Dopaminergic modulation of pair bonds and song preference learning in the female zebra finch

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June 2023

A thesis submitted to McGill University in partial fulfillment of the requirements of the degree of Master of Science in Biology

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Abstract

Social bonds play a central role in the sexual selection, survival, and reproductive success of many social animals. In some species, vocal communication plays an additional role in establishing and maintaining social bonds and mate preferences. Zebra finches (*Taeniopygia guttata*) form lifelong pair bonds and rely heavily on learned auditory signals for social recognition and decision-making. Brain areas responsible for auditory learning and song perception are additionally densely innervated by dopamine (DA) inputs. While past findings have highlighted the role of DA in pair bonding, there is still limited knowledge about how dopamine works in auditory systems as well as other forebrain areas to mediate pair bonding behaviors in female zebra finches. Here, we investigated the degree to which dopaminergic inputs to higher-order auditory processing areas are important for forming preferences in pair-bonded female zebra finches. We observed that female zebra finches showed a significant relationship between the degree to which their preference shifted with two weeks of cohabitation and the number of dopaminergic inputs present in the caudomedial nidopallium (NCM). In a second study, we investigated the topography of DA inputs to another region important for social behaviors and bonding, the nucleus accumbens (NAc). We found that the ventral tegmental area (VTA) and substantia nigra pars compacta (SNc) send dopaminergic projections to the NAc at both its rostral and caudal regions, confirming analogous neurocircuitry as previously observed mammalian models. Taken together, our findings give new insight into how dopaminergic projections from the VTA are mediating preferences observed in female zebra finches.

Résumé

Les liens sociaux jouent un rôle central dans la sélection sexuelle, la survie et le succès reproductif de nombreux animaux sociaux. Chez certaines espèces, la communication vocale joue un rôle supplémentaire dans l'établissement et le maintien des liens sociaux et des préférences des partenaires. Les diamant mandarins (*Taeniopygia guttata*) forment des couples pour la vie et utilisent des signaux auditifs appris pour la reconnaissance sociale et la prise de décisions. Les zones cérébrales responsables pour l'apprentissage auditif et de la perception des chansons sont innervées par dopamine (DA). Bien que les recherches antérieures montrent l'évidence du rôle de la DA dans l'établissement des liens entre les paires, il existe encore peu de preuves sur la façon dont la DA s'agit dans les systèmes auditifs ainsi que dans d'autres zones du cerveau antérieur pour médire les comportements d'établissement de liens entre les paires chez les diamants mandarins femelles. Ici, nous avons étudié comment les entrées dopaminergiques dans les zones de traitement auditif d'ordre supérieur sont importantes pour la formation des préférences. Nous avons observé que les femelles présentaient une relation significative entre le comment elles ont changé leurs préférences après l'établissement de liens entre les paires et le nombre d'entrées dopaminergiques présentes dans le nidopallium caudomédial (NCM). Nous avons aussi étudié la topographie des entrées de DA dans une deuxième région importante pour les comportements sociaux et l'attachement, le noyau accumbens (NAc). Nous avons constaté que l'aire tegmentale ventrale (VTA) et la substantia nigra pars compacta (SNc) envoient des projections dopaminergiques au NAc dans ses régions rostrale et caudale, ce qui confirme l'existence d'une neurocircuiterie analogue à celle observée dans les modèles de mammifères. En tout, nos résultats donnent un nouvel aperçu de la façon dont les projections dopaminergiques de la VTA médisent les préférences observées chez les femelles diamant mandarin.

Acknowledgments

I would first like to acknowledge and thank my incredible supervisor Dr. Sarah C. Woolley for her support and continuous guidance throughout all the stages of preparing this thesis. I would also like to extend my thanks to my amazing committee members, Dr. Tomoko Ohyama and Dr. Jonathan Britt for their insightful knowledge and feedback on my project. I am also thankful to all of the members in the Sakata and Woolley labs for all of their help with bird care and maintenance. Additionally, I would like to extend a special thank you to Madison McLaughlin, for her help with designing the terminal counting protocol and tissue processing as well as Marlo Naish, for all of her help with terminal counting. This project would not have been possible without the support of the Natural Sciences and Engineering Research Council of Canada (NSERC), who financed my project through Dr. Woolley.

Preface and Contribution of Authors

The two projects described in this thesis will later be used in manuscripts to be submitted to peer-reviewed journals for publication. The first project described in Chapter 1 discusses the effect of dopaminergic inputs from midbrain regions to higher auditory processing areas on mate song preference. Dr. Woolley and I designed the experimental setup, I performed the injection surgeries and perfusions, Madison McLaughlin helped with behavioral testing and tissue processing, and Marlo Naish helped with terminal counting. I trained the birds for the behavioral testing, processed the data, and analyzed the data. Dr. Woolley provided guidance on data processing and analysis. The second chapter of this thesis focuses on using retrograde labeling to better understand the neuroanatomy of dopaminergic projections to forebrain regions in the female zebra finch hypothesized to play a role in preference. Dr. Woolley and I designed the experiment. I performed the retrograde tracer injections, conducted the perfusions, processed the tissue, and analyzed the images. Dr. Wooley provided guidance on data processing and analysis.

Introduction

The formation of social bonds is a key factor in the survival and reproductive success of many social animals (Alberts 2019; Holt-Lunstad et al., 2010). In monogamous species, reproduction often involves the formation of a pair bond (Kleiman, 1977). In some species, vocal communication mediates mate preference and can act to establish and maintain social bonds (Narins et al., 2006; Zann & Bamford, 1996). However, the neural areas required for auditory signal processing and how they affect the sensory processing of signals responsible for mate choice are largely understudied. Thus, understanding the mechanisms underlying signal perception and preference can help us better understand the role preference plays in shaping and being shaped by social experiences.

Songbirds are an excellent model to study the role that vocal communication plays in pair bonds and female preference. Zebra finches (*Taeniopygia guttata*) rely heavily on learned auditory signals for social recognition and decision-making (Riebel, 2009). Female zebra finches use learned songs produced by males to assess and recognize individuals and select mates (Woolley & Doupe, 2008). Moreover, mating with an individual male leads to the formation of life-long pair bonds and learned preferences for the mate's song that can be maintained for months to years (Woolley & Doupe, 2008; Zann & Bamford, 1996). These song preferences are hypothesized to be mediated, in part, by catecholamine release into the auditory cortex (Barr et al., 2021; Happel, 2016; Miranda & Liu, 2009). In particular, dopaminergic neurons can integrate information about the salience or reward value of a stimulus with feedback from higher-order brain areas to associate specific sensory information together with a behavioral context (Bromberg-Martin & Hikosaka, 2010; Horvitz, 2000; Wise, 2004).

Dopamine acts as a critical modulator of learning and motivation in natural behaviors (Berke, 2018). It has previously been implicated in the display of courtship behaviors, including those observed in zebra finches (Alger et al., 2011; Bharati & Goodson, 2006; Goodson et al., 2009). Moreover, it plays a role in mediating pair bond formation in voles and the pleasure derived from music in humans (Aragona et al., 2006; Salimpoor et al., 2003). For example, previous studies in monogamous prairie voles have shown that dopamine transmission in the nucleus accumbens (NAc) mediates mate preferences, specifically by promoting pair bond formation through the activation of D2 dopamine receptors (Aragona et al., 2006). Additionally, subcutaneous injections of D2 receptor agonists have been shown to induce song preferences in unpaired females, suggesting a similarity behind neural mechanisms for zebra finch pair-bond formation and previous rodent models (Day et al., 2019).

Here, we used two distinct approaches to address the role of dopamine in song preference learning and pair-bond formation. Chapter 1 examined the role of dopamine in song preference formation by testing the degree to which dopamine depletion in a secondary auditory processing area impacts pair bonding and song preference following mating. In Chapter 2, we investigated the inputs to the nucleus accumbens, focusing specifically on dopaminergic inputs. We injected retrograde tracer into the nucleus accumbens to confirm dopaminergic projections from the midbrain to the NAc and to uncover the topographic organization of the dopaminergic inputs from the midbrain. Taken together, these studies contribute to our understanding of how dopamine may interact with different higher-order processing systems to encode song preferences in female zebra finches.

Chapter 1 – Effects of dopaminergic inputs in the secondary auditory areas on the formation of mate song preferences

Background

Female zebra finches evaluate the quality of the songs performed by males and use these songs for recognition and mate selection (Miller, 1979; Riebel 2009**)**. In addition, females display species-typical song preferences, including preferences for courtship (directed) over non-courtship (undirected) songs, and for songs from tutored males over untutored males (Clayton 1988; Miller, 1979; Woolley & Doupe, 2008). Beyond these species-typical preferences, females also vary in their preferences for the songs of individual males, and this variation may be a consequence of individual experiences. For example, females develop strong and long-lasting preferences for the songs of individuals with whom they interact, in particular for the songs of their father or a mate (Miller, 1979; Riebel et al., 2002; Woolley & Doupe, 2008). These individual preferences are learned and result from experiences either during development or as adults (Clayton 1988; Miller, 1979; Woolley & Doupe, 2008).

Regions in the secondary auditory pallium, such as the caudomedial nidopallium (NCM) and caudomedial mesopallium (CMM) show responses to songs over other non-song sounds (Bailey et al., 2002; Mello et al., 2004). Moreover, the NCM has been shown to play a role in song perception, discrimination, and learning, and is hypothesized to be a location for auditory memory in female birds (Gentner et al., 2001; Tomaszycki & Blaine, 2014). Temporary inactivation of the NCM using lidocaine has been shown to affect female zebra song discrimination, as well as male affiliation. (Tomaszycki & Blaine, 2014). In females, a number of studies have found greater activity in both the CMM and NCM in response to categorically preferred songs such as courtship songs (Chen et al., 2016; Van Ruijssevelt et al., 2018; Woolley

& Doupe, 2008). Based on these data, we hypothesize that the auditory pallium, in particular the NCM, may be involved in mate song recognition and song preference learning in female zebra finches.

In songbirds, dopaminergic neurons in the ventral tegmental area (VTA) have been implicated in the processing of rewarding stimuli (Bharati & Goodson, 2006; Charlier et al., 2005). Dopaminergic cells in the caudal VTA of male birds are involved in incentive and reward regulation, and even demonstrate differential activation during singing depending on the song context (Goodson et al., 2009). Studies in rats have shown that pairing VTA stimulation with the playback of a tone leads to the expanded representation of that tone in the auditory cortex (Bao et al., 2001). Moreover, dopaminergic neurons in the VTA and substantia nigra pars compacta (SNc) send projections to the NCM in songbirds, an avian analog of the human secondary auditory cortex (Mello et al. 1998; Pinaud & Terleph, 2008). The NCM is strongly innervated by catecholaminergic inputs (Barr et al., 2021; Van Ruijssevelt et al., 2018; von Eugen et al., 2020). Pharmacological manipulations of a general dopamine agonist or D1 receptor agonist in the NCM has shown to alter song preferences; suggesting a connection between dopaminergic activity in the NCM and general song preference (Barr et al., 2021).

Given that the NCM appears to be involved in song memory formation in both males and females and the potential role of the NCM and VTA in song preference formation and expression, we investigated the degree to which dopamine plays in manipulating song preferences. Specifically, whether dopamine projections to the NCM are responsible for encoding a female's preference for their mate. To this end, Chapter 1 aimed to gain a better understanding of the role that dopamine inputs to the NCM play on female mate preferences. Here, we depleted dopaminergic inputs in the NCM prior to the introduction of a mate to test if

the formation of mate song preference would be hindered once the pair bond had been established. This allowed us to observe the role of dopaminergic inputs in the NCM on female mate preference formation in an active behavioral context.

Research Question

While previous work highlighted a role for dopamine in the NCM in inducing plasticity in female song preferences (Barr et al., 2021), it is unclear whether dopaminergic inputs to the NCM also mediate mate song preference – specifically whether these inputs are significant in the formation of female preferences for a mate's song. Chapter 1 aims to bridge the gap between what is known about female zebra finch song preference and the effects of dopaminergic activity in the NCM in order to gain a better understanding of the degree to which dopaminergic activity in the NCM mediates mate song preferences. Specifically, we investigated the degree to which dopaminergic inputs to a secondary auditory area (NCM) are important for the formation of preferences in female zebra finches that are forming pair bonds with a male mate. We tested whether depleting dopaminergic inputs in the NCM of female birds prior to introducing them to two weeks of cohabitation with a male prevented song preference formation. We hypothesized that females with depleted dopaminergic inputs to the NCM would not show an increased preference for their mate's song after mating.

Methods

Animals.

All zebra finch females used in this study (N = 15, > 90 days post-hatch) were raised with both parents and all siblings until 60 days of age, then moved into same-sex group cages in a colony. Bird care and procedures followed all Canadian Council on Animal Care guidelines and were approved by the Animal Care Committee of McGill University

Stereotaxic surgery.

6-hydroxydopamine (6-OHDA) is a hydroxylated analog of dopamine, which causes neuronal damage mainly due to oxidative stress (Blandini et al., 2008). The use of 6-OHDA-induced dopaminergic depletion is highly reproducible and has been used in different areas of the brain to selectively eliminate dopaminergic fibers and cell bodies (Blandini et al., 2008; Schober, 2004). Additionally, 6-OHDA has been previously shown to successfully reduce presynaptic DA input to song nuclei in zebra finches males (Miller et al., 2015; Tanaka et al., 2018).

6-OHDA was stereotaxically injected directly into the NCM of anesthetized female zebra finches to deplete dopaminergic inputs from the ventral tegmental area (VTA) and substantia nigra pars compacta (SNc). Surgery was performed as previously described (Barr et al., 2021; Woolley et al., 2014). Briefly, at least 30 minutes prior to surgical procedures, females were given an analgesic (Metacam, Boehringer Ingelheim, USA) and deprived of food and water. Before surgery, females received an intramuscular injection of 0.03mg/g Ketamine and 0.0015mg/g Midazolam and were placed alone in a cage with a heat lamp for up to 10 minutes for anesthetic induction. Females were then placed in a stereotaxic frame fitted with a beak bar and anesthesia was maintained with a steady flow of 0-2% isoflurane vapor. We then subcutaneously injected lidocaine under the scalp for local anesthesia before making an incision

to expose the skull above the general area of the y-sinus. After removing the top layer of the skull, the coordinates for the y-sinus were obtained and used as the origin point with which to calculate the coordinates of the left and right NCM, where the injection occurred.

The injections consisted of bilateral injections of 11.8 mg 6-OHDA-HBr/ml (i.e., 8 mg freebase 6-OHDA/ml; Hello Bio, Princeton, NJ, USA) and 2 mg ascorbic acid/ml (stabilizer) in a 0.9% NaCl solution. Injections of 50nl of the solution were made at 1mm and 2mm deep, in the posterior portion of the NCM to minimize effects on adjacent regions. Control birds received a sham injection of vehicle (2 mg ascorbic acid /ml in 0.9% NaCl). All injections were made using a pressure-based system (Drummond Nanoject III, Broomall, PA, USA) at a rate of 10nl/sec. After the injections, the incision site was closed with tissue adhesive, and the bird was allowed to recover from anesthesia under a heat lamp. All females were given 6-7 days to recover post-injection prior to preference testing. Injections were administered prior to any preference testing to ensure any observed changes in preference resulted from pair bonding with a mate and not from 6-OHDA injections.

Song stimuli.

All male song stimuli were directed song samples recorded from males (N = 14) from our colony at McGill University. Recordings were obtained in sound-attenuating chambers (TRA Acoustics, Cornwall, Ontario) by exposing males to stimulus females (not used in this experiment). Songs were recorded at 44.1 kHz using sound-triggered recording software Sound Analysis Pro (SAP; Tchernichovski et al., 2000). Song stimuli used for string pull preference tests were a selection of 10-15 recordings of directed songs from each male, free of noise and female calls, that were a representative sample of varying song duration, number of bouts, and number of introductory notes from each male's repertoire.

Preference testing.

To quantify female preferences for the songs of specific males before and after pair-bonding, we used a two-choice assay. Previous studies show that females can demonstrate significant song preferences in a two-choice assay (Barr et al., 2021). Furthermore, because trials are unrewarded and are self-initiated by the females, the assay provides a readout not only of preference but also of motivation. In this assay, female zebra finches were placed in a cage containing two strings. When pulled, each string triggered the playback of a song from a single male zebra finch through an adjacent speaker, such as the song of their mate for one string and the song of an unknown male for the other string. To account for side bias, the song triggered by each string was switched following 1 hour of free pulling, which meant that females with a song preference would need to switch which string they preferentially pulled. Females were given access to activated strings for free pulling for 7 hours/day for 5 days for the initial preference test and 7 hours/day for 3-4 days after the two weeks of cohabitation.

Pair bonding observations.

Following the initial preference testing, females were paired with their less-preferred males. Male-female pairs were housed together in a single cage. Two separate male-female pairs were housed in separate cages within the same sound-attenuating chamber separated by an opaque barrier. Females from each pair could therefore hear, but not see or physically interact with, the male in the neighboring cage. This allowed us to provide females with passive exposure to a non-mate's song. The pairs were observed once a week for an hour to record any pair bond behavior (e.g. clumping, preening, nest building). All pairs were displaying nest-building behavior by the end of the second week.

Perfusion and immunocytochemistry.

Following the second round of preference testing, females were perfused then immunocytochemistry was performed to confirm the depletion of dopamine terminals. Females were anesthetized through isoflurane inhalation and subsequently transcardially perfused with 25ml of saline containing 12mg of heparin, followed by 150ml of 4% paraformaldehyde. Perfused brains were kept for 24 hours in 4% paraformaldehyde, then transferred to 30% sucrose for cryoprotection. Brains were then sliced into 40 μm coronal sections and stored in 0.025M phosphate-buffered saline solution containing sodium azide.

Immunocytochemistry was performed as previously described (Barr & Woolley, 2018; Barr et al., 2021). Briefly, one set of every third section was washed, blocked with donkey serum, then incubated in a primary antibody solution containing sheep anti-tyrosine hydroxylase (1:1000 dilution; Novus Biologicals, Centennial, CO, USA) for 48 hours. Next, sections were incubated for 2h in a secondary antibody solution containing donkey anti-sheep conjugated to Alexa Fluor 488 (to label TH; 3ul/ml; Life Technologies, Eugene, OR, USA). Sections were mounted onto chromium-aluminum gelatin-coated slides and cover-slipped with DAPI (ProLong Gold Antifade Reagent with DAPI; Life Technologies, Eugene, OR, USA). A second set of every third section was run alongside the first set and underwent identical immunocytochemistry protocol except the addition of the primary sheep anti-tyrosine hydroxylase antibody was omitted. This “secondary-only” set was used as a baseline when calculating the threshold for dopaminergic terminal fluorescence.

Imaging and cell terminal counting.

The primary region of interest imaged was the NCM. As the brains were sliced coronally, the NCM was imaged separately in medial and lateral areas to encompass the range of the 6-OHDA injection. Compound images overlaying TH labeled terminals and DAPI stained nuclei were taken for both hemispheres of each region (medial NCM, lateral NCM) with a 40x objective of a Zeiss Axio Imager upright microscope and an AxioCam MRm Zeiss camera (Carl Zeiss, Germany).

The total number of dopaminergic terminals per section was calculated for each section using FIJI particle analysis. First, the secondary-only set was run through a Sobel edge detector to select for sharp changes in intensity in the image. The pixel threshold for each secondary-only image was acquired by choosing a pixel value cutoff at 0.00%, where every pixel less than the value is considered foreground, and every pixel greater than that value is considered background. The mean pixel threshold of all secondary-only images was calculated and used as the baseline threshold for cell terminal counting.

Each primary antibody-containing image was also first run through a Sobel edge detector to select for sharp changes in intensity in the image. Next, the mean pixel threshold of the corresponding secondary-only set was applied, and the images were converted into binary images. Finally, watershed separation was applied to split connected terminals into separate ones and particle analysis was run to count the total amount of terminals present on each image. This process was applied to both lateral and medial sections of each brain.

The location of each injection site was noted, and the overall spread of depletion was counted for each hemisphere of each bird, to quantify the degree, if any, of dopaminergic

depletion that occurred. We quantified terminals for both 6-OHDA treated and control birds. Quantification was performed blind to bird ID and treatment.

Analysis.

For preference tests, we only included hours in which the female had pulled the strings on both sides, to ensure that the bird had experienced both song options. For each hour that reached the criterion, the mate was assigned a value of 1, the unfamiliar male a value of 0, and the distribution of pulls was bootstrapped with replacement (10,000 iterations) to obtain 95% confidence intervals. The mean bootstrap value for each hour was used as a "preference index" and single-trial confidence intervals (CIs) for one song over the other (95% CI). The average across all trials was used to calculate the female's overall preference index. Females were defined as having a "preference" if both the mean and the confidence intervals did not overlap with 0.5 (the probability of having no preference).

To compare the dopaminergic depletion of terminals in the NCM, we compared the mean dopaminergic fluorescence of 6-OHDA injected and control females. To this end, we used a mixed-effects model with treatment (6-OHDA vs. control) as independent variables, individual ID nested in immunocytochemistry batch as a random variable and mean dopaminergic fluorescence of the medial and lateral sections as the dependent variables. Next, to examine whether the 6-OHDA injections affected mate song preference formation, we conducted a full factorial design with treatment (6-OHDA vs. control), epoch (pre-mating vs. post-mating), and their interaction as independent variables, individual ID nested in immunocytochemistry batch as a random variable, and string pull distribution as the dependent variable. All models were conducted using a restricted maximum likelihood approach with unbounded variance components.

To investigate the relationship between treatment and preference strength, we ran a contingency analysis. To this end, we conducted a contingency analysis with preference (unfamiliar vs. no preference vs. mate) as the response variable and treatment (6-OHDA vs. control) as the predictor variable for both testing epochs (pre-mating, post-mating). We also ran a contingency analysis to investigate the total change in preference strength. We ran a contingency analysis with final preference change (unfamiliar vs. no preference vs. mate) as the response variable and treatment (6-OHDA vs. control) as the predictor variable.

To investigate the relationship between preference and dopaminergic terminal fluorescence, we ran a bivariate analysis. We conducted the bivariate analysis with the initial preference, post-mating preference, and shift in preference as the dependent variables and the mean dopaminergic fluorescence as the independent variable, across all birds. All statistical analyses were completed using programs from JMP Pro 16 (SAS, Cary, NC, USA) or custom-written Matlab code (Mathworks, Natick, MA, USA).

Results

6-OHDA injections cause dopaminergic depletion in the NCM

We confirmed dopaminergic depletion in the NCM by comparing the mean dopaminergic terminal fluorescence s between control and 6-OHDA injected females. We observed that 6-OHDA injected females had significantly lower levels of TH fluorescence in the medial NCM (closer to the injection site) in comparison to control females ($F(1, 13.06) = 7.975$, $p = 0.0143$) (Fig 1A). In contrast, in the lateral NCM, farther from the injection site there were no significant differences between 6-OHDA control injected females ($F(1,12.96) = 1.603$, $p = 0.2278$) (Fig 1B).

6-OHDA injections show no significant effects on mate preference formation

Next, we looked at the degree to which dopamine depletions in the NCM affected song preferences, including the formation of mate song preference. To this end, we tested the effects of treatment (6-OHDA vs. control) and mating (pre vs. post-mating) on the mean bootstrap preference for each female. We found no significant effect of mating epoch ($F(1,13) = 1.1698$, $p = 0.2991$), treatment ($F(1,13) = 0.0327$, $p = 0.8592$, or the interaction between mating and treatment on preference formation ($F(1,13) = 0.3786$, $p = 0.5489$) (Fig 2).

Dopaminergic activity shows significant interaction with preference shift

One aspect of the preference data that stood out was that while the two treatments showed similar preferences for the mate's song after mating, there were differences between the groups in the strength of preferences before mating. In particular, control females were more likely to show significant preferences for a song prior to mating than 6-OHDA-treated females (chi-square = 4.857, p=0.0275). In fact, 6-OHDA-treated females did not show significant

preferences for either song on the initial preference tests (Fig 3A). Because of the difference in pre-mating preference, these data indicate that control females may have shown greater changes in preference than 6-OHDA females.

To address this more directly, we first tested whether there were differences between control and 6-OHDA-treated females in the degree of shift in preference before and after mating. While control females showed greater, positive changes in preference, these were not significantly greater than 6-OHDA treated females. We also tested whether control females were more likely to show categorical switches in preference toward the mate's song, either from no preference or a preference for the unfamiliar song to a preference for the mate's song. We found there was a trend for control females to move toward a preference for the mate's song, while 6-OHDA females more frequently strengthened their preferences for the unfamiliar song (chi-square = 5.14, p=0.0763; Fig 3B).

Finally, given the variation in the dopaminergic innervation into the NCM in both control and 6-OHDA, we tested whether there was a correlation between either the overall preferences or the amount of change in preference and the degree of dopaminergic innervation either within each treatment group or across all individuals. We found that, after mating, there was a trend towards stronger preferences for the mate's song in individuals with higher levels of dopaminergic innervation (Rsquare = 0.227, p=0.0723). In addition, we observed a significant correlation between the amount of dopaminergic terminal fluorescence and the degree of shift in the preference. Females that had greater levels of dopamine innervation also showed greater changes in preference (Pearson's $r(15) = 0.266$, $p = 0.0489$) (Fig 3C).

Figure 1

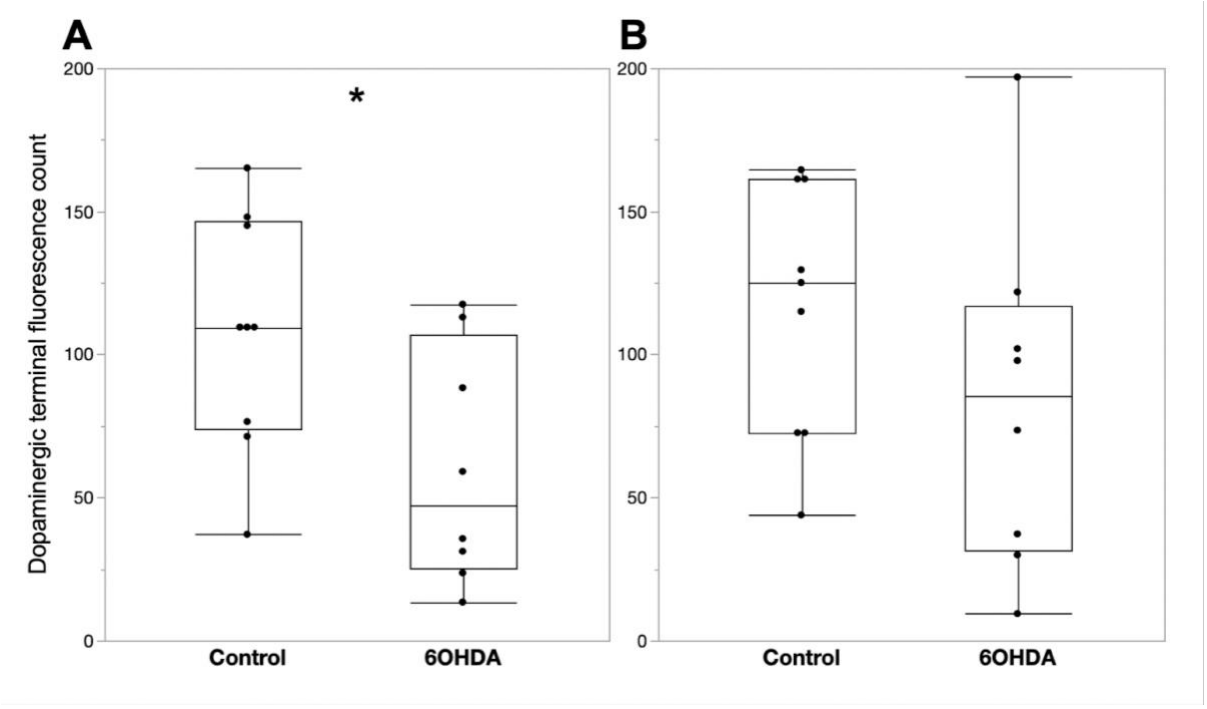

Figure 1: Mean dopaminergic terminal fluorescence in the medial (**A**) and lateral (**B**) NCM of control and 6OHDA females. Points are individual birds. * indicates $p<0.05$.

Figure 2

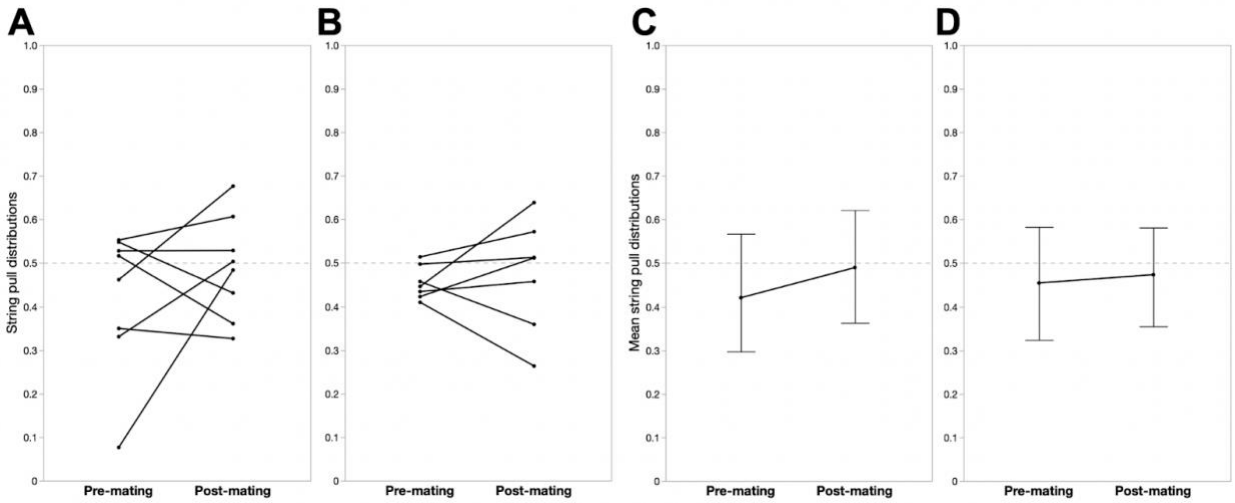

Figure 2: String pulls distributions over time. Connected points represent Pre-mating and Post-mating string pull distribution for individual birds. Pre-mating represents the initial preference before mate introduction, and Post-mating represents the re-tested preference after mate introduction and pair bonding. Values approaching 1.0 represent a preference for the mate's song, and values approaching 0 represent a preference for the "unfamiliar male" song. **A.** String pull distribution before and after mating in all control females. **B.** String pull distribution before and after mating in all 6-OHDA females. **C.** Mean string pull distribution before and after mating in control females. **D.** Mean string pull distribution before and after mating in 6-OHDA females. Vertical lines represent 95% confidence intervals for each point

Figure 3

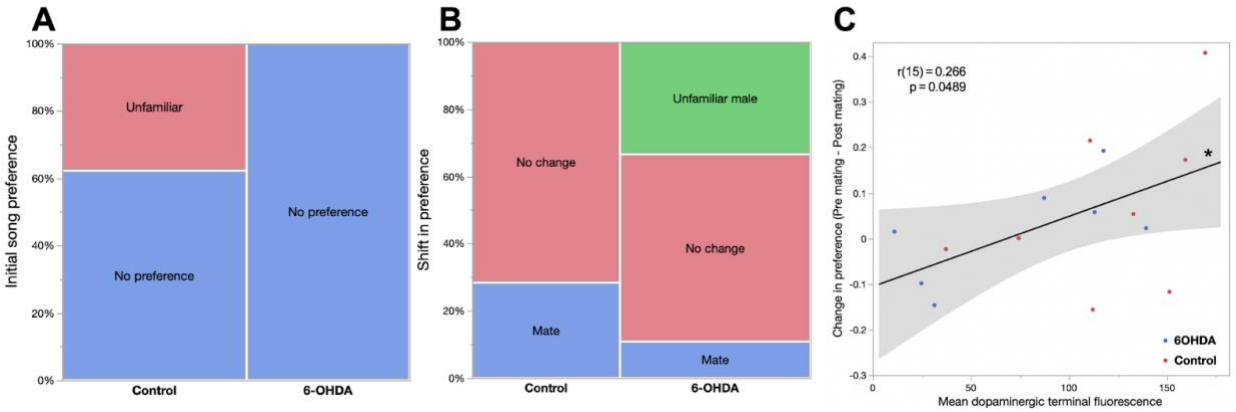

Figure 3: A. Mosaic plot of initial song preference for both treatments. **B.** Mosaic plot of the overall shift in song preference observed in both treatments. **C.** Correlation between relative mean dopaminergic terminal fluorescence and change in preference across all females used in the study. Individual points depict mean dopaminergic terminal fluorescence across both hemispheres (medial and lateral) in relation to a bird's change in preference. The black line indicates the linear fit of data. Grey shading indicates the confidence region for the fitted line.

Chapter 2 – Dopaminergic projections from the ventral tegmental area to the nucleus accumbens

Background

Pair bonding behaviors in mammals include affiliation, proximity maintenance, duration, and synchrony (Bales et al., 2021). Studies of pair bonding in mammalian models, specifically prairie voles, have focused on a wider diversity of behaviors, including partner preference between mates, selective aggression toward conspecific strangers but not their partner, and bi-parental care of offspring (Gobrogge & Wang, 2016). The nucleus accumbens (NAc) has been a region of interest in mammalian models, hypothesized to influence a variety of motivated behaviors associated with pair bonding (Aragona et al., 2006; Liu & Wang, 2003). Similarly, zebra finch pair behaviors are characterized by close contact, allopreening, and synchronized behaviors, however, studies of pair bonding in songbirds have focused primarily on mate-song preference (Silcox & Evans, 1982). In order to gain a better understanding of the broader circuitry underlying pair bond formation in zebra finches, we aimed to expand studies of pair bonding to non-auditory areas, such as the nucleus accumbens. However, one challenge has been the lack of detailed neuroanatomy and connectivity for the nucleus accumbens in songbirds. Thus, as a first approach to studying other regions that may be implicated in pair bonding, we aimed to better delineate the topography and connectivity of the NAc.

In the introduction of this thesis, we summarized findings in mammalian species that highlighted brain regions beyond auditory processing areas where dopaminergic modulation was responsible for mate preferences (Aragona et al., 2006). Moreover, our data from Chapter 1 demonstrated that the degree of dopaminergic innervation of the NCM was correlated with greater plasticity in preference expression. Specifically, individuals with greater dopaminergic

innervation in the NCM showed greater changes in preference following mating. Taken together, we were interested in investigating other regions in the zebra finch brain that may receive dopaminergic inputs from the VTA and how dopaminergic modulation of these non-auditory regions could affect mate preference formation and pair bonding.

The NAc is a brain region well characterized in mammals to mediate reward associated with motivation and incentivized learning (Balthazart and Absil, 1997; Pontieri et al., 1995). In humans, activity in the nucleus accumbens is correlated with how much individuals like a new, unfamiliar piece of music. Songs that individuals are willing to pay more to listen to in a listening task also evoke greater activity in the nucleus accumbens (Salimpoor et al., 2003). Moreover, the mammalian NAc can be subdivided into its rostral pole (ACR), accumbens shell (ACS), and accumbens core (ACC) components, each with functionally different roles (Salgado & Kaplitt, 2015). Similarly, the songbird NAc also has distinct rostral core and caudal shell components that have been theorized to be functionally distinct as well (Carrillo & Doupe, 2004; Montagnese et al., 1993; Montagnese et al., 2015). Therefore, one area of interest that could partly mediate mate and song preferences in zebra finches is the nucleus accumbens.

Dopaminergic modulation in the NAc has been found to influence a variety of motivated behaviors across taxa. For example, dopamine transmission in the rostral shell of the NAc is capable of promoting pair bond formation in prairie voles (Aragona et al., 2006). Moreover, the experience of pleasure while listening to music in humans has also been linked to dopamine release in the NAc (Salimpoor et al., 2011). Dopaminergic cells in the VTA of other avian models such as the Japanese quails (*Coturnix japonica*) are known to send additional projections to forebrain areas essential to the regulation of affiliative behavior (Balthazart and Absil, 1997; Durstewitz et al., 1999). However, the dopaminergic innervation in the forebrain areas of zebra

finches, specifically the NAc, and its influences on pair-bonding behaviors are still poorly understood.

Previous work in zebra finches focusing on dopaminergic transmission in regions where the nucleus accumbens is situated has hinted at a potential role that the NAc may play in pair-bonding behaviors. Studies focused on the ventral medial striatum found that paired males and females showed higher levels of dopamine than in unpaired birds (Banerjee et al., 2013). Moreover, PET scans in female zebra finches have also shown differential dopaminergic activity in the striatum in response to a mate's song in comparison to a non-mate's song. Specifically, females showed higher levels of dopaminergic activity in the dorsal striatum in response to their mate's song (Tokarev et al., 2017). Work focusing on the NAc of female zebra finches has additionally shown differential dopamine receptor expression in the NAc depending on the mate's song context; showing higher levels of D1 receptor expression when exposed to partners who sang more courtship songs and higher levels of D2 receptor expression when their partners sang less courtship song (Alger et al., 2022). Given that dopamine transmission in the NAc is involved in pair bonding in mammalian models, and previous literature in mated zebra finches has observed differential dopaminergic activity in or near the NAc, we are interested in uncovering the potential role that the NAc could be playing in pair bonding and mate song preferences in female zebra finches.

Given that dopaminergic activity in the NAc plays a role in mate preference in mammals, it is crucial to first map out the dopaminergic projections to the NAc in zebra finches. Furthermore, since dopaminergic activity in the NCM has also been implicated in female mate song preference, it is imperative to gain a better understanding of the organizational structure of the dopaminergic neurons in the VTA (Barr et al., 2021). To this end, Chapter 2 aimed to gain a

better understanding of the topography of the VTA in relation to any inputs it may be sending to the NAc. Here, we injected a retrograde tracer into the NAc to gain a better sense of how the dopaminergic neurons in the VTA are organized. This allowed us to determine whether similar organizational features characterize dopaminergic circuitry important to mate and mate song preference.

Research Question

While the role of dopamine transmission in the NAc in pair bonding has been well characterized in mammals, we still don't know much about the connectivity of the nucleus accumbens in female zebra finches nor the role that it may play in pair bonding and mate preferences. Given that we know that the VTA sends dopaminergic projections to the NAc in other avian models, it is important to uncover any neuronal relationship that these areas may share in zebra finches. In this chapter, we aimed to begin dissecting the topography of the nucleus accumbens in female zebra finches using retrograde tracers to map the origin of the dopaminergic inputs in the NAc in order to better understand the topography of both the VTA and NAc. We hypothesized that given previous literature in other avian models, we should also see neurons in the VTA sending dopaminergic projections to the NAc.

Methods

Animals.

Zebra finch females used in this chapter were either raised in a cage with both parents and siblings (N = 2, >90 days post-hatch) or were raised with only the mother and siblings (N = 2, >90 days post-hatch). Once 60 days of age, all females (N = 4) were housed in same-sex group cages in our colony. Bird care and procedures followed all Canadian Council on Animal Care guidelines and were approved by the Animal Care Committee of McGill University

Neuroanatomical tracing.

Surgery was performed as previously described (Barr et al., 2021; Woolley et al., 2014). Briefly, at least 30 minutes prior to surgical procedures, females were given an analgesic (Metacam, Boehringer Ingelheim, USA) and deprived of food and water. Before surgery, females received an intramuscular injection of 0.03mg/g Ketamine and 0.0015mg/g Midazolam and were placed alone in a cage with a heat lamp for up to 10 minutes for anesthetic induction. Females were then placed in a stereotaxic frame fitted with a beak bar and anesthesia was maintained with a steady flow of 0-2% isoflurane vapor. We then subcutaneously injected lidocaine under the scalp for local anesthesia before making an incision to expose the skull above the general area of the y-sinus. After removing the top layer of the skull, the coordinates for the y-sinus were obtained and used as the origin point with which to calculate the coordinates of the rostral (left) and caudal (right) NAc.

The injections consisted of a lysine-fixable Texas-red conjugated dextran (3000 kDa; 10%; Life Technologies, Eugene, OR, USA), diluted in a 0.025% Phosphate Buffer Saline solution. Injection volumes for both the rostral and caudal NAc contained 25-50 nl of tracer. Injections were spaced at 2-minute intervals to avoid backflow along the pipette. All injections

were made using a pressure-based system (Drummond Nanoject III, Broomall, PA, USA) at a rate of 1nl/sec. After the injections, the incision site was closed with tissue adhesive, and the bird was allowed to recover from anesthesia under a heat lamp. All females were given 5-6 days to recover post-injection prior to perfusion.

Perfusion and immunocytochemistry.

Birds were perfused 5-6 days following the tracer injections. Females were anesthetized through isoflurane inhalation and subsequently transcardially perfused with 25ml of saline containing 12mg of heparin, followed by 150ml of 4% paraformaldehyde. Perfused brains were kept for 24 hours in 4% paraformaldehyde, then transferred to 30% sucrose for cryoprotection. Brains were then sliced into 40 μm coronal sections and stored in 0.025M phosphate-buffered saline solution containing sodium azide.

Immunocytochemistry was performed as previously described (Barr & Woolley, 2018; Barr et al., 2021). Briefly, one set of every third section was washed, blocked with donkey serum, then incubated in a primary antibody solution containing sheep anti-tyrosine hydroxylase (1:1000 dilution; Novus Biologicals, Centennial, CO, USA) for 48 hours. Next, sections were incubated for 2h in a secondary antibody solution containing donkey anti-sheep conjugated to Alexa Fluor 488 or Alexa Fluor 350 (to label TH; 3ul/ml or 6 ul/ml respectively; Life Technologies, Eugene, OR, USA). Sections were mounted onto chromium-aluminum gelatin-coated slides and cover-slipped with Prolong Gold antifade reagent (Life Technologies, Eugene, OR, USA).

The regions of interest imaged were the VTA, SN, and NAc. Due to the two functionally different neuronal populations in the VTA, the rostral and caudal portions were imaged separately (Goodson et al., 2009; Matheson & Sakata, 2015; Barr et al., 2021). Compound

images overlaying TH labeled and Dextran labeled neurons were taken for both hemispheres of each region (rostral VTA, caudal VTA, SNc) with a 40x objective of a Zeiss Axio Imager upright microscope and an AxioCam MRm Zeiss camera (Carl Zeiss, Germany). Compound images overlaying TH and Dextran near the NAc injection sites were also imaged respectively to record the location of the retrograde tracer injection and confirm the proper targeting of the injection.

Analysis.

To compare Dextran colocalization in TH-cells across regions and birds, we first computed the mean percent of TH cells colocalized with Dextran for each imaged region (rostral VTA, caudal VTA, SNc) for each bird. Next, we analyzed Dextran colocalization with TH cells across our injection sites, specifically the rostral NAc core, caudal NAc core, rostral NAc shell, and caudal NAc shell. To do this, we used a full factorial design with our image regions (rostral VTA vs. caudal VTA vs. SNc) as independent variables, bird ID as random variables, and the mean percent TH cells colocalized with Dextran as the dependent variable. We then analyzed the Dextran colocalization with TH cells across our imaged regions of interest. We used a full factorial design with our injection locations (rostral NAc core vs. caudal NAc core vs. rostral NAc shell vs. caudal NAc shell) as independent variables, bird ID as random variables, and the mean percent TH cells colocalized with Dextran as the dependent variable.

We also analyzed the non-colocalized Dextran-labeled neurons across all regions. To this end, we first computed the mean percent of Dextran-labeled neurons that were not colocalized with TH for each imaged region for each bird. Next, we used a full factorial design with our injection locations, image regions, and their interaction as independent variables, bird ID as random variables, and the mean percent of Dextran cells not colocalized with TH cells as the

dependent variable. All models used a restricted maximum likelihood approach with unbounded variance components. All post hoc tests were conducted using Tukey's HSD with $\alpha < 0.05$. All statistical analyses were completed using programs from JMP Pro 16 (SAS, Cary, NC, USA).

Results

VTA and SNc send differential dopaminergic projections to the NAc core and shell

We first investigated the anatomical connectivity of the zebra finch dopaminergic midbrain to the rostral and caudal components of the nucleus accumbens (NAc). Retrograde tracer injections of Dextran (Texas-red conjugated dextran n = 4) were made into either the rostral or caudal region of the NAc and we quantified the retrograde labeling in the ventral tegmental area (VTA) and substantia nigra pars compacta (SNc), where we stained neurons positive for the DA-synthetic enzyme Tyrosine Hydroxylase (TH) (Fig. 4). Across all injection sites, we observed consistent labeling in the rostral VTA (rVTA), caudal VTA (cVTA), and SNc. However, there was significant variation in tracer expression across the midbrain dopaminergic nuclei, indicating some topography in the projections to the nucleus accumbens.

When injections were targeted to the caudal NAc core (n = 2), we observed retrogradely labeled TH neurons in the rVTA, cVTA, and SNc (Fig 5). We then quantified the colocalization of the tracer in each of these regions and found a significant interaction effect between regions ($F(2, 27.1) = 6.9254$, $p = 0.0037$). Post hoc analyses using Tukey's HSD demonstrated that colocalization in the rVTA and SNc was significantly higher than colocalization in the cVTA ($p = 0.0044$, $p = 0.0248$) while colocalization levels in the rVTA and SNc were similar ($p = 0.4893$) (Fig 5D).

Rostral NAc shell injections (n = 3) resulted in retrogradely labeled TH neurons throughout all regions (Fig 6). Colocalization of the tracer in each of these regions showed a significant interaction effect between regions ($F(2, 9) = 15.5578$, $p = 0.0012$). Post hoc analyses using Tukey's HSD demonstrated that colocalization in the SNc was significantly higher than

colocalization in the rVTA and cVTA ($p = 0.003$, $p = 0.0045$), but not between rVTA and cVTA ($p = 0.5207$) (Fig 6D).

When targeting the rostral NAc core ($n = 1$), we observed retrogradely labeled TH neurons in all regions (Fig 7). Colocalization of the tracer in each of these regions found no significant interaction effect between regions ($F(2,9) = 0.3991$, $p = 0.6822$) (Fig 7D). Moreover, our targets in the caudal NAc shell ($n = 1$) also showed colocalization in all regions (Fig 8). Colocalization of the tracer in each of these regions found no significant interaction effect between regions ($F(2,19) = 1.6118$, $p = 0.226$) (Fig 8D).

Given our sample size, small injection volumes, and targeting, we consider these results to underestimate the total amount of retrogradely labeled dopaminergic and non-dopaminergic neurons in the VTA/SNc regions. Although coverage was partial, our results identify the previously undescribed organization of projections from the VTA and SNc to the rostral and caudal NAc in female zebra finches.

Variation in tracer colocalization in the VTA across different NAc tracer targets

Next, we sought to compare the amount of colocalization of the retrogradely labeled cells between VTA/SNc regions across different injection site targets (Fig 9). We observed a significant interaction when comparing colocalization in the rVTA ($F(3,15) = 17.8275$, $p < 0.0001$) (Fig 9A). Post hoc analyses using Tukey's HSD demonstrated that colocalization of the tracer in the rVTA was significantly higher in NAc caudal core targets than colocalization in NAc rostral and caudal shell targets ($p = 0.0011$, $p < 0.0001$).

We also observed a significant interaction when comparing colocalization in the cVTA ($F(3,24) = 28.918$, $p < 0.0001$) (Fig 9B). Post hoc analyses using Tukey's HSD also demonstrated that colocalization of the tracer in the cVTA was significantly higher in NAc

caudal shell targets than colocalization in NAc rostral shell and caudal core targets (ps. < 0.0001). Moreover, Tukey's HSD post hoc analyses showed that colocalization of the tracer in the cVTA was also significantly higher in NAc rostral core targets than colocalization in NAc rostral shell and caudal core targets ($p = 0.0007$, $p = 0.0163$). There were no significant interactions between injection targets in the SNc ($F(3,1.737) = 0.376$, $p = 0.7857$) (Fig 9C).

Additional non-colocalized retrogradely labeled neurons in the rVTA

We observed additional Dextran-labeled neurons that were not colocalized with TH+ neurons throughout all sections. We then quantified the total number of neurons that were not colocalized with TH+ neurons in the rVTA, cVTA, and SNc (Fig 10), and observed a significant interaction between these regions ($F(2,62.45) = 6.7942$, $p = 0.0021$). Post-hoc analyses using Tukey's HSD showed that there was a significantly higher level of non-colocalized, Dextran-labeled neurons in the rVTA than the cVTA ($p = 0.0014$) and a marginally higher amount of non-colocalized neurons in the rVTA than the SNc ($p = 0.0697$). We did not observe any significant interactions of the injection location on the amount of non-colocalized Dextran neurons observed ($F (3,3.035) = 1.4084$, $p = 0.3913$).

Figure 4

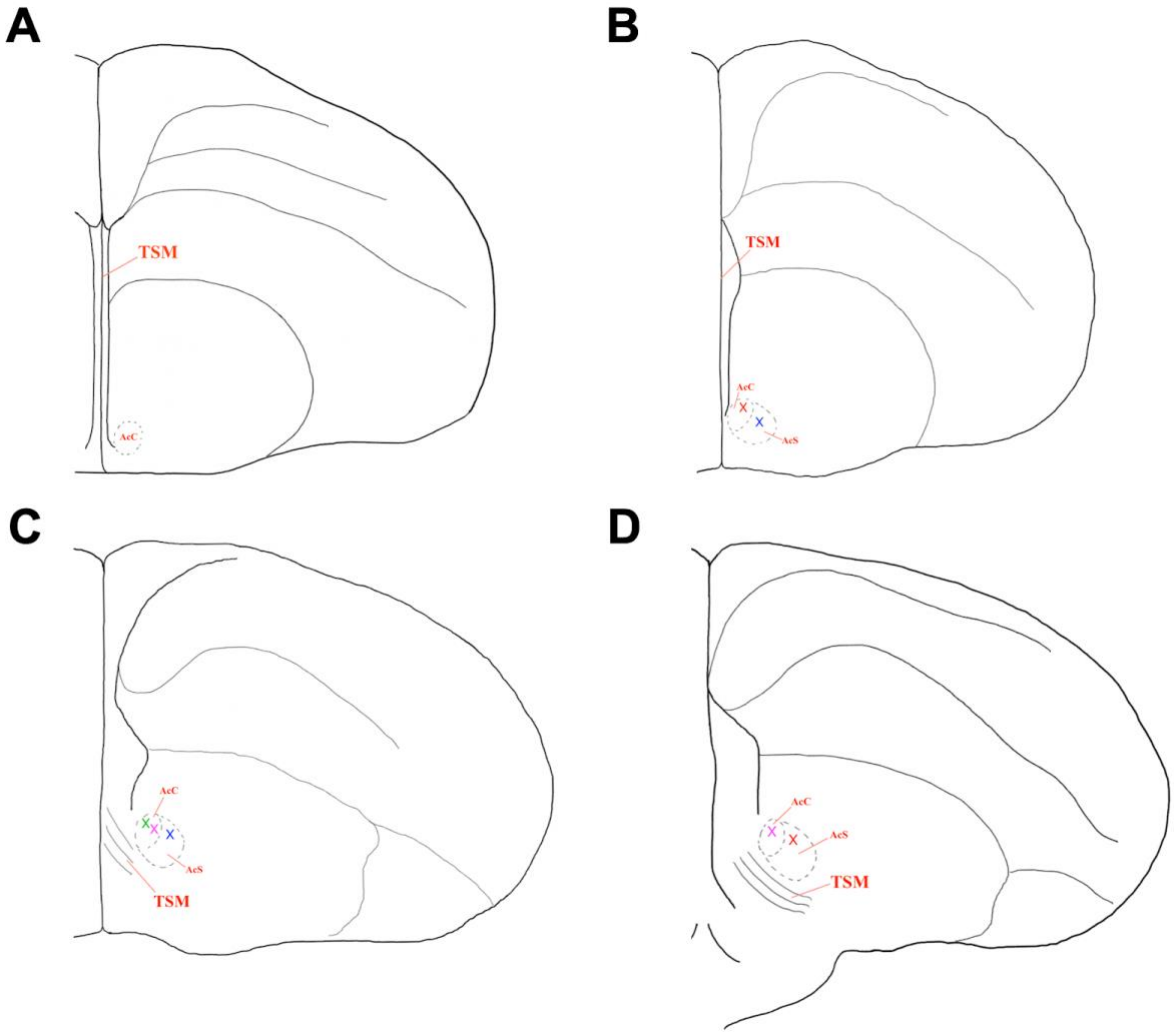

Figure 1: Diagram of the Dextran injection sites in the rostral (**A,B**) and caudal (**C,D**) regions of the NAc. Injection sites are indicated by the 'x', different colors represent injections in different birds.

Figure 5

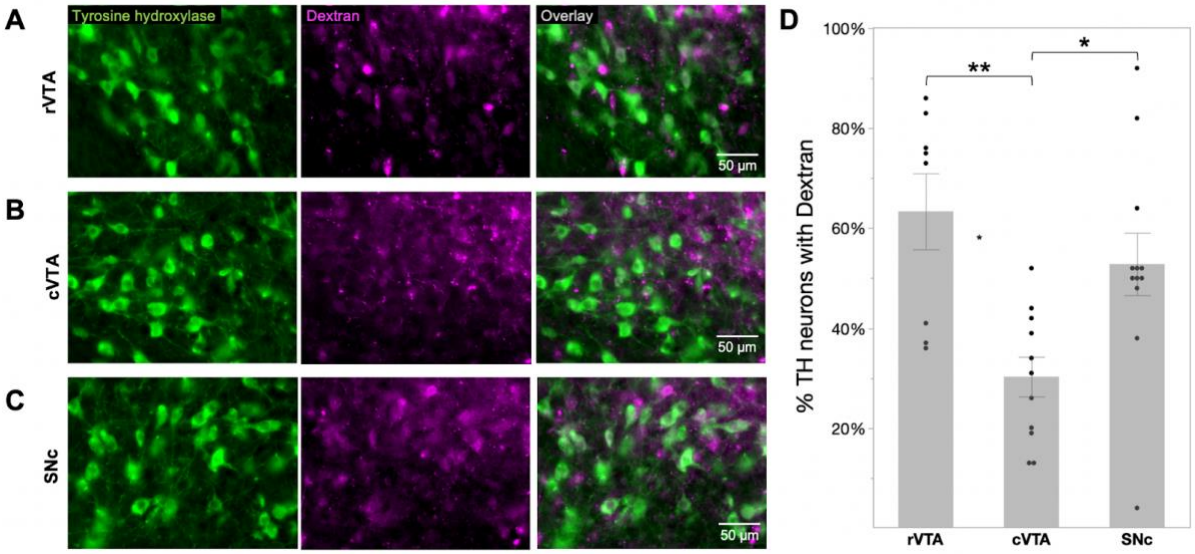

Figure 5: A-C. Representative images of TH expression (green; left), Dextran expression (magenta; middle), and their co-expression (right) in the rostral VTA (**A**), caudal VTA (**B**) and Substantia Nigra pars compacta (**C**) in birds who received Dextran injections in the caudal core of the NAc. **D**. There was substantial retrograde labeling in TH neurons in both portions of the VTA and SNc. Points indicate individual section counts. Error bars represent the standard error on the mean. ** indicates $p < 0.01$; * indicates $p < 0.05$.

Figure 6

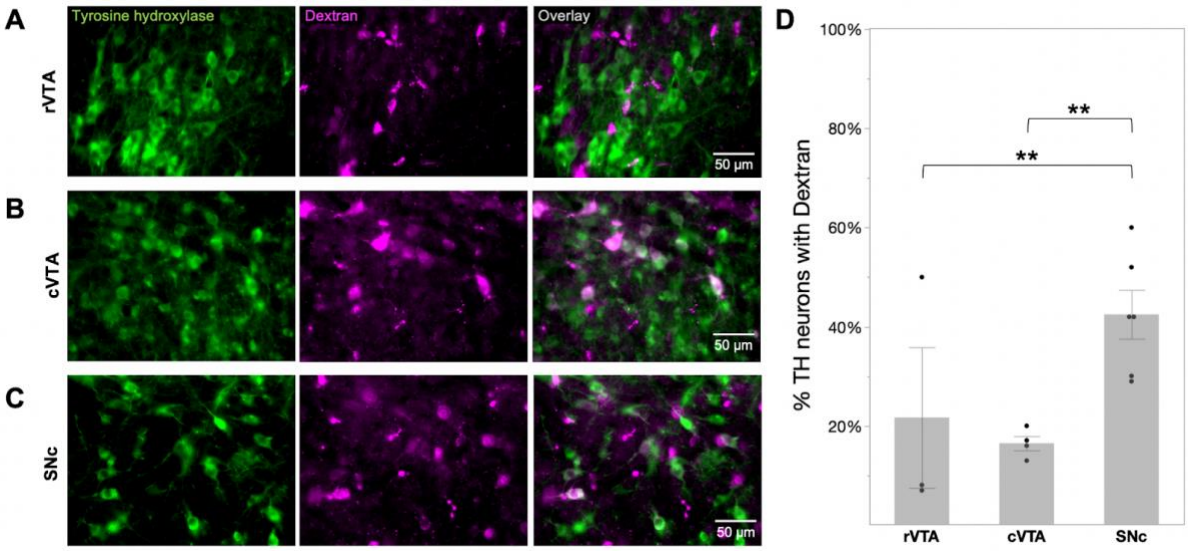

Figure 6: A-C. Representative images of TH expression (green; left), Dextran expression (magenta; middle), and their co-expression (right) in the rostral VTA (**A**), caudal VTA (**B**) and Substantia Nigra pars compacta (**C**) in birds who received Dextran injections in the rostral shell of the NAc. **D**. There was substantial retrograde labeling in TH neurons in both portions of the VTA and SNc. Points indicate individual section counts. Error bars represent the standard error on the mean. ** indicates $p < 0.01$.

Figure 7

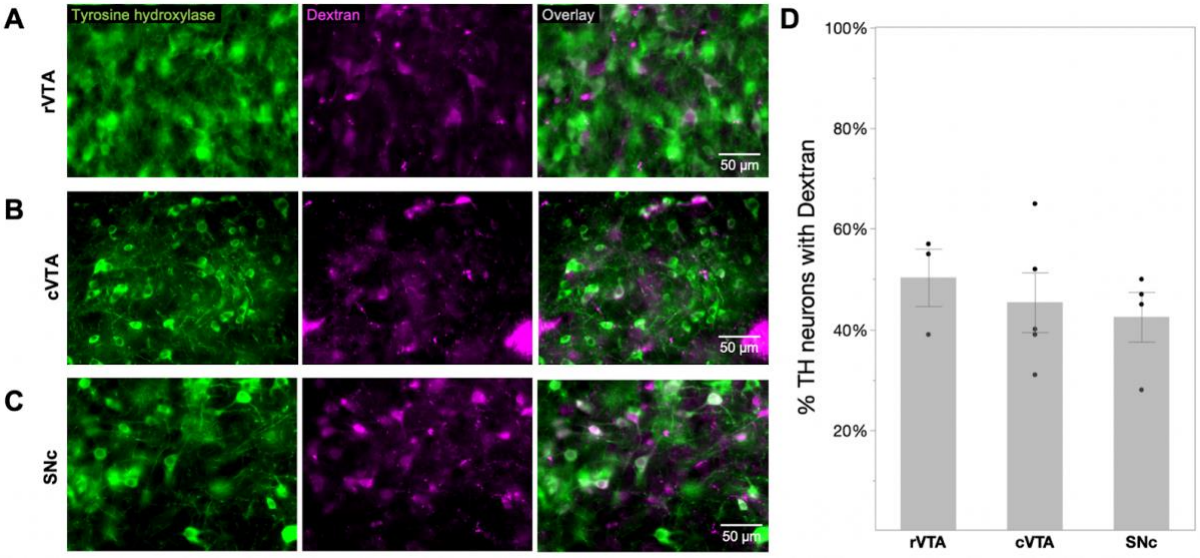

Figure 7: A-C. Representative images of TH expression (green; left), Dextran expression (magenta; middle), and their co-expression (right) in the rostral VTA (**A**), caudal VTA (**B**) and Substantia Nigra pars compacta (**C**) in birds who received Dextran injections in the rostral core of the NAc. **D**. There was substantial retrograde labeling in TH neurons in both portions of the VTA and SNc, but no significant differences between regions. Points indicate individual section counts. Error bars represent the standard error on the mean.

Figure 8

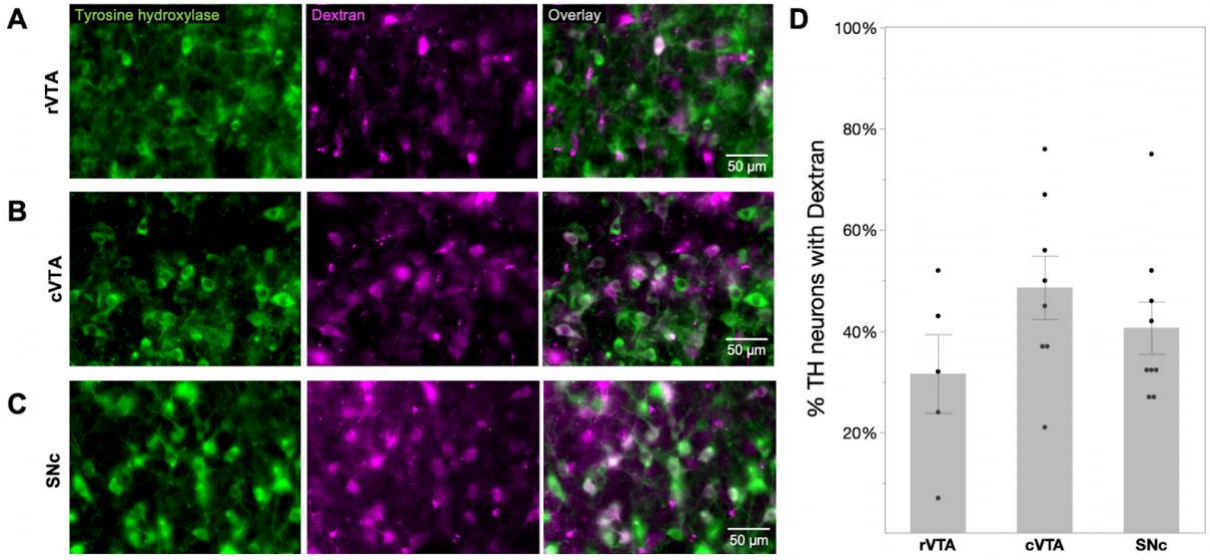

Figure 7: A-C. Representative images of TH expression (green; left), Dextran expression (magenta; middle), and their co-expression (right) in the rostral VTA (**A**), caudal VTA (**B**) and Substantia Nigra pars compacta (**C**) in birds who received Dextran injections in the caudal shell of the NAc. **D**. There was substantial retrograde labeling in TH neurons in both portions of the VTA and SNc, but no significant differences between regions. Points indicate individual section counts. Error bars represent the standard error on the mean.

Figure 9

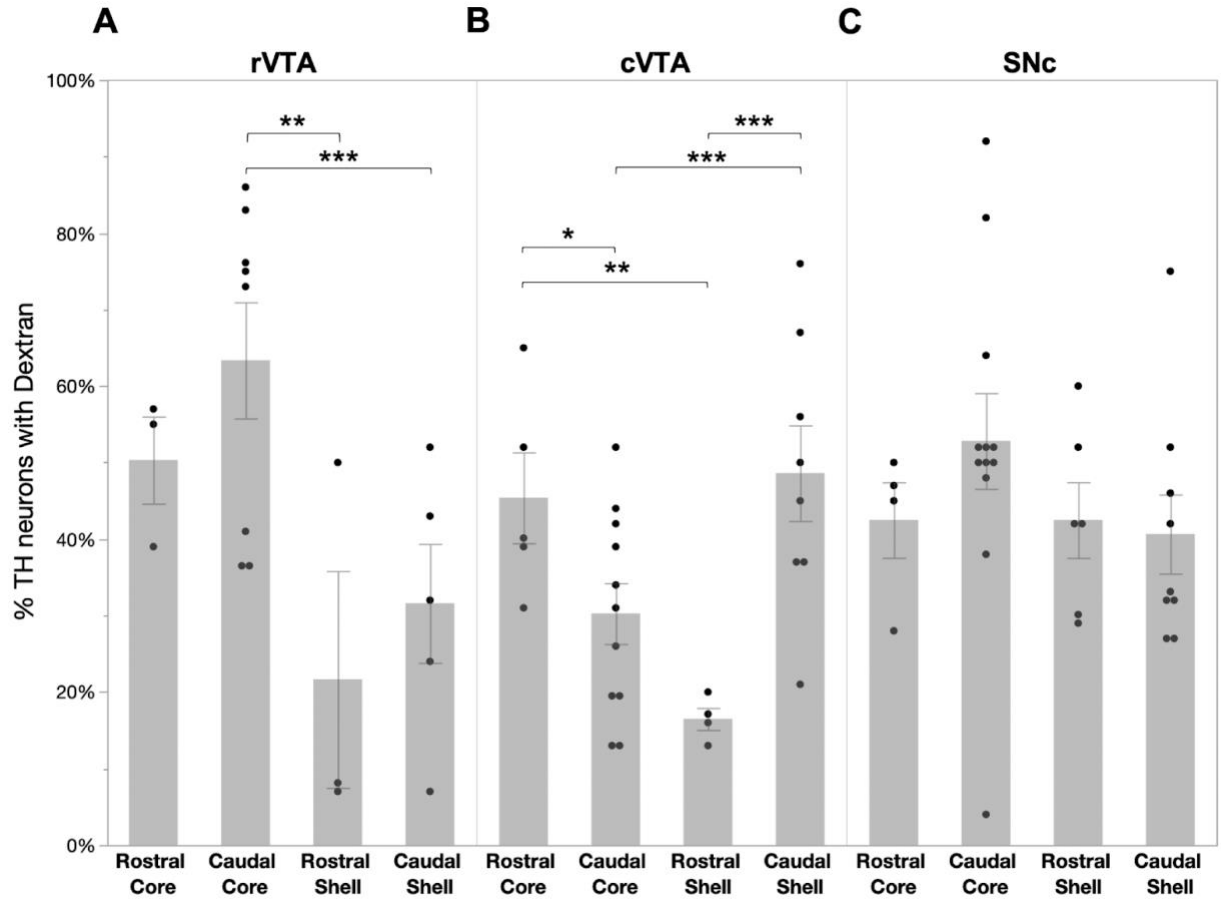

Figure 9: A-C. Percent of TH-immunoreactive cells colocalized with Dextran in the rostral VTA (**A**), caudal VTA (**B**), and substantia nigra par compacta (**C**). Grey bars represent the mean of each injection site. Points represent each individual section quantified. Error bars represent the standard error on the mean. *** indicates $p < 0.0001$; ** indicates $p < 0.01$; * indicates $p < 0.05$.

Figure 10

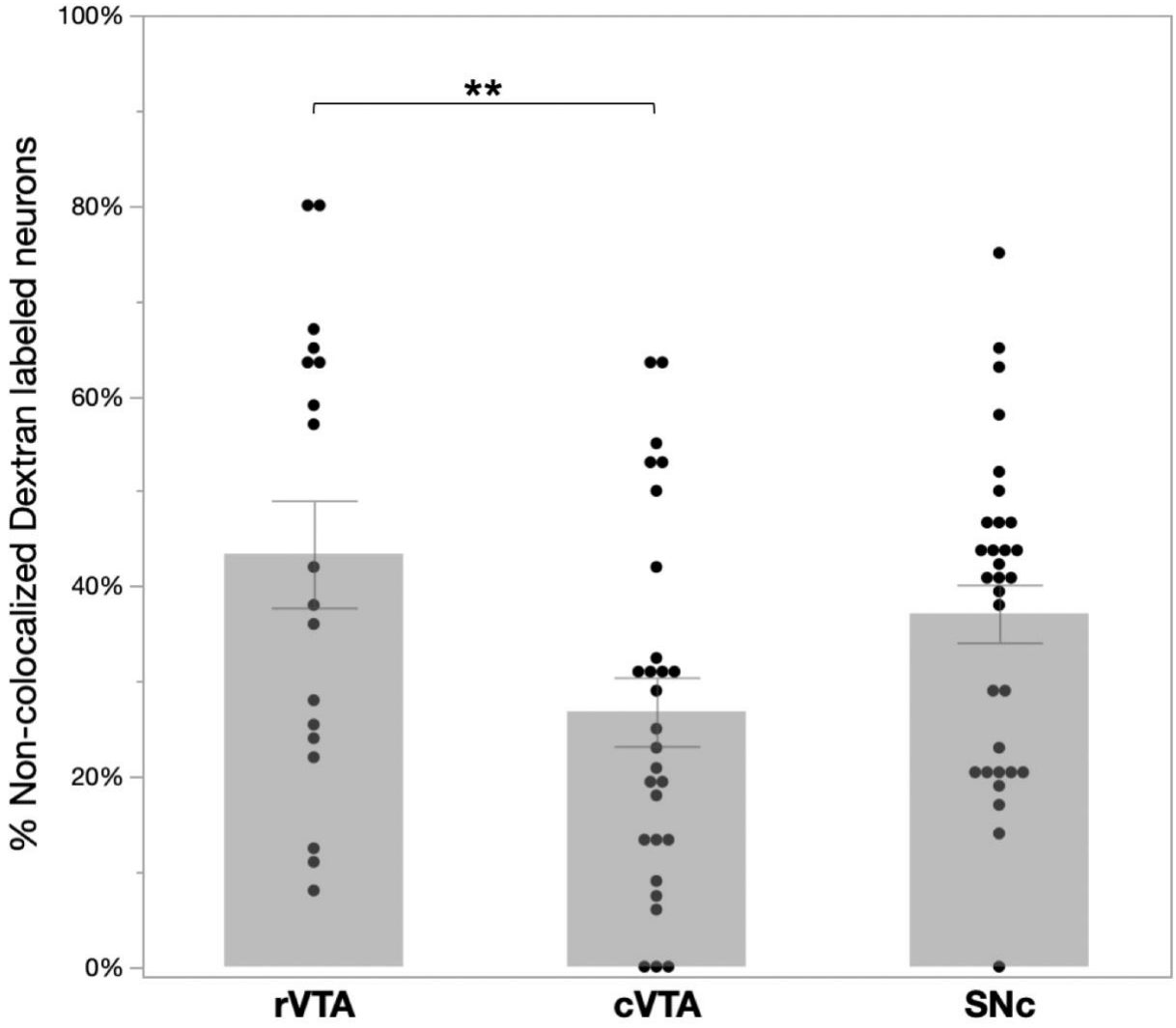

Figure 10: Percent of Dextran labeled cells non-colocalized in the rostral VTA (rVTA), caudal VTA (cVTA), and substantia nigra par compacta (SNc). Grey bars represent the mean of each region. Points represent each individual section quantified. Error bars represent the standard error on the mean. ** indicates $p < 0.01$.

Discussion

The formation of social bonds is a key factor in the survival and reproductive success of many social animals (Alberts 2019; Holt-Lunstad et al., 2010). In some species, acoustic signals are important for establishing and maintaining bonds (Zann & Bamford 1996). Songbirds are an excellent model for studying social bonding, they can form lifelong monogamous pairs and use acoustic cues in pair formation and maintenance (Riebel, 2009; Zann & Bamford 1996). We hypothesize that dopamine is a key modulator in the formation and maintenance of bonds, both in the auditory system as well as in areas such as the nucleus accumbens and social behavior network. However, there is limited knowledge of about how the acoustic communication signals are important for pair bonding are mediated by dopamine (DA). Here, we used dopamine depletion with a neurotoxin and neuroanatomical tracing to investigate dopaminergic contributions to song preference learning.

Understanding the role that dopamine plays in signal perception and preference can lend insight into the role preference plays in shaping social experiences. Although the influence of dopamine on pair-bonding behaviors in mammals has been well documented, less is known about the role of DA in pair-bonding and the connections of dopaminergic neurons in zebra finches (Alger et al., 2011; Aragona et al., 2006; Bharati & Goodson, 2006; Goodson et al., 2009). Our experiments sought to uncover the role of DA in a behavioral context and better understand the overall dopaminergic innervation to other regions that may play a role in mediating mate preferences. The results of our experiments expand upon current zebra finch neuroanatomy and highlight a potential influence of dopamine on plasticity in mate song preference. Moreover, our findings lend new insight into the influence of dopaminergic activity on pair bonding and song preference learning observed in zebra finches.

In Chapter 1, we manipulated dopamine in a secondary auditory area in female zebra finches to investigate the role of dopamine in auditory regions on song preferences. Specifically, we injected the neurotoxin 6-hydroxydopamine (6-OHDA) into the caudomedial nidopallium (NCM) of female zebra finch and tested their preferences for their mate's song both before and after pairing. We showed that the use of 6-OHDA injections in the NCM significantly decrease dopaminergic terminal fluorescence in the medial NCM (closer to the injection site). Our injections were contained to the medial NCM, as we found no differences in the dopaminergic terminal fluorescence in the lateral NCM between 6-OHDA-injected birds and controls. While 6-OHDA has primarily been used to reduce DA in the striatum, previous studies utilizing 6-OHDA injections in zebra finches have primarily targeted dopaminergic inputs in male zebra finch song nuclei, therefore our injections in auditory regions in zebra finches present a novel area to utilize these injections (Blandini et al., 2008; Miller et al., 2015; Schober 2004; Tanaka et al., 2018). Since 6-OHDA is highly oxidative, we used a mild dose in order to maintain cell integrity in the NCM (Schober 2004). We stained all our sections with DAPI to ensure that our injections didn't cause cell death in the NCM while also optimizing the amount of DA terminals we depleted. We observed little to no cell death in our NCM sections but our sections still require quantification to get a better sense of the extent of cell death that may have been caused by our injections. Taken together, our data suggest that although the injection did target a portion of the NCM, the overall spread of the injection may not have been sufficient to deplete enough dopaminergic inputs present in the NCM to evoke a change in behavior.

Control females also received the same surgery as the 6-OHDA experimental birds thus they also received injections of a vehicle control before mating. While previous studies have shown that injecting control birds with a vehicle control has no effect on behavior, it is possible

that the surgery or the injection of a vehicle in the NCM may have caused small lesions to the NCM leading to behavioral changes different from what we had predicted (Hoffman et al., 2016; Miller et al., 2015). Future work should focus on further optimizing the 6-OHDA injections in order to maximize depletions of dopaminergic terminals to gain a better understanding of how to create better 6-OHDA depletion models in the NCM of female zebra finches.

We also observed that there were no significant differences between the overall preference change between 6-OHDA treated females and control females. This could be accounted for by many small but significant changes in our experimental methods compared to previous studies. For example, unlike previous work (Wall & Woolley, 2023), our control females did not significantly prefer the mate's song over an unfamiliar song. Previous studies have shown that string pull assays are a sufficient method to test song preference in female zebra finches by providing a means to quantify their motivation to hear a particular song over another (Barr et al., 2021; Wall & Woolley, 2023). For this experiment, we adjusted the testing protocol compared to previous studies. In particular, our preference tests were conducted over the course of 3-5 days, for several hours at a time, both before and after pairing. Moreover, we tested females prior to pairing and paired them with the male that produced their lesser preferred song. While we observed nest-building behavior in all of our test pairs, it is possible that by pairing females with males whose song they preferred less, behavioral data may have differed from what was expected. As such, these factors may have played a role in influencing behaviors in the control females, resulting in no preference shift observed in control females.

Finally, we found that the degree of dopaminergic innervation of the NCM was correlated with greater plasticity in preference expression. Individuals with greater dopaminergic innervation in the NCM showed greater changes in preference following mating. These findings

emphasize the role that dopamine release in the NCM might be playing in synaptic plasticity and consolidating the memory of song in the NCM. Previous work in our lab has shown that dopamine agonist infusions in the NCM have the ability to induce plasticity by shifting song preferences and our findings here suggest that depleting these dopaminergic inputs in the NCM also works to introduce variation in the plasticity observed in mate song preferences (Barr et al., 2021). Taken together, these data suggest that the ability of an individual bird to form a pair bond and a preference for their mate's song may be related to the strength of the dopaminergic inputs coming into the NCM and that this relationship should be examined in the future.

In Chapter 2, we sought to map out the neuroanatomy of other structures associated with pair bonding in relation to dopaminergic neurons. Specifically, we injected retrograde tracer into regions of the nucleus accumbens (NAc) of female zebra finches to observe the topography of the dopaminergic neurons in the midbrain that may be projecting there. Our findings confirm that the dopaminergic neurons in the ventral tegmental area (VTA) and substantia nigra pars compacta (SNc) send projections to the rostral and caudal core and shell components of the NAc in zebra finches. Moreover, we observed differential topographical organization of inputs from the VTA to different regions of the NAc. Such topography is in line with previous data from mammalian models, where they observed graded topographic organization of projections from the VTA to the different regions of the NAc (Ikemoto, 2017; Rodríguez-López et al., 2017). Given the role of the NAc in pair bonding behaviors in mammals, understanding the topography and organization of dopaminergic projections to the NAc will provide a foundation for further study of the role of dopamine in the NAc in mediating mate preference in songbirds (Balthazart & Absil, 1997; Bjorklund & Dunnett, 2007; Durstewitz et al., 1999).

When comparing between injection sites, we found that the injections into the caudal core of the NAc resulted in the highest colocalization of tyrosine hydroxylase positive (TH+) neurons with the Dextran tracer. Previous studies have linked the NAc caudal core to the regulation of cue-elicited approach and avoidance decisions in rats (Hamel et al., 2017). Given this connectivity between the rostral VTA (rVTA) and the NAc caudal core, it would be interesting to study whether both the caudal core and the rVTA are involved in similar approaches and avoidance decisions in songbirds. We also observed higher colocalization between the tracer and TH+ neurons in the caudal VTA (cVTA) in injections targeting the rostral core and caudal shell. Previous work in zebra finch females has indicated that dopaminergic neurons in the cVTA show higher activation in response to a preferred song in comparison to non-preferred songs and silence (Barr & Woolley, 2018; Barr et al., 2021). Our data also indicated relatively low levels of colocalization in the rostral shell. Previous literature in voles has shown that dopamine transmission within the rostral shell promotes pair bond formation (Aragona et al., 2006). As such, we hypothesize that during the pair bonding process, there could be a strengthening of the connections or changes in dopamine release leading to the formation of mate preferences. It is unclear whether the circuitry in the NAc observed in voles is conserved in zebra finches as well. Future work should focus on the circuitry between the VTA and NAc rostral core, rostral shell, and caudal shell to gain a better understanding of how song preference and pair bonding may be mediated by these regions.

We also observed additional neurons in the SNc that send comparable projections to every region of the NAc. While little literature surrounds dopaminergic projections from the SNc to the NAc in songbirds, previous tracing studies in the pigeon (*Columba livia*) have demonstrated that these connections exist in the avian brain (Husband & Shimizu, 2011).

Moreover, previous retrograde labeling in the NCM has also demonstrated dopaminergic projections from the SNc to the NCM, which is also involved in mate preference (Barr et al., 2021). Therefore, our findings in the SNc could be indicating that both the VTA and SNc could be influencing NAc activity simultaneously through dopamine release during pair bonding. Future work focusing on the projections from the SNc to the NAc could give us better insight into how these projections may be shaping preference.

Our findings also confirm the presence of additional retrograde labeled cells that did not colocalize with TH+ neurons in the midbrain. Specifically, we observed a higher amount of non-colocalized, Dextran-labeled cell bodies in the rVTA when compared to cell bodies in the cVTA. Previous work in mammalian models has indicated that the majority of non-dopaminergic projections to the NAc are GABAergic and the remainder glutamatergic (Margolis et al., 2006; Yamaguchi et al., 2011). Thus, we hypothesize that the additional Dextran labeled cells we observed in the VTA and SNc should be GABAergic and glutamatergic neurons. Previous retrograde tracing in rats has also indicated that while the majority of projections from the VTA to the NAc are ipsilateral there still exist some contralateral projections between the VTA and NAc (Breton et al., 2019). Since all of our injections were bilateral, we do not know the extent of any contralateral projections from the VTA to the NAc. Future studies are encouraged to look into how the VTA may be sending non-dopaminergic and contralateral projections to the NAc.

While our current findings clarify the general topography of the VTA/SNc and their respective projections to the different regions of the NAc, it would also be interesting to see if biological sex, developmental experience, and social interaction influence the structural connectivity between these regions. Our tracing study used both females who had never been exposed to male songs along with normally-reared females. Given the small sample size and

limited overlap in injection sites between birds from different rearing conditions, we are unable to show any rearing-dependent differences. Moreover, all of the females used in this study were never mated, therefore we have yet to compare the neurocircuitry of these regions between unmated females and mated females. It will be interesting for future work to examine these connections on a larger scale, using more birds from various social and developmental conditions to better understand how sex, developmental and experiential influences may shape the connections between the midbrain and NAc.

In summary, our findings provide new insight into the role of the dopamine system and give us a clearer idea of how it may be acting to mediate mate preferences observed in zebra finch females. Our results from Chapter 1 highlight the potential of dopamine-inducing neuronal plasticity in the NCM when forming song preferences. While our findings in the NCM do not prove our hypothesis, we uncovered a potential role of dopamine in the NCM in mediating mate song preference through plasticity. This study also presents a new method to quantify dopaminergic terminal fluorescence that can be used in the future to observe terminals. Moreover, our results from Chapter 2 support evidence from other avian and mammalian models and conserved neurocircuitry in the NAc, confirming the presence of dopaminergic innervation from the VTA/SNc to NAc in zebra finches.

Conclusion

This study sought to investigate the role that dopamine plays in forming mate song preferences and mediating mate preferences observed in female zebra finches, a crucial behavior necessary for reproductive success. We first studied the effects of dopaminergic input depletion in higher auditory processing areas in females on mate song preference formation. Female zebra finches showed a significant relationship between how willing they were to switch their preference to the number of dopaminergic inputs present in the NCM. To this end, it appears that the amount of dopaminergic inputs in the NCM may modulate mate song preferences however how dopaminergic inputs influence mate song preference formations remains unknown. In the second part of this study, we explored mate preference beyond an auditory preference to confirm other reward pathways mediated by dopamine release. Doing so, we found that the VTA and SNc send projections to the NAc at both its rostral and caudal regions, confirming analogous neurocircuitry as previously observed mammalian models. Taken together, our findings highlight the critical role that dopamine plays in pair bonding and get a better idea of how dopaminergic projections from the VTA may be playing a role in mediating preferences observed in female zebra finches.

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