have a variety of effects on neighboring cells, from inhibition to excitation [Kang et al., 1998; Parpura et al., 1994; Fellin, 2009]. Therefore, the increased receptor RNA in male astrocytes or other neuronal subtypes may activate inhibitory cells, which could then dampen PFC activity. This finding complements the idea introduced in Chapter 2 of this dissertation that the Yerkes-Dodson relationship may be shifted higher in females compared to males.

While there were sex differences observed in cell-type distribution of β adrenoceptor RNA and the total number of β2 adrenoceptor RNA labeled cells, the total number of β1 adrenoceptor RNA labeled cells was similar in females and males. If neither NE release nor adrenoceptor RNA in the PFC revealed sex differences, the behavioral sex differences observed in previous chapters of this dissertation could have been related to receptor behavior that this first step in receptor
quantification did not assess. There could be differences in receptor expression (see 4.7 Future Studies).

4.6 Conclusion

This study confirms sex differences in NE signaling in the PFC. Specifically, these results have identified significantly more β1 adrenoceptor RNA labeled glutamatergic cells in the PFC of females and β1 adrenoreceptor RNA labeled non-glutamatergic cells in the PFC of males. Additionally, more β2 adrenoceptor RNA labeled glutamatergic cells were found in the PFC of males compared to females. These findings may contribute to the sex differences, or lack thereof, in decision-making behavior observed in previous chapters of this dissertation and suggest that NE activation of astrocytes or other neuronal subtypes may be dampening PFC activity in males compared to females. This sex difference was previously overlooked in studies that did not mention females, indicating that females should be included in NE-PFC research and investigated as a unique test group from males, as well as considered for unique interventions upon decreased task performance.
Figure 4.13. Summary schematic. Females had comparable NE projections and release sites in the PFC to males. RNA for NE receptors tested here was more highly concentrated on glutamatergic cells than GABAergic cells or cells with neither marker. There was a significant sex difference, as males had more adrenoceptor RNA on non-glutamatergic cells than females, females had more β1 adrenoreceptor RNA labeled glutamatergic cells compared to males, and males had more β2 adrenoreceptor RNA labeled glutamatergic cells compared to females.

4.7 Future studies

The present RNA results suggest that the β adrenoceptor antagonist study in Chapter 3 should be repeated with more specific pharmacological targets. Considering that β1 and β2 adrenoceptors are present in greater proportions of female and male glutamatergic cells respectively, potential sex differences in decision-making behavior after blocking both of those receptors could have been obscured. Therefore, future studies could involve independent betaxolol and ICI118,551 administration to specifically block β1 and β2 adrenoceptors respectively before female and male performance of the 2AFC decision-making task.

Having determined that there are sex differences in PFC adrenoceptor RNA, future studies should investigate the role of adrenergic signaling in PFC cellular activity. Since density of RNA encoding adrenoceptors is only a proxy for adrenoceptors expressed in the PFC, further investigations would assess β adrenoceptor internalization using flow cytometry. Flow cytometry was not conducted in the present study because receptors are highly dynamic, so that level of specificity would need to be applied to resting state, baseline performance, LC manipulations, and pharmacology. This is an exciting future direction, but would be quite an undertaking as a first step in the investigation of β adrenoceptors in the PFC.

These investigations open the door for development of sex-specific interventions for NE-regulated behavior such as decision-making. Future studies are required to determine how astrocytes or other neuronal subtypes respond to adrenoceptor activity. Activating astrocytes could have a variety of downstream outcomes depending on the chemicals they release and the cells they act on [Kang et al., 1998; Parpura et al., 1994; Fellin, 2009]. Therefore, future investigations could administer a β1
adrenoceptor agonist into the PFC of an anesthetized rat via microiontophoresis, allowing for calcium imaging to determine local astrocyte response. A follow up electrophysiological recording should be completed for female and male PFC neurons after microiontophoresis of β agonists to see if they increase or decrease activity. Similar studies have been previously conducted, but these proposed experiments would add cell type specificity to the findings [Bunney and Aghajanian, 1976].

A future direction of this research includes electrophysiological recordings from the PFC of females and males to determine increases or decreases to cellular activity upon NE binding. However, RNA proxies for that information are an achievable first step toward that knowledge and can be collected simultaneously with the β adrenoceptor data that the present experiments were designed to probe.
CHAPTER 5

GENERAL DISCUSSION

5.1 Main findings

This dissertation examined sex differences in noradrenergic regulation of decision-making through a set of functional, pharmacological, and anatomical experiments that compared female and male Long-Evans rats. Each chapter assessed a different aspect of rodent brains and behavior to begin to probe the root of sex differences in LC-NE signaling to the PFC. Previous studies investigating sex differences in LC/NE-PFC regulation of decision-making behavior are lacking. Therefore, the findings reported in this dissertation contribute to the literature by adding female comparisons of LC-NE regulation of decision-making to years of male overrepresentation in brain and behavior research [Beery and Zucker, 2011; Woitowich et al., 2020].

The goal of Chapter 2 was to investigate the relationship between LC activity and decision-making behavior by comparing the performance of female and male Long-Evans rats in the 2AFC decision-making task before and after chemogenetic activation of the LC. Results revealed decreased performance in females compared to males, indicating that females are more sensitive to increased LC activity than males. These results suggest that sex differences in sensitivity to LC activity may contribute to sex differences in decision-making behavior.

The goal of Chapter 3 was to determine if the sex differences in LC regulated decision-making behavior are NE dependent. To investigate this question, female and male Long-Evans rats were tested in the same 2AFC decision-making task before and after pharmacologically increasing the amount of NE available in the brain. Results revealed decreased performance in males compared to females, suggesting that NE may be involved in producing sex differences in decision-making behavior, although perhaps driven by different underlying factors than the sex differences observed after LC manipulations. This indicates that males are more sensitive to increased available NE than females. Further experiments investigated the role of β adrenergic receptors in this NE activity by
pharmacologically blocking them before task completion. Results of those experiments revealed decreased performance in both females and males, suggesting that β adrenergic receptors play a role in regulating decision-making behavior in both sexes.

The goal of Chapter 4 was to compare NE projections to a main regulator of decision-making behavior, the PFC, in female and male Long-Evans rats. A three-pronged approach was taken to address this topic: 1) compare density of NE projections to the PFC across females and males, 2) compare density of NE release sites in the PFC across females and males, and 3) compare cell-type distribution of RNA coding for NE receptors in the PFC across females and males. Results revealed that females had more glutamatergic and fewer non-glutamatergic PFC cells labeled with β1 receptor RNA, while males had more glutamatergic PFC cells labeled with β2 receptor RNA. This suggests a potential role of β receptors in regulating behavioral sex differences of LC-PFC decision-making observed in previous chapters. Additionally, there may be β1 receptors on interneurons or other neural subtypes that inhibit PFC activity in males but not females.

This research adds to the sex differences previously observed in the LC and provides a basis for understanding the sex differences observed in decision-making behavior [Bangasser et al., 2011; Bangasser et al., 2013; Pinos et al., 2001; Busch et al., 1997; Ohm et al., 1997]. The extent of anatomical sex differences in NE circulation is currently unknown. Sex differences in LC projections to the PFC, NE release sites in the PFC, and/or NE receptors in the PFC may contribute to behavioral sex differences. The present experiments aim to fill the knowledge gap linking sex differences in decision-making and sex differences in LC-NE circuitry.

5.2 Discussion of functional, pharmacological, and anatomical sex differences in noradrenergic regulation of decision-making

5.2.1 Discussion of the role of increased LC activity in female and male decision-making

The long-term goal of this dissertation was to outline sex differences in LC-NE signaling that may impact decision-making. The overarching objective of this dissertation was to compare female and
male behavioral responses to manipulations of the LC-NE system, as well as anatomical substrates that might underlie these behaviors. The hypothesis was that female rats can manage a higher amount of LC activity than males before it impacts their decision-making performance. Based on that hypothesis, it was predicted that the same increase in LC activity across females and males would decrease decision-making performance in males more than females because females are better suited for higher LC activity.

To achieve that goal and test that hypothesis, first the role of increased LC activity in female and male decision-making had to be determined. Previous studies suggest that an optimal level of LC activity can promote focused attention and decision making in a Yerkes-Dodson relationship [Gamo et al., 2010; Aston-Jones and Cohen, 2005; Arnsten, 2011]. However, anatomical sex differences in LC structure and sex differences in behavioral pathologies in which NE is implicated suggest that the Yerkes-Dodson relationship between decision-making and LC activity may vary across sexes [Bangasser et al., 2011; Bangasser et al., 2013; Pinos et al., 2001; Busch et al., 1997; Ohm et al., 1997]. Therefore, the manipulations in this chapter were investigated through a dose-response curve to assess different points along that Yerkes-Dodson relationship.

To directly investigate this relationship between LC activity and decision-making, Chapter 2 of this dissertation activated LC cells through a chemogenetic manipulation before performance of the 2AFC decision-making task. This experiment resulted in greater disruption of female decision-making behavior than male. Further, LC activation with DREADDs showed no impact on motivation, sucrose consumption, and locomotion. These findings suggest that females are more sensitive to increased LC activity than males. However, considering the small sample size, and unclear impacts of this manipulation on LC activity these results should be validated in future investigations.

5.2.2 Discussion of the role of noradrenergic signaling in female and male decision-making
Limited female data exists regarding the effect of ATM on decision-making behavior. One recent study compared the effects of ATM on a gambling task in females and males and found an improvement in performance across both sexes, but additional research in this area is needed [Chernoff et al., 2021]. In line with the results reported here, previous studies have found that males decrease performance in the progressive ratio motivation task after administration of high doses of NE reuptake inhibitor ATM [Higgins et al., 2020]. Further, comparative adrenergic receptor studies in females and males are missing. Studies in males have found that β receptor antagonists decrease performance in behavior such as the working memory task [Ramos et al., 2005; Ramos et al., 2008]. The present study filled those gaps in the literature and investigated the role of NE in decision-making using two pharmacological manipulations: 1) increasing synaptic NE levels and 2) blocking the impact of NE on β adrenoceptors. The hypothesis was that males are more sensitive to increases in NE than females and that females are more sensitive to decreased availability of β adrenoceptors than males with regard to decision-making performance. Based on that hypothesis, it was predicted that the same increase in NE across females and males would decrease decision-making performance in males more than females and the same decrease in β adrenoceptor availability across females and males would decrease decision-making performance in females more than males because females are better suited for higher LC activity. These studies were achieved by administering NE reuptake inhibitor ATM and β adrenoceptor blocker propranolol in females and males before performance of the 2AFC task, progressive ratio task, and sucrose consumption test to determine the behavioral effects of noradrenergic signaling on decision-making, motivation, and reward consumption.

In the present study, ATM disrupted male 2AFC performance more than females. Further investigations showed that ATM also reduced motivation and sucrose consumption in males but not females. This suggests that males are more sensitive to increased NE availability than females.
However, given the additional effects on motivation and consumption behavior, the decision-making results may have been influenced by those underlying factors.

This is the opposite trend from the previous Chapter chemogenetically increasing LC activity before 2AFC performance in which changes were only seen in females (Chapter 2). While male performance decreased upon ATM manipulation, female performance decreased to a much lower degree and in some cases remained unchanged, suggesting that females and males may have different thresholds for NE availability before behavior is impacted.

Additionally, artificially blocking NE receptors with propranolol disrupted decision-making performance and sucrose consumption, but not in a sex-dependent manner. When β adrenoceptors were artificially blocked, both female and male decision-making behavior in the 2AFC task was disrupted.

Collectively the pharmacological studies suggest that male decision-making behavior was more sensitive than female to increases in NE signaling, but performance in both sexes was likely dependent on β adrenoreceptors. However, in addition to changes in decision-making, NE manipulations that led to a reduction in engagement in the 2AFC task also led to reduced interest in reward consumption, which may have been an underlying factor.

In the context of the optimal behavioral performance outlined by the Yerkes-Dodson relationship, excess signaling via adrenoceptors could lead to hyperarousal and inability to focus on a task, while blocking them could lead to hypoarousal and inability to attend to a task [Gamo et al., 2010; Aston-Jones and Cohen, 2005; Arnsten, 2011]. The 2AFC findings reported here complement this idea. This study also confirms that manipulating NE signaling can reveal sex differences in decision-making behavior.

5.2.3 Discussion of substrates of NE-PFC innervation across female and male rats

Several behavioral studies have now identified sex differences in the impact of LC-NE manipulations on decision-making (Chapters 2-3). However, the substrates that may underly these
sex differences have yet to be investigated. The connections between the LC-NE system and the PFC are well understood in males, but that understanding is not fully extended to females. Chapter 4 compared NE-PFC in females and males via three avenues: 1) NE projections to the PFC, 2) NE release sites in the PFC, and 3) NE receptors in the PFC. Differences in any of these measures may contribute to differences in behavioral sensitivity to NE observed across sexes in Chapters 2 and 3 of this dissertation.

The LC-NE neurons release neuromodulatory peptides into the PFC via direct projections [Chandler et al., 2014; McGaughy et al., 2008]. Further, sex differences have been found in cortical inputs to the LC, however outputs from the LC to the cortex have not yet been investigated [Sun et al., 2020]. Given the role of the PFC in decision-making, it is likely that sex differences in NE signaling to the PFC, likely from the LC, contribute to the sex differences observed in Chapters 2-3.

Therefore, this chapter included experiments designed to anterogradely trace NE axons in the PFC of females and males to fill that gap in comparative anatomy. Further, synaptic NE release sites exist along the axon and their quantification cannot necessarily be predicted purely by axon density [Aoki et al., 1997]. Therefore, this chapter additionally quantified NE boutons in the PFC. However, NE released from those boutons into the PFC could produce a variety of cellular effects depending on the receptors and cells on which it acts [Waterhouse et al., 1982; Huang et al., 1996; Ji et al., 2008]. Therefore, this chapter labeled the RNA coding for glutamatergic cells, GABA-ergic cells, and two subtypes of β adrenergic receptors in the PFC. The hypothesis of this chapter was that a more fortified neural infrastructure for LC-NE function in the female brain compared to the male brain not only exists in the brainstem, but also extends to NE projections and/or their binding sites in prefrontal target regions. Thus, predicted outcomes were more axons and/or receptor RNA in the female brain compared to the male brain.
No significant differences in adrenergic axonal density in the PFC were found between females and males. Further, a similar number of release sites were identified in females compared to males after NE bouton quantification in the PFC. However, females were found to have more β1 adrenoceptor RNA on glutamatergic cells, while males had more β1 adrenoceptor RNA on other cells. This means that while the overall number of cells labeled with β1 adrenoceptor RNA was similar between sexes, female β1 adrenoceptor RNA in the PFC was biased toward projection neurons and male β1 adrenoceptor RNA in the PFC was biased toward other cells. Additionally, males had more β2 adrenoceptor RNA on glutamatergic cells than females.

Overall, significant sex differences were found in the distribution of NE receptors across different cell-types in the PFC, and not in NE-PFC projections. This suggests that anatomical sex differences in NE axonal density or release sites within the PFC are unlikely to be the substrate for sex differences in decision-making behavior. It is more likely that a different mechanism like the increased receptor RNA in male astrocytes or other neuronal subtypes may be activating inhibitory cells, which could then dampen PFC activity. Thus, these results may contribute to an explanation for sex differences observed in decision-making behavior from earlier chapters of this dissertation.

5.3 Proposed mechanisms

Results from Chapter 2 indicated that females are more sensitive to increased LC activity than males. In contrast, results from Chapter 3 indicated that males are more sensitive to increased NE availability than females. These findings may seem contradictory because the LC is the main source of NE for cortical regions like the PFC that regulate decision-making behavior investigated in Chapters 2 and 3. However, several possible explanations exist.

First, the results of Chapter 2 also suggested that decision-making behavior was not driven by altered motivation or sucrose consumption. On the contrary, results from Chapter 3 suggested that the decision-making behavior investigated in the context of ATM may have been influenced by decreased motivation and reward consumption. Therefore, the findings from Chapter 2 and Chapter
3 seem to be different behavioral results that may be regulated by different underlying mechanisms. NE also acts in regions of the brain more involved in hunger cues like the hypothalamus.

Those mechanisms will need to be investigated in future experiments. Potential mechanisms include the other neurotransmitters released by the LC aside from NE. While the LC is the main source of NE to the PFC, it does not exclusively release NE. Other neurotransmitters such as galanin, glutamate, dopamine, and neuropeptide Y were likely influenced by Chapter 2 increasing LC activity but not necessarily by Chapter 3 increasing NE availability [Holets et al., 1988; Kempadoo et al., 2016]. They still might have been affected by Chapter 3 manipulations, as the increased competition for adrenoceptors may have influenced which receptors these other neurotransmitters like dopamine bound to. However, previous unpublished experiments conducted by the Vazey lab compared the behavioral effect of ATM and methylphenidate, which acts on both NE and dopamine, and found that they have opposing effects in performance of the 2AFC task. Therefore, while we cannot rule out its activity, it is unlikely that dopamine entirely regulated the effects observed after ATM administration. Ultimately, a manipulation like the overall LC activation in Chapter 2 yielded different results from a more targeted manipulation of one specific neurotransmitter released from the LC in Chapter 3, and this might be related to the activity of other neurotransmitters also released by the LC.

Another potential explanation was that the different outcomes between Chapters 2 and 3 may be the result of increased NE release in the PFC of males compared to females. While females were more sensitive to an increase in LC activity, males were more sensitive to direct NE manipulations. This was investigated in Chapter 4, which assessed density of axons and boutons and found no sex differences. But NE release may have been investigated in this dissertation with too wide a lens. It is possible that the sex differences in response to NE observed in Chapter 3, where blocking NE reuptake by ATM lead to greater deficits in males than females, could have been driven by increased NE release per bouton in males compared to females. Previous studies in males have
reported greater NE release from the LC to the PFC than to other brain regions [Chandler et al., 2014]. If the male LC releases more NE than the female LC, it could contribute to an explanation for Chapter 3 findings. Since those more nuanced attributes of NE release were not investigated in Chapter 4, future studies are still needed.

The other seemingly contradictory finding in this dissertation was the lack of sex differences in decision-making behavior upon propranolol administration in Chapter 3. Since LC anatomy and LC and NE regulated behavior are different between females and males, it is surprising that antagonizing NE receptors would not yield sex differences in the same behavior. However, Chapter 4 RNA results provided some insights that may help explain these findings.

In Chapter 4, males had more overlap between β2 adrenergic receptor RNA and glutamatergic RNA, while females had more overlap between β1 adrenergic receptor RNA and glutamatergic RNA. It is possible that β2 and β1 adrenergic receptors have some opposing effects in female and male PFCs. If β2 adrenoceptors are more excitatory in the male PFC and β1 adrenoceptors are more excitatory in the female PFC, then a non-selective antagonist of both β1 and β2 adrenoceptors would have similar behavioral effects on females and males despite different underlying mechanisms. The tools exist to test this potential explanation in future experiments using selective antagonists such as betaxolol and ICI118,551.

Additionally, Chapter 4 revealed that males had more potential for β1 adrenergic receptor RNA on inhibitory PFC cells compared to females. This sex difference may underly Chapter 3 findings, but would have to be electrophysiologically assessed in future studies to determine if NE binding to β receptors leads to inhibition of PFC cells or not.

Collectively, these findings support two compatible potential hypotheses:

1. Female deficits from LC manipulations may be driven primarily by co-released neurotransmitters rather than NE.
2. Male deficits in NE manipulations may be driven primarily by male brains releasing more NE per site than females.

These are testable hypotheses for future studies. Overall, the experiments outlined above provide new insights into the LC-NE system and its regulation of decision-making in female and male rats, while at the same time opening the door to even more experiments that can build on this foundation.

Figure 5.1. Proposed mechanisms for female (left) and male (right) noradrenergic regulation of decision-making behavior. RNAscope results suggest that males may have more noradrenergic inhibitory activity (red x circles) in the PFC than females (Chapter 4). This would mean that females have more excitatory activity (starbursts) in the PFC than males, which may put them farther along the Yerkes-Dodson curve than males and push them out of optimal decision-making performance upon artificial increases to LC activity (Chapter 2). In contrast, males may have more NE (black dots) release per vesicle, putting them farther along the Yerkes-Dodson curve than females and pushing them out of optimal decision-making performance upon increases to NE availability (Chapter 3). Considering these opposing effects, it is also possible that neurotransmitters other than NE (grey dots) released by the LC play a role in the sex differences observed in Chapter 2.

5.4 Implications for behavior

There is currently a gap in research about sex differences in the neural regulation of most biological functions including decision-making. These experiments have provided support to the idea that females and males may have different thresholds for LC activation before behavior is impacted. Going above that activity level could lead to hyperarousal and inability to focus on a task, while going below that activity level could lead to hypoarousal and inability to attend to a task. These findings have clinical implications for individuals whose baseline decision-making leans toward hyperarousal, such as those diagnosed with ADHD or anxiety disorders. Understanding sex differences in the neural mechanisms regulating decision-making behavior has the potential to advance the development of more specialized therapies for disorders such as ADHD that manifest
differently in females and males. Ultimately, the positive impact of these experiments was an advanced understanding of decision-making regulation in females and males.

5.5 Potential limitations and future directions

These experiments investigated the LC-NE system and its relation to decision-making in females and males. However, there are still many other factors at play and this is in no way a comprehensive analysis of every aspect of the LC-NE system. Therefore, further investigations are needed to fill the remaining gaps in our understanding of the LC-NE system, including those outlined below.

5.5.1 Limitations in assessment of the role of increased LC activity in female and male decision-making

For example, the LC projects NE throughout the brain and the experiments outlined in Chapter 2 globally activated the LC. This means that behavioral effects observed in Chapter 2 were not exclusively driven by LC projections to the PFC. The results could have been colored by altered anxiety levels from the LC projections to the amygdala, altered memory retrieval from the LC projections to the hippocampus, or any number of other LC activities. Previous investigators have found decreased performance of a set-shifting task upon targeted LC-mPFC activation that was not observed upon global LC activation [Cope et al., 2019]. This does not invalidate the experiment above, because many modern pharmaceuticals are systemic in nature, so the experiments outlined above provide a realistic understanding of how systemic alterations to the LC-NE system can affect females and males differently. But a future experiment could fill this gap by manipulating only PFC-projecting LC cells. A retrograde virus could be infused into the PFC of female and male rats. In the same surgery, the flex hm3Dq virus could be infused into the LC. After recovery, behavioral analysis of the 2AFC task could be assessed before and after injections of CNO to increase the activity of PFC-projecting LC cells, similar to what has been done previously [Cope et al., 2019]. These findings are expected to support the findings from Chapter 2 and take them one step further to show that females decrease performance upon smaller artificial increases (lower doses of CNO)
in PFC-projecting LC cell activity than males. If anything, a stronger effect would be anticipated, in line with previous findings [Cope et al., 2019].

However, there are still other manipulations that could have increased LC activity. Optogenetic manipulations could have been used instead of chemogenetic manipulations. Optogenetics are temporally more precise but stimulate phasic activity, while chemogenetics stimulate tonic activity [Goutaudier et al., 2019]. Previous studies have concluded that phasic activity is involved in maintaining focused attention, while tonic activity is more involved in behavioral flexibility [Aston-Jones and Cohen, 2005]. Thus, the complex decision-making cognitive load that the 2AFC task presents may be more suited for combining both optogenetic and chemogenetic manipulations. A simpler task like the sustained attention, signal detection tasks or go/no-go paradigm might be better suited for optogenetic LC manipulation.

Another potential limitation is that NE release may or may not have increased in relation to increases to the LC. Previous studies suggest that this is true, but an exciting future direction would be to compare female and male NE release using high performance liquid chromatography before and after CNO injection [Svensson, 1987]. This would allow for quantification of NE in the PFC before and after LC activation to determine if there is more NE in the PFC after LC activation and how that increase compares across sexes. Ultimately, the set of experiments outlined in this dissertation provided clear evidence for the effects of specific manipulations to the LC system and the NE system. Future investigations into the exact relationship between them remain exciting avenues of research.

Additionally, previous literature suggests that LC-NE signaling is reduced upon feeding [Sciolino et al., 2022]. This may play a role in attenuating the intended manipulations in relation to a sucrose-water reward consumption task. However, that study concluded that satiation influences LC-NE activity and the rats in the present investigation were mildly food restricted to avoid satiation during task performance [Sciolino et al., 2022]. A further control for satiation was the sucrose consumption
test, which revealed that the animals were not satiated from the much smaller volume of reward received during 2AFC and progressive ratio testing.

5.5.2 Limitations in assessment of the role of noradrenergic signaling in female and male decision-making

As the experiments in Chapter 2 did not specifically target the LC cells projecting to the PFC, experiments in Chapter 3 also did not target PFC cells for pharmacological manipulation. This can also be addressed in a future experiment involving the same ATM and propranolol injections directly into the PFC instead of administered systemically. This experiment would allow the response of NE activity to be assessed specifically in the PFC. Those more precise experiments were not conducted in Chapter 3 because the aims of this dissertation were to understand the underpinnings of behavior in the whole interconnected body, rather than isolating systems that might work differently when put into context.

Considering the results of Chapter 4, it could be valuable to repeat the pharmacological experiments of Chapter 3 with selective β adrenoceptor blockers such as betaxolol and ICI118,551. The non-selective β blocker used in Chapter 3 might have obscured sex differences in decision-making performance guided by different distribution of β1 and β2 receptors on different cell-types in females and males.

5.5.3 Limitations in assessment of substrates of NE-PFC innervation across female and male rats

Had the female PFC shown greater axon and/or bouton density than the male PFC, further investigations would still be necessary to better understand NE release in the PFC of females compared to males. Females could have more diffuse NE release, while male NE release is more targeted near receptors, indicating that females have more homogenous release, while males operate under more of an all or nothing system. In that case, males would have clustered NE release
near axon terminals potentially increasing binding to low affinity α1 adrenoceptors, which would likely underly behavioral sex differences.

Chapter 4 investigated the number of NE release sites but did not quantify how much NE could be released per bouton. Future experiments could use electron microscopy to assess the amount of NE present in each vesicle in pursuit of answers to that question. Neurotransmitters could be quantified in female and male PFCs rather than adrenergic axons and boutons.

Further, the β adrenoceptors were quantified on excitatory and inhibitory cells, but it still can’t be concluded whether the overall effect in the PFC is excitatory or inhibitory because NE is adaptable for different circumstances and can act as an excitatory or inhibitory neurotransmitter. The RNAscope experiment is still informative of sex comparisons in these cell types and has added valuable knowledge to build off in future research. However, future directions could include electrophysiological recordings of individual PFC cells in response to the pharmacological manipulations outlined above.

Another limitation of note is that RNA that codes for adrenoceptors is not equivalent to the expression of adrenoceptors on the cell surface. As noted above, an exciting future direction could assess receptor expression using flow cytometry.

5.6 Concluding remarks

Previous studies identified anatomical sex differences in the LC which inspired this dissertation. The first investigations in the present set of experiments aimed to understand the behavioral effects of those previously identified sex differences. Once it had been determined that LC related sex differences in behavior exist, further investigations were completed to pursue the mechanism linking sex differences in anatomy to behavior. The most logical hypothesis was that the sex differences in anatomy and behavior were related and driven by the main neurotransmitter released by the LC: NE. Additional behavioral experiments used a manipulation of NE rather than LC. However, NE is not the only neurotransmitter released by the LC. This neurotransmitter diversity
may explain why the behavioral effects of manipulating the LC and manipulating NE did not reveal the same sex differences. Interestingly, while NE manipulations did not reveal the same sex differences as LC manipulations, they did reveal sex differences nonetheless. Subsequently, this dissertation investigated previously unexplored downstream avenues of NE release and activity in a key decision-making regulator: the PFC. Preliminary investigations revealed no sex differences in NE projections or release sites. However, potential for increased LC-NE axon density in females remains a possibility underlying the differential impact of LC and NE manipulations. The present investigations analyzed two different measures innervation, axon density and varicosities. Future investigations could assess additional different metrics such as NE density within a vesicle. Further analysis of NE activity in terms of potential binding sites on adrenoreceptors revealed sex differences that may impact behavioral observations after LC manipulation.

After conducting these experiments, several new sex differences related to LC-NE regulation of decision-making behavior are known. Females are more sensitive to increased LC activity, males are more sensitive to increased NE availability, and females have more β1 adrenergic receptors on glutamatergic cells in the PFC while males have more β2 adrenergic receptors on glutamatergic cells in the PFC. Additionally, many new avenues for future investigation have been identified. Ultimately, these experiments provide a clearer starting point for a more personalized treatment approach to address sexually differentiated decision-making behaviors such as ADHD.
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