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How Do Adult Songbirds Learn New Sounds? Using Neuromodulators to Probe the Function of the Auditory Association Cortex

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HOW DO ADULT SONGBIRDS LEARN NEW SOUNDS? USING NEUROMODULATORS TO PROBE THE FUNCTION OF THE AUDITORY ASSOCIATION CORTEX

A Dissertation Presented

by

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“

As I walk this road will I ever
Find that solution to my resolution
And as I take each step will there be that
Real comprehension of my redemption
What should I do now and where do I find how
To find myself…
Define myself...

”

Kaleidoscope – Transatlantic

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ABSTRACT

HOW DO ADULT SONGBIRDS LEARN NEW SOUNDS? USING NEUROMODULATORS TO PROBE THE FUNCTION OF THE AUDITORY ASSOCIATION CORTEX

MAY 2020

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The ability to associate sounds and outcomes is vital in the life history of many species. Animals constantly assess the soundscape for cues associated with threats, competitors, allies, mates or prey, and experience is crucial for those associations. For vocal learning species such as humans and songbirds, learning sounds (i.e. perception and association learning) is also the first step in the process of vocal learning. Auditory learning is thought to depend on high-order cortical brain structures, where sounds and meaning are bound. In songbirds, the caudomedial nidopallium (NCM) is part of the auditory association cortex and is known to be involved in sound learning and perception. During songbird development, NCM plays a role in song learning, but in adulthood, NCM's role is less clear and a matter of controversy in the literature. Furthermore, NCM is a site of action of neuromodulators including neuroestradiol (E2) and dopamine (DA). E2 is known to be produced by NCM neurons that contain the enzyme aromatase, which converts testosterone into E2. E2 production is also known to increase in the NCM during social interactions, and exogenous E2 modulates neuronal firing, but its effects on auditory behavior have not been pinpointed. Effects of E2 within the mammalian and

avian hippocampus had been previously reported to support spatial learning. My main goal in this dissertation was to clarify the role of NCM in adult zebra finches (*Taeniopygia guttata*). Towards this end, I developed experiments in which I manipulated and thus documented the effects of two neuromodulatory systems, E2 and DA. I first examined the role of E2 in auditory-dependent behavior. For this, I developed a novel operant conditioning task with social reinforcement. Using this task, I showed that inhibiting E2 production within NCM during learning impairs acquisition of auditory associations. However, after the learning process was completed, I found that E2 production and even NCM activity were no longer required for maintaining high auditory performance, suggesting that NCM does not play a role in memory retrieval or auditory discrimination in adults. These findings led me to develop the hypothesis that E2 in NCM modulates online associative learning signals. In mammals, plasticity in virtually all learning-related brain regions is dependent on dopamine (DA) regulation and E2-DA interactions have been reported in several of these regions. Much is known about DA signaling in brain areas involved in decision-making and reinforcement learning. I here review the literature on motor and, especially, sensory cortical regions and provide a comprehensive review of the current knowledge of DA's roles in cortical regions involved in sensory and motor learning, paying especial attention to non-mammalian vertebrates. I found that this literature is surprisingly limited in mammals, and often non-existent in non-mammalian vertebrates. Then, I hypothesized that E2 could be operating on dopaminergic (DAergic) signaling in NCM, in which D1 receptor (D1R) mRNA had been reported. Since there were no data on the anatomical and functional effects of these receptors, I investigated whether D1R protein could be detected and D1R-mediated

signaling modulated synaptic plasticity in NCM. Specifically, I found that D1R protein is prevalent in NCM neurons, especially in aromatase-, GABA-, and parvalbumin-positive neurons. Activating D1R *in vitro* reduced the amplitude of spontaneous GABAergic and glutamatergic currents and increased the frequency of the latter. Similarly, activating D1R *in vivo* reduced firing of putative-inhibitory interneurons, but increased firing of putative-excitatory projection neurons. Finally, I showed that D1R activation disrupted stimulus-specific adaptation of NCM neurons, a phenomenon reflective of active auditory memory formation. In conclusion, this dissertation advances the literature by providing direct evidence that E2 production within the auditory cortex affects sensory learning, potentially by tapping into the DAergic system, which itself modulates plasticity mechanisms associated with learning and memory. I propose that these findings could apply to other vertebrates that contain aromatase and DA receptors in their auditory cortex, including humans.

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CHAPTER 1

INTRODUCTION

Language learning in humans requires mastering how to receive and send signals under different frameworks, such as syntax, grammar, semantics and phonemes. Hence, the modern synthesis of spoken language posits that it is inherently multifactorial, involving highly complex human-exclusive features (e.g. semantics, grammar), but also, more primary components that are not exclusive to humans. Theoretical and empirical work has led to the hypothesis that some of these components, including vocal and auditory learning, have evolved not just in humans but also in other vertebrates, such as songbirds. Studying vocal and auditory learning in songbirds can therefore provide insight into the mechanisms of sensorimotor integration as well as spoken language [Jarvis, 2019]. More broadly, auditory learning can be vital to many species, independently of vocal learning ability. Animals constantly assess their environment for cues associated with threats, competitors, allies, mates or prey, and experience plays a large role in forming those associations. For example, individual recognition by vocal patterns has been reported in a variety of animals, including humans, dolphins, penguins and songbirds (Order Passeriformes: suborder Passeri) and vocalizations are used to identify mates, neighbors/strangers, group members and parents/offspring [Aubin and Jouventin, 1998; Sayigh et al., 1998; Gentner et al., 2000; Goodwin and Podos, 2014]. These behaviors are dependent on associative learning mechanisms. For learning of spoken language, in particular, the first step in the learning process is to make associations between complex sounds and their meaning. The neural circuits that support

vocal learning are currently being unpacked and studied, and associative learning in general is often thought to rely on high-order cortical brain structures.

Songbirds have become valuable models for understanding vocal learning, an ability that depends on the acquisition and subsequent reproduction of sounds for communication [Petkov and Jarvis, 2012; Mello, 2014]. At the onset of their lives, songbirds begin intensive auditory learning, after which singing behavior itself emerges. Subsequently, auditory learning continues to aid in other critical processes such as individual recognition, mate selection and sound-outcome associations [Gentner et al., 2000; Verzijden et al., 2012].

In songbirds, the caudomedial nidopallium (NCM) is a secondary auditory region considered analogous to Wernicke's area, the center for speech comprehension in humans [Bolhuis et al., 2010]. Many features of NCM are consistent with a role in active auditory memory formation, such as stimulus-specific adaptation, juvenile song memorization and adult sound association learning [Jarvis et al., 1995; Chew et al., 1996; London and Clayton, 2008; Bell et al., 2015]. Additionally, the songbird NCM is an important target of a wide variety of neuromodulators, such as norepinephrine [Ikeda et al., 2015; Lee et al., 2018], nitric oxide [Wallhäusser-Franke et al., 1995], neuroestradiol [Saldanha et al., 2000] and dopamine [Matragrano et al., 2012]. NCM therefore possesses many features that support cellular and neural circuit plasticity mechanisms related to the processing and association of complex sounds.

From an evolutionary standpoint, the NCM seems to be highly conserved in its structure among sauropsids. In non-avian sauropsids (Reptilia), auditory centers are positioned in a very similar anatomical location as in birds within the dorsoventricular

ridge (DVR) [Foster and Hall, 1978]. Therefore NCM's origins are suggested to have been present in the common sauropsid ancestor [Butler et al., 2011]. By contrast, E2 production within NCM by the enzyme aromatase seems to be a feature of the songbird lineage, but not of non-songbirds such as ring doves or budgerigars [Metzdorf et al., 1999]. Since aromatase [Yague et al., 2006] is also abundant in the human temporal cortex, E2 signaling in the songbird auditory cortex can provide important comparative insights into the effects of E2 on auditory learning. Currently, the functional consequences of cortical E2 signaling are largely unexplored for any species.

Available information on NCM's specific functions in songbirds is conflicting. Data from one research group have suggested that NCM lesions do not affect juvenile song learning or adult operant auditory learning, but do impact recovery from a reinforcement-driven song plasticity paradigm [Canopoli et al., 2014; Canopoli et al., 2017]. These findings contrast with findings strongly implicating NCM in vocal learning [London and Clayton, 2008; Tsoi et al., 2014; Yanagihara and Yazaki-Sugiyama, 2016] and with a few reports of NCM's supporting adult auditory plasticity [Jarvis et al., 1995; Bell et al., 2015]. Therefore, studies that can clarify NCM's function are warranted, especially those aimed at understanding the potential role(s) of NCM in adult songbirds once song learning is completed.

Studies carried out previously by our group showed that E2 production is elevated locally in the adult songbird NCM during social interactions and auditory/visual playbacks [Remage-Healey et al., 2008; Remage-Healey et al., 2012; Remage-Healey et al., 2013], but the behavioral implications of NCM E2 fluctuations have not been clarified. In adult males, blockade of NCM E2 synthesis eliminates the natural

phonotactic preference for their own songs [Remage-Healey et al., 2010]. However, phonotaxis experiments cannot disentangle auditory discrimination, learning, motivation, and memory retrieval, and thus the precise function of E2 signaling in NCM remains uncertain.

My dissertation project aimed to better understand neuromodulatory mechanisms underlying adult auditory learning. Studying songbirds, I present findings that clarify the role of the adult auditory association cortex (NCM). The central hypothesis of my thesis is that dopamine (DA) and estradiol (E2) interact to modulate auditory learning and memory mechanisms in the adult NCM.

In Chapter 2 (published in the journal *Hormones & Behavior*), I present a novel behavioral tool to assess auditory learning in songbirds using social reinforcement, which circumvents two important caveats of commonly used behavioral paradigms: food restriction and complete social isolation. Using this tool, I show that E2 participates in auditory learning in adults, such that pharmacologically blocking its production within NCM slows acquisition of accurate sound-outcome associations. In this same chapter, I further show that the NCM activity is only required in the process of learning, but no longer required after sounds have been learned. Finally, I place these findings into the context of how, in mammalian hippocampus, estrogens regulate spatial learning. Comparisons between avian and mammalian results may help frame our understanding of this newfound role of estrogens in regulating sensory learning.

In the following chapters, I focus on the question of what types of learning signals are potentially modulated by E2 in the adult NCM. In mammals, virtually all brain regions involved in promoting goal-directed behaviors rely on DA signals [Happel,

2016]. Many decades of intense research have been dedicated to understanding how DA signals govern decision-making in the mammalian cortex. However, I thoroughly reviewed the literature on motor and, especially, sensory cortical regions and found that this literature is surprisingly limited in mammals, and often non-existent in non-mammalian vertebrates. Thus, in Chapter 3, I present a comprehensive review of the current knowledge of DA's roles in cortical regions involved in sensory and motor learning. This review pays especial attention to non-mammalian vertebrates as an attempt to highlight an important gap in understanding of the evolution of DA signaling in motor/sensory learning systems.

The role of DA signaling has been examined in the songbird brain in the context of reinforcement learning for song production and motivation to sing [Leblois et al., 2010; Schmidt and Ding, 2014; Matheson and Sakata, 2015; Chen et al., 2016], but it has been far less studied in the context of auditory processing and learning. Matragrano et al. [2012] reported that hearing song rapidly increases production of DA in songbird auditory regions, especially in the NCM. Importantly, NCM shows extensive presence of mRNA for dopamine receptors [Kubikova et al., 2010], but their function remains unexplored.

In Chapter 4, I present findings on the anatomy and physiology of DA receptors in NCM. First, through immunofluorescence I describe the DA D1 receptor-positive (D1R+) neuron distribution in NCM and show that most aromatase-positive neurons are also D1R+. I further characterize these neurons by showing that D1R neurons are predominantly inhibitory. I then test the function of these receptors both *in vitro* and *in vivo*. *In vitro*, in whole-cell patch clamp recordings, I find that D1R activation reduces

the amplitude of GABAergic and glutamatergic spontaneous currents but increases the frequency of glutamatergic currents. *In vivo*, I employed a novel neuronal recording device coupled with a retrodialysis probe; my findings corroborate the *in vitro* findings, such that D1R activation reduces the firing of putative-GABAergic neurons, while increases the firing of putative-glutamatergic neurons. Importantly, D1R activation disrupts neuronal auditory short-term memory formation in NCM neurons, which suggests that DA plays a key role in sound memory formation. Finally, I place these findings into the context of how DA signaling is involved in encoding signals related to goal-directed behavior, and how DA acts in concert with E2 in several brain regions to promote neural plasticity.

Overall, my dissertation supports the hypothesis that NCM is a key brain region for auditory plasticity and learning in adult zebra finches and provides new evidence that E2 and DA modulate this process. The significance of each study is discussed in detail in the individual chapters. How these findings connect, and what future directions can follow are discussed in my final chapter, Final Considerations.

CHAPTER 2

AUDITORY LEARNING IN AN OPERANT TASK WITH SOCIAL REINFORCEMENT IS DEPENDENT ON NEUROESTROGEN SYNTHESIS IN THE MALE SONGBIRD AUDITORY CORTEX

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2.1 Abstract

Animals continually assess their environment for cues associated with threats, competitors, allies, mates or prey, and experience is crucial for those associations. The auditory cortex is important for these computations to enable valence assignment and associative learning. The caudomedial nidopallium (NCM) is part of the songbird auditory association cortex and it is implicated in juvenile song learning, song memorization, and song perception. Like human auditory cortex, NCM is a site of action of estradiol (E2) and is enriched with the enzyme aromatase (E2-synthase). However, it is unclear how E2 modulates auditory learning and perception in the vertebrate auditory cortex. In this study we employ a novel, auditory-dependent operant task governed by social reinforcement to test the hypothesis that neuro-E2 synthesis supports auditory learning in adult male zebra finches. We show that local suppression of aromatase activity in NCM disrupts auditory association learning. By contrast, post-learning performance is unaffected by either NCM aromatase blockade or NCM pharmacological inactivation, suggesting that NCM E2 production and even NCM itself are not required for post-learning auditory discrimination or memory retrieval. Therefore, neuroestrogen synthesis in auditory cortex supports the association between sounds and behaviorally relevant consequences.

2.2 Introduction

The ability to associate sounds and outcomes is vital to the life history of many species. Animals constantly assess the environment for cues associated with threats, competitors, allies, mates or prey, and experience plays a large role in forming those associations. For example, individual recognition by means of vocalizations has been reported in a variety of animals, including humans, dolphins, penguins and songbirds (Order Passeriformes: suborder Passeri) and it can be used to identify mates, neighbors/strangers, group members and parents/offspring [Aubin and Jouventin, 1998; Sayigh et al., 1998; Gentner et al., 2000; Goodwin and Podos, 2014]. These behaviors are dependent on associative learning mechanisms in higher-order cortical regions where sounds and meaning are bound.

Songbirds are valuable models for understanding vocal learning, an ability that depends on the acquisition and subsequent reproduction of sounds for communication [Petkov and Jarvis, 2012; Mello, 2014]. At the onset, songbirds begin intensive auditory learning, after which singing behavior itself emerges. Subsequently, auditory learning continues to aid individual recognition, mate selection and sound-outcome associations [Gentner et al., 2000; Verzijden et al., 2012].

The avian caudomedial nidopallium (NCM) is a region of secondary auditory cortex [Bolhuis et al., 2010] that both processes songs and associates songs and behaviorally-relevant consequences [Jarvis et al., 1995]. Neural activity in NCM reflects the association between song and reward/punishment outcomes in an operant paradigm [Bell et al., 2015].The songbird NCM is also target of neuromodulators, including norepinephrine [Ikeda et al., 2015; Lee et al., 2018], nitric oxide [Wallhäusser-Franke et al., 1995], dopamine [Matragrano et al., 2012] and neuroestradiol (E2) [Saldanha et al.,

2000]. E2 rapidly enhances the NCM neural responses to songs in zebra finches [Remage-Healey et al., 2010]. Since both aromatase (E2-synthase) [Yague et al., 2006] and E2 receptors [González et al., 2007] are abundant in the human temporal cortex, E2 signaling in the songbird auditory cortex can provide important comparative insights. Currently, however, the functional consequences of cortical E2 signaling are unclear for any species.

E2 production is elevated locally in the songbird NCM during social interactions and auditory/visual playbacks [Remage-Healey et al., 2008; Remage-Healey et al., 2012; Remage-Healey et al., 2013], but the behavioral implications of NCM E2 fluctuations have not been clarified. In males, blockade of NCM E2 synthesis eliminates the natural phonotactic preference for their own songs [Remage-Healey et al., 2010]. However, phonotactic experiments do not disentangle auditory discrimination, learning, motivation, and memory retrieval, thus the precise function of E2 signaling in NCM remains uncertain.

The current study introduces a low-cost operant behavioral paradigm to assess auditory learning and discrimination/retrieval. The former can be inferred by evaluating the trajectory of subjects' performance when exposed to novel stimuli. The latter can be inferred by evaluating performance in response to previously learned stimuli. Our approach relies on visual social reinforcement, without depriving animals of food, water, or regular social contact. Since all trials are initiated by subjects, social motivation can be inferred by quantifying the number of trials initiated. Using this task, we evaluated whether E2 synthesis inhibition systemically (oral administration) as well as locally in NCM affects auditory performance. We predicted that blocking E2 synthesis would affect

auditory learning by impairing discrimination ability. Systemic treatments did not markedly affect learning rates or post-learning performance, but reduced motivation to engage in the learning task. By contrast, we found that blockade of E2 synthesis in the NCM does not affect post-learning performance but markedly reduces learning rates for new sound-outcome pairs.

In vertebrates, effects of neuro-E2 on spatial memory formation had been previously demonstrated in hippocampus [Bailey and Saldanha, 2015; Luine, 2016; Tuscher et al., 2016; Gervais et al., 2018; Paletta et al., 2018]. Our work provides evidence that in sensory pallial cortex [Wang et al., 2010; Jarvis et al., 2013; Briscoe et al., 2018], local E2 synthesis modulates sensory associative memory formation.

2.3 Material and methods

2.3.1 Animals

Birds came from the University of Massachusetts Amherst colony (14:10 hour light-dark cycle). Birds were not actively breeding (single-sex cages). A total of nine males successfully completed all training and testing sessions and were employed in two pharmacology experiments. Five of these animals were used for systemic administration experiments and five were cannulated (including one that had previously participated in the systemic treatment experiment). All procedures were in accordance with the Institutional Animal Care and Use Committee at the University of Massachusetts Amherst.

2.3.2 Behavioral apparatus

. Behavioral automation code was custom-made in Python for control of Raspberry Pi computers (Raspberry Pi foundation). The script was designed to run daily

and automatically without the need for constant human input or monitoring. Detailed instructions for assembly and methodology are part of a separate methods study (Macedo-Lima and Remage-Healey, in preparation).

A male and female were first housed in the same cage inside a sound attenuation chamber (Eckel Industries) for 1-2 weeks, then put in adjacent cages separated by a sheet of opaque polarized glass. The male's cage contained an infrared beam break sensor mounted on a semi-opaque acrylic rectangle behind which a red LED was positioned.

Subsequently, training sessions took place 7 days/week at 2-3 hours after lights-on. Training stages were modified from Gess et al. [2011]: 1) introduction to the polarized glass mechanism, 2) training to operate the infrared switch, and finally 3) the GO/NO-GO protocol. These training steps are described in detail in the supplementary methods.

2.3.3 GO/NO-GO procedure

The GO/NO-GO procedure is summarized in Figure 2.1a. Birds initiated all trials, triggering the infrared beam. Immediately, a ~65 dB tone (see Stimuli section) was played from the speaker pseudorandomly associated with a contingency (GO or NO-GO; detailed below). If the bird pecked again within a two second interval, it would receive a consequence (reward or punishment). If a GO tone had been played, the glass would turn transparent for 6 seconds, resulting in a HIT (i.e., period of visual engagement with the adjacent social partner through the transparent barrier); if a NO-GO tone had been played, a loud (~75 dB) 2-second burst of white noise would play (punishment) and a 16 second inactivation (time-out) period would follow, resulting in a FALSE ALARM. If the

bird did not respond within 2 seconds a 6-second inactivation period would follow resulting in a MISS (GO trial) or CORRECT REJECTION (NO-GO trial).

Initial training had three stages with 90, 75 and 50% GO-trial rates. In order to continue to the next stage, the bird was required to respond (HITS + FALSE ALARMS) to more than half the trials initiated. Our rationale for doing this was to keep a high reward rate until birds "understood" they had to peck twice to get rewarded. Correctness was not evaluated until GO-trial rate was lowered to 50% because it would be confounded by the higher GO ratio.

When 50% reward chance stage was reached, correct responses were monitored daily by the formula: $\%\ \text{Correct} = \frac{\#\text{HITS} + \#\text{REJECTIONS}}{\#\text{TRIALS}}$. The learning threshold we employed was: 70% Correct on two consecutive days (modified from Gess et al. [2011]). Birds needed to reach criterion with two successive tone sets (described below) in order to move to the testing stage.

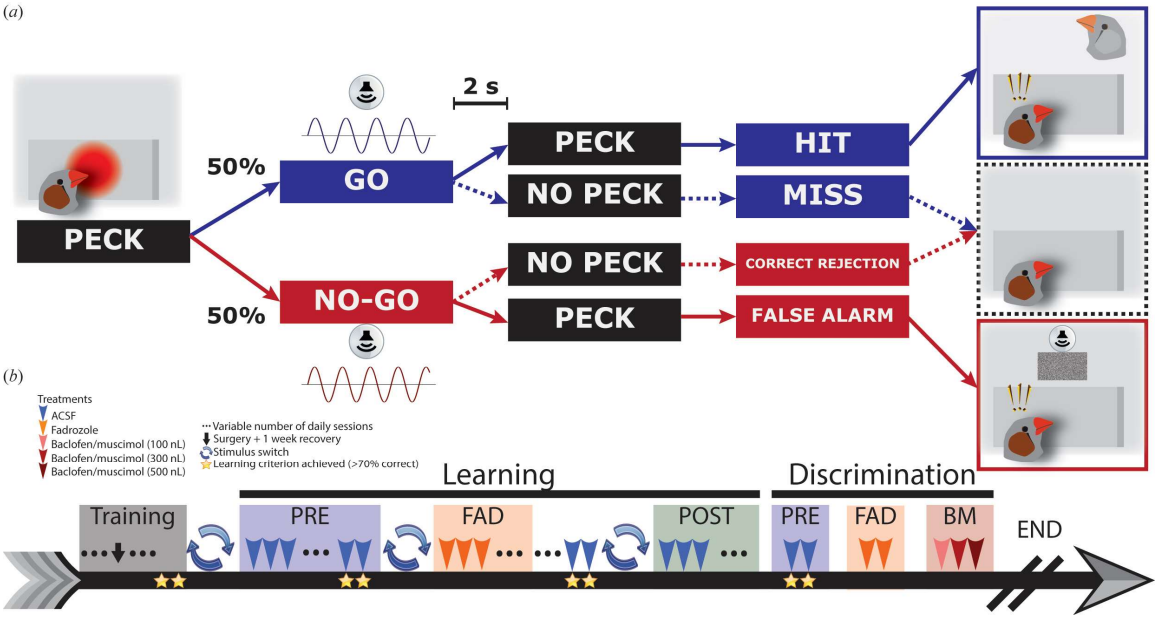

Figure 2.1: Auditory discrimination GO/NO-GO procedure and behavioral testing timeline.

(a) Auditory discrimination GO/NO-GO procedure. Birds initiate all trials. Solid arrows indicate trajectories that yield consequences (reward or punishment); dotted arrows indicate trajectories that do not yield consequences. Possibilities after a GO-trial are indicated by blue arrows, and the ones after a NO-GO-trial by red arrows. The bird has 2 seconds to respond after the stimulus is played. Reward consists of the activation of the polarized glass and visual access to the other bird for 6 seconds, while punishment consists of a loud burst of white noise and the switch being inoperant for 16 seconds. Lack of response to stimuli does not yield consequences. (b) Behavioral testing timeline. Go/No-go testing happened daily for 4 hours. Once birds reached learning criterion (>70% correct on two consecutive days) during training, treatments and the Learning stage started. Sound stimuli were switched (absolute and relative frequencies; see Methods) when birds reached criterion, except for during the Discrimination stage when learned tones were maintained. Shaded boxes represent the treatment stages in Figures 2.2, 2.5 (Learning), 2.3 and 2.6 (Discrimination).

2.3.4 Stimuli and testing stages

NCM neurons respond to song, white noise and pure tones, and show adaptation to those in the awake state, consistent with active memory formation [Chew et al., 1996]. In view of this, we developed a within-subject protocol well-suited for pharmacological manipulations using pure tones, which enabled objective control over discrimination difficulty.

Pure tones (2 seconds in duration) were generated in Adobe Audition 2014 (Adobe). Fade-in/fade-out filters were applied to first and last 100 ms of the tones. Digital sound level was normalized to -25 dB and amplified to ~65 dB in the behavioral booths. A two-second white noise burst was generated in the same software, but digital sound level was set to -5 dB which, after the same amplification factor used for tones, resulted in a sound pressure level of ~75 dB in the behavioral booths.

The range of frequencies (2-4 kHz) was selected based on the zebra finch audibility curve [Okanoya and Dooling, 1987] and natural vocalization range [Elie and Theunissen, 2016].The 200 Hz discrimination interval was intended to balance the facility of discrimination and of association learning. Importantly, maintaining this

frequency gap across treatment conditions ensured that discrimination difficulty would be consistent, facilitating comparison across treatments.

For GO/NO-GO training and testing, 5 pairs of pure tones separated by 200 Hz were randomly ordered (random.org). For the first training round with the first tone pair, the association between lower or higher tone and either GO or NO-GO trial was also randomized (e.g. lower tone with GO trials). After reaching learning criterion, the contingencies switched to the next randomized (random.org) tone pair. Different absolute frequencies, and the association between tone frequency and trial type was the opposite from the prior "rule" (e.g. if GO trials were the lower they became the higher tone). This switch in both absolute and relative frequencies associated with trial type requires that animals switch from the previous association rule and yields a new training curve.

In cannulation experiments, birds underwent NCM cannulation surgery (detailed below) after reaching criterion with the first tone pair. After recovery from surgery, they were retrained with the first tone pair (50% reward rate) until reaching/maintaining criterion, then trained with a new tone pair. Finally, after reaching criterion with the latter, the Learning stage would begin (see below). GO/NO-GO trial ratio was always 50%.

Pharmacology experiments were divided in two stages: Learning and Discrimination (Figure 2.1b). For the Learning stage, when birds reached criterion, a new tone pair was used. This stage had three treatments: (1) PRE: birds were given vehicle. Treatment continued daily until birds reached learning criterion (2) FAD: birds were given the aromatase inhibitor fadrozole (FAD; detailed below); treatment was repeated

daily until birds reached criterion or initiated a similar number of trials as in PRE (range 5-15 days in our sample); at least 2 days of vehicle treatment were administered after FAD treatment to allow for drug clearance [Alward et al., 2016]; this treatment was repeated until criterion was reached or maintained for 2 days. (3) POST: vehicle was administered until criterion was reached.

For the Discrimination stage, which followed the Learning stage, the most recently learned tone pair was maintained to assess effects of treatments on post-learning performance. This stage unfolded over three phases: (1) PRE: the last two days (above learning criterion) of the POST treatment (vehicle) from the Learning stage were used as baseline. (2) FAD: FAD treatment was administered for 2 days to test for effects on performance independent of learning. In the cannulation experiments, one animal could only be treated for 1 day, because the cannula started to detach, and treatment had to be suspended. Following initial indications that FAD treatment was not impacting post-learning performance, we tested whether NCM function itself would affect it. Therefore, for 3 cannulated animals, (3) BM followed: baclofen/muscimol (GABA receptor A/B agonists) treatment was administered for 2 days to test for effects of NCM inhibition on post-learning performance.

We note that this paradigm does not precisely dissect post-learning performance into components such as auditory discrimination or memory retrieval. We label this stage as “Discrimination” for simplicity.

2.3.5 Cannulation surgery

Cannulas (Plastics One) were custom designed for bilateral NCM targeting. Guide, injection and dummy cannulas were 22, 28 and 28 G respectively. Guide cannulas

measured 4 mm below pedestal and dummy/injection cannulas projected 300 µm below the guide. Interbarrel distance was 1.5 mm.

After reaching learning criterion for the first time in the GO/NO-GO training stage, birds were implanted with guide and dummy cannulas. Dust caps were not used. Birds were food deprived for 30 minutes, anesthetized with isoflurane and placed in a stereotaxic apparatus. Cannula placement was at 1.1 mm anterior, ~0.7 bilateral and 1.5 ventral respective to the midsagittal sinus; head was tilted forward at a 50° angle. Skull around the cannula was covered with Metabond (C&B), and dental cement was applied to secure the cannula to the skull.

Birds were allowed to recover for 1 week after surgery in their behavioral cages adjacent to their partners' cage with the smart glass turned transparent. After 1 week, GO/NO-GO training resumed. The same previously learned tone pair was used to certify auditory regions were intact. After reaching/maintaining criterion, the second tone pair was used, and, after reaching criterion this time, GO/NO-GO Learning stage started.

To confirm cannula placement (Supplementary figure 2.6), the animal was transcardially perfused with cold 0.1 M phosphate buffered saline followed by 4% paraformaldehyde. Brain was postfixed in the same fixative overnight in 4 °C, cryoprotected in 30% sucrose and sectioned at 40 µm in a cryostat. Sections were mounted with Prolong Diamond (ThermoFisher). Images were taken at 10x magnification with a confocal microscope (Nikon A1 Spectral Detector Confocal) and stitched.

2.3.6 Drugs and treatment

In cannulation experiments, in PRE and POST conditions, birds were injected with artificial cerebrospinal fluid (vehicle; in mM: 199 NaCl, 26.2 $NaHCO_3$, 2.5 KCl, 1 NaH_2PO_4, 1.3 $MgSO_4$, 2.5 $CaCl_2$, 11 Glucose; pH 7.4); in the FAD condition, fadrozole (Novartis; 2 mM) was dissolved in ACSF the day before the first day of treatment and injected.

Concentrations of 100 μM have been established to modulate acute changes in neuronal activity when administered via retrodialysis [Remage-Healey et al., 2010] and similar intracerebral injections (~150 mM) of vorozole, another aromatase inhibitor, affect Japanese quail behavior [Seredynski et al., 2013]. Importantly, FAD targets aromatase with high specificity in rat [Browne et al., 1991] and zebra finch tissues [Wade et al., 1994].

Our rationale for increasing the concentration of previously published results using retrodialysis [Remage-Healey et al., 2010] is the following. A key feature of retrodialysis drug delivery is the ability to maintain concentrations throughout the duration of the infusion. Most likely, the same cannot be achieved with a single, acute (bolus) injection. Maximum concentration would only be present at the beginning right after injection and would decay due to clearance and diffusion. Furthermore, since our behavioral testing occurred over 4 hours, we were unsure about how long our single injection would remain in bioactive concentrations. Therefore, we tried to overcome the unpredictability about tissue concentration and bioactivity duration in our treatments by increasing the dose previously used for retrodialysis 20-fold.

Treatments were administered via bilateral cannulas (Plastics One; see supplementary methods), daily, 10 minutes before testing. Bilateral dummy cannulas

were removed, and injection cannulas were inserted (28 G). Animals were handled for less than 2 minutes. Injection cannulas were coupled to tubing and to two 15 µL Hamilton syringes mounted on a syringe pump (Harvard Apparatus PHD2000). A 500 nL volume was injected over one minute for all conditions.

Following initial indications that FAD treatment was not impacting post-learning performance, we tested whether NCM function itself would affect it. Therefore, In a subset of animals (n=3), a 1 mM baclofen-0.1mM muscimol (BM; GABA receptor A/B agonists) solution in ACSF was injected to test for effects of NCM inhibition on post-learning performance and for potential drug leakage into primary auditory regions (field L), which could potentially affect sound perception. 100, 200 and 500 nL volumes were administered over 3 successive days. The same cocktail was verified efficacious for silencing auditory forebrain neurons in electrophysiological recordings in anaesthetized birds (Supplementary material).

In addition to central treatments, systemic treatments were performed to test whether our task was sensitive to other aromatase-containing nuclei [Saldanha et al., 2000], many of which known to be involved in the social behavior network, such as the medial amygdala, midbrain and medial preoptic area [Newman, 1999].

For the systemic treatment experiments, treatments were administered by mouth with a pipette 1 hour before testing. In the PRE and POST conditions, birds were given vehicle (0.9% saline in distilled water). In the FAD condition, fadrozole (30 µg in 30 µL in vehicle; dose of ~2 µg/g body weight) was given. Animals were handled for less than a minute. Similar systemic doses were shown to inhibit aromatase and impact behavior in

zebra finches [Wade et al., 1994; Rensel et al., 2013]. Following the treatment, a 1-hour timer was set for the start of the behavioral assay.

2.3.7 Statistics and software

Behavioral data were analyzed through custom-made Python and R scripts. The code supporting this article was uploaded to a public repository (github.com/HealeyLab).

For single-factor comparisons, the normality of the data was assessed by Shapiro-Wilk tests. When test results indicated violation of normality, data were analyzed by non-parametric tests (Wilcoxon or Friedman tests). Alternatively, parametric tests (one and two-sample t-tests and repeated measures ANOVAs) were used. Effect sizes were estimated by Cohen's d (d; for t and Tukey tests), r-statistic (r; for Wilcoxon tests), η^2 (for ANOVAs) and the Kendall's W (KW; for Friedman tests).

Zebra finches exhibit on average differential learning curves and strategies towards GO vs. NO-GO trials in a food reward paradigm [Anand and Nealen, 2019]. Therefore, in addition to % Correct, which combines both trial types, we computed hit (HR) and rejection rates (RR) with the formulas: $\mathrm{HR}=100\times\frac{\#\mathrm{HITS}}{\#\mathrm{GO\ TRIALS}}$; $\mathrm{RR}=100\times\frac{\#\mathrm{REJECTIONS}}{\#\mathrm{NO\text{-}GO\ TRIALS}}$. Whole-day performances (HR, RR and % Correct) were compared between the first and the last day of each treatment in the Learning stage. To control for length of each treatment, the last day was capped at the earliest day each subject reached criterion (above 70% Correct on two consecutive days) across PRE and FAD treatments (e.g. if subject reached criterion on day 4 during PRE, but on day 8 during FAD, the first day of FAD was compared with day 4 of FAD). Data were analyzed by 2-way repeated measures ANOVA with Treatment and Day as factors.

Because whole-day performance analyses do not consider individual variability in the number of trials, performance was also evaluated by logistic regression [Cox, 1958]. For each treatment condition, raw trial-by-trial performances were concatenated and binarized, i.e. correct responses were scored as 1; incorrect responses as 0. Because birds initiated all trials, to correct for the number of trials across treatments the maximum number of trials included for analysis was capped at the minimum number of trials performed by each bird across the PRE and FAD drug treatments (e.g. if a bird performed 350 trials during PRE, 200 during FAD and 250 during POST, only the first 200 trials of each treatment were included). Then, a logistic regression curve was fit for each treatment and their odds-ratio, area-under-curve (AUC) and intercept were compared. Learning rates before and after cannulation surgery (presurgery vs postsurgery; no pharmacological treatment) were analyzed in a similar way, as was post-learning performance (PRE vs FAD in the Discrimination stage). A higher odds-ratio can be interpreted as a faster rate of change towards predominantly correct responses. A higher AUC can be interpreted as a predominance of correct responses. A lower intercept can be interpreted as an initial deficit in performance. We analyzed total trials (Hit=1, Rejection=1, Miss=0, False-alarm=0), only GO (Hit=1, Miss=0) and only NO-GO trials (Rejection=1, False-alarm=0) separately.

At the beginning of the first day after a change in tones, birds need to extinguish the previous association in order to learn the new one, a process often called reversal learning. To infer reversal learning capacity, we compared performances at the beginning of the first day of each treatment to performances at the end of the previous day. We restricted performance measures to the first 50 trials of each treatment's first day and

compared those with the last 50 trials of the previous day, which always consisted of vehicle treatment (or no treatment for PRE). Data were analyzed by 2-way repeated measures ANOVA with Treatment and Day as factors.

We quantified response bias according to Macmillan and Creelman [1990]: $c = -0.5 \times [Z(HR) + Z(FAR)]$, where HR is the hit rate, FAR is the false alarm rate as described above and Z is the inverse cumulative distribution value at those probabilities. Positive values suggest a bias towards not responding to tones, negative values suggest a bias towards responding to tones, and zero suggests the lack of bias. We applied this formula to the whole-day trial analyses (first vs last day of treatments) and to the reversal learning data (50 trials of the first day of each treatment).

To control for the number of trials on response bias, we performed a similar analysis as described above for logistic regressions. To create a response bias regression, we scored Hits and False Alarms as 1, and Misses and Rejections as 0 and performed logistic regressions. Higher values on these curves indicate higher probability of indiscriminate response.

Because the entire task was self-initiated, each animal's motivation to engage in the task was inferred by the daily number of trials. Because the number of days during the Learning stage varied across animals, each animal's daily trial numbers were averaged for statistical testing.

Statistical analyses of pharmacological data do not include washout periods (POST) because of the variable duration of the FAD treatment among animals, which could result in unpredictable differences in clearance or long-term effects of aromatase inhibition.

Statistical significance was accepted when $p < 0.05$. All statistical results are included in tables (Tables 2.1-2.2, supplementary tables 2.3-2.4).

2.4 Results

2.4.1 Aromatase inhibition in NCM impairs learning of new sounds

Each treatment's first vs last day (whole-day) performance is shown in Figure 2.2a-c (statistical results in Table 2.1). Analyzing all trials combined (GO + NO-GO), FAD treatment reduced % Correct (Figure 2.2a). Analyzing GO trials only, there was a trend for an interaction between treatment and day of training, underlay by a mean decrease in hit rates on day 1 due to FAD (Figure 2.2b). In NO-GO trials, there was a trend for a decrease in rejection rates (Figure 2.2c). Therefore, these results suggest that daily FAD treatments in NCM reduce the rate auditory learning in whole-day performances, possibly affecting GO trials on the first day, and NO-GO trials throughout the treatment.

Because whole-day performance analyses do not encompass individual variability in the number of trials, we looked at the same data using logistic regressions on the raw trial-by-trial performance (see Methods). In the all-trial learning curves (GO + NO-GOS), FAD reduced the area under the curve (AUC) without affecting the odds-ratio or the intercept (Figure 2.2d). GO trial curves did not significantly change with treatment, but there was a trend for a lower intercept due to FAD (Figure 2.2e). The detriment in learning was attributed to learning deficits in NO-GO trials specifically, in which FAD reduced the AUC, but not the odds-ratio or the intercept (Figure 2.2f). These results show that daily FAD treatments in NCM impair acquisition of sound-outcome associations, particularly in NO-GO trials.

Because of the trends observed on day 1 of the Learning stage during FAD, particularly in GO trials, we restricted our analyses to the beginning of treatments. In this stage, animals must extinguish the previous sound-outcome association, i.e. reversal learning. Reversal learning capacity was inferred by comparing the first 50 trials of each treatment vs the 50 last trials of the day before (i.e., prior tone-pair regime, see Methods). FAD reduced reversal learning of overall trial % Correct on the first day (Figure 2.2g) and reduced hit rates in the GO trials (Figure 2.2h), but not NO-GO trial rejection rate (Figure 2.2i). Since reversal learning reflects extinction of the previous association, the results on reversal learning impairments in GO trials indicate that FAD in NCM affects the extinction of the previous 'rule' for relative frequency (e.g. higher tone associated with NOGO) that became newly associated with GO trials.

Because we observed different trends in hit and rejection rates due to treatment (FAD reduced hit rates only initially while reducing rejection rates throughout), we analyzed response bias (i.e., indiscriminate responses to both stimuli). There was a trend for an interaction between day and treatment on whole-day response bias, underlay by a more negative (i.e. stronger) response bias on the last day due to FAD (Supplementary figure 2.8a). However, after controlling for the number of trials in the logistic regressions, this effect was not significant (Supplementary figure 2.8b). Finally, in the reversal learning analyses, response bias did not significantly change (Supplementary figure 2.8c). These results suggest that the reduction in rejection rates due to FAD did not systematically result in an increase in response bias (indiscriminate responses to GOs and NO-GOs).

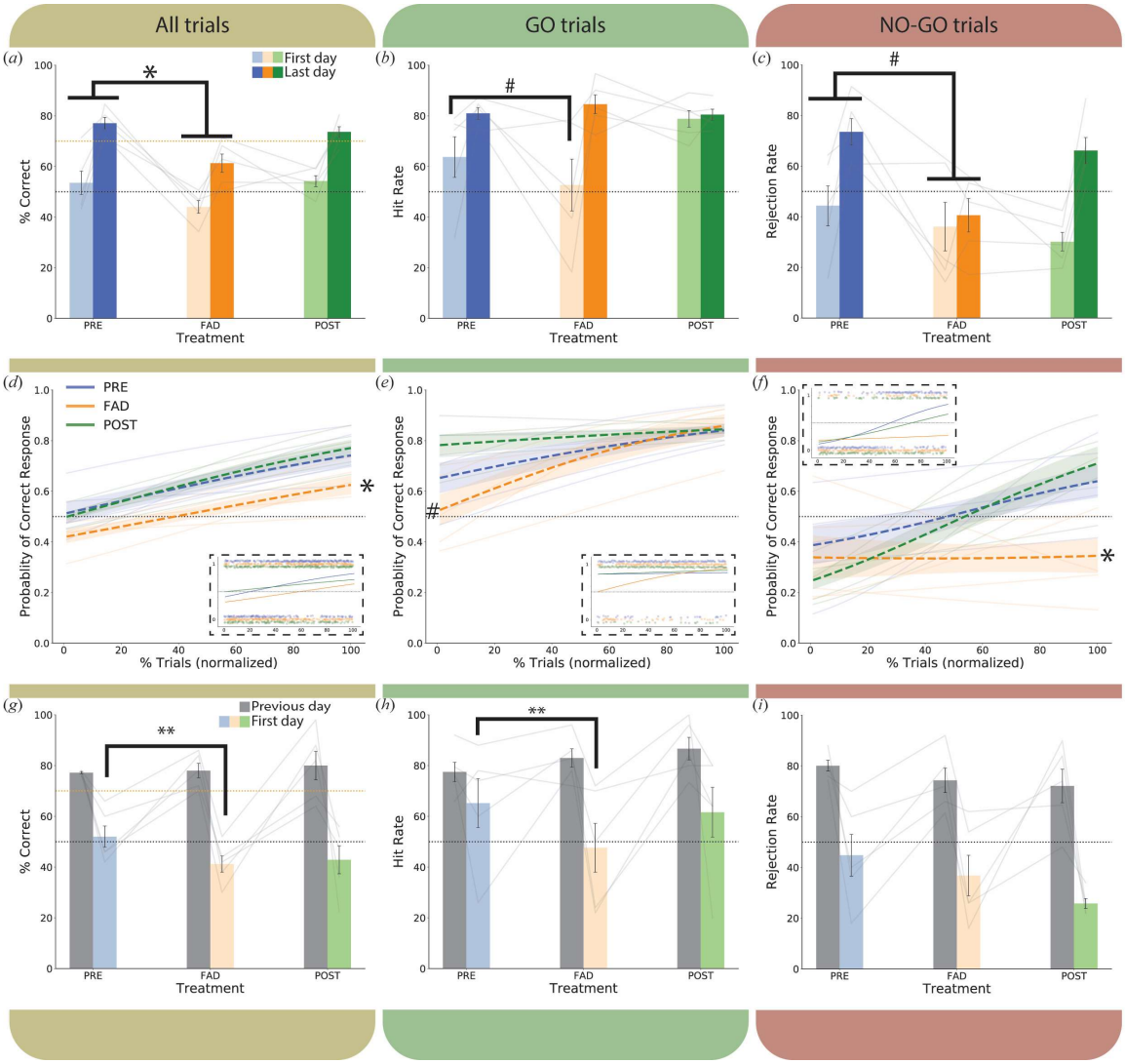

Figure 2.2: NCM fadrozole (FAD) injections impair performance in an auditory learning task.

Performance was analyzed using combined (left), GO (middle) or NO-GO trials (right). (a-c) First vs last day whole-day performances. FAD decreased combined trial performance (a). There was a trend for a decrease in hit rates on the first day (b) and a trend for an overall reduction in rejection rates (c). (d-f) Logistic regressions. FAD decreased the area under the curve of the combined-trial performance (d). There was a trend for a reduction in the intercept of the GO trials (e). FAD reduced the area under the curve of the NO-GO trial performance (f). Insets in d-f are all from one representative subject. Dots around 0 and 1 represent incorrect and correct responses respectively, which are the data used for the logistic regressions. (g-i) Reversal learning (first 50 trials of day one versus last 50 of previous day). FAD impaired performance on the first day of combined trials (g) and of GO trials (h), but not NO-GO trials (i). *$p < 0.05$; **$p < 0.01$; $^{\#}p < 0.1$.

Table 2.1: Intra-NCM fadrozole experiments

Figure number	Testing stage	Descriptive	Test	n	Factor	Results and effect size	Post-hoc
2.2a	Learning	Whole-day % Correct	2-way RM-ANOVA (PRE vs FAD)	5	Treatment	**$F(1,16) = 11.684$, $p = 0.016$, $\eta^2 = 0.252$**	-
					Day	**$F(1,16) = 25.194$, $p = 0.002$, $\eta^2 = 0.544$**	-
					Interaction	$F(1,16) = 3.519$, $p = 0.138$, $\eta^2 = 0.076$	-
2.2b	Learning	Whole-day Hit rate	2-way RM-ANOVA (PRE vs FAD)	5	Treatment	$F(1,16) = 0.977$, $p = 0.352$, $\eta^2 = 0.049$	-
					Day	$F(1,16) = 6.190$, $p = 0.068$, $\eta^2 = 0.309$	-
					Interaction	$F(1,16) = 3.735$, $p = 0.089$, $\eta^2 = 0.187$	-
2.2c	Learning	Whole-day Rejection rate	2-way RM-ANOVA (PRE vs FAD)	5	Treatment	$F(1,16) = 7.550$, $p = 0.051$, $\eta^2 = 0.262$	-
					Day	$F(1,16) = 6.556$, $p = 0.063$, $\eta^2 = 0.227$	-
					Interaction	$F(1,16) = 5.464$, $p = 0.080$, $\eta^2 = 0.189$	-
2.2d	Learning	Combined-trial logistic regression	Paired t-test (PRE-FAD)	5	Area-under-curve	**$t_4 = 2.787$, $p = 0.049$, $d = 0.737$**	-
			Paired t-test (PRE-FAD)	5	Odds-ratio	$t_4 = 1.115$, $p = 0.327$, $d = 0.263$	-
			Paired t-test (PRE-FAD)	5	Intercept	$t_4 = 2.065$, $p = 0.108$, $d = 0.721$	-
2.2e	Learning	GO-trial logistic regression	Paired t-test (PRE-FAD)	5	Area-under-curve	$t_4 = 0.910$, $p = 0.414$, $d = 0.248$	-
			Paired t-test (PRE-FAD)	5	Odds-ratio	$t_4 = -1.345$, $p = 0.250$, $d = -0.623$	-
			Paired t-test (PRE-FAD)	5	Intercept	$t_4 = 2.261$; $p = 0.087$, $d = 0.609$	-
2.2f	Learning	NO-GO-trial logistic regression	Paired t-test (PRE-FAD)	5	Area-under-curve	**$t_4 = 2.930$, $p = 0.043$, $d = 0.742$**	-
			Paired t-test (PRE-FAD)	5	Odds-ratio	$t_4 = 1.540$, $p = 0.198$, $d = 0.749$	-
			Paired t-test (PRE-FAD)	5	Intercept	$t_4 = 0.360$, $p = 0.737$, $d = 0.268$	-
2.2g	Learning	Reversal % Correct	2-way RM-ANOVA (PRE vs FAD)	5	Treatment	$F(1,16) = 4.545$, $p = 0.100$, $\eta^2 = 0.046$	-
					Day	**$F(1,16) = 71.178$, $p = 0.001$, $\eta^2 = 0.726$**	-
					Interaction	**$F(1,16) = 15.721$, $p = 0.017$, $\eta^2 = 0.160$**	Previous day: $t_{6.7} = -0.289$, $p = 0.781$, $d = -0.158$ **First day: $t_{6.7} = 3.907$, $p = 0.006$, $d = 1.16$**
2.2h	Learning	Reversal Hit rate	2-way RM-ANOVA (PRE vs FAD)	5	Treatment	$F(1,16) = 4.270$, $p = 0.073$, $\eta^2 = 0.125$	-
					Day	$F(1,16) = 5.760$, $p = 0.074$, $\eta^2 = 0.169$	-
					Interaction	**$F(1,16) = 15.460$, $p = 0.004$, $\eta^2 = 0.454$**	Previous day: $t_{6.7} = -1.319$, $p = 0.224$, $d = -0.592$ **First day: $t_8 = 4.241$, $p = 0.003$, $d = 0.731$**
2.2i	Learning	Reversal Rejection rate	2-way RM-ANOVA (PRE vs FAD)	5	Treatment	$F(1,16) = 1.218$, $p = 0.291$, $\eta^2 = 0.25$	-
					Day	**$F(1,16) = 34.02$, $p < 0.001$, $\eta^2 = 0.692$**	-
					Interaction	$F(1,16) = 0.032$, $p = 0.862$, $\eta^2 = 0.001$	-
2.3b	Discrimination	Combined-trial logistic regression	Paired t-test (PRE-FAD)	5	Area-under-curve	$t_4 = -1.787$, $p = 0.148$, $d = -0.483$	-
			Wilcoxon test (PRE-FAD)	5	Odds-ratio	$V = 11$, $p = 0.438$, $r = 0.422$	-
			Wilcoxon test (PRE-FAD)	5	Intercept	$V = 4$; $p = 0.438$, $r = -0.422$	-
2.3c	Discrimination	Combined-trial logistic regression	RM-ANOVA (PRE vs BM100nL vs BM200nL vs BM500nL)	3	Area-under-curve	$F(3,6) = 0.507$, $p = 0.692$, $\eta^2 = 0.188$	-
			RM-ANOVA (PRE vs BM100nL vs BM200nL vs BM500nL)	3	Odds-ratio	$F_{3,6} = 0.04$, $p = 0.99$, $\eta^2 = 0.016$	-
			Friedman test (PRE vs BM100nL vs BM200nL vs BM500nL)	3	Intercept	$\chi^2 = 0.403$, $p = 0.615$, $KW = 0.120$	-

2.4.2 NCM aromatase inhibition does not affect performance with previously-learned sounds

To test whether the FAD effects on learning were due to impairments in post-learning performance, after animals eventually reached learning criterion in the POST condition (i.e. daily ACSF injections), FAD was given again, but tone-contingency pairs were not changed. Whole-day performances are shown in Figure 2.3a. FAD treatment did not affect whole-day performances (RM-ANOVA; PRE vs FAD; all $p > 0.293$).

Similarly, performance logistic curves were not different between PRE and FAD (Figure 2.3b). Further, hit and rejection rates (Figure 2.3b insets), as well as response bias (not shown), were all unchanged (all $p > 0.110$). These results indicate that FAD in NCM does not affect performance with previously-learned sounds.

Since neuroestrogen synthesis inhibition had no effect on post-learning performance we were curious about the extent that NCM activity itself is necessary for this ability. To address this possibility, we silenced NCM neuronal activity pharmacologically with baclofen/muscimol (BM). In 3 animals BM was given following the FAD treatment above in 3 different injection volumes (100, 200 and 500 nL). Whole-day performances are shown in Figure 2.3a. BM treatment did not affect whole-day performances (RM-ANOVA; PRE vs BM; all $p > 0.175$).

Similarly, performance logistic curves were not different among PRE and BM treatments (Figure 2.3c). Further, hit and rejection rates (Figure 2.3c insets), as well as response bias (not shown), were all unchanged (all $p > 0.102$). Therefore, NCM does not appear to be necessary for the performance with previously-learned tones.

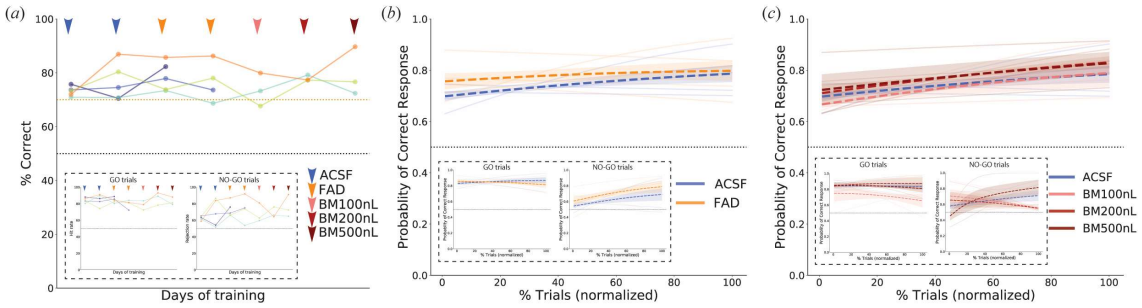

Figure 2.3: Discrimination of previously learned tones is not impaired by NCM fadrozole (FAD) and baclofen/muscimol (BM) injections.
BM was administered in 3 increasing volumes: 100, 200 and 500 nL. Neither drug affected discrimination of previously learned tones. Daily performance (different colors are different animals) is shown in (a); logistic regressions in (b, c). Insets in (a-c) show GO and NO-GO trial performances.

2.4.3 NCM aromatase inhibition did not affect motivation

Daily trial initiation counts are shown in Supplementary Figure 2.5. NCM FAD treatment did not affect mean daily trial initiation, indicating that motivation to engage in the task was unaffected by aromatase inhibition within NCM.

2.4.4 NCM cannulation damage transiently reduced auditory learning

After undergoing at least one round of learning in the GO/NO-GO task with 50% GO-trial rate, birds (n=5) were implanted with bilateral cannulas targeting NCM (Supplementary figure 2.6). One week after surgery GO/NO-GO training resumed and the previous (before surgery) tone pair was employed to certify auditory function was intact. After reaching/maintaining criterion (range 2-6 days in our sample), a new tone pair was used to verify learning capacity. Comparison between whole-day performances before and after surgery during new tone learning is shown in Supplementary figure 2.7 (statistical results in supplementary Table 2.3). Neither % Correct (Supplementary figure 2.7a) or hit rate (Supplementary figure 2.7b) differed between pre- and post-surgery. However, there was a decrease in rejection rate (RR) in the post-surgery (Supplementary

figure 2.7c). Therefore, NCM cannulation surgery affected whole-day performance by decreasing RR, resulting in a more indiscriminate response to NO-GOs.

Whole-day performance analyses do not consider variability in the number of trials, while logistic regressions do take this into account. Combined trial (GO + NO-GO) -logistic learning curves were not different when comparing pre vs. post-surgery (Supplementary figure 2.7d), and neither were GO learning curves (Supplementary figure 2.7e), but surgery reduced the AUC of the NO-GO learning curve (Supplementary figure 2.7f).

Regarding response bias, there was no effect in the whole-day analyses (Supplementary Figure 2.4a). However, when correcting for the number of trials, there was an increase in the AUC of the response bias logistic curve (Supplementary figure 2.9b). Therefore, NCM cannulation, after 1 week-recovery, impaired learning of NO-GO trials, without significantly affecting GO trials and the overall learning, resulting in an increase in response bias (when controlling for the number of trials performed). These results are consistent with NCM's role in auditory learning. Importantly, this effect returned to before-surgery levels by the time drugs were delivered > 2 weeks after surgery (compare to PRE values in Figure 2.2).

Because for some birds the pre-surgery tones were their first exposure to tone learning, we could not analyze reversal learning.

We observed that whole-day performance generally returned to before-surgery levels ~2-3 weeks later (compare supplementary figure 2.7 with main Figure 2.2). Specifically, % Correct did not differ, hit rates increased and rejection rates did not change (supplementary Table 2.3). Furthermore, there was a trend for a more negative

response bias (compare supplementary figures 2.8 and 2.9). When adjusting for the total number of trials (logistic regressions), no differences were found in either all-trial, GO, NO-GO or response bias curves' parameters (not shown; all $p > 0.095$).

2.4.5 Systemic aromatase inhibition did not affect post-learning performance but reduced motivation

Systemic FAD (orally administered) before the task reduced the combined-trial whole-day learning (Figure 2.4a; statistical results in Table 2.2). While hit rates are not affected (Figure 2.4b), there was a trend for a reduction in rejection rates (Figure 2.4c). However, when correcting for the number of trials, FAD did not significantly affect the combined-trial learning curves (Figure 2.4d), the GO learning curves (Figure 2.4e) or the NO-GO learning curves (Figure 2.4f). These results indicate that daily oral FAD treatment before the task might impact daily learning, but this effect is not observed when correcting for the number of trials performed.

In the reversal learning analyses, FAD did not impact all-trial reversal learning (Figure 2.4g), but there was a trend for an increase in hit rates (Figure 2.4h). There was also a trend for a decrease in rejection rates on the first day of FAD (Figure 2.4i). Therefore, oral FAD treatment did not systematically impact reversal learning.

Because we observed trends in both hit and rejection rates due to treatment changing in different patterns, we analyzed response bias. In whole-day analyses there was no effect of FAD on response bias (Supplementary figure 2.11a; statistical results in supplementary Table 2.4). Similarly, in the logistic regressions, FAD did not change most of the curve parameters, except for inducing a trend for a higher intercept under FAD (Supplementary figure 2.11b). Finally, in the reversal learning analyses, FAD produced a

trend for an increase in response bias on the first day (Supplementary figure 2.11c). Therefore, systemic FAD treatment did not systematically impact response bias.

Daily trial initiation counts are shown in Supplementary figure 2.12; systemic FAD treatment reduced mean daily trial initiation, consistent with a reduction in motivation with systemic aromatase inhibition.

After animals learned in the POST condition, FAD was given again, but tones were not changed. Whole-day performances are shown in Figure 2.5a. FAD did not affect whole-day performance (% Correct, HR and RR all $p > 0.205$). Likewise, combined-trial performance logistic curves were not different between PRE and FAD (Figure 2.5b) nor were GO and NO-GO curves (insets; all $p > 0.280$), indicating that the systemic FAD treatments did not impact the performance with previously-learned stimuli.

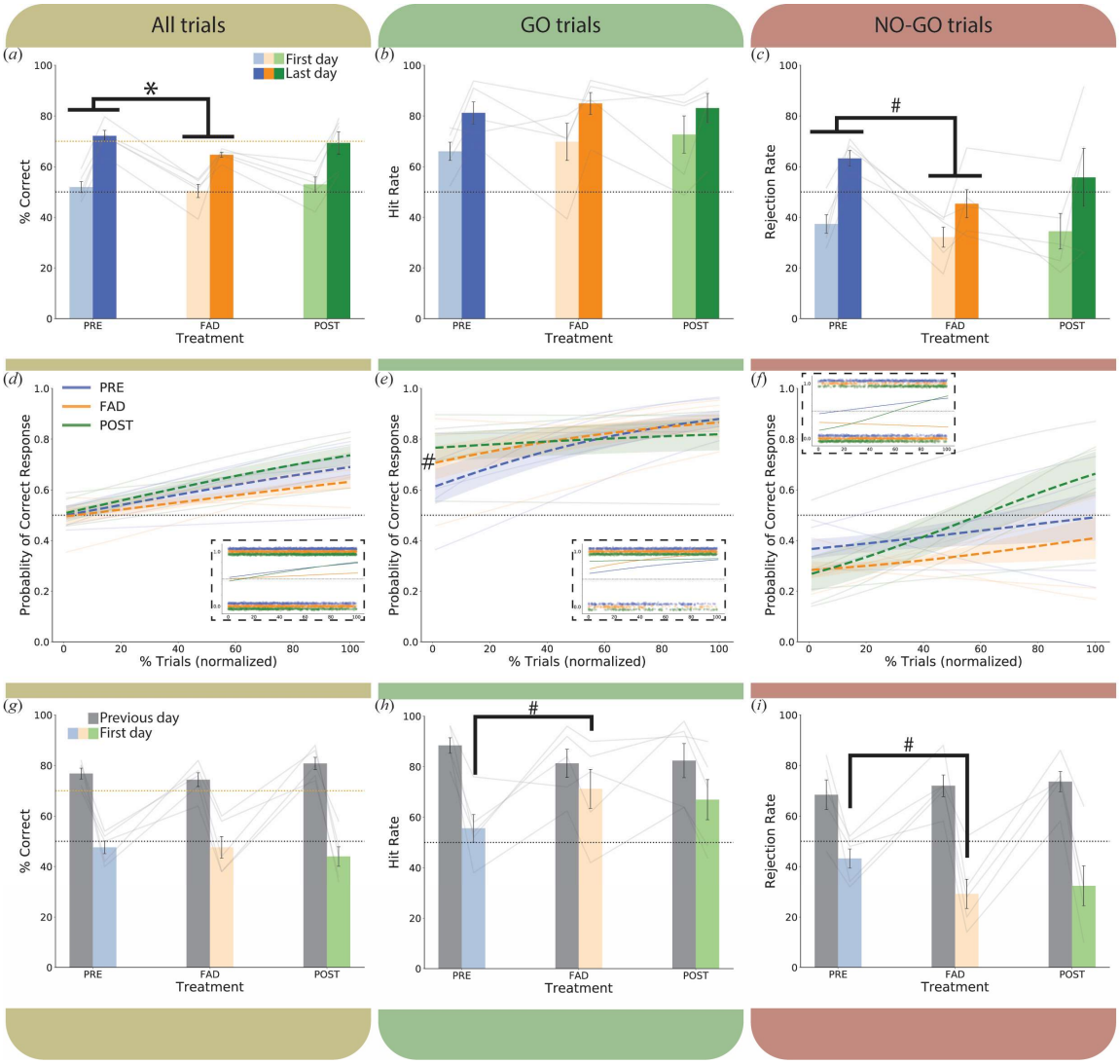

Figure 2.4: Oral fadrozole (FAD) administrations before auditory learning task. Performance was analyzed using combined (left), GO (middle) or NO-GO trials (right). (a-c) First vs last day whole-day performances. FAD decreased combined-trial whole-day performance (a). Hit rates were unchanged, but (b) there was a trend for an overall reduction in rejection rates (c). (d-f) Logistic regressions. FAD did not affect any learning curves parameters, except for producing a trend for a higher intercept in the GO trials due to FAD. Insets in d-f are all from one representative subject. Dots around 0 and 1 represent incorrect and correct responses respectively, which are the data used for the logistic regressions. (g-i) Reversal learning (first 50 trials of day one vs last 50 of previous day). FAD did not affect combined trials (g), but there were trends for an increase in hit rates on the first day (h) and for a decrease in rejection rate on the first day (i). *$p < 0.05$; $^{\#}p < 0.1$.

Table 2.2: Systemic fadrozole experiments

Figure number	Testing stage	Descriptive	Test	n	Factor	Results and effect size	Post-hoc
2.4a	Learning	Whole-day % Correct	2-way RM-ANOVA (PRE vs FAD)	5	Treatment	**$F(1,16) = 5.927$, $p = 0.031$, $\eta^2 = 0.054$**	-
					Day	**$F(1,16) = 87.519$, $p < 0.001$, $\eta^2 = 0.801$**	-
					Interaction	$F(1,16) = 2.567$, $p = 0.135$, $\eta^2 = 0.024$	-
2.4b	Learning	Whole-day Hit rate	2-way RM-ANOVA (PRE vs FAD)	5	Treatment	$F(1,16) = 1.366$, $p = 0.307$, $\eta^2 = 0.054$	-
					Day	**$F(1,16) = 13.958$, $p = 0.020$, $\eta^2 = 0.549$**	-
					Interaction	$F(1,16) = 0$, $p = 0.996$, $\eta^2 = 0$	-
2.4c	Learning	Whole-day Rejection rate	2-way RM-ANOVA (PRE vs FAD)	5	Treatment	$F(1,16) = 4.881$, $p = 0.058$, $\eta^2 = 0.112$	-
					Day	**$F(1,16) = 23.475$, $p = 0.001$, $\eta^2 = 0.538$**	-
					Interaction	$F(1,16) = 2.476$, $p = 0.154$, $\eta^2 = 0.057$	-
2.4d	Learning	Combined-trial logistic regression	Paired t-test (PRE-FAD)	5	Area-under-curve	$t_4 = -1.401$, $p = 0.234$, $d = 0.395$	-
			Paired t-test (PRE-FAD)	5	Odds-ratio	$t_4 = 0.771$, $p = 0.484$, $d = 0.354$	-
			Paired t-test (PRE-FAD)	5	Intercept	$t_4 = 0.149$, $p = 0.889$, $d = 0.108$	-
2.4e	Learning	GO-trial logistic regression	Wilcoxon test (PRE-FAD)	5	Area-under-curve	$V = 3$, $p = 0.313$, $r = -0.541$	-
			Paired t-test (PRE-FAD)	5	Odds-ratio	$t_4 = 0.997$, $p = 0.375$, $d = 0.378$	-
			Paired t-test (PRE-FAD)	5	Intercept	$t_4 = -2.523$, $p = 0.065$, $d = -0.334$	-
2.4f	Learning	NO-GO-trial logistic regression	Paired t-test (PRE-FAD)	5	Area-under-curve	$t_4 = 1.250$, $p = 0.279$, $d = 0.480$	-
			Paired t-test (PRE-FAD)	5	Odds-ratio	$t_4 = 0.002$, $p = 0.999$, $d = 0.001$	-
			Paired t-test (PRE-FAD)	5	Intercept	$t_4 = 1.343$, $p = 0.250$, $d = 0.442$	-
2.4g	Learning	Reversal % Correct	2-way RM-ANOVA (PRE vs FAD)	5	Treatment	$F(1,16) = 0.109$, $p = 0.758$, $\eta^2 = 0.001$	-
					Day	**$F(1,16) = 87.110$, $p < 0.001$, $\eta^2 = 0.904$**	-
					Interaction	$F(1,16) = 0.286$, $p = 0.621$, $\eta^2 = 0.003$	-
2.4h	Learning	Reversal Hit rate	2-way RM-ANOVA (PRE vs FAD)	5	Treatment	$F(1,16) = 0.372$, $p = 0.575$, $\eta^2 = 0.008$	-
					Day	**$F(1,16) = 21.529$, $p = 0.010$, $\eta^2 = 0.472$**	-
					Interaction	**$F(1,16) = 17.413$, $p = 0.014$, $\eta^2 = 0.381$**	Previous day: $t_{5.19} = 0.949$, $p = -0.931$ First day: $t_{5.19} = -2.085$, $p = 0.089$, $d = 0.631$
2.4i	Learning	Reversal Rejection rate	2-way RM-ANOVA (PRE vs FAD)	5	Treatment	$F(1,16) = 0.642$, $p = 0.468$, $\eta^2 = 0.008$	-
					Day	**$F(1,16) = 62.779$, $p < 0.001$, $\eta^2 = 0.795$**	-
					Interaction	$F(1,16) = 4.206$, $p = 0.074$, $\eta^2 = 0.053$	-
2.5b	Discrimination	All-trial logistic regression	Wilcoxon test (PRE-FAD)	5	Area-under-curve	$V = 8$, $p = 1$, $r = 0.060$	-
			Paired t-test (PRE-FAD)	5	Odds-ratio	$t_4 = -0.727$, $p = 0.508$, $d = -0.281$	-
			Paired t-test (PRE-FAD)	5	Intercept	$t_4 = 1.004$; $p = 0.372$, $d = 0.236$	-

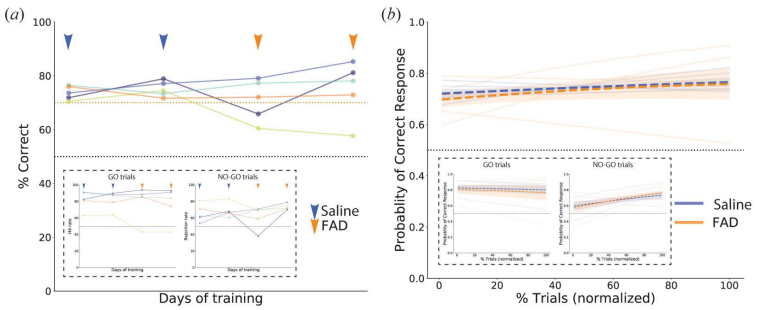

Figure 2.5: Discrimination of previously learned tones is not impaired by oral fadrozole.
Whole-day combined-trial performance is shown in (a); combined-trial logistic regressions in (b, c). Insets show GO- and NO-GO-trial performances.

2.5 Discussion

In this study we employ a novel behavioral task for the study of auditory learning with social reinforcement in zebra finches. Our main finding is that neuroestrogen synthesis is important for auditory learning, but not post-learning performance, in adult males. More specifically, our findings can be summarized as follows: inhibiting aromatase locally in the auditory association cortex (caudomedial nidopallium; NCM) is sufficient to impair learning of new sounds, but it does not impair performance after sounds are learned. We provide evidence that neuroestrogen production in a cortical region is important for sensory learning.

Our data indicate that NCM, and more specifically aromatase activity within NCM, plays a key role in the online process of associating sounds with behaviorally relevant consequences in adult songbirds, consistent with previous observations regarding NCM function [Jarvis et al., 1995; London and Clayton, 2008; Bell et al., 2015], and with our findings that NCM cannulations transiently (~1 week after surgery) impaired learning of new sounds (Supplementary figure 2.7). However, once the learning process is completed, NCM might no longer be required for active discrimination (see Figure 2.3). We note that our task does not allow for precisely dissecting post-learning performance

into its contributing features, i.e. discrimination and memory retrieval. Therefore, we propose that the adult NCM is acting in the encoding/consolidation process of auditory associations, rather than in their retrieval or in sound discrimination after learning. This is reminiscent of hippocampal function, which is required for encoding, consolidation and retrieval of early/detailed memories but not involved in the retrieval of sedimented memories [Wiltgen et al., 2010]. Interestingly, recent studies in the mammalian and avian hippocampus have also observed a role for estrogens in learning, both locally-produced and from the circulation [Bailey and Saldanha, 2015; Luine, 2016; Tuscher et al., 2016; Gervais et al., 2018; Paletta et al., 2018].

Blocking E2 production in the adult zebra finch NCM had been previously shown to impair phonotactic preference towards the bird's own song [Remage-Healey et al., 2010]. However, sound preference experiments do not provide a complete answer regarding the nature of the behavior, since they conflate auditory memory, discrimination, and preference. Our behavioral task provides the opportunity to dissect different components of auditory-dependent behaviors. Our results suggest that NCM itself and E2 production within NCM are likely involved in auditory memory formation processes, rather than post-learning discrimination/retrieval. We hypothesize that in the phonotaxis study mentioned above, blocking E2 production in NCM might have affected some aspect of auditory memory formation, e.g. spatial associations, or perhaps sound valence signaling.

Developmentally, auditory learning serves several important purposes in songbirds. Juveniles need to form auditory memories of their tutor song in order to develop their own song. After song crystallization, auditory learning is no longer required

for song production but continues to be relevant for environmental sound-consequence associations and individual recognition [D'Amelio et al., 2017; Elie and Theunissen, 2018]. Indeed, in adults, NCM lesions impair the phonotactic preference for tutor song over novel conspecific song [Gobes and Bolhuis, 2007]. Neuronal responses in awake restrained zebra finches show adaptation to sounds played repeatedly, which is an indication of active memory formation [Chew et al., 1996]. In spite of these features, data from one group have suggested NCM lesions do not affect juvenile song learning or adult auditory learning, but do impact recovery from a reinforcement-driven song plasticity paradigm [Canopoli et al., 2014; Canopoli et al., 2017]. These findings contrast with our present results (both from aromatase inhibition and from cannulation surgery damage) and previous findings implicating NCM in vocal learning [London and Clayton, 2008; Tsoi et al., 2014; Yanagihara and Yazaki-Sugiyama, 2016] and adult auditory learning [Jarvis et al., 1995; Bell et al., 2015]. The lack of comparable effects on vocal/auditory learning found in the lesion studies might have been due to NCM regeneration [see Fig 2 in Canopoli et al., 2014; our data] or some other unknown lesion-reactive plasticity mechanism.

E2 modulates neuronal activity in the NCM of male zebra finches as young as 25 days post-hatch, even before singing onset [Vahaba et al., 2017], but unilateral FAD infusions in NCM during tutoring do not impair song learning. This E2 production blockade during juvenile tutoring increased neuronal firing to tutor song playback when the same animals became adults [Vahaba et al., 2019]. During song playback, E2 production is increased in the adult NCM [Remage-Healey et al., 2008], but is reduced in the juvenile NCM followed by an immediate increase after song playback [Chao et al.,

2014]. Assuming that sound-outcome encoding is a general feature of NCM which extends to song-reinforcement learning in juveniles, our data indicate that E2 production in NCM is important during adult auditory learning, predicting an opposite result in the juvenile NCM during a similar auditory learning task. Interestingly, some areas directly implicated in juvenile song learning and production also contain aromatase fibers and/or neurons, such as HVC and HVC shelf [Ikeda et al., 2017]. Future research should address whether E2 production/signaling directly in these cortical areas support song learning in juveniles, and what other roles they are playing in adults, in which these areas mostly support song production.

To modulate goal-directed auditory learning, E2 could be acting in concert with and/or modulating reinforcement signals, such as midbrain dopamine (DA) release. In striatum and preoptic area of both birds and mammals, E2 and DA systems interact [Becker, 1990; Lammers et al., 1999; Balthazart et al., 2002; Tozzi et al., 2015] and DA and E2 can interact with the same receptors [Olesen and Auger, 2008; Tozzi et al., 2015]. In the mammalian auditory cortex, DA signaling regulates auditory learning [Schicknick et al., 2012] and DA is controlled by steroid hormones in zebra finch NCM [Matragrano et al., 2011; Rodríguez-Saltos et al., 2018]. We have recent evidence that the majority of aromatase-expressing neurons in NCM also express dopamine receptors (unpublished observations). Therefore, studying E2 and DA interactions in the auditory cortex could provide important network and cellular mechanisms for the behavioral phenomena observed here.

We show that systemic FAD reduces motivation to engage in a socially motivated task. Concordantly, in other birds, aromatase inhibition has been linked to reductions in

social behavior measures. In adult Japanese quail, systemic and whole-brain aromatase inhibition have been shown to reduce socio-sexual motivation, such as proximity to an opposite sex individual [Seredynski et al., 2013; de Bournonville et al., 2016], attributed to aromatase in the medial preoptic area in males [de Bournonville et al., 2019]. Furthermore, in male canaries, motivation to sing was reduced by systemic FAD [Alward et al., 2016]. We note that the dose of FAD used in our experiments was 15-fold lower and administered orally vs intraperitoneally comparing to Alward et al. [2016]. In our paradigm, higher oral doses might be needed to observe effects on learning, but those might produce a further reduction in motivation, which can obscure interpretation of learning data.

It is possible that the trends for increases in response bias due to FAD treatment reflect an impairment of impulse control. Interestingly, an involvement of aromatization/E2 in impulsive behavior has been suggested in humans [Smith et al., 2014] and rodents [Svensson, 2010; Bayless et al., 2013]. Future implementations of our behavioral task could help illuminate the comparative aspects of this proposed relationship between neuroestrogens and impulsive behavior in songbirds and other species.

One caveat is that our study was only performed in male songbirds. Data from our group have shown that the same dose of oral fadrozole used here did not impair immediate early gene EGR1 expression in the female NCM, but it did in males [Krentzel et al., 2019]. Furthermore, unlike in males, NCM electrophysiological responses in females were unaffected by modulation of G-protein coupled E2 receptors [Krentzel et al., 2018]. It is possible that, since females also possess an ovarian source of E2, drugs

that affect E2-signaling are needed in higher doses to produce an effect. Finally, in males, NCM contains higher density of aromatase fibers than in females (although not of aromatase neurons), which points towards a higher dependency on local aromatization in males [Saldanha et al., 2000; Peterson et al., 2005]. Nevertheless, females are also motivated to engage in the same task (unpublished observation) which provides an interesting avenue of future research, particularly for the exploration of sex differences in the role of aromatase in auditory learning.

Estrogens are important modulators of auditory function in vertebrates, including humans [for review see Caras, 2013]. In women, auditory sensitivity, working memory, and speech perception in noise are modulated by estrogens, both endogenous [Walpurger et al., 2004; Al-Mana et al., 2010; Sao and Jain, 2016] and exogenous [Kilicdag et al., 2004]. In seasonally-breeding songbirds, estrogens, in particular 17β-estradiol (E2), are known to be higher during the breeding season and shift auditory responses in the periphery [Lucas et al., 2007] and also likely in the forebrain [Caras et al., 2012; Caras et al., 2015]. Indeed, in the opportunistically-breeding zebra finch, E2 infusions in the NCM rapidly increase neuronal responses to song stimuli while FAD infusions change neuronal firing patterns [Remage-Healey et al., 2010]. Our results build on this literature by unveiling the functional consequences of neuroestrogen production in the auditory association cortex, which helps clarify the difference between peripheral effects reported in the literature vs central effects reported here. It is possible that in the periphery (e.g. hair cells), E2 enhances sound sensitivity and detection, while central E2 may be affecting neuronal plasticity and sound memory formation.

Studies in humans had previously linked infant E2 levels with increased language capacity [Wermke et al., 2014; Schaadt et al., 2015], but a causal relationship has not been tested. An interesting question is whether, like in songbirds, E2 production by the auditory cortex [Yague et al., 2006] impacts language learning, or more broadly, auditory learning in humans during development and/or adulthood.

In conclusion, we demonstrate that aromatase activity in the secondary auditory cortex of adult songbirds is important for auditory learning, but not post-learning auditory performance. Furthermore, we show that NCM itself might not be required for online auditory performance with previously learned sound-outcome associations. Our findings suggest that NCM E2 (and NCM itself) plays a role in the pairing between sounds and behaviorally relevant consequences, and that this signal is likely distributed and stored in other brain regions after the initial association.

Effects of neuro-E2 production had been previously demonstrated in mammalian and avian hippocampus [Bailey and Saldanha, 2015; Luine, 2016; Tuscher et al., 2016; Gervais et al., 2018; Paletta et al., 2018]. Our study builds on this literature by providing direct evidence that E2 production within the auditory cortex affects sensory learning. We hypothesize that these findings could apply to other vertebrates that contain aromatase in their auditory cortex, including humans.

2.6 Acknowledgments

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2.8 Supplementary methods

2.8.1 Behavioral task

Before GO/NO-GO, birds were introduced to the polarized glass mechanism and trained to operate the switch. Details of these tasks are provided below.

2.8.1.1 Introduction

So that birds were introduced to the function of the polarized glass, this stage consisted of turning the glass transparent for the duration of 6 seconds at pseudorandom intervals between 30 and 60 seconds. This training stage lasted for 4 hours and was repeated for two days.

2.8.1.2 Shaping

Following the two days of introduction, birds were trained to operate the switch. In the first day of shaping, the switch was baited with egg food supplement (Quiko Exotic) attached to a red tape. When birds ate from the tape, the infrared beam would be triggered, and the glass would turn transparent for 6 seconds. The red LED would turn on

to signal when the switch was active and turned off when the glass was transparent and when the training was over.

The performance files generated by the software were constantly monitored. If the number of activations were higher than ~30, the tape was removed, and training continued. If the activation count was not higher than 100 in 4 hours, shaping would be extended for 7-8 more hours (until lights-off), and the same rationale was used the next day. Shaping was repeated daily until birds achieved the following 2 criteria two days in a row: (1) consistently triggered the beam without the tape (more than 100 activations) and (2) did so during a 4-hour trial. If (1) was achieved during an extended trial, a 4-hour trial (without tape) was applied the following day. After two consecutive days of achieving both criteria, the next training stage was started in the following day.

2.8.2 Anaesthetized electrophysiological recordings

Recording drives were made in-house and consisted of 8 tetrodes (4x25 µm NiCr wires; Sandvik; impedance adjusted to 200-300 kΩ by gold-plating) arranged in a bundle coupled to a microdialysis probe (CMA11; Harvard Apparatus) and routed into a custom-made circuit board (modified from Open Ephys board by Daniel Pollak; Advanced Circuits). Before recording, wires were dipped in DiI-594 (ThermoFisher) for electrode placement confirmation. Recordings were sampled at 20 kHz using Intan Technologies amplifier and evaluation board (RHD2000; courtesy of Joseph Bergan).

Two female zebra finches were used for this experiment. Females were used instead of males because this experiment was originally part of another project. They were retrieved from our aviary, anaesthetized with isoflurane, implanted with headposts secured with dental cement and craniotomies were performed exposing the brain

overlaying the auditory lobule. The following day, they were deeply anaesthetized with 20% urethane (3x 30 μL intramuscular injections; 40 min intervals) and head-fixed for recordings. The recording drive was inserted in the craniotomies with the wires targeting the auditory lobule (Field L/NCM), and the probe just lateral to that (caudocentral nidopallium/caudomedial mesopallium). Coordinates (relative to midsagittal sinus) were 0.5-0.9 mm lateral, 1.1 mm anterior, and 1-2.0 mm ventral. Head was tilted forward at a 45° angle. Both hemispheres were used for recordings. Once a stable recording site was achieved, drug infusions at 2 μL/min took place using a microdialysis pump (Harvard Apparatus PHD2000).

Recordings were common median filtered and 300 Hz high-pass filtered. Single unit sorting was performed with Kilosort [Pachitariu et al., 2016]. Sorting results were manually curated and only well-isolated units (high signal-to-noise ratio; no contamination with other units; segregation in waveform PCA space) were used for these analyses (15 from left; 3 from right hemisphere).

Supplementary video. Operant task with social reinforcement. The first section of the video is of a GO trial resulting in a HIT; the second section is of a NO-GO trial resulting in a FALSE ALARM. Note that this animal activates the switch while perching on the switch itself, while other animals trigger it from the wood perch in front of the switch. Also note that this bird sings when he gains visual access to the female. Finally, note in the NO-GO trial that the white noise burst is aversive enough to cause the animal to leave the perch, but he promptly returns after the noise ceases.

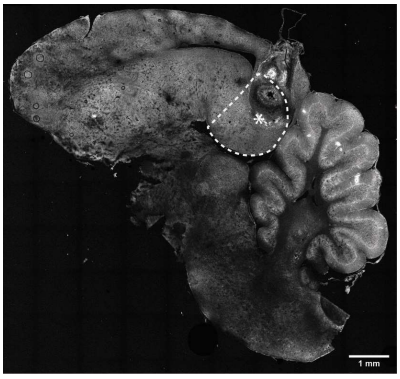

Figure 2.6: Supplementary – NCM cannula placement confirmation.
Dotted lines are an approximation of NCM's anatomical boundaries. Asterisk marks the tip of the cannula.

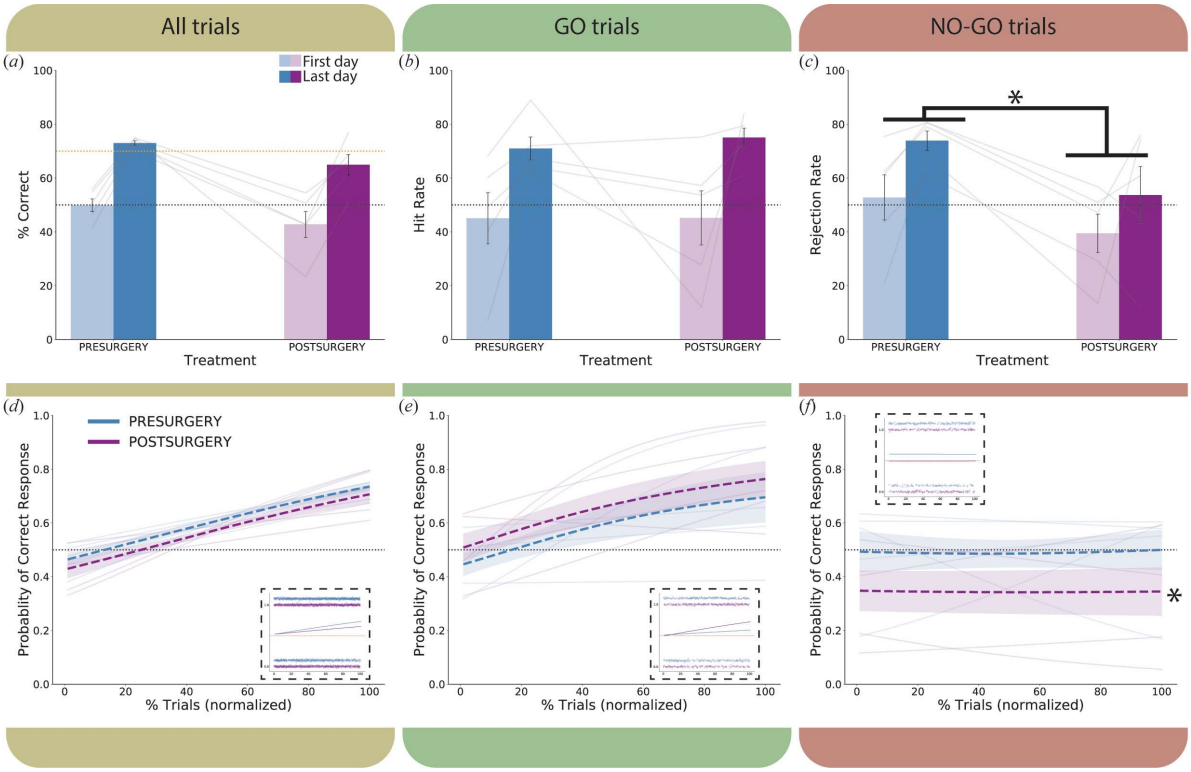

Figure 2.7: Supplementary – One week after cannulation surgery, animals show mild impairments in sound learning.
(a-c) Comparing first vs last day performances, neither all-trial (a) or hit rate (b) are significantly affected, but rejection rates are significantly decreased after surgery (c). Logistic learning curves are shown in (d-f). All-trial (d) and GO-trial (e) curves are not affected but, NO-GO-trial's (f) area under curve is reduced after surgery. Insets in d-f are all from one representative subject. Dots around 0 and 1 represent incorrect and correct responses respectively, which are the data used for the logistic regressions. *$p < 0.05$.

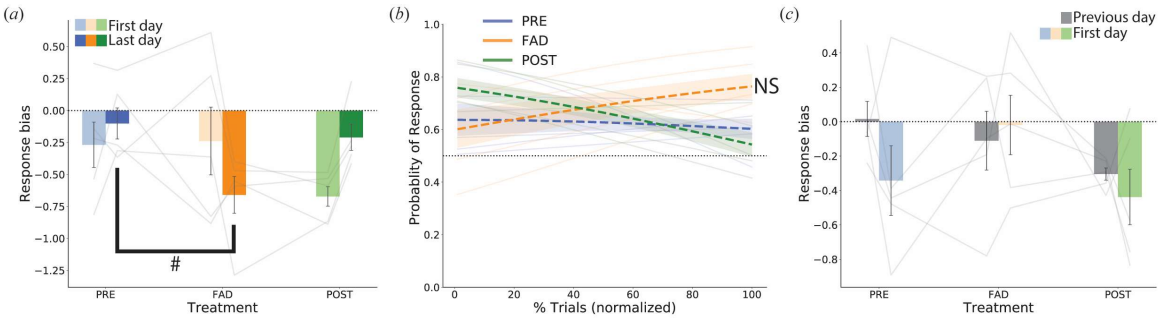

Figure 2.8: Supplementary – Response bias analyses during NCM fadrozole (FAD) administrations before auditory learning task.
(a) First vs last whole-day response bias analyses. There is a trend for an increase of response bias on the last day of training with FAD treatments. (b) Indiscriminate response (hit or false alarm) logistic curves are not significantly changed with FAD. (c) Reversal response bias (first vs previous day of each treatment) is not significantly changed with FAD. #p < 0.1

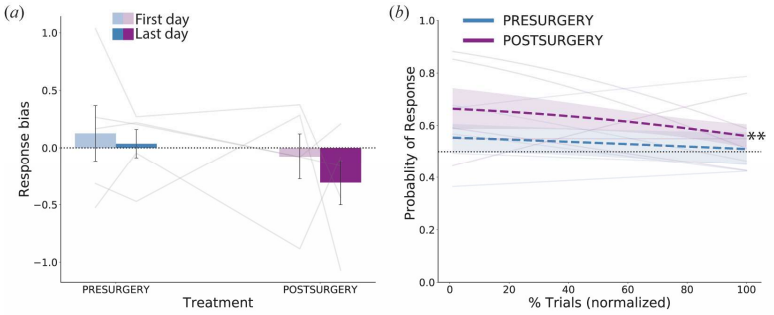

Figure 2.9: Supplementary – Response bias during auditory learning before vs one week after NCM cannulation surgery.
(a) First vs last day response bias are not significantly changed. Negative values indicate bias towards responding, while positive values indicate bias towards non-responding to tones. b) Indiscriminate response (hit or false alarm) logistic curve's area-under-curve is increased following surgery, indicating an increase in response bias. **p < 0.01.

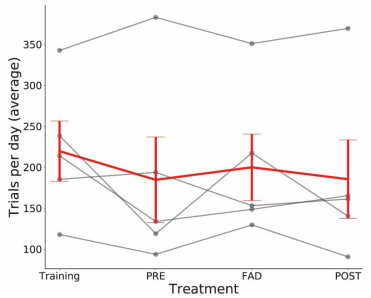

Figure 2.10: Supplementary – Average daily trial initiation is not affected by NCM fadrozole injections.
Training data correspond to post-surgery learning trials without handling and injections.

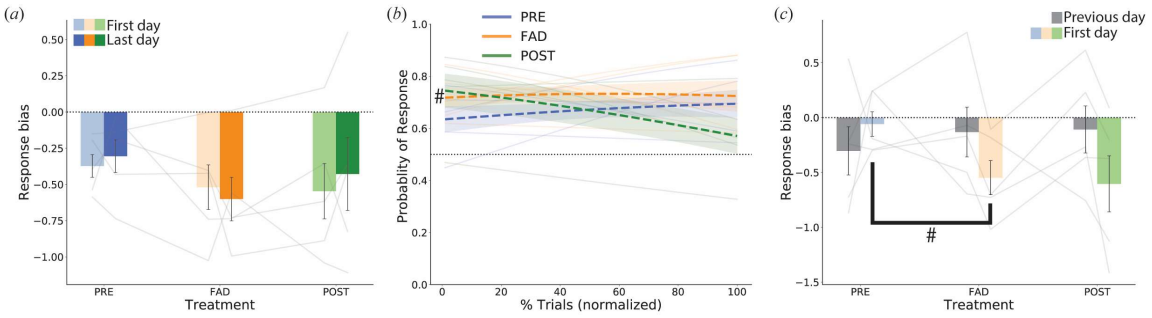

Figure 2.11: Supplementary – Response bias analyses during oral fadrozole (FAD) administrations before auditory learning task.
Negative values (a,c) indicate bias towards responding, while positive values indicate bias towards non-responding to tones. (a) FAD did not significantly affect first vs last whole-day response bias analyses. (b) Indiscriminate response (hit or false alarm) logistic curves are not significantly changed with FAD, but there was a trend for a higher intercept due to FAD. (c) Reversal response bias (first vs previous day of each treatment). There was a trend for a more negative (stronger) response bias on the first day of FAD. #$p < 0.1$.

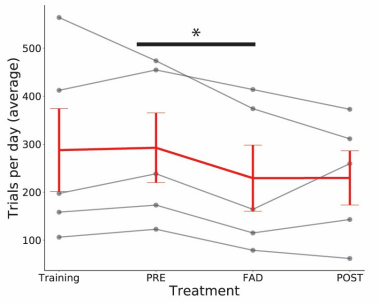

Figure 2.12: Supplementary – Average daily trial initiation was reduced by oral fadrozole administration.
Training data correspond to learning trials without handling and injections. *$p < 0.05$

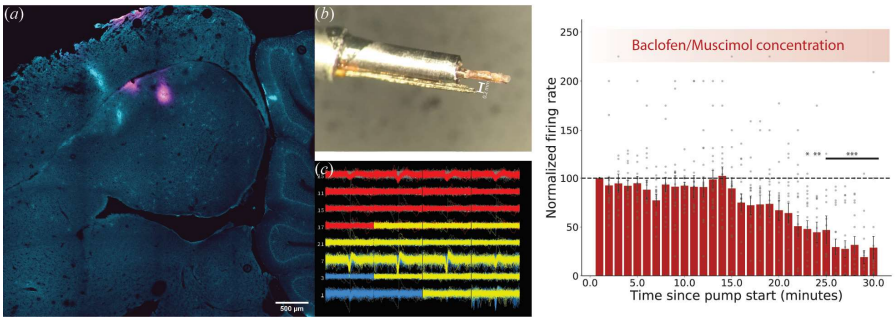

Figure 2.13: Supplementary – Baclofen/muscimol (1/0.1 mM) retrodialysis reduces spontaneous activity of single units in the auditory lobule.

(a) Electrode tracts in magenta (DiI-594); background fluorescence in cyan. (b) Tetrode (8x) array coupled with microdialysis probes for drug delivery. (c) Example neurons recorded in different tetrodes. (d) Firing rate of 17 single units was decreased after baclofen/muscimol infusion. Values were normalized by the first minute. Statistically significant decreases were observed starting at 23 minutes after the start of the microdialysis pump (RM-ANOVA; Time: $F_{29,493} = 6.975$; $p < 0.001$; Dunnett post-hoc test vs first minute). *$p<0.05$; **$p<0.01$; ***$p<0.001$.

Table 2.3: Supplementary – Intra-NCM fadrozole experiments

Supplementary figure number	Testing stage	Descriptive	Test	n	Factor	Results and effect size	Post-hoc
2.7a	Pre- vs post-surgery learning	Whole-day % Correct	2-way RM-ANOVA	5	Treatment	$F(1,16) = 2.567$, $p = 0.149$, $\eta^2 = 0.024$	-
					Day	**$F(1,16) = 95.432$, $p < 0.001$, $\eta^2 = 0.895$**	-
					Interaction	$F(1,16) = 0.059$, $p = 0.822$, $\eta^2 = 0.001$	-
2.7b	Pre- vs post-surgery learning	Whole-day Hit rate	2-way RM-ANOVA	5	Treatment	$F(1,16) = 0.067$, $p = 0.805$, $\eta^2 = 0.003$	-
					Day	**$F(1,16) = 10.566$, $p = 0.017$, $\eta^2 = 0.514$**	-
					Interaction	$F(1,16) = 0.101$, $p = 0.768$, $\eta^2 = 0.005$	-
2.7c	Pre- vs post-surgery learning	Whole-day Rejection rate	2-way RM-ANOVA	5	Treatment	**$F(1,16) = 8.758$, $p = 0.042$, $\eta^2 = 0.434$**	-
					Day	$F(1,16) = 2.942$, $\eta^2 = 0.146$, $p = 0.162$	-
					Interaction	$F(1,16) = 0.578$, $p = 0.489$, $\eta^2 = 0.029$	-
2.7d	Pre- vs post-surgery learning	Combined-trial logistic regression	Paired t-test	5	Area-under-curve	$t_4 = 1.554$, $p = 0.195$, $d = 0.603$	-
			Paired t-test	5	Odds-ratio	$t_4 = -0.081$, $p = 0.940$, $d = -0.027$	-
			Paired t-test	5	Intercept	$t_4 = 0.920$, $p = 0.410$, $d = 0.266$	-
2.7e	Pre- vs post-surgery learning	GO-trial logistic regression	Paired t-test	5	Area-under-curve	$t_4 = -1.125$, $p = 0.324$, $d = -0.654$	-
			Paired t-test	5	Odds-ratio	$t_4 = -0.249$, $p = 0.816$, $d = -0.088$	-
			Paired t-test	5	Intercept	$t_4 = -1.308$, $p = 0.261$, $d = -0.394$	-
2.7f	Pre- vs post-surgery learning	NO-GO-trial logistic regression	Paired t-test	5	Area-under-curve	**$t_4 = 3.722$, $p = 0.020$, $d = 0.605$**	-
			Paired t-test	5	Odds-ratio	$t_4 = 0.347$, $p = 0.746$, $d = 0.101$	-
			Paired t-test	5	Intercept	$t_4 = 1.982$, $p = 0.119$, $d = 0.451$	-
Compare supplementary figure 2.7a with main figure 2.2a	Presurgery vs PRE (ACSF) learning	Whole-day % Correct	2-way RM-ANOVA	5	Treatment	$F(1,16) = 2.873$, $p = 0.165$, $\eta^2 = 0.045$	-
					Day	**$F(1,16) = 50.889$, $p = 0.002$, $\eta^2 = 0.804$**	-
					Interaction	$F(1,16) = 0.010$, $p = 0.925$, $\eta^2 = 0.000$	-
Compare supplementary figure 2.7b with main figure 2.2b	Presurgery vs PRE (ACSF) learning	Whole-day Hit rate	2-way RM-ANOVA	5	Treatment	**$F(1,16) = 19.328$, $p = 0.002$, $\eta^2 = 0.498$**	-
					Day	**$F(1,16) = 8.658$, $p = 0.042$, $\eta^2 = 0.223$**	-
					Interaction	$F(1,16) = 1.762$, $p = 0.221$, $\eta^2 = 0.045$	-
Compare supplementary figure 2.7c with main figure 2.2c	Presurgery vs PRE (ACSF) learning	Whole-day Rejection rate	2-way RM-ANOVA	5	Treatment	$F(1,16) = 0.580$, $p = 0.489$, $\eta^2 = 0.023$	-
					Day	$F(1,16) = 15.742$, $p = 0.017$, $\eta^2 = 0.631$	-
					Interaction	$F(1,16) = 1.746$, $p = 0.339$, $\eta^2 = 0.047$	-
Compare supplementary figure 2.8a with supplementary figure 2.4a	Presurgery vs PRE (ACSF) learning	Whole-day Response bias	2-way RM-ANOVA	5	Treatment	$F(1,16) = 5.910$, $p = 0.072$, $\eta^2 = 0.350$	-
					Day	$F(1,16) = 0.058$, $p = 0.822$, $\eta^2 = 0.003$	-
					Interaction	$F(1,16) = 1.670$, $p = 0.266$, $\eta^2 = 0.099$	-
2.8a	Learning	Whole-day response bias	2-way RM-ANOVA (PRE vs FAD)	5	Treatment	$F(1,16) = 3.826$, $p = 0.086$, $\eta^2 = 0.193$	-
					Day	$F(1,16) = 0.465$, $p = 0.533$, $\eta^2 = 0.023$	-
					Interaction	$F(1,16) = 4.727$, $p = 0.061$, $\eta^2 = 0.238$	-
2.8b	Learning	Response bias logistic regression	Paired t-test (PRE-FAD)	5	Area-under-curve	$t_4 = -2.039$, $p = 0.111$, $d = -0.388$	-
			Paired t-test (PRE-FAD)	5	Odds-ratio	$t_4 = -1.550$, $p = 0.196$, $d = -0.540$	-
			Paired t-test (PRE-FAD)	5	Intercept	$t_4 = 0.500$; $p = 0.643$, $d = 0.186$	-
2.8c	Learning	Reversal response bias	2-way RM-ANOVA (PRE vs FAD)	5	Treatment	$F(1,16) = 0.480$, $p = 0.508$, $\eta^2 = 0.032$	-
					Day	$F(1,16) = 0.519$, $p = 0.511$, $\eta^2 = 0.034$	-
					Interaction	$F(1,16) = 2.508$, $p = 0.152$, $\eta^2 = 0.166$	-
2.9a	Pre- vs post-surgery learning	Whole-day Response bias	2-way RM-ANOVA	5	Treatment	$F(1,16) = 3.616$, $p = 0.116$, $\eta^2 = 0.307$	-
					Day	$F(1,16) = 0.363$, $p = 0.580$, $\eta^2 = 0.023$	-
					Interaction	$F(1,16) = 0.391$, $p = 0.550$, $\eta^2 = 0.020$	-
2.9b	Pre- vs post-surgery learning	Response bias logistic regression	Paired t-test	5	Area-under-curve	**$t_4 = -4.817$, $p = 0.009$, $d = -0.622$**	-
			Paired t-test	5	Odds-ratio	$t_4 = 0.615$, $p = 0.572$, $d = 0.284$	-
			Paired t-test	5	Intercept	$t_4 = -1.807$, $p = 0.145$, $d = -0.587$	-
2.10	Learning	Mean daily trial initiation	Wilcoxon test (PRE-FAD)	5	Trials/day	$V = 6$, $p = 0.813$, $r = -0.181$	-

Table 2.4: Supplementary – Systemic fadrozole experiments

Supplementary figure number	Testing stage	Descriptive	Test	n	Factor	Results and effect size	Post-hoc
2.11a	Learning	Whole-day Response bias	2-way RM-ANOVA	5	Treatment	$F(1,16) = 4.218$, $p = 0.108$, $\eta^2 = 0.245$	-
					Day	$F(1,16) = 0.004$, $p = 0.952$	-
					Interaction	$F(1,16) = 0.490$, $p = 0.522$, $\eta^2 = 0.028$	-
2.11b	Learning	Response bias logistic regression	Paired t-test (PRE-FAD)	5	Area-under-curve	$t_4 = -1.272$, $p = 0.272$, $d = -0.401$	-
			Paired t-test (PRE-FAD)	5	Odds-ratio	$t_4 = 0.355$, $p = 0.740$, $d = 0.201$	-
			Paired t-test (PRE-FAD)	5	Intercept	$t_4 = -2.254$, $p = 0.087$, $d = -0.441$	-
2.11c	Learning	Reversal response bias	2-way RM-ANOVA (PRE vs FAD)	5	Treatment	$F(1,16) = 0.496$, $p = 0.508$, $\eta^2 = 0.032$	-
					Day	$F(1,16) = 0.213$, $p = 0.665$, $\eta^2 = 0.014$	-
					Interaction	$F(1,16) = 4.565$, $p = 0.095$, $\eta^2 = 0.295$	-
2.12	Learning	Mean daily trial initiation	Paired t-test (PRE-FAD)	5	Trials/day	$t_4 = 5.960$, $p = 0.004$, $d = 0.357$	-

CHAPTER 3

DOPAMINE MODULATION OF MOTOR AND SENSORY PERFORMANCE AMONG VERTEBRATES

Manuscript in preparation
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3.1 Abstract

Goal-directed learning is a key contributor to evolutionary fitness in animals. Over the past decades we have learned much about the neural mechanisms that mediate learning, and the neuromodulator dopamine (DA) is commonly implicated. In higher order cortical regions, most of what is known about DA's role is derived from few cases, in brain regions involved in motivation and decision-making, while significantly less is known about DA's potential role in regulating structures involved in improving an animal's motor performance and sensory sensitivity in pursuing rewards or avoiding punishments (e.g. motor/sensory cortices). Moreover, rodent and primate research represents over 95% of publications in the field, while little beyond basic anatomy is known in other vertebrate groups. This significantly limits our general understanding of how DA signaling systems have evolved and allowed organisms to adapt to their environments. This review takes a pan-vertebrate view of the literature on the role of DA in motor/sensory cortical regions, highlighting, when available, research on non-mammalian vertebrates. We provide an evolutionary perspective on DA function and emphasize that DA-induced plasticity mechanisms are widespread across all cortical systems resulting in motor/sensory adaptations. Continued research progress in a wide span of vertebrates will be crucial to further our understanding of how the DA system can

persist or change in face of evolutionary pressures, and how it can become disrupted in a neural disease context. Important gaps in the current literature are also identified.

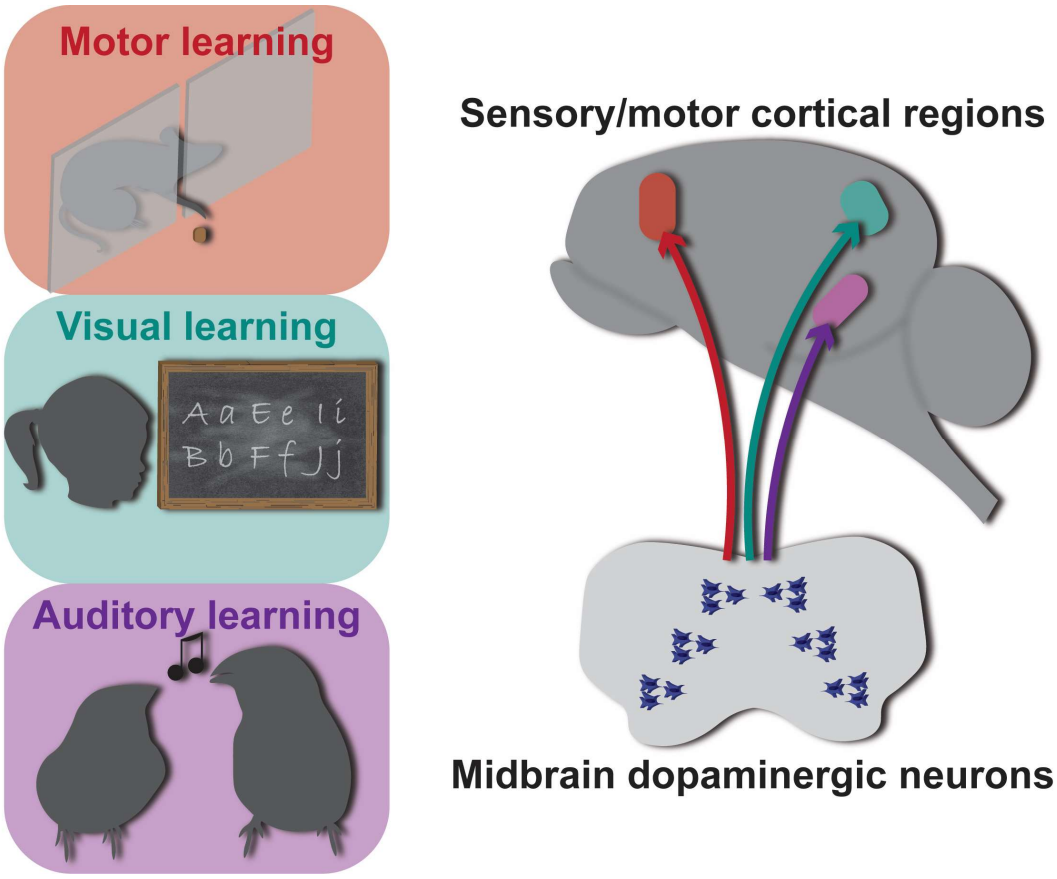

Figure 3.1: Plasticity effects of dopamine onto sensory/motor cortex (graphical abstract)
Highly complex phenomena such as motor, visual and auditory learning depend on midbrain dopamine signaling directly onto motor/sensory cortical structures. The color pattern in panels, arrows and brain structures (ellipses) indicate the specificity of the dopamine projections and their effects.

3.2 Introduction

Research on dopamine (DA) has a rich history. This molecule was first described as a neurotransmitter and suggested to be involved Parkinson's disease six decades ago by Arvid Carlsson [Carlsson et al., 1958], part of a research trajectory for which he was awarded the 2000 Nobel Prize. Parallel to Carlsson's studies, Peter Olds and James

Milner published pioneering research on the electrical stimulation of "pleasure centers" in the rodent brain, which included basal centers for DA action [Olds and Milner, 1954].These seminal findings have driven a large research effort focused on reinforcement processing and motor disorders in the basal ganglia and in the role of DA therein. Two decades later, DA was found also to be present in the rat cortex, detected after ablation of the noradrenergic system [Thierry et al., 1973; Berger et al., 1974].

The years that followed saw an explosion of research on DA, and effects in many brain regions have since been discovered. In mammals, the majority of the DA has been found to be produced in midbrain and hypothalamus, and four main projection pathways have been identified: the tuberoinfundibular, nigrostriatal, mesolimbic and mesocortical pathways [Björklund and Dunnett, 2007]. The latter comprises projections from the midbrain (e.g. tegmentum, substantia nigra) to virtually all cortical regions. An interesting feature of the mammalian cortical DA system is that the density of cortical DA projections generally decreases from anterior to posterior, being the highest in the frontal lobe and lowest in the occipital lobe [Descarries et al., 1987].The current body of literature seems to parallel the anatomy, that is, the areas with the most DA fibers have received the lion's share of research attention. As such, many recent reviews have concentrated on the wealth of data about DA effects on the prefrontal cortex (PFC), mediating phenomena such as reinforcement processing, motivation and attention [e.g. Clark et al., 2014; Chaua et al., 2018; Thiele and Bellgrove, 2018; Weele et al., 2018]. Interestingly, to this point, a recent study proposed that hominid evolution was significantly shaped by striatal DA and its role in sociality [Raghanti et al., 2018]. By contrast, relatively few address DA's direct effects on other cortical regions [motor:

Vitrac and Benoit-Marand, 2017; sensory: Jacob and Nienborg, 2018]. Another large body of literature has focused on learning and behavioral plasticity due to dopaminergic (DAergic) effects on striatum/nucleus accumbens circuits [Cerovic et al., 2013; Sulzer et al., 2016; Gallo, 2019; Woolley, 2019]. Our goal in this review is to address some of the gaps in the literature, specifically by examining effects of DA on cortical regions that modify motor/sensory performance and direct (local) actions of DA in these regions, rather than by means of indirect input from the PFC, striatum or other subcortical regions. Additionally, this review synthesizes available data about DAergic effects on cortical systems in non-mammalian vertebrate species (see below), in order to provide an evolutionary perspective on DA function in less studied cortical systems.

3.3 Cortical DA as a reinforcement-dependent, performance-enhancing signal

After experiencing a rewarding situation, for example, many animals will attempt to replicate the same conditions in which such reward was received so it can experience the reward again in the future. This process requires not only the positive experience signal associated with the reward, but also the encoding of environmental conditions that accompanied or preceded it. Intrinsic motivation to seek subsequent reinforcement can lead to improvements in motor and sensory performance.

Reinforcement learning (be it reward or punishment) is known to recruit several brain regions to signal valence and form memories about environmental information. Notably, the ventral tegmental area (VTA) is a key region in this process. It integrates inputs from areas that process multimodal information such as the prefrontal cortex (e.g. decision-making; attention) and the dorsal striatum (e.g. sensory and motor information) [Beier et al., 2015]. The VTA then sends DA-containing projections to regions such as

the striatum, the nucleus accumbens and the cortex. The firing of these DAergic neurons is intimately linked to reinforcement processing and inducing changes in the target regions [e.g. Menegas et al., 2018]. Reinforcement and decision-making networks that include VTA, striatum, nucleus accumbens and prefrontal cortical structures appear to be necessary for the installation of the reward-seeking (and punishment-avoiding) behaviors. VTA projections to motor and sensory cortices are associated with improving and fine-tuning responses to the environment in order to achieve the desired behavioral outcome more efficiently [McGann, 2015].

It is important to note that the findings mentioned so far are derived mostly from experiments in laboratory rodents and primates. As studies on other vertebrate species have emerged, it has become evident that this system can be involved in many goal-directed behaviors, such as learning of song in birds [Woolley, 2019]. To fully appreciate the deep evolutionary history of DA signaling, it is also important to derive lessons from work on invertebrate species, which have a rich history of studies on DA regulation of sensory, motor, learning and other processes [Verlinden, 2018]. The phylogenetic "myopia" focusing on rodents and primates, as noted by Brenowitz and Zakon [2015], greatly hinders our understanding of the evolution of nervous systems and may be preventing scientific breakthroughs sparked by studying non-traditional organisms [Manger et al., 2008; Carlson, 2012; Remage-Healey et al., 2017]. To this point, in an excellent review on the evolution of DA systems in chordates, Yamamoto and Vernier [2011] emphasize that tracing back the origin of DA systems in vertebrates and how they evolved is an important step to better understand brain DA functions and how they can become maladaptive in a disease context. As an illustration of these biases, the literature

on the roles of DA in the reinforcement processing and decision-making circuitry is vast as compared to the one on the role of DA in improving motor/sensory performance. A non-exclusive PubMed search with the keywords *dopamine AND (accumbens OR striatum OR "prefrontal cortex")* returns ~43,000 results, while *dopamine AND ("motor cortex" OR "auditory cortex" OR "somatosensory cortex" OR "visual cortex")* returns only ~1300 results, a ~33-fold discrepancy. In a similar way, the literature that includes the term rodent exceeds the literature that includes any other non-mammal species ~29-fold, while including the term monkey exceeds the same literature ~14-fold – *dopamine AND brain AND rodent NOT (reptile OR bird OR fish OR amphibian)* returns ~58,000 items; *dopamine AND brain AND monkey NOT (reptile OR bird OR fish OR amphibian)* returns ~28,000 items; *dopamine AND brain AND (reptile OR bird OR fish OR amphibian)* returns ~2,000.

A key distinction for understanding DA modulation concerns indirect versus direct effects of DA on cortical regions. For simplicity, in this review, we classify as indirect those effects of DA on the striatum or other subcortical structures, which in turn may modify their connections with the cortex; or actions on the prefrontal cortex, which can modify other cortical regions by affecting top-down attentional and motivational mechanisms. For example, DA in the prefrontal cortex has been found to modulate attention through its connections to the visual cortex [Noudoost and Moore, 2011]. By contrast, we classify direct effects as actions of DA-containing fibers that synapse directly on the cortical region in focus.

Molecularly, DA binds to a plethora of different receptors, with modulatory actions (seconds to minutes timescales) that can increase or decrease of circuit

excitability [reviewed by Beaulieu and Gainetdinov, 2011]. In the current review, we will mostly refer to the D1 class of receptors (D1a, D1b, D1c, D1d) as simply D1 or D1-like, and to the D2 class (D2, D3, D4) as D2 or D2-like.

The ratio of D1- to D2-like receptors and their expression in different cell types largely accounts for whether a neural circuit's excitability will be activated or suppressed due to the effects of DA. Such effects are achieved by modulating the activity of the excitatory (glutamatergic) or inhibitory (GABAergic) fast neurotransmission of downstream neurons. These effects are now known to be highly nuanced, often referred to D1- or D2-dominated states, respectively linked to high activation-high robustness-low flexibility and low activation-high perturbation-high flexibility states [Durstewitz and Seamans, 2008].

It is important to note that generalizing DA action across structures should be done with caution. For example, inhibition of either D1 or D2 receptors in motor cortex can impair motor learning and long-term potentiation [Molina-Luna et al., 2009], which contradicts (or at least complicates) the "textbook definition" of D1 and D2 receptor effects' being mutually-antagonistic, as is often assumed for the striatum/accumbens [Beaulieu and Gainetdinov, 2011].

It should also be pointed out that while the VTA provides the largest source of DA to the mammalian cortex, it is not the sole source. To illustrate, substantia nigra pars compacta neurons send diffuse projections into the cortex [Gaspar et al., 1992]. Additionally, locus coeruleus neurons co-release norepinephrine and DA throughout the cortex [Devoto et al., 2005], and DA can bind to adrenergic receptors [Cornil and Ball,

2008]. This perspective will become increasingly important as we learn more about how cortical sensory and motor circuits are shaped by DAergic inputs.

Across vertebrates, cortical systems vary markedly in macro-anatomy, but share fundamental features including embryonic origin, connectivity, function, and gene expression patterns. Through identifying shared patterns, homologues to the mammalian six-layered pallial cortex have been hypothesized in all major vertebrate groups, although in fishes telencephalic homology is less clear [Bruce and Neary, 1995; Dugas-Ford et al., 2012; Pfenning et al., 2014; Yamamoto and Bloch, 2017; Tosches et al., 2018]. In mammals, the neocortex is the outermost structure of the telencephalon, while in other vertebrates, cortical structures may be distributed in discrete nuclei across the pallium. We adopt in this review the nomenclature "cortex/cortical structure" to refer to the identified/proposed cortical homologues to mammalian cortical pallium in other vertebrates (Table 3.1).

As some caveats, there are no conclusive data on specific cortical motor/sensory subdivisions of fish, amphibian and reptile telencephalon; in birds, motor regions besides the vocal production nuclei are largely unknown and data on taste cortical homologues in any non-mammalian species are essentially nonexistent. However, even when homology is unclear or not present, cortical structures that share similar principles due to convergent evolution can be equivalently informative for understanding general principles of such systems and the evolutionary pressures that mold them [Katz, 2019].

As part of our review we ask the following questions: since DA is present in motor and sensory cortices, what roles does DA play in these structures? Do the principles of DA effects in motor/sensory cortices mirror those in reinforcement

processing centers and the basal ganglia? Furthermore, are DA's effects generalizable across other vertebrates? How did this modulatory system evolve?

3.4 Dopamine-induced plasticity in motor cortex

The context and timing of DA signaling is crucial for behavioral plasticity. Prior to the key association of cues with consequences, DA signaling by VTA is purely subsequential, that is, the signal follows reward consumption. However, with repetition of the pairing of environmental conditions with consequences, DA signaling starts to anticipate the consequence, and then becomes a predictive signal. If a prediction or expectation about the consequence is violated, DA neurons will now signal when the consequence was predicted to happen and will inform the direction of this violation (good or bad surprise), by increasing or decreasing their firing rates, respectively. The literature classically refers to this "algorithm" as reward prediction error [Barto et al., 1981; Schultz et al., 1997]. This algorithm not only signals **when** an animal should make a decision to pursue a reward, but also **how** to achieve this reward with more ease or accuracy. For example, this algorithm is implemented when repetition of a motor pattern to achieve reinforcement leads to fine-tuning of the movement. Therefore, the DAergic signals that are used to reinforce the decision-making network could also be used to reinforce and tune motor performance.

3.4.1 DA fiber and receptor distribution suggest topographical specificity

The VTA is the major DAergic projection to the mammalian primary motor cortex (M1) [Scheibner and Törk, 1987] and most M1-projecting VTA neurons project solely to the motor cortex (i.e. no collaterals) (Hosp et al., 2015; Hosp et al., 2011). In the rat M1, axons containing tyrosine hydroxylase (TH; enzyme upstream of the DA

production chain) that lack DA β-hydroxylase (DBH; enzyme that converts DA into norepinephrine) are abundant [Berger et al., 1985]. Similar findings were reported in macaques [Noack and Lewis, 1989; Williams and Goldman-Rakic, 1993] and humans, where TH fibers are particularly dense in layers I and V/VI [Gaspar et al., 1989].

DA receptors of both D1 and D2 families are present in M1. In macaques, D1 receptors are mainly found in layers I-III and V-VI while D2 is more concentrated in layer V [Lidow et al., 1990; Lidow et al., 1991]. Together with the TH fiber distribution, these findings suggest some topographical specificity of DAergic projections in M1. Superficial (layer I) projections may target mainly D1 receptors, while deeper (layer V) projections target both D1 and D2 receptors.

3.4.2 DA effects in motor cortex cannot be predicted by effects in other regions

Like the PFC, the rat M1 *in vitro* exhibits both long-term depression and potentiation (LTD and LTP) [Hess and Donoghue, 1996]. After a motor skill learning task, the rat M1 shows stronger local connections (field potentials) *in vitro*, which suggests that LTP can be induced by behavioral training [Rioult-Pedotti et al., 1998]. However, unlike in the PFC, D1 and D2 receptors in M1 can be synergistic for LTP induction. Molina-Luna et al. [2009] have shown that blocking either D1 or D2 receptors prevents electrical tetanus-induced LTP formation in M1 *in vitro*. These findings are seemingly in contrast with data on prefrontal cortex (PFC) slices, where D1 but not D2 receptors are involved in *in vitro* electrical tetanus-induced LTP formation [Huang et al., 2004]. Notably, however, both receptors are involved in spike timing-dependent LTP in the PFC [Xu and Yao, 2010]. These results reflect the diversity of DAergic mechanisms in shaping neuronal plasticity among different cortical areas.

Experiments in humans suggest that DA can have local effects within the motor cortex. L-DOPA (DA precursor) enhanced performance in a training-dependent motor learning task in both young [Flöel et al., 2005] and elderly adults, and increased DA release (measured by displacement of radioligands) in the elderly [Floel et al., 2008]. Using transcranial magnetic stimulation (TMS), Ziemann et al. [1997] found that motor cortex activity can be modulated by systemic D2 receptor agonists and antagonists, effects potentially due to local actions of DA. Meintzschel and Ziemann [2006] expanded these findings in another training-dependent motor task, where a D2 agonist enhanced learning while an antagonist decreased it. These data confirm that DAergic function is important for goal-directed, practice-dependent motor plasticity in humans, but in order to clarify whether these effects are direct or indirect (e.g. via striatum or prefrontal cortex) more targeted approaches are needed.

3.4.3 DA effects in non-mammalian motor cortex are largely unexplored

Midbrain DAergic nuclei that participate in reward-seeking and learning are a highly conserved feature in all vertebrates [reviewed by Martínez-García and Lanuza, 2018], which implies that research in other organisms could greatly inform about how these circuits operate in mammals and vice versa. Here, we summarize the relatively scant literature on the effects of DA on non-mammalian motor systems (also see table 3.2).

As in mammals, TH-containing projections are clearly evident in the proposed cortical homologues [gecko: Smeets et al., 1986; Iberian ribbed newt: González and Smeets, 1991; zebra finch: Bottjer, 1993; African cichlid fish: O'Connell et al., 2011a;

túngara frog: O'Connell et al., 2011b] and DA receptors [zebra finch: Kubikova et al., 2010; African cichlid fish: O'Connell et al., 2011a].

Data are lacking for DA receptors in reptile and amphibian cortex, but receptor presence has been inferred by the expression of DARPP-32, a protein commonly associated with D1 receptors in mammals [gecko: Smeets et al., 2001; túngara frog: O'Connell et al., 2011b]. The extent to which DARPP-32 receptors and D1 receptors in non-mammals are associated is unknown and deserves investigation. In fact, in zebra finch telencephalon these two sets of receptors do not always colocalize in neurons (own unpublished observations). To the best of our knowledge, the function of this system has only been explored in the vocal motor cortex songbird (suborder Passeri).

3.4.4 DA in the song control/production cortical system in songbirds

The song system has been studied intensively in songbirds, but regarding the effects of DA, striatal regions have received greater attention than cortical regions. The songbird striatum contains a region (Area X) dedicated to song learning of and song motor plasticity. Area X receives massive DAergic inputs from VTA [Lewis et al., 1981] and plays a vital role in song learning in development [Sohrabji et al., 1990]. In the context of song production, a reward prediction learning function arriving in Area X from DAergic neurons in the avian VTA was identified [Gadagkar et al., 2016]. Optogenetic manipulations of these fibers, as well as pharmacological manipulations of DA receptors in Area X affected both adult song plasticity and juvenile song learning [Hisey et al., 2018]. The effects of DA on this striatal system have been reviewed elsewhere [Kubikova and Košťál, 2010; Simonyan et al., 2012].

In the avian cortex (pallium), the sensorimotor region HVC (acronym is the proper name) and the motor region robust nucleus of the arcopallium (RA) are two crucial areas for song learning and production. They contain dense TH fiber tracts [Bottjer, 1993] derived mainly from the mesencephalic central gray, but also from VTA [Appeltants et al., 2000; Appeltants et al., 2002]. Concordantly, both motor cortical regions express mRNA for D1A, D1B (D1 family), D2 and D3 (D2 family) receptors. D1B and D3 receptors are expressed in higher intensity in HVC and RA, as compared to the surrounding brain regions [Kubikova et al., 2010].

Few studies have examined DAergic physiology in the songbird vocal motor cortex. Adult male European starlings (*Sturnus vulgaris*) exposed to high quality song playbacks (presumed higher quality competitors) sang more and showed decreased levels of DA metabolites in HVC but not RA. [Salvante et al., 2010]. *In vitro*, RA projection neurons exhibited increased resting membrane potential and firing rate in response to DA and a D1 agonist, while a D2 agonist had no such effect [Liao et al., 2013]. In HVC, DA projections from the periaqueductal gray to HVC are crucial for song learning during development in zebra finches. This signal seems to convey social context and/or motivation in the juveniles when engaging with live tutors. VTA projections to HVC are significantly less dense in juveniles (as in adults), but their function was not explored [Tanaka et al., 2018].

In sum, DA effects on motor cortex are evident in mammals and are related to goal-directed, practice-dependent motor skill improvement. There are limited parallels in non-mammalian vertebrates, with notable recent progress in birdsong production and learning. It would be interesting to know, from an evolutionary standpoint, to what extent

DA is involved in cortical motor plasticity in other vertebrates. For instance, behaviors such as flight or the perfection of hunting skills are examples of natural goal-directed motor learning that likely involve the DAergic system in motor areas, and which could thus provide important future study opportunities.

3.5 Dopamine-induced plasticity in the visual cortex

Across vertebrates, the visual system plays a crucial role in the optimization of visual information processing during reinforcement learning. For example, in macaques reinforcement following a visual stimulus has been shown to increase visual performance, such that larger rewards produced faster reaction times in a saccade visual task, and neural activity in the DAergic midbrain during the task reflected strong reward prediction error encoding [Nomoto et al., 2010].

DAergic fibers in the primary visual cortex (V1) were first shown in cats [Törk and Turner, 1981] and later shown to be present in rats [Descarries et al., 1987; Phillipson et al., 1987] and primates, including humans [Phillipson et al., 1987; Berger et al., 1988]. Their region-specificity resembles that in motor cortex, where most DAergic fibers can be found in deeper layers (V and VI), and at a lesser density in layer I [Descarries et al., 1987; Berger et al., 1988]. Receptor binding assays corroborate the TH fiber architecture, since D1 receptor binding is strongest in the layers I-III and V/VI, while D2 binding is strongest in layer V of the macaque visual cortex [Lidow et al., 1990].

3.5.1 Sparse DAergic fiber presence yet clear effects in visual cortex

There is evidence that DA acts locally within V1, but data are not as abundant as in the motor cortex. On the whole, the mammalian cortex has an anterior-posterior gradient of decreasing DA content and projections. Located in the posterior-most

occipital lobe, the mammalian primary visual cortex is the target of the sparsest DAergic projections [Descarries et al., 1987] and shows the lowest detectable DA content of all regions in the cortex [Brown et al., 1979]. It is important to note that higher-order visual association cortical areas such as the rhinal and the posterior parietal cortices are localized more anteriorly. Nevertheless, stimulus-evoked DA release in rat visual cortical areas could be detected by microdialysis [Müller and Huston, 2007]. Furthermore, in the cat V1, local application of DA decreased neural activity in deeper layers, but not in superficial layers [Reader, 1978]. A similar effect was observed in the rat V1 [Gottberg et al., 1988]. Interestingly, both these studies observed mostly decreases in neuronal firing following DA application. When using specific agonists, a D1 agonist decreased, but a D2 agonist mildly increased firing rates (both applied in deeper layers) [Gottberg et al., 1988]. It is possible that in V1, D1- vs. D2-receptor activation could yield different effects contrary to the majority of other systems studied (i.e. associated with different g-protein-coupled signaling pathways). Alternatively, D1-D2 receptor distribution might differ – e.g. D1 receptors may be present in mostly inhibitory interneurons in the deeper cortical layers and thereby shape overall excitability.

In the macaque rhinal cortex, an associative region that integrates multimodal sensory information, D2 receptors seem to be essential for learning of visual cues. Reducing the production of D2 receptors specifically in this region, Liu et al. [2004] showed that macaques were no longer able to learn visual cues that predicted rewards. Moreover, fMRI data from awake rhesus macaques showed that visual cortical regions are strongly modulated by reward prediction error signaling which could be attenuated by a systemic D1 receptor antagonist [Arsenault et al., 2013].

3.5.2 Visual cortex activity in primates can be mediated by DA in the PFC

Studies in anesthetized macaques report that effects of DA signaling on visual performance can happen through DA modulation of prefrontal regions [Noudoost and Moore, 2011; Zaldivar et al., 2018] but not through direct V1 manipulations [Zaldivar et al., 2014]. Different types of anesthesia produce different effects on VTA firing rates/patterns and possibly DA release, which could render effects of exogenous DA difficult to interpret [reviewed by Marinelli and McCutcheon, 2014]. Therefore, it appears that local effects of DA in the monkey visual cortex warrant more attention and should not be definitively ruled out.

Research with human subjects also points towards an important indirect prefrontal-mediated effect on visual cortex activity. Transcranial magnetic stimulation (TMS) on the visual cortex (V5/MT) normally disrupts visual performance. However, activating both D2 and D1 receptors simultaneously prevented TMS-induced interference of visual performance, but no such effect was observed with a D2 agonist alone. Because none of the treatments affected local visual cortex excitability by TMS, the authors assumed the effects are mediated by prefrontal effects on visual performance [Yousif et al., 2016].

In view of these studies, it is plausible that DA modulation in the visual cortex is secondary or residual in primates, acting mainly on frontal circuits. Alternatively, local effects of DA on the visual cortex could be unveiled through tasks that involve more specific visual learning or conditioning.

3.5.3 Anatomical data support DA modulation in non-mammalian visual cortex

In non-mammalian vertebrates, some interesting visual plasticity phenomena correlate with anatomical substrates of DAergic systems. However, in many cases conclusive experimental data that would solidify such a link are lacking. Birds are the only non-mammal group in which the visual cortical system has been explored in detail.

In songbirds, there is correlational evidence that DA plays a role in visual recognition. Avian visual processing is distributed between two separate cortical regions: the entopallium and the hyperpallium. Lesions of the thalamo-recipient entopallium impaired performance of birds trained to identify a feeder based on visual pattern identification [Watanabe et al., 2008]. Lesions of the hyperpallium (another higher-order visual association cortical region) impaired performance of birds trained to identify feeders based on location [Watanabe et al., 2011] – the authors suggest that the entopallium and hyperpallium are analogous to the ventral and dorsal streams of mammalian visual processing, which process "what" and "where" objects are, respectively [Goodale and Milner, 1992]. In Indian house crows (*Corvus splendens*), neuron activation in the hyperpallium (entopallium was not sampled), VTA and SNc were implicated in spatial and visual pattern recognition performance [Taufique and Kumar, 2016]. These three studies point to these two cortical areas as important loci for visual learning and memory. The crow study further suggests that midbrain DAergic nuclei play a role in this process, but determining the causal relationship requires further experimentation.

Anatomical work further strengthens this hypothesis. The hyperpallium in the zebra finch brain is a hotspot of D1-like receptors, but is largely devoid of D2-like receptors. Conversely, the entopallium can be anatomically delineated by the absence of

DA receptors [Kubikova and Košťál, 2010]. TH fibers seem to follow a similar pattern in the canary (*Serinus canaria*) [Appeltants et al., 2001]. Interestingly, the entopallium has been proposed to be homologous to mammalian cortical layer IV, and the hyperpallium to layers IV and/or V [Dugas-Ford et al., 2012]. Because mammal visual cortex layer IV is virtually devoid of DA signaling, while layer V is not, DA signaling in these cortical areas of songbirds seems to map onto this homology hypothesis.

Outside of songbirds, there is evidence suggesting that DA could be affecting visual imprinting in chickens. Domestic chicks (*Gallus gallus*) can be experimentally imprinted on artificial objects (colored rotating cubes or cylinders) and thereafter display attraction behaviors towards them. The anterior mesopallium, a polysensory cortical region, is involved in visual imprinting and its neurons respond selectively to imprinted visual (but also auditory) stimuli [Nicol et al., 1995]. TH fibers are present in this region [Metzger et al., 1996], as are D1-like receptors [Schnabel et al., 1997]. However, to our knowledge there have been no experimental manipulations of DA to test its effects on visual imprinting (but see next section for auditory).

Among other vertebrate groups, data on DA function in visual cortical regions are even rarer and only reported in amphibians and reptiles. In the common toad (*Bufo bufo*), prey-catching strategy could be modified by systemic D1/D2 agonist apomorphine [Glagow and Ewert, 1999], but it is not clear whether the visual system itself is directly responsible for this. The medial pallium was suggested as a visual cortical target in amphibians [Scalia, 1976; Kicliter, 1979] and this area contains DA/TH fibers in a newt [González and Smeets, 1991] and gymnophionan [González and Smeets, 1994] but not in anurans [González et al., 1993; O'Connell et al., 2011b]. In red-eared slider turtles

(*Trachemys scripta*), a visual cortical region called the dorsal cortex shows spatial adaptation (plasticity) and encodes information about spatial and temporal features [Fournier et al., 2018]. TH fibers and DARPP-32-positive cell bodies can be detected in this region in Tokay gekkos (*Gekko gecko*) [Smeets et al., 2001]. These studies identify potential anatomical substrates for studying DAergic modulation of visual inputs in amphibians and reptiles and could be an interesting area to pursue evolutionary and comparative questions.

3.6 Dopamine-induced plasticity in the auditory cortex

Associating sounds with consequences is an important survival trait in many species. Cues associated with, for example, competitors vs allies or predators vs prey are constantly being surveilled. Additionally, many animals rely on auditory recognition of individuals for social interactions [Aubin and Jouventin, 1998; Sayigh et al., 1998; Gentner et al., 2000; Goodwin and Podos, 2014]. The auditory system is highly plastic and able to form complex auditory associations, which makes it a highly attractive system for studying neuromodulation.

3.6.1 DA signaling locally in auditory cortex supports auditory learning

As with other cortical systems in mammals, DAergic projections in the mammalian auditory cortex are mainly from VTA and SNC [Budinger et al., 2008] and are stratified, mostly in layers I and V/VI [Campbell et al., 1987]. A detailed quantification of DA receptor distribution in the auditory cortex seems to be lacking, but qualitative inspections of rat brains suggest a distribution pattern that parallels that in other cortical regions – D1 in layers I-III and V/VI and D2 mostly present in layer V [Boyson et al., 1986].

The first pieces of evidence that DA and VTA play a role in auditory plasticity were shown in rodents. In an auditory conditioning task with Mongolian gerbils (*Meriones unguiculatus*), levels of homovanillic acid (DA metabolite) detected via microdialysis increased significantly in auditory cortex only during the first day of the task, which suggests that DA signaling locally is important during the initial stages of learning [Stark and Scheich, 1997]. VTA was shown to exert a profound effect on the organization of the primary auditory cortex (A1). Pairing VTA stimulation with pure tone playbacks, Bao et al. [2001] observed an enhanced representation of the paired tone in the anesthetized rat A1, an effect that could be blocked by combined systemic D1 and D2 receptor antagonists.

The importance of local DAergic signaling in the auditory cortex for auditory learning was demonstrated formally through lesions of DAergic signaling with 6-hydroxy-DA in rats [Kudoh and Shibuki, 2006] and by local infusions of D1 modulators in Mongolian gerbils [Schicknick et al., 2008; Schicknick et al., 2012]. The relevance of D2 receptors locally still needs to be confirmed experimentally.

LTP can be induced by electrical tetanus in the rat primary A1 [Kudoh and Shibuki, 1994], but to our knowledge LTP modulation in A1 by DA has not been directly tested. This surprising gap is an important area for future research.

Local DA effects were also demonstrated in monkeys. Recordings from the auditory cortex in long-tailed macaques (*Macaca fascicularis*) showed clear evidence of reward prediction error signaling [Brosch et al., 2011]. VTA to auditory cortex synapses were modulated by D1 receptor inhibitor injected either systemically or in the auditory cortex [Mylius et al., 2014].

In humans, DA-dependent auditory plasticity has been hypothesized. Daily L-DOPA treatment enhanced association of objects with new (artificial) words presented in audio form [Knecht et al., 2004], and a D1/D2 receptor agonist decreased it [Breitenstein et al., 2006]. This suggests that increased availability of a DA precursor is beneficial, while persistent activation of DA receptors is prejudicial for auditory associative learning. Therefore, controlled timing of phasic DA release might be the mechanism by which humans engage in auditory learning takes place. Whether such effects are intrinsic to the auditory cortex awaits confirmation.

3.6.2 Anatomical substrate of DAergic signaling in avian auditory association cortex

The DAergic midbrain has been shown to be engaged after exposure to behaviorally relevant sounds in a variety of vertebrates [midshipman fish: Petersen et al., 2013; spadefoot toad: Burmeister et al., 2017; e.g. zebra finch: Barr and Woolley, 2018], but its involvement in cortical plasticity has only been explored in birds.

Auditory imprinting seems to be DA-dependent since it can be blocked by systemic D2 antagonist in chicks [Gruss and Braun, 1996; Gruss et al., 2003], but no data on cortical manipulations is yet available.

In songbirds, TH/DAergic fibers are conspicuous across the secondary auditory cortex (NCM and the caudomedial mesopallium, CMM) but not the primary auditory cortex, field L2 [Reiner et al., 1994]. DA receptors follow a similar pattern: D1-like receptors are abundant in NCM and CMM, and D2-like receptors are abundant in CMM but not in NCM. Neither receptor is evident in the thalamo-recipient Field L, primary auditory cortex [Kubikova et al., 2010]. In female white-throated sparrows (*Zonotrichia albicollis*), exposure to conspecific song increased the activation (phosphorylation) of TH

fibers and the levels DA metabolites in the caudomedial nidopallium (NCM), part of the songbird auditory association cortex [Matragrano et al., 2012]. Furthermore, in juvenile zebra finches, song tutoring increased VTA activation [Chen et al., 2016]. These studies show that DA is likely involved in plasticity mechanisms triggered by auditory events in songbird cortex. Since NCM is an area involved in auditory associations, DA likely facilitates synapse strengthening for the formation of auditory memories, although this possibility is currently untested.

3.7 Dopamine-induced plasticity in the olfactory cortex

In dynamic environments, the ability to associate meaning to odors is an important evolutionary adaptation present in virtually all vertebrates. Multiple brain areas have been implicated in odorant processing and associative learning, including the olfactory bulb, olfactory tubercle (part of the striatum), medial amygdala, orbitofrontal cortex, entorhinal cortex, piriform cortex and others. DAergic signaling is pervasive in these systems. Of note, the olfactory bulb contains one of the major DA-producing cell groups in vertebrates, whose neurons are continuously generated throughout the lifespan [Hinds, 1968; Pérez-Cañellas and García-Verdugo, 1996; Kornack and Rakic, 2001; but see Bergmann et al., 2012]. DA/GABAergic interneurons regulate the activity of local olfactory projection neurons (mitral/tufted cells), a feature that seems to be evolutionarily conserved across vertebrates [Hsia et al., 1999; Davison et al., 2004; Kawai et al., 2012] and even invertebrates [Perk and Mercer, 2006]. The role of these neurons has been intensely studied and recently reviewed elsewhere [Pignatelli and Belluzzi, 2017]. Of note, some external tufted cells can also be DAergic in rats [Halász et al., 1981], macaques and humans [Smith et al., 1991], but these do not seem to project outside of the

olfactory bulb. Rather, they modulate the activity of periglomerular cells [De Saint Jan et al., 2009].

While DA effects on the olfactory bulb and olfactory striatum [Zhang et al., 2017] are known, the role of DA in other olfactory cortical regions has been relatively understudied.

3.7.1 Does DA in the piriform cortex facilitate olfactory memory formation?

The olfactory bulb projection neurons send diffuse projections to the piriform cortex (PC), which is proposed as the olfactory memory association cortex [Haberly, 2001; Wilson and Sullivan, 2011; Bekkers and Suzuki, 2013]. It receives dense DAergic projections from VTA and locus coeruleus with an unconventional anterior-to-posterior increase in density in the rat. DA fibers are more abundant in deeper layers II and III (PC is a paleocortical region with only 3 layers) [Datiche and Cattarelli, 1996]. D1 receptors are most abundant in layer II and more prevalent than D2 receptors, which are virtually absent [Boyson et al., 1986; Santana et al., 2009].

The effects of DA on PC activity are poorly understood but reports confirm modulation by both D1 and D2 receptor action. In rat PC *in vitro*, D2 receptor agonists and antagonists decreased and increased, respectively, DA release and turnover, presumably by activating D2 autoreceptors located on axon terminals [Bannon et al., 1983; Plantjé et al., 1987]. *In vivo*, local injections of D1/D2 agonists in tandem, but not separately, increased the firing rate of PC neurons and impaired social interactions in rats. Interestingly, the same treatment did not impair short-term olfactory memory formation [Zenko et al., 2011].

Data from human studies also point to an involvement of the PC in olfactory learning. MRI data reveal a progressively higher activation to the conditioned stimulus in this area with olfactory Pavlovian conditioning [Gottfried et al., 2002], but data on DA effects have not yet been reported.

3.7.2 The olfactory entorhinal cortex is affected by local DA

Another cortical region directly modulated by DA is the entorhinal cortex (EC). In macaque EC, TH fibers are considerably dense, especially in the medial portion, called the olfactory EC [Akil and Lewis, 1993]. DA reduced layer V pyramidal neuron excitability in rat lateral EC, *in vitro*, through D1 but not D2 receptors [Rosenkranz and Johnston, 2006]. Increased synaptic DA through a reuptake inhibitor impaired LTP and LTD at PC to EC synapses in awake rats [Caruana et al., 2007]. Rodent behaviors such as the Coolidge effect or individual recognition through olfactory cues are impaired by EC, but not hippocampus lesions [Bannerman et al., 2001; Bannerman et al., 2002; Petrulis and Eichenbaum, 2003], and could be under the regulation of DA.

In sum, both PC and EC are important sites of olfactory processing and learning in mammals, and both are directly modulated by DAergic projections from VTA [Aransay et al., 2015]. Specific data in humans are lacking, but a similar function is likely.

3.7.3 The PC is conserved across vertebrates

The three-layered PC is thought to be a highly evolutionarily conserved structure, reminiscent of the similarly three-layered reptilian/avian lateral olfactory cortex [Aboitiz et al., 2002]. It is reasonable to suggest, therefore, that the effects of DA on olfactory plasticity in non-mammals might be similar as those reviewed above. Concordantly,

olfactory cortical regions contain significant TH and/or DARPP-32 protein immunoreactivity in non-mammals [ball python: Smeets, 1988; e.g. canary: Appeltants et al., 2001; túngara frog: O'Connell et al., 2011b; lungfish: López and González, 2017]. In birds, the piriform cortex [Rieke and Wenzel, 1978; Reiner and Karten, 1985] seems to contain both D1 and D2 receptors, although more detailed investigation was not performed [Kubikova et al., 2010]. No studies were found on DA modulation of olfactory function in non-mammals.

3.8 Dopamine-induced plasticity in the taste cortex

3.8.1 DA in the insular cortex modulates taste learning

In many species, taste learning is crucial for survival. Formation of aversion memory to toxic or spoiled foods after negative consequences from a first contact ensures that animals do not make the same mistake in the future. Taste responses converge in the insular cortex (IC), along with other experiences such as enteroception, addiction and complex emotional reactions, being described as a hub for integrating several systems [Gogolla, 2017]. Interestingly, the IC has a bidirectional connection with VTA, which could be the anatomical substrate through which the IC both receives and modulates reward processing signals [Ohara et al., 2003].

Taste learning has been shown to involve DA signaling in the rodent IC. In the rat, D1 receptors expression is greater in deep layer VI, followed by layer II; D2 receptors are mostly concentrated in layer V [Gaspar et al., 1995]. In DA-depleted mice, LTP in the IC can be rescued by a DA reuptake inhibitor [Moreno-Castilla et al., 2016]. Inhibition of D1 receptors locally in the rat IC before a taste aversion task impaired memory formation, but not retrieval of a previously learned association [Berman et al.,

2000]. Activation of D1 but not D2 receptors in mice leads to phosphorylation of the subunit NR2 of the NMDA receptor in both IC and hippocampus [David et al., 2014], which is an attractive mechanistic model for how taste memory formation takes place.

While a local effect to the IC is evident in rodents, human data on DA and taste responses only exist for subcortical structures. For example, hunger and food modulate extracellular DA in the striatum [Volkow et al., 2002; Wang et al., 2004; Wang et al., 2014]. Systemic amphetamine, which boosts DA release, changes IC responses to sucrose in women [Melrose et al., 2016], but it is not possible to infer whether DA directly modulates the IC. To our knowledge there are no available studies that explore the role of DA in human taste learning, although the human IC is known to receive reward prediction error signals [Preuschoff et al., 2008]. In sum, DA is known to modulate responses and taste learning in rodents, with an important effect on the IC. However, data on human taste learning, and more specifically, on the involvement of DA in the process, are lacking.

3.8.2 Taste learning in non-mammals is an open field of study

Taste learning has been reported in other vertebrate groups. Aversion learning to food laced with lithium-chloride (an emetic) could be induced in several lizard species, but not in frogs or salamanders [Paradis and Cabanac, 2004]. Curiously, this type of learning could be successfully induced in goldfish, and was impaired by whole telencephalon or dorsomedial telencephalon lesions [Martín et al., 2011]. As it could also be successfully induced in birds [bobwhite quail: Wilcoxon et al., 1971; buteo hawk: Brett et al., 1976], it is possible that taste aversion learning was a secondary loss in amphibians. We could not find further published data on DA effects, or more broadly, on

the neural mechanisms of taste processing in non-mammalian vertebrate cortex. Given the extreme diversity in feeding habits across vertebrates, this area of study could bring interesting insight on how taste processing systems evolved.

3.9 Dopamine-induced plasticity in the somatosensory cortex

3.9.1 DA projections and receptor distribution in somatosensory cortex is species-specific within mammals

DAergic fibers in the somatosensory cortex (SSC) originate mostly from VTA and seem to follow the traditional pattern found in other neocortical regions. They are denser in layer I in squirrel monkeys [Lewis et al., 1987], but denser in deep layer VI in rats [Descarries et al., 1987]. DA receptors somewhat map onto these distributions: D1 receptors are denser in superficial layers I-III, while D2 receptors are denser in area V in Rhesus macaques [Lidow et al., 1991], while in the rat, D1 receptors are denser in deeper layers V-VI, and D2 in layer V [Gurevich and Joyce, 2000]. These differences might arise due to these species' natural histories and further strengthen the argument for the importance of avoiding generalizations, and for studying a broad range of organisms to gain true insight the evolution of neural systems.

3.9.2 DA signaling modulates damage-reactive plasticity in somatosensory cortex

Reports of local effects of DA in the SSC suggest that DA plays a key role in plasticity, especially when there is a need for cortical reorganization after an injury. Intracortical injections of either D1 or D2 antagonist increased the responses of the sensorimotor cortex (transition between somatosensory and motor cortices) to muscle stimulation in anesthetized rats [Hosp et al., 2011a]. Furthermore, peripheral nerve transection progressively increased levels of DA metabolites in the rat somatosensory

cortex, suggesting that the increase in DA signaling might be related to the reorganization of the cortex when sensory information from an appendage is eliminated [Jiménez-Capdeville et al., 1996]. Similarly, unihemispheric stroke induction in rats increased DA levels in the contralateral hemisphere, and D2 antagonist prevented recovery of nociceptive response in the weeks following the stroke. In the event of a stroke, the contralateral hemisphere is thought to undergo reorganization in order to aid in physical recovery and compensation, with DA involved in this process [Obi et al., 2018].

Interestingly, DA receptors may also play an organizational role in the SSC. In the developing rat, D3 receptors are transiently highly expressed in layer IV and correlate with the development of the barrels (cortical representation of the whiskers) [Gurevich and Joyce, 2000]. These authors did not examine the origin of the DA input to this system, nor did they follow up with DA manipulations. These would be interesting topics to explore, since this system is known for being experience- and critical period-dependent for proper development [Erzurumlu and Gaspar, 2012].

Finally, spike timing-dependent plasticity in corticostriatal synapses can be prevented by D1 antagonist and modulated by D2 antagonist [Pawlak and Kerr, 2008]. Whether these effects are due to cortical versus striatal receptors has not been explored.

3.9.3 DA modulates human somatosensory cortex excitability

Somatosensory learning in humans is an important component of the formation of pain memories and plays a role in learning of textures and patterns. Braille reading is an example of highly specialized tactile learning that requires plasticity in the SSC [Debowska et al., 2016]. However, human data exploring DA function on the SSC could only be found for Parkinson patients, likely justified by the well-characterized

degeneration of the DA systems and consequent motor and sensory impairments. Systemic DAergic medication may alter perception of tactile stimuli [Nelson et al., 2012; Nelson et al., 2018] and may reduce somatosensory cortex excitability in Parkinson patients [Palomar et al., 2011]. However, it might be premature to extrapolate these findings to healthy humans, since in Parkinson's patients, the DAergic system has been deteriorating. For instance, one of these studies reports that somatosensory cortex excitability is lower in Parkinson's patients than in healthy controls [Nelson et al., 2018]. Studies examining the effects of DAergic drugs on healthy humans are needed for a better understanding of these effects under wide-ranging physiological conditions.

3.9.4 DA receptors and fibers are present in non-mammal somatosensory cortex

Somatosensory cortical regions have been mapped in a variety of non-mammalian vertebrates [leopard frog: Kicliter, 1979; crocodile: Pritz and Northcutt, 1980; e.g. rockfish: Ito et al., 1986; pigeon: Wild, 1987]. The same regions are known to express TH/DAergic fibers [gecko: Smeets et al., 1986; Iberian ribbed newt: González and Smeets, 1991; pigeon: Wynne and Güntürkün, 1995; African cichlid fish: O'Connell et al., 2011a; túngara frog: O'Connell et al., 2011b]. Still, no studies could be found in which DA systems were manipulated and their effects on somatosensory plasticity observed. However, future comparative work in this system seems promising given the emerging anatomical literature. For example, phenomena such as electrosensory learning in electric fish or toxic food aversion learning in general should engage somatosensory cortical regions and potentially rely on DA signaling.

3.10 Final considerations

In view of the literature discussed here, it is evident that DA plays roles in virtually all mammalian cortical circuits, but systems can vary remarkably at both molecular and anatomical levels. For example, variation occurs in the cortical layer distribution of receptors and DAergic fibers, and in the receptor activation effects and plasticity outcomes (facilitation/inhibition). Additionally, in different species these factors may vary with evolutionary history. Because of this, the current perspective emphasizes that findings in one cortical system do not necessarily generalize to other systems or across species. We were particularly surprised to find that, after reviewing the literature, the presence of LTP modulation by DA in auditory, visual, and olfactory (piriform) cortical systems are commonly assumed but rarely demonstrated directly, or even tested. It is thus an open question as to whether the fundamental properties of neural systems plasticity observed in the PFC/striatum extend to other cortical modalities.

Our review also illustrates that there is a strong anatomical basis – DA fibers and receptor distributions – to hypothesize that cortical DA effects are widespread across vertebrates. However, there are limited reports on this subject, which highly constrains our understanding about how these circuits adapted and evolved. Since the anatomy of cortical structures is widely variant among vertebrates – e.g. three layered, nucleated, six layered, super vs subventricular –, understanding how projections from evolutionarily conserved midbrain structures (VTA/SNc) have reshaped with evolving cortical projection targets could be helpful for understanding midbrain-cortex synapse formation and these synapses contribution to behavior in healthy and diseased brain states.

DA release is frequently under the control of, or acting in concert with, other neuromodulator systems. Much is known about interactions between the DA system and

other traditional neuromodulators such as norepinephrine [reviewed by Xing et al., 2016], oxytocin [reviewed by Baskerville and Douglas, 2010] and acetylcholine [reviewed by De Kloet et al., 2015]. Less explored are interactions between the DA system and steroid hormones. In mammalian and avian striatum and preoptic area, estradiol (E2) and DA interact to regulate one another's production, release and receptor expression [Becker, 1990; Lammers et al., 1999; Balthazart et al., 2002; Tozzi et al., 2015]. In the mammalian striatum, E2 infusions rapidly increase dopamine release [Xiao et al., 2003], and DA agonists reverse the decrease in LTP induced by an E2-production inhibitor [Tozzi et al., 2015]. In fact, there is some evidence that DA and E2 could bind to the same receptors [Olesen and Auger, 2008; Tozzi et al., 2015].

Specifically, in cortex, data about possible interactions between DA and steroids are scarcer. In male rats, gonadectomy and hormone replacements affected DA levels in the PFC, but not the motor cortex [Aubele and Kritzer, 2011]. In healthy normocycling women, E2 levels are associated with DA neurotransmission in the PFC, and both systems interact to regulate working memory [Jacobs and D'Esposito, 2011]. In the songbird secondary auditory cortex (NCM), TH fiber density and DA release seem to be under control of steroid hormones [Matragrano et al., 2011; Rodríguez-Saltos et al., 2018], but direct evidence of functional interaction is lacking. Studying E2 and DA interactions in the auditory cortex is an interesting avenue of research and of potential relevance to human auditory function, since both aromatase (E2-synthase) [Yague et al., 2006] and E2 receptors [González et al., 2007] are abundant in the human temporal cortex.

Finally, in this review we identify important gaps in the DA research literature regarding effects on mammalian motor and sensory cortices and on non-mammalian vertebrate cortices. Overall, it is clear that DA-induced plasticity mechanisms are widespread across all cortical systems and induce motor/sensory adaptations to achieve behavioral goals more efficiently. Furthermore, studying basal vertebrates might prove crucial for advancing our understanding of (I) how the DA system can change in face of evolutionary pressures, (II) what other functions this system might express and (III) how it contributes mechanistically in a neural disease context.

3.11 Acknowledgments

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3.12 Conflict of interest

The authors declare no conflict of interest.

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Table 3.1: Cortical homology across vertebrates

Modality	Group	Cortical area	Key references
Motor	Amphibians	?	-
	Reptiles	DVR*	[Distel, 1978]
	Birds	HVC; RA (vocal)	[Nottebohm et al., 1976; Nottebohm and Arnold, 1976]
Visual	Amphibians	Medial pallium	[Kicliter, 1979]
	Reptiles	Dorsal cortex	[Gusel'nikov et al., 1972; Fournier et al., 2018]
	Birds	Hyperpallium; Entopallium	[Hodos and Karten, 1970; Pettigrew and Konishi, 1976; Watanabe et al., 2011]
Auditory	Amphibians	Medial pallium	[Northcutt and Ronan, 1992]
	Reptiles	DVR*	[Foster and Hall, 1978]
	Birds	Field L; NCM; CMM	[Karten, 1968; Kelley and Nottebohm, 1979; Vates et al., 1996]
Olfactory	Amphibians	Lateral pallium	[Hoffman, 1963; Scalia et al., 1968]
	Reptiles	Lateral cortex	[Goldby, 1937]
	Birds	Piriform cortex	[Rieke and Wenzel, 1978; Reiner and Karten, 1985]
Gustatory	Amphibians	?	-
	Reptiles	?	-
	Birds	?	-
Somatosensory	Amphibians	Medial pallium	[Kicliter, 1979]
	Reptiles	DVR*	[Pritz and Northcutt, 1980]
	Birds	Hyperpallium apicale; anterior nidopallium	[Wild, 1987]

CMM: caudomedial mesopallium; DVR: dorsoventricular ridge; NCM: caudomedial nidopallium; RA: robust nucleus of the arcopallium; *Specific subdivisions within the DVR are unexplored. Fishes were not included in this table since cortical homology parallels with other vertebrates are largely unclear.

Table 3.2: Anatomical distribution of dopaminergic markers across vertebrate telencephalons

Area (cortical)	Group	Tyrosine hydroxylase	D1-like receptors	D2-like receptors	Key references
Motor	Amphibians	?*	?*#	?	-
	Reptiles	?*	?*#	?	-
	Birds	+(HVC; RA)	+(HVC; RA)	+(HVC; RA)	[Bottjer, 1993; Kubikova et al., 2010]
	Mammals	+	+	+	[Scheibner and Törk, 1987; Lidow et al., 1990; Lidow et al., 1991]
Visual	Amphibians	+(newt/gymnophionan: medial pallium) −(anurans: medial pallium)	?#*	?	[González and Smeets, 1991; González et al., 1993; González and Smeets, 1994; O'Connell et al., 2011b]
	Reptiles	+(dorsal cortex)	?#*	?	[Smeets et al., 1986]
	Birds	+(hyperpallium) −(entopallium)	+(hyperpallium) −(entopallium)	−(hyperpallium) −(entopallium)	[Metzger et al., 1996; Schnabel et al., 1997; Appeltants et al., 2001; Kubikova et al., 2010]
	Mammals	+	+	+	[Törk and Turner, 1981; Lidow et al., 1990]
Auditory	Amphibians	?*	?*#	?	-
	Reptiles	?*	?*#	?	-
	Birds	+(NCM; CMM) −(Field L2)	+(NCM; CMM) −(Field L2)	+(CMM) −(NCM; Field L2)	[Reiner et al., 1994; Kubikova et al., 2010]
	Mammals	+	+	+	[Boyson et al., 1986; Campbell et al., 1987; Budinger et al., 2008]
Olfactory	Amphibians	+(lateral pallium)*	?*#	?	[González and Smeets, 1991; O'Connell et al., 2011b]
	Reptiles	+(lateral cortex)*	?*#	?	[Smeets et al., 1986; Smeets, 1988; Smeets et al., 2001]
	Birds	+	+	+	[Appeltants et al., 2001; Kubikova et al., 2010]
	Mammals	+	+	+	[Boyson et al., 1986; Datiche and Cattarelli, 1996; Santana et al., 2009]
Gustatory	Amphibians	?*	?*#	?	-
	Reptiles	?*	?*#	?	-
	Birds	?	?	?	-
	Mammals	+	+	+	[Gaspar et al., 1995; Ohara et al., 2003]
Somatosensory	Amphibians	+(newt/gymnophionan: medial pallium) −(anurans: medial pallium)	?*#	?	[González and Smeets, 1991; González et al., 1993; González and Smeets, 1994; O'Connell et al., 2011b]
	Reptiles	?*	?*#	?	-
	Birds	+(hyperpallium apicale) +(anterior nidopallium)	+(hyperpallium apicale) +(anterior nidopallium)	+(hyperpallium apicale) +(anterior nidopallium)	[Reiner et al., 1994; Kubikova et al., 2010]
	Mammals	+	+	+	[Descarries et al., 1987; Lewis et al., 1987; Lidow et al., 1991]

CMM: caudomedial mesopallium; DVR: dorsoventricular ridge; NCM: caudomedial nidopallium; RA: robust nucleus of the arcopallium; ?: No data were found; *: In reptiles and amphibians, there are reports of markers in the DVR, but specific subdivisions within the DVR are unknown; #: There is data on the presence of DARPP-32 protein, but whether DARPP-32 and D1 receptors are colocalized in non-mammals is unknown. Fishes were not included in this table since cortical homology parallels with other vertebrates are largely unclear.

CHAPTER 4

DOPAMINE D1 RECEPTOR SIGNALING DRIVES AUDITORY PLASTICITY IN THE SONGBIRD AUDITORY CORTEX

Manuscript in preparation
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4.1 Abstract

Learning to process and interpret sounds is one of the first steps in the process of vocal learning and is thought to depend on high-order cortical brain structures, where sounds and meaning are associated. In songbirds, the caudomedial nidopallium (NCM) is part of the auditory association cortex and is important for sound learning and perception throughout life. NCM is also site of action of neuromodulators including neuroestradiol (E2) and dopamine (DA). E2 plays a role in association learning in NCM, but how it affects learning circuits is unknown. We hypothesized that DA regulates learning circuits, perhaps by interacting with local E2 signaling in NCM. Here, using male and female adult zebra finches, we first show that NCM D1 receptor (D1R) protein is found in ~33% of neurons containing E2-synthase (aromatase). NCM D1R-expressing neurons are predominantly GABAergic (~61%) and most parvalbumin+ neurons contain D1Rs (~55%). We show that pharmacologically activating D1Rs with SKF-38393 *in vitro* reduces the amplitude of GABAergic currents and glutamatergic currents, but also increases the frequency of the latter. Corresponding *in vivo* results show that D1R activation reduces the firing of putative fast-spiking interneurons, while it increases the firing of putative excitatory projection neurons. Finally, D1R activation renders neurons unable to further adapt to novel stimuli, and most likely places them into a pre-adapted

state. Our data suggest that D1Rs modulate learning and memory in the songbird sensory cortex.

4.2 Introduction

Vocal and auditory learning have evolved in humans and other organisms, such as songbirds. Studying these features in songbirds can provide insight into the mechanisms that enable spoken language [Jarvis, 2019]. The first step in the spoken language learning process is to make associations about complex sounds, which engages high-order cortical brain structures, where sounds and meaning are bound.

In songbirds, the caudomedial nidopallium (NCM; Fig. 4.1A) is a high-order, secondary auditory region considered analogous to the center for speech comprehension in humans, Wernicke's area [Bolhuis et al., 2010]. Neuronal responses in awake restrained zebra finch NCM show stimulus-specific adaptation to sounds played repeatedly, consistent with active memory formation [Chew et al., 1996]. The NCM is an important target of a wide variety of neuromodulators, such as norepinephrine [Ikeda et al., 2015; Lee et al., 2018], nitric oxide [Wallhäusser-Franke et al., 1995], neuroestradiol [Saldanha et al., 2000; Macedo-Lima and Remage-Healey, 2020] and dopamine (DA) [Matragrano et al., 2012]. NCM therefore is a highly plastic structure involved in the processing, and perhaps association, of complex sounds.

In addition to neuromodulators, NCM is rich in NMDA receptors [Saldanha et al., 2004], which are classically regarded as key players in cellular memory formation processes, such as long-term potentiation (LTP) and depression [Lüscher and Malenka, 2012]. Stimulus-specific adaptation in NCM neurons has been extensively studied, and it reflects familiarity with songs, as well as song-consequence associations [Chew et al.,

1996; Bell et al., 2015; Lu and Vicario, 2017]. However, the receptors involved and neuromodulatory mechanisms underlying associations between sound and context in higher-order circuits like NCM have not been elucidated.

Dopaminergic (DAergic) fibers permeate the secondary auditory regions NCM and the caudomedial mesopallium (CMM), but not the thalamo-recipient auditory region, Field L [Reiner et al., 1994]. DA receptor mRNA maps onto this architecture, such that D1 receptors (D1Rs) are abundant in NCM and CMM, and D2 receptors are abundant in CMM but not in NCM. Neither receptor is evident in the primary auditory cortex Field L [Kubikova et al., 2010]. DA signaling seems to be a key distinctive feature between secondary versus primary auditory cortex in songbirds, providing an anatomical circuit-basis for DA-dependent auditory learning.

Functionally, the role of DA and DAergic midbrain nuclei have been examined in the songbird brain in the context of reinforcement learning for song production and motivation to sing [Leblois et al., 2010; Schmidt and Ding, 2014; Matheson and Sakata, 2015; Gadagkar et al., 2016]. Much less is known about how DA signaling might regulate auditory processing and association. Hearing song rapidly increases production of DA in songbird auditory regions, especially in the NCM [Matragrano et al., 2012]. Accordingly, DA signaling has been proposed as a key component of NCM plasticity, related to the encoding and processing of song.

In this study, we hypothesized that DA signaling via D1 receptors (D1Rs) promotes NCM neural circuit plasticity. We obtained both anatomical and physiological evidence to test this hypothesis. First, using immunofluorescence, we show that there is high rate of coexpression of D1R and aromatase (neuroestradiol-synthase) in NCM

neurons and that most D1R-expressing neurons are inhibitory. Furthermore, we show that activating D1R decreases the amplitude of GABAergic, while it increases the frequency of glutamatergic currents *in vitro*. In awake birds, we extend these findings, such that activating D1Rs decreases the firing of putative-inhibitory interneurons, while it increases the firing of putative-excitatory projection neurons, and that stimulus-specific adaptation to complex signals (songs) is regulated by D1Rs. These findings inform several circuit models we present for NCM regulation by DA.

DA signaling modulates auditory association learning in primary cortex [Bao et al., 2001; Schicknick et al., 2008; Schicknick et al., 2012; Reichenbach et al., 2015]. In these studies, DA activation was paired with simple stimuli such as pure tones and sweeps. However, there is limited work on the role of DA in processing complex auditory signals, in high-order cortical structures. In humans, systemic DA-enhancing treatments enhanced auditory language learning [Breitenstein et al., 2004; Knecht et al., 2004]. Here, we show in a songbird that DA signaling specifically in a high-order sensory cortex can modulate the encoding of complex auditory signals by shifting inhibitory-excitatory balance.

4.3 Material and methods

4.3.1 Animals

A total of 31 adult (> 90 days old) zebra finches (24 males) were used in this study. Birds came from the University of Massachusetts Amherst colony (14:10 hour light-dark cycle) and were not actively breeding (single-sex cages). All procedures were in accordance with the Institutional Animal Care and Use Committee at the University of Massachusetts Amherst.

4.3.2 Immunofluorescence, imaging and quantification

Four males and three females were used for immunofluorescence experiments, to characterize the phenotype of D1 receptor-positive (D1R+) cells. Briefly, animals were taken from the aviary, deeply anesthetized and perfused with ice-cold phosphate buffered saline followed by room-temperature phosphate-buffered 4% paraformaldehyde. Brains were extracted, postfixed in the same fixative overnight, cryoprotected in 30% sucrose and frozen until processing. With a cryostat, 40 µm parasagittal sections were made and sampled in 4 subseries collected in cryoprotectant solution and stored at –20 °C until processed.

Tissue from 2 males and 2 females was processed for D1R, aromatase (ARO) and tyrosine hydroxylase (TH) triple immunofluorescence; tissue from all 4 males and 3 females was processed for D1R, GABA and parvalbumin (PV) triple immunofluorescence.

Sections containing NCM were selected and transferred from cryoprotectant to phosphate buffer (PB) and washed 3x15 min. They were then incubated in 10% normal goat serum (NGS; Vector Labs) in 0.3% Triton-X in PB (PBT) for 2 h. Primary antibody solutions (Table 4.1) were prepared in 10% NGS in 0.3% PBT. To confirm antibody specificity, in a subset of sections the D1R antibody was preincubated for 1 h with blocking peptide (Fig. 4.1B). Sections were incubated with primary antibodies for 1 h at room temperature, followed by 2 days at 4 °C. Then, sections were washed 3x15 min in 0.1% PBT and transferred to secondary antibody solutions (goat polyclonals; ThermoFisher; 1:200) prepared in 0.3% PBT. Finally, sections were washed in 3x10 min in 0.1% PBT and kept in the same solution in the fridge until mounted (1 or 2 days later) and coverslipped with ProLong Diamond with DAPI (ThermoFisher).

Images were taken with a confocal microscope (Nikon A1si). First, NCM was localized and a 4x4 large image was taken at 10x magnification. Then, using only the DAPI channel, the microscope stage was digitally controlled and moved to selected locations on the 10x images, at the ventral and dorsal posterior edges of NCM (Fig. 4.1C). Then, 15 µm (1 µm step size) z-stack images were taken at 60x magnification, starting from the top-most surface of the section. Two sections per hemisphere per animal were imaged. All laser intensities were maintained uniform across all images within experiments.

D1R antibody penetration noticeably decayed at ~5 µm deep into the tissue, therefore only the top 5 µm of each z-stack was quantified. Cell counts were performed manually by a blinded experimenter using Fiji (ImageJ; NIH). Briefly, color histograms were set individually for each image so that background was predominantly dark and only strong signals were counted. Only antibody localization around the nuclei (DAPI) was included. Only cells with large, ovoidal nuclei (presumably neurons) were counted. Antibody quantification was done using the z-stack, while DAPI quantification was done using the z-max-projection image.

Table 4.1: Primary antibody table

Antibody	Type	Host	Dilution	Company
anti-D1DR	Polyclonal	Guinea pig	1:100	Alomone Labs
D1DR peptide	-	Rat	1:10	Alomone Labs
anti-Aromatase	Polyclonal	Rabbit	1:2000	Azac
anti-Tyrosine Hydroxylase	Monoclonal	Mouse	1:2000	Immunostar
anti-GABA	Polyclonal	Rabbit	1:1000	Sigma
anti-Parvalbumin	Monoclonal	Mouse	1:10000	Millipore

4.3.3 *In vitro* whole-cell patch clamp

4.3.3.1 Recordings

Fifteen males were used for slice recordings across two experiments. We focused these experiments on males to further understand the mechanisms of a previous behavioral study [Macedo-Lima and Remage-Healey, 2020]. We note that we did not observe systematic sex differences in the immunofluorescence and *in vivo* electrophysiology finding. However, we do not discard the possibility of sex differences.

After swift decapitation, the top of the skull was resected and the head was immediately immersed in a Petri dish filled with ice-cold carbogen-aerated cutting solution (0-Mg^{2+} cutting; in mM: 222 glycerol, 25 $NaHCO_3$, 2.5 KCl, 1.25 NaH_2PO_4, 0.5 $CaCl_2$, 34 glucose, 0.4 ascorbic acid, 2 Na_2-pyruvate, 3, myoinositol. Standard: idem except 25 glucose and 3 $MgCl_2$; ~320 mOsm/kg, pH 7.4). In the Petri dish, the cerebellum was resected and the brain was removed from skull. Then, brain was removed from the cutting solution and placed on an ice-cold Petri dish lid covered with KimWipe (KimTech). The lateral forebrain of both hemispheres was cut parasagittally to yield flat lateral surfaces, and cerebral hemispheres were bisected. The lateral edges of each hemisphere were then dabbed dry on the KimWipe, glued (cyanoacrylate) to the cutting stage and immediately immersed in the vibratome (Leica VT1000S) chamber filled with ice-cold carbogen-aerated cutting solution. Slices were cut at 250-300 µm starting from the medial edge which contains NCM. NCM does not have clearly defined lateral boundaries, but song-inducible gene expression experiments indicate strong responses that extend ~1 mm from the midline [Mello and Clayton, 1994], thus only the first three sections (750-900 µm) were used for recordings. After cutting, slices were transferred to 37 °C carbogen-aerated 0-Mg^{2+} or standard recording solution (0-Mg^{2+}; in mM: 111

NaCl, 25 $NaHCO_3$, 2.5 KCl, 1.25 NaH_2PO_4, 2 $CaCl_2$, 28 glucose, 0.4 ascorbic acid, 2 Na-pyruvate, 3 myo-inositol; standard: idem except 25 glucose and 3 $MgCl_2$; ~320 mOsm/kg, pH 7.4). After a 30-minute recovery at 37 °C and a 30-minute stabilization at room temperature, recordings started. All recordings were performed at room temperature.

Recording pipettes (borosilicate glass) were pulled with a vertical pipette puller (Narishige PC-10) and had a tip resistance of 4-7 MΩ when submerged in the recording solution and backfilled with either K-gluconate- or CsCl-based solution, for excitatory (EPSC) and inhibitory (IPSC) postsynaptic currents recordings, respectively (*K-Gluconate*: in mM: ~95 K-gluconate, 20 KCl, 0.1 $CaCl_2$, 5 HEPES, 5 EGTA, 3 MgATP, 0.5 NaGTP, 20 creatine-phosphate disodium; osmolarity adjusted to ~295 mOsm/kg with K-gluconate; pH 7.4; *CsCl*: in mM: ~120 CsCl, 8 NaCl, 10 TEA-Cl, 0.2 EGTA, 10 HEPES, 2 MgATP, 0.2 NaGTP; osmolarity adjusted to ~295 mOsm/kg with CsCl; pH 7.4). Internal solutions also contained 0.1% AlexaFluor-488-hydrazide (ThermoFisher) and 0.1% Neurobiotin (Vector) for "online" and post hoc detection (see below) of the cell, respectively.

Cells were identified with an inverted microscope (Nikon FN-1) with DIC optics. Recordings were made with an EPC-10 amplifier and recorded and compensated (series resistance, slow/fast capacitance) with PatchMaster software (HEKA). Liquid junction potential was automatically subtracted. Traces were digitized at 20 KHz. Recordings were made in voltage clamp mode at -70 mV. After whole-cell configuration was achieved, cells were allowed to stabilize for 5 min. Then, baseline drug cocktails (bicuculline or DNQX) were delivered and allowed to act for a minimum of 2 min. Drug-

containing solutions were gravity-delivered and flow-matched to a ~2 mL/min peristaltic pump (Cole-Palmer MasterFlex L/S) outtake. Tissue chamber capacity was ~1 mL. Recording quality was constantly monitored between recording blocks and recordings were aborted if series resistance compensation rose above 40 MΩ.

Nine males were used for AMPA/kainate/NMDA EPSCs (sEPSCs) recordings. For sEPSC isolation, bicuculline (20 μM; Santa Cruz) was added to the 0-Mg^{2+} recording solution. For most recordings, after bicuculline was delivered and allowed to take effect, a 1-min baseline recording was made, but for a few cells (9 out of 25), a 7-min rundown recording followed. Then, ±-SKF-38393 hydrochloride (10 or 50 μM; abcam) [Ding and Perkel, 2002] was delivered and a 7-min recording was timed to the start of the delivery. Maximum concentration of drug is estimated to have been achieved within 1 min. Finally, a 10-min washout recording was timed to the start of SKF-38393 clearance, and complete washout is estimated to have been achieved within 1 min. Finally, to confirm the nature of the currents, D-AP-5 (50 μM; abcam, Santa Cruz or Alomone) was delivered and currents were monitored for ~5 min.

Six males were used for spontaneous GABA IPSCs (sIPSCs) recordings. For sIPSC isolation, DNQX (20 μM; Tocris) was added to the standard recording solution. Recordings were made similar to EPSC recordings, except different cells were used for SKF (n=7) and rundown experiments (n=4). After a 1-min baseline recording, either SKF-38393 (10 μM) or nothing (rundown) was added to the recording solution and currents were monitored for 7 min. Then, a 10-min washout (or continued rundown) followed. Finally, to confirm the nature of the currents, bicuculline (20 μM) was added to the recording solution and currents were monitored for ~5 min.

After recordings were completed, the recording pipette was slowly retrieved, and slices were drop-fixed overnight in 4% paraformaldehyde in PB. Then, they were transferred to cryoprotectant solution and kept at -20 °C until processed.

4.3.3.2 Analyses

Recordings were analyzed in IgorPro 6. All traces were downsampled (5x) and lowpass filtered at 500 Hz.

In sEPSC experiments, for amplitude measurements, cells were only included (n=9) in the analysis if series resistance did not change by more than 20% from baseline values. For frequency recordings, all cells (n = 25) were analyzed, as recording quality fluctuations are not expected to interfere with their detection, due to their high amplitude (>50 pA; noise band ~5 pA). Currents were thresholded and manually curated with NeuroMatic [Rothman and Silver, 2018]. After curation, currents were automatically measured by custom IgorPro code.

For sIPSC recordings, cells whose series resistance changed more than 20% from baseline were excluded from all analyses. For each cell, one template current was manually selected and spontaneous PSCs were automatically detected using a spontaneous current detection algorithm [Clements and Bekkers, 1997] implemented by Dr. Geng-Lin Li for IgorPro. After detection, all IPSCs were measured automatically by custom IgorPro code.

For imaging cells containing neurobiotin, slices were washed 3x15 min in PB and incubated in 10% NGS in 0.3% PBT for 2 h. Sections were then incubated with rabbit anti-aromatase diluted 1:2000 in 10% NGS in 0.3% PBT for 1 h at room temperature, followed by 2 days at 4 °C. Then, sections were washed 3x15 min in 0.1% PBT and

transferred to 0.3% PBT containing goat anti-aromatase at 1:200 and Streptavidin-DyLight-488 (Vector) at 1:200. Finally, sections were washed in 3x10 min in 0.1% PBT and kept in the same solution in the fridge until mounted (1 or 2 days later) and coverslipped with ProLong Diamond with DAPI (ThermoFisher). Cells were imaged using a confocal microscope (Nikon A1si) at 20 and 100x magnification.

4.3.4 *In vivo* awake head-fixed electrophysiology

4.3.4.1 Headpost implantation and craniotomy surgery

Five males and four females were retrieved from the single-sex cages and implanted with headposts. Briefly, under isoflurane anesthesia, custom-made headposts were lowered on the top of the beak and secured with dental cement. Markings over the lateral-anterior edges of NCM (1.1 lateral, 1.4 anterior) were performed, large craniotomies over NCM were made and meninges were resected. A small craniotomy was made in an anterolateral part of the skull for the implantation of a silver ground wire using cyanoacrylate. Craniotomies were sealed with Kwik-Cast (WPI), and birds were allowed to recover from anesthesia. Recordings were performed within 4 days of surgery.

4.3.4.2 Retrodialysis-microdrive (RetroDrive) fabrication

Custom retrodialysis probe-coupled multielectrode drives (RetroDrives) were assembled in house. RetroDrives consisted of a circular printed circuit board (PCB; Sunstone Circuits) soldered to a 36-pin connector (Omnetics; A79026-001), and a 5 mm 17G guide tube (stainless steel; Component Supply Company). A strand of 28G enameled copper magnet wire (Remington Industries) was soldered to the PCB ground. Three polyimide tubes (2x 198 µm; 1x 100 µm diameter) were inserted through the guide tube, glued side-by-side on the wall of the guide tube and cut to be flush with the guide

tube. Tetrodes were made by twice-folding and twisting polyimide-coated NiCr tetrode wire (Sandvik) with a tetrode spinner (LabMaker). Tetrodes were inserted through the larger polyimide tubes (4 in each) and pinned to the PCB. A single reference wire (50 µm polyimide-coated NiCr wire; Sandvik) was inserted through the smaller polyimide tube and pinned to the PCB. Wires were glued to the top of the polyimide tubes. A microdialysis cannula (CMA8011085; Harvard Apparatus) was glued adjacent to the polyimide tubes containing tetrodes, such that a minimum of 3 mm of cannula protruded from the guide tube. Tetrodes were cut with tetrode-cutting scissors (FST 14058-11) to ~0.5 mm from the tip of the cannula. Reference wire was cut to a similar length at an acute angle. When 1 mm membrane probes (CMA8011081; Harvard Apparatus) were inserted, the tips of the probe and wires were offset by ~0.5mm. Importantly, the horizontal distance between probe wires were ~0.2 mm. Finally, tetrodes were gold-plated to 200-250 kΩ impedance and all wires and pins were covered with liquid electrical tape (Gardner Bender) and allowed to dry. RetroDrives were confirmed to successfully operate in NCM using baclofen/muscimol delivery to locally silence neurons within minutes in an earlier study [Macedo-Lima and Remage-Healey, 2020].

4.3.4.3 Recording protocol

On the day of the recording, a microdialysis probe was perfused with artificial cerebrospinal fluid (aCSF; described below) using a microinjection pump (PHD2000, Harvard Apparatus). RetroDrive wires were dipped in 6.25% DiI (ThermoFisher) in 200-proof ethanol for visualization of electrode tracks. Then, the animal was comfortably restrained and head-fixed. The Kwik-Cast was removed from the craniotomy over one of the hemispheres. Animal and RetroDrive grounds were connected using alligator clips.

The microdialysis probe was inserted through the cannula and the RetroDrive was lowered to NCM (~1.5-2 mm from brain surface). Importantly, tetrodes were positioned medial to the probe such that wires were ~0.5 mm lateral from the stereotaxic zero.

Recordings were made while animals listened to auditory stimuli and aCSF (PRE) followed by SKF-38393 (SKF) followed by aCSF (POST) were infused during song playback to assess within-subject the effects of SKF on responses to auditory stimuli (described in detail below). Recordings were completed within 4 hours of restraint.

Recordings were made from both hemispheres in different days. When recording in the first hemisphere was completed, the craniotomy was resealed with Kwik-Cast and the animal was returned to the home cage. Within 2 days, the second hemisphere recording was made, after which the animal was overdosed with isoflurane and decapitated. The brain was drop-fixed and cryoprotected in 30% sucrose in 10% formalin, and frozen until cutting. Cryostat sections were obtained at 40 μm and imaged to confirm location of wires and probe.

Recordings were amplified and digitized by a 32-channel amplifier and evaluation board (RHD2000 series; Intan technologies) and sampled at 30 kHz using Intan software. An Arduino Uno was connected to the recording computer to deliver TTL pulses to the evaluation board's DAC channel bracketing the beginning and end of the audio stimuli (described below) to optimize detection. Audio playback and TTL pulses were controlled by a custom-made MatLab script which also controlled the Arduino and sent a copy of the audio analog signal to the evaluation board ADC channel.

4.3.4.4 Stimuli

Zebra finch songs were obtained from multiple databases (http://ofer.sci.ccny.cuny.edu/song_database), therefore unlikely to have been familiar to our subjects. Twenty-four song files from unrelated birds were bandpass filtered at 0.5-15 kHz and trimmed to include two consecutive motifs without introductory notes in Adobe Audition (Adobe) and mean amplitude-normalized to 70 dB in Praat [Boersma and van Heuven, 2001]. Songs were randomly and equally split into two sets, then into 3 subsets containing 4 songs each. For each animal, 1 set was used per hemisphere and, within a hemisphere recording, 1 subset was used per treatment. This was done to ensure that for each treatment birds listened to novel stimuli, because NCM neurons exhibit stimulus-specific adaptation [Chew et al., 1996]. Importantly, there was no consistent difference among neuronal responses to different subsets, as a function of the responses to each stimulus and within each neuron (Mixed-effects GLM/ANOVA: Subset: $F_{5,2}$=0.539, p=0.746). Therefore, responses across treatments are comparable as they were presumed to reflect responses to novel stimuli.

Each playback session consisted of 4 conspecific songs, repeated 30 times each in pseudorandomized order. Interstimulus interval was pseudorandom within the interval 5±2 s. Audio pressure was amplified to ~65 dB as measured by a sound level meter (RadioShack). Playback trial duration lasted ~20 min.

Recordings were made from each hemisphere on different days. For each animal's first recording, the starting hemisphere was randomized in the first subjects, then counterbalanced between sexes. The stimulus set was also initially randomized, then

counterbalanced across sexes and hemispheres, but the subset selected for each treatment was always randomized (www.random.org).

4.3.4.5 Drugs and treatment

SKF-38393 (abcam) aliquots were made in double-distilled water (20 mM; 10 µL) [Schicknick et al., 2012] and kept at -20 °C. On the day of the experiment, one aliquot was added to 1 mL (final concentration 0.2 mM as in [Schicknick et al., 2012]) of previously frozen aCSF aliquots (in mM: 199 NaCl, 26.2 $NaHCO_3$, 2.5 KCl, 1 NaH_2PO_4, 1.3 $MgSO_4$, 2.5 $CaCl_2$, 11 Glucose, 0.15 bovine serum albumin; pH 7.4). All aliquots were filtered before being loaded into the RetroDrive. Treatment and playback timeline are described in Fig 5. After each round of playback, treatment syringes were switched and a 30 min infusion period elapsed. Retrodialysis speed was set to 2 µL/min [as in Remage-Healey et al., 2010; Remage-Healey and Joshi, 2012; Vahaba et al., 2017].

4.3.4.6 Analyses

Sound playback was detected using a custom audio convolution algorithm in MatLab.

Recordings were highpass filtered at 300 Hz and common-median filtered in MatLab (MathWorks). Single-unit sorting was done with Kilosort [Pachitariu et al., 2016]. Sorting results were manually curated in Phy (https://github.com/cortex-lab/phy) and only well-isolated units (high signal-to-noise ratio; low violation of refractory period; low contamination with other units; segregation in waveform PCA space) were used.

After sorting, for each single-unit, 2000 waveforms were selected pseudorandomly and measured (peak-to-peak duration and ratio; Fig 4a) in MatLab. All further data processing was done in Python and R.

Baseline firing rates were calculated using 500 ms preceding each stimulus playback trial. Within each treatment condition, baseline and stimulus firing rates were averaged across stimuli and trials. Peristimulus time histograms (PSTHs) were generated using 10-ms time bins.

Z-scores were calculated by the formula $Z = \frac{Mean(S) - Mea\ (B)}{\sqrt{Var(S) + Var(B) - 2(Cov(S,B))}}$, where S and B are the stimulus and baseline firing rates across stimulus trials, respectively. After computing z-score by stimulus, those were averaged to yield a single z-score per unit per treatment.

Adaptation rates were calculated using trials 6-25, which is the approximate-linear phase of the adaptation profile in NCM [Phan et al., 2006]. For each stimulus, the stimulus firing rate across trials was normalized by the firing on trial 6 (set to 100%). Then, a linear regression was calculated between trials 6 and 25. For each treatment, the minimum (steepest) adaptation slope across stimuli was used for each unit.

Latency to respond to stimuli were calculated as in Ono et al. [2016]. Briefly, for each stimulus, 5-ms PSTH were generated and convolved with a 5-point box-filter. The latency to respond to a stimulus was the time after stimulus onset in which the filtered PSTH rose above 3 standard deviations of the average preceding baseline period (100 ms). If threshold was not crossed within 400 ms, that stimulus was excluded from analyses.

4.3.5 Experimental Design and Statistical Analyses

All statistical analyses and plotting were performed using libraries for R and Python, respectively.

Our general statistical approach was to perform general linear modeling (GLM; 'lme4' R package) for multiple-factor analyses followed by ANOVA with Kenward-Roger's degree-of-freedom approximation. For single-factor analyses, we performed GLM and assessed normality and homoscedasticity of residuals (Shapiro-Wilk and Levene tests, respectively). If data violated either of those tests, data were log-transformed when possible (non-negative, non-zero data) and GLM/ANOVA was run on transformed data. When log-transformation was not possible (i.e., zeroes in the dataset) or data still violated normality-homoscedasticity assumptions, non-parametric analyses were performed (Kruskal-Wallis followed by Dunn's post-hoc tests, Friedman test or Wilcoxon signed rank tests).

For immunofluorescence data, quantifications of the two sections belonging to the same hemisphere and animal were averaged. Data were analyzed by mixed-effects GLM followed by ANOVA, with Hemisphere and Area (dorsal vs ventral) as repeated factors over Subjects. For the GABA-D1-PV analyses, tissue from the right hemisphere of one female was excluded because NCM could not be confidently localized (off-plane section). Although animals of both sexes were used, we did not have power to detect sex differences. Nevertheless, no qualitative sex differences were observed. To test coexpression proportions, chi-square tests were performed on the total sum of cells per label to form a 2x2 contingency table (e.g. ARO+/ARO– vs D1R+/D1R–). Pearson's z-scored residuals were analyzed to obtain corresponding one-tailed p-values.

For patch clamp recordings, minute-by-minute-binned data were analyzed by mixed-effects GLM followed by ANOVA, using Time bins (60 seconds) as a repeating factor over Cells, and Treatment or Dose as independent factor. Bin 1 corresponded to the

minute before SKF treatment; bins 2-8 corresponded to the SKF treatment. Washout was excluded from these analyses and is presented for visualization in all figures. When main factors were significant, post-hoc Dunnett tests were used to compare treatment bins with bin 1 (control). When interactions were significant, single-factor repeated-measures ANOVA were performed in each Experiment individually, followed by post-hoc Dunnett tests.

For *in vivo* recordings, cell types were classified using 2-D hierarchical clustering ('stats' R package) on peak-to-peak duration vs ratio measurements. The optimal number of clusters was determined using the package Nbclust [Charrad et al., 2014] with the gap statistic method. After clustering, each unit's auditory responsiveness was tested by Wilcoxon tests (30x baseline vs 30x stimulus trials per song during aCSF infusion). Cells responsive to at least one song were included in all following analyses. Before-drug (PRE) differences among cell types were tested using Kruskal-Wallis tests and Dunn's post-hoc tests with Benjamini-Yekutieli false-discovery rate adjustments. Treatment data were analyzed by mixed-effects GLM followed by ANOVA using Treatment (repeated) and Cell type as factors; Tukey post-hoc tests were used when ANOVA was significant. Due to statistical power limitations, we performed separate analyses excluding Cell type and including Hemisphere and Sex as factors. However, no systematic sex or hemisphere differences with treatment were detected. Washout (POST) was excluded from analyses.

4.4 Results

4.4.1 Aromatase and D1-receptor proteins are coexpressed by NCM neurons

We found that D1-receptor-protein-positive (D1R+) neurons are often found coexpressing aromatase, representing 29.6 and 35.4% of the aromatase+ (ARO+)

neuronal subpopulation in dNCM and vNCM respectively (Fig. 4.1C). Moreover, the population of D1R+/ARO+ neurons represents 6.6 and 10% of the neuronal population (DAPI nuclei) in dNCM and vNCM, respectively. A chi-square test analyzing these proportions yielded a significant relationship between D1R and ARO counts ($\chi^2(1)=22.210$, $p<0.001$), with double-labeled cells significantly more frequent than expected (ARO+/D1R+ Pearson's residual=3.609, $p<0.001$).

Interestingly, we frequently observed tyrosine hydroxylase+ (TH+) fibers enveloping D1R+/ARO+ neurons (Fig. 4.1D; not formally quantified).

D1R+ neurons were observed in similar densities and proportions (over DAPI) between hemispheres and between vNCM and dNCM. However, we found that vNCM contains significantly higher percentage of ARO+ neurons (Hemisphere: $F_{1,9}=0.105$, $p=0.753$; Area: $F_{1,9}=9.179$, $p=0.014$, Hemisphere*Area: $F_{1,9}=0.435$, $p=0.526$). Interestingly, vNCM also had a higher percentage of double-labeled D1R+/ARO+ (Hemisphere: $F_{1,9}=1.707$, $p=0.224$; Area: $F_{1,9}=11.211$, $p=0.009$, Hemisphere*Area: $F_{1,9}=2.182$, $p=0.174$), but not of D1R–/ARO+ (Hemisphere: $F_{1,9}=0.165$, $p=0.694$; Area: $F_{1,9}=2.310$, $p=0.163$, Hemisphere*Area: $F_{1,9}=0.014$, $p=0.909$). Of note, dorsal NCM (dNCM) contained higher density of DAPI nuclei (in $10^3\times$cells/mm^3; dNCM: 776±31.3; vNCM: 642±22.4; Hemisphere: $F_{1,9}=0.267$, $p=0.618$; Area: $F_{1,9}=18.411$, $p=0.002$, Hemisphere*Area: $F_{1,9}=0.000$, $p=1.000$), the same regional pattern as recently demonstrated in juveniles [Vahaba et al., 2020]. This potentially underlies the increase in the percentage of ARO+ and D1R+/ARO+ neurons we observed (since neither of these densities change). Together these data show that D1R protein is prevalent in NCM

neurons and that D1R+ neurons represent a significant population of NCM neurons.

Moreover, ARO+/D1R+ neurons are also prevalent, particularly in vNCM.

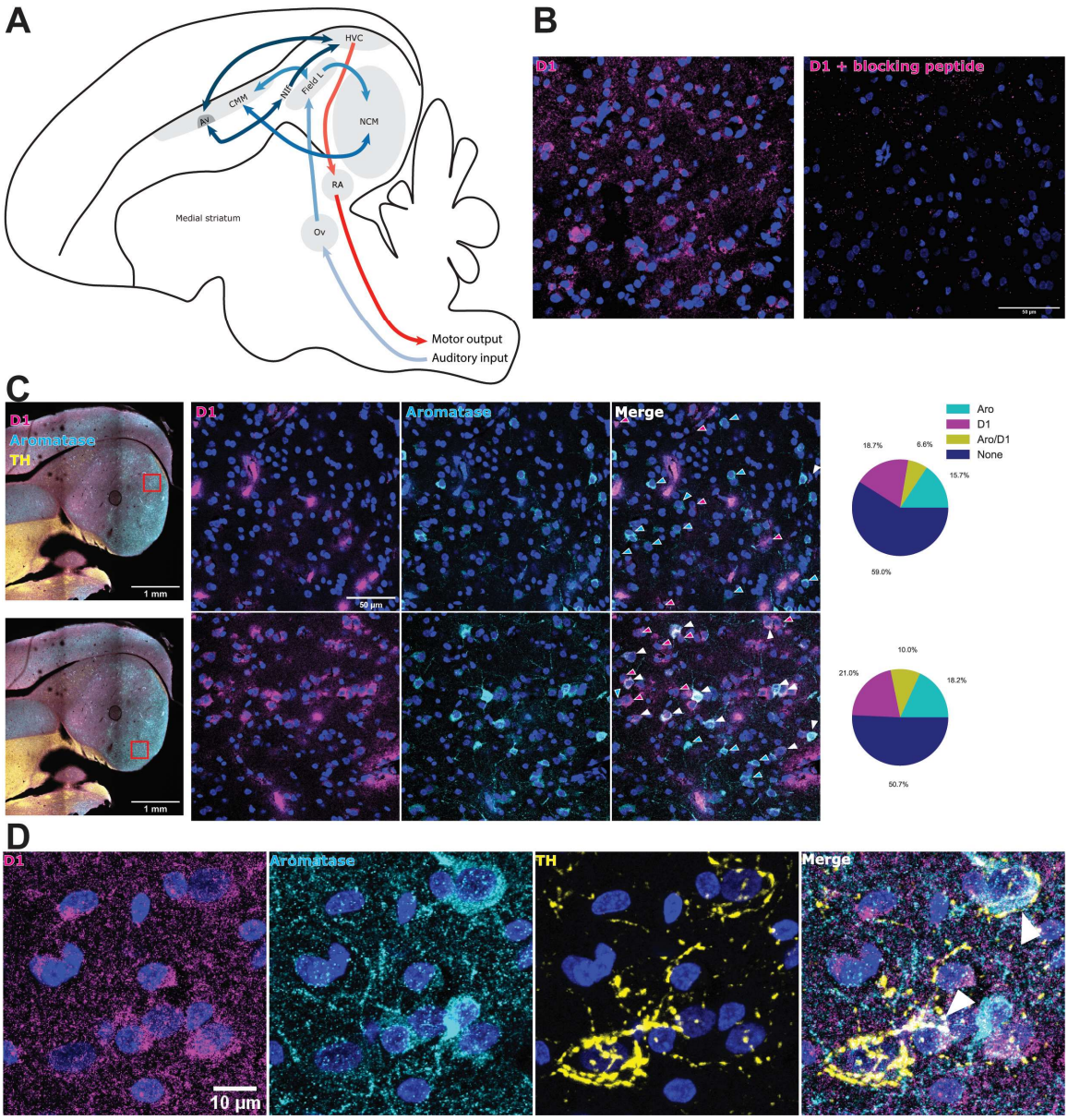

Figure 4.1: Aromatase and D1 receptor (D1R) proteins are coexpressed by NCM neurons.

(A) Songbird auditory circuits. Lighter-to-darker colors illustrate the trajectory of auditory (blue) and motor (red) information. (B) Specificity confirmation of the anti-D1R antibody used here. Preincubation with blocking peptide virtually eliminated cellular staining. (C) Triple immunofluorescence staining for D1R, aromatase and tyrosine hydroxylase (TH). Magenta and cyan arrows indicate single-labeled D1R and aromatase+ neurons, respectively. White arrows indicate double-labeling. (D) TH fibers are frequently found in association with double-labeled neurons. Note that fibers tightly envelop double-labeled soma.

4.4.2 D1R+ neurons are predominantly GABA+

We found that the majority (58.7 and 64.2%) of D1R+ neurons are GABA+, and these colabeled neurons represent 21.9 and 26.4% of the neuronal population in dNCM and vNCM, respectively. The reciprocal is also true, such that 54.6% (dNCM) and 56.5% (vNCM) of GABA+ neurons also contain D1R (Fig. 4.2A). A chi-square test analyzing these proportions yielded a significant relationship between D1R and GABA counts ($\chi^2(1)=759.6$, $p<0.001$), with double-labeled cells significantly more frequent than expected (GABA+/D1R+ Pearson's residual=17.791, $p<0.001$). Similarly, we observed that the majority (~55%) of parvalbumin+ (PV+) neurons also express D1R, combining vNCM and dNCM (Fig. 4.2B), and these represent ~4% of the NCM neuronal population (Fig. 4.2A). A chi-square test analyzing these proportions yielded a significant relationship between D1R and PV counts ($\chi^2(1)=29.3$, $p<0.001$), with double-labeled cells significantly more frequent than expected (PV+/D1R+ Pearson's residual=4.044, $p<0.001$).

We did not find differences in the densities or percentages (over DAPI) of D1R+, GABA+ or D1R+/GABA+ neurons between hemispheres or areas. However, a greater percentage of D1R+ neurons contained GABA in the left hemisphere and in the vNCM (Hemisphere: $F_{1,10.265}=10.542$, $p=0.005$; Area: $F_{1,14.040}=10.617$, $p=0.006$, Hemisphere*Area: $F_{1,14.040}=1.173$, $p=0.297$). Altogether, these results show that D1R+ neurons are predominantly GABA+ and represent a significant subpopulation in NCM. Of note, our data show that the majority of PV+ neurons express D1Rs, which suggests this subpopulation is of particular interest for dopamine modulation of auditory processing.

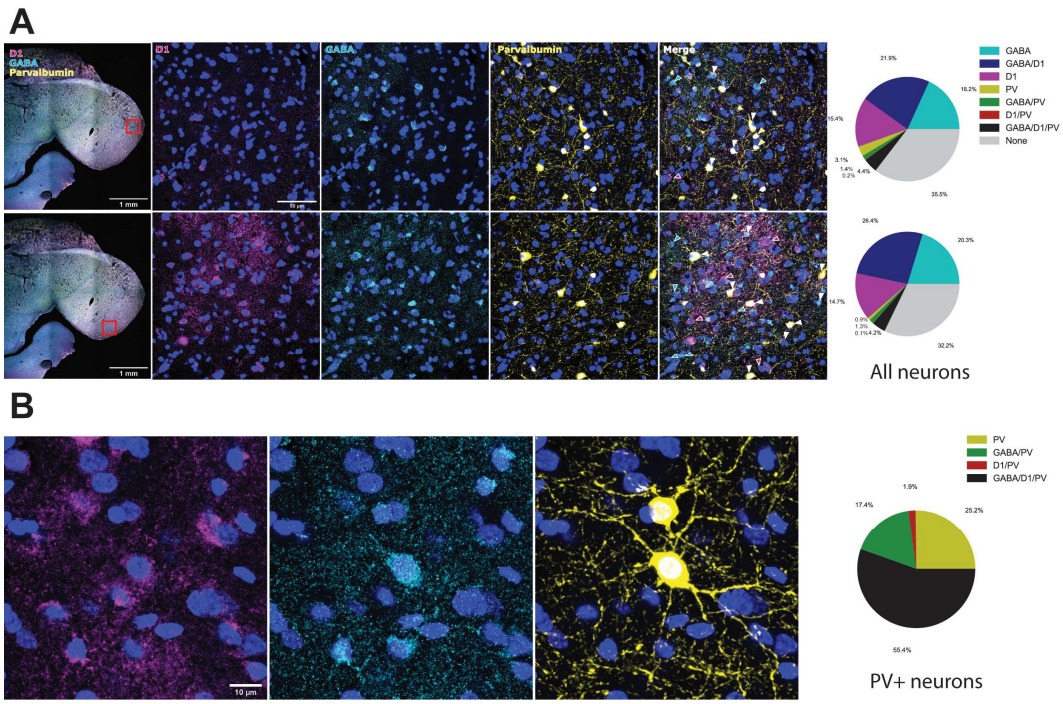

Figure 4.2: D1 receptor (D1R)-containing neurons are predominantly GABA+. (A) Triple immunofluorescence stain for D1R, GABA and parvalbumin (PV). Magenta, cyan and yellow arrows indicate single-labeled D1R, GABA and PV+ neurons, respectively. White arrows indicate triple-labeling. (B) The majority of PV+ neurons also express GABA and D1Rs.

4.4.3 D1R activation reduces the amplitude of GABAergic currents in NCM *in vitro*

We recorded spontaneous postsynaptic currents from neurons in NCM *in vitro* (Fig. 4.3A). Inhibitory currents were isolated with the AMPA receptor antagonist DNQX (sIPSCs; Fig. 4.3B). In separate sets of cells, we either applied 10 µM SKF or nothing (rundown) to the bath.

For amplitude measurements, GLM analyses comparing treatments (SKF or rundown) detected a significant interaction between Time and Treatment (log-transformed data: Time: $F_{7,63}$=3.219, p=0.006; Treatment: $F_{1,9}$=0.337, p=0.576; Time*Treatment: $F_{7,63}$=2.706, p=0.016). Examining each experiment individually, SKF-38393 significantly reduced the sIPSC amplitude (RM-ANOVA on log-transformed data:

$F_{7,42}$=5.716, p<0.001; Dunnett's post-hoc test vs before-treatment: p<0.05 between minutes 4-7 of SKF). Rundown experiments did not show the same reduction (RM-ANOVA on log-transformed data: $F_{7,21}$=0.790, p=0.603). For frequency measurements, GLM analyses contrasting treatments did not detect significant differences between SKF-38393 and rundown, but detected a significant reduction of sIPSC frequency over time (Time: $F_{7,63}$=4.531, p<0.001; Treatment: $F_{1,9}$=0.042, p=0.843; Time*Treatment: $F_{7,63}$=0.265, p=0.965; Dunnett's post-hoc test vs before-treatment: p<0.05 between minutes 5-7). These results show that SKF-38393 treatment significantly reduced the amplitude of GABAergic currents. The reduction in frequency observed in SKF-38393 treatments did not differ from rundowns indicating that, regardless of treatment, the number of detected sIPSCs decayed over time.

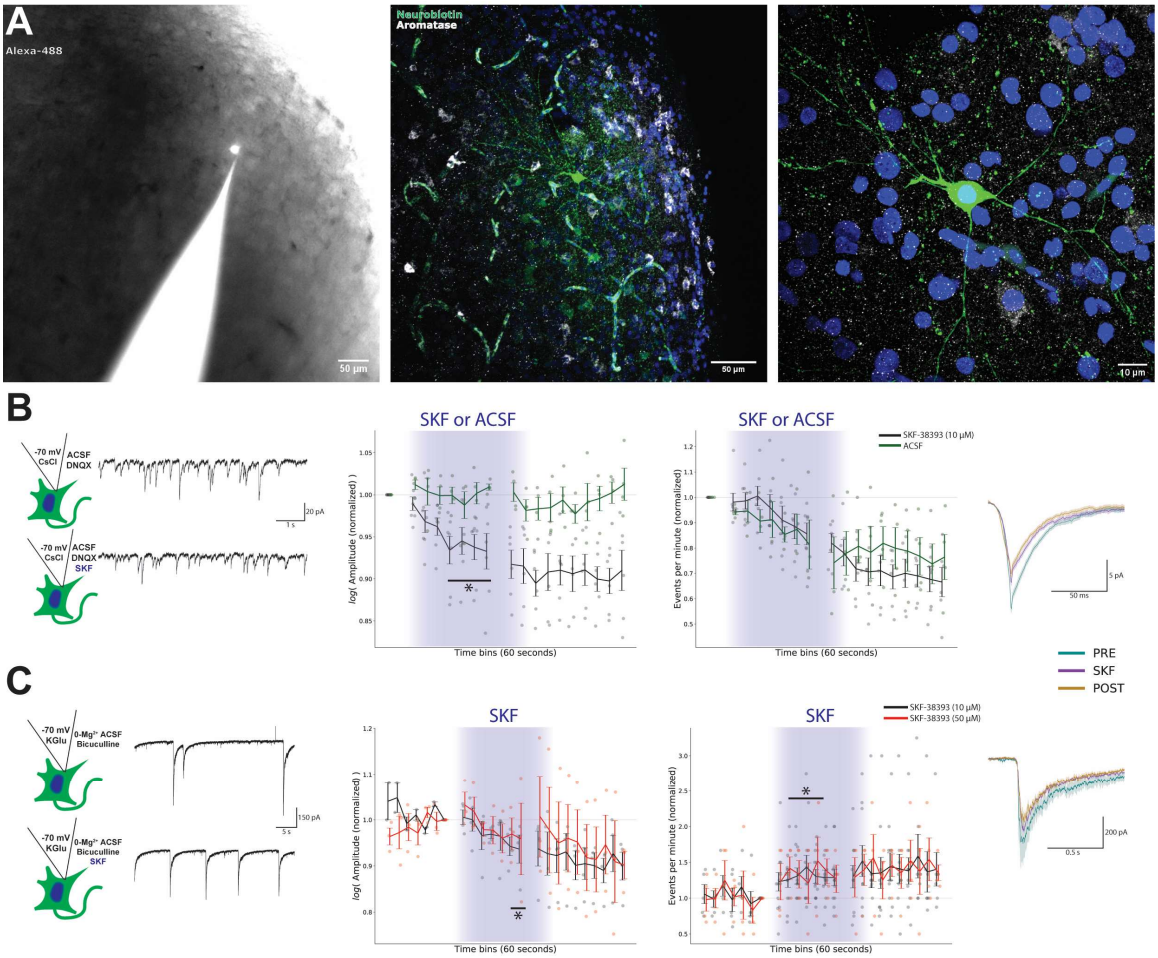

Figure 4.3: D1R activation reduces spontaneous GABAergic (sIPSC) and glutamatergic (sEPSC) currents amplitude but increases sEPSC frequency. (A) Representative neuron in NCM visualized during (left; Alexa-488) and after recording (Neurobiotin-Streptavidin-488 stain; aromatase stain to confirm NCM location). (B) Representative sIPSCs recording from NCM neuron, before and during 10 µM SKF-38393 infusion (left panel). Amplitude of sIPSCs (middle panel) was significantly different from rundowns (aCSF) and reduced from baseline by SKF (4-7 min). Frequency (right panel) was also reduced but did not differ from rundown experiments (5-7 min). Waveforms show representative sIPSC (mean±SEM during 1 min) before (PRE), on the last minute of (SKF) and on the last minute after (POST) drug infusion. (C) Representative AMPA/NMDA/Kainate sEPSCs recording from NCM neuron, before and during 10 µM SKF-38393 infusion (left panel). Amplitude of sEPSCs (middle panel) was significantly reduced by 10 and 50 µM (no dose difference) SKF (6-7 min). However, frequency (right panel) was increased by SKF (2-5 min). In these experiments, rundowns were done before treatment and did not significantly change. Waveforms show representative sEPSC (mean±SEM during 1 min) before (PRE), on the last minute of (SKF) and on the last minute after (POST) drug infusion.

4.4.4 D1R activation reduces the amplitude but increases the frequency of glutamatergic currents in NCM *in vitro*

Excitatory NMDA/AMPA/Kainate currents (sEPSCs) were isolated in 0-Mg^{2+} bath containing the $GABA_a$-receptor antagonist bicuculline (Fig. 4.3C). For these experiments we used two doses (10 and 50 μM) of SKF-38393 in different sets of cells. In these experiments we performed rundown experiments before SKF-38393 treatment in a subset of cells (n=4 for amplitude; n=8 for frequency; see methods). Both amplitude and frequency of sEPSCs were stable during 7 minutes before treatment (RM-ANOVA: Amplitude: $F_{6,24}$=0.673, p=0.673; Frequency: $F_{6,48}$=1.200, p=0.323).

For amplitude measurements after SKF-38393 treatment, we performed a GLM analysis on the effect of different doses of SKF-38393 over time. These analyses showed a reduction in the amplitude of sEPSCs over time due to treatment, but no difference between doses (Time: $F_{7,35}$=4.292, p=0.002; Dose: $F_{1,5}$=0.009, p=0.930; Time*Dose: $F_{7,35}$=0.427, p=0.878; Dunnett's post-hoc test vs before-drug: p<0.05 on minute 7 of SKF). For frequency measurements, GLM analyses showed a trend for a change in sEPSC frequency (Time: $F_{7,98}$=1.998, p=0.063; Dose: $F_{1,14}$=0.163, p=0.163; Time*Dose: $F_{7,98}$=0.499, p=0.833).

Since no effects of dose were observed, we combined the datasets and performed simpler model analyses for increased power. A 1-way RM-ANOVA showed a decrease in amplitude of sEPSCs due to SKF-38393 treatment (log-transformed; $F_{7,42}$=4.868, p<0.001; Dunnett's post-hoc test vs before-drug: p<0.05 between minutes 6-7 of SKF). Further, this analysis demonstrated an increase in frequency of sEPSCs due to SKF-38393 treatment ($F_{7,105}$=2.819, p=0.010; Dunnett's post-hoc test vs before-drug: p<0.05

between minutes 2-5 of SKF). These experiments show that SKF-38393 treatment reduces the amplitude but increases the frequency of sEPSCs.

In summary, D1R activation *in vitro* reduced the amplitude of both GABA and glutamatergic spontaneous currents but increased the frequency of the latter. These findings establish the prediction that D1R activation *in vivo* causes differential effects depending on cell type, namely downregulate and upregulate GABAergic and glutamatergic neuron firing, respectively.

4.4.5 Cell type separation based on waveform measurements in *in vivo* recordings

We isolated 107 single-units from 9 adult birds in awake head-fixed recordings using the RetroDrive. We measured peak-to-peak duration and ratio of each unit and analyzed the data using an unsupervised hierarchical clustering algorithm (see methods; Fig. 4.4A). The gap-statistic results show that the variance in clustering is better and more parsimoniously explained by 4 clusters. The classification commonly used in the literature of narrow- and broad-spiking neurons in songbird cortex uses only peak-to-peak duration and a division boundary of ~0.4 ms [Schneider and Woolley, 2013; Yanagihara and Yazaki-Sugiyama, 2016; Vahaba et al., 2017; Aurore et al., 2019]. Our data provides evidence of 2 further subdivisions; therefore, we named our clusters to extend the previous classification: NS1 and NS2 – narrow-spiking; peak-to-peak < 0.4 ms; and BS1 and BS2 – broad-spiking; peak-to-peak > 0.4 ms.

Following clustering, non-auditory-responsive cells were excluded from the analyses (see methods) and 92 units were further analyzed. This sample consisted of 21 (L) and 23 (R) units from females and 34 (L) and 14 (R) units from males. Representative PSTHs and adaptation slopes of a NS1 and a BS1 are shown in Fig. 4.4B.

Cell type classification reflected on differences in physiology phenotypes assessed during aCSF infusion (Fig. 4.4C). Cell types differed in baseline firing rates (Kruskal-Wallis test: $\chi^2(3)=24.554$, $p<0.001$; Dunn's post-hoc: $p<05$ in NS1–BS2 and NS2–BS2), stimulus firing rates (Kruskal-Wallis test: $\chi^2(3)=23.199$, $p<0.001$; Dunn's post-hoc: $p<05$ in NS1–BS1, NS1–BS2 and NS2–BS2), z-scores (Kruskal-Wallis test: $\chi^2(3)=20.682$, $p<0.001$; Dunn's post-hoc: $p<05$ in NS1–BS1 and NS1–BS2), adaptation slopes (Kruskal-Wallis test: $\chi^2(3)=8.488$, $p=0.037$; Dunn's post-hoc: all $p>0.05$), latencies to respond (Kruskal-Wallis test: $\chi^2(3)=12.715$, $p=0.005$; Dunn's post-hoc: $p<05$ in NS1–BS2) and in the % of songs responded to (Kruskal-Wallis test: $\chi^2(3)=17.994$, $p<0.001$; Dunn's post-hoc: $p<05$ in NS1–BS1 and NS1–BS2). Therefore, the 4 cell types clustered by waveform shape in our recordings also differ in physiological profile. NS1 cells have symmetrical and narrow action potentials, high firing rates and z-scores, as well as fast response latencies and lower selectivity, which all parallel features of mammalian cortical high-firing inhibitory interneurons. All other cell types show statistically similar physiological features among themselves, with the exception that BS cells are statistically more selective than NS1. However, they do differ in waveform shape: NS2 cells have asymmetrical and narrow spike shapes; BS1 cells have asymmetrical and broad spike shapes; and finally, BS2 cells have the most asymmetrical and broadest spike shapes.

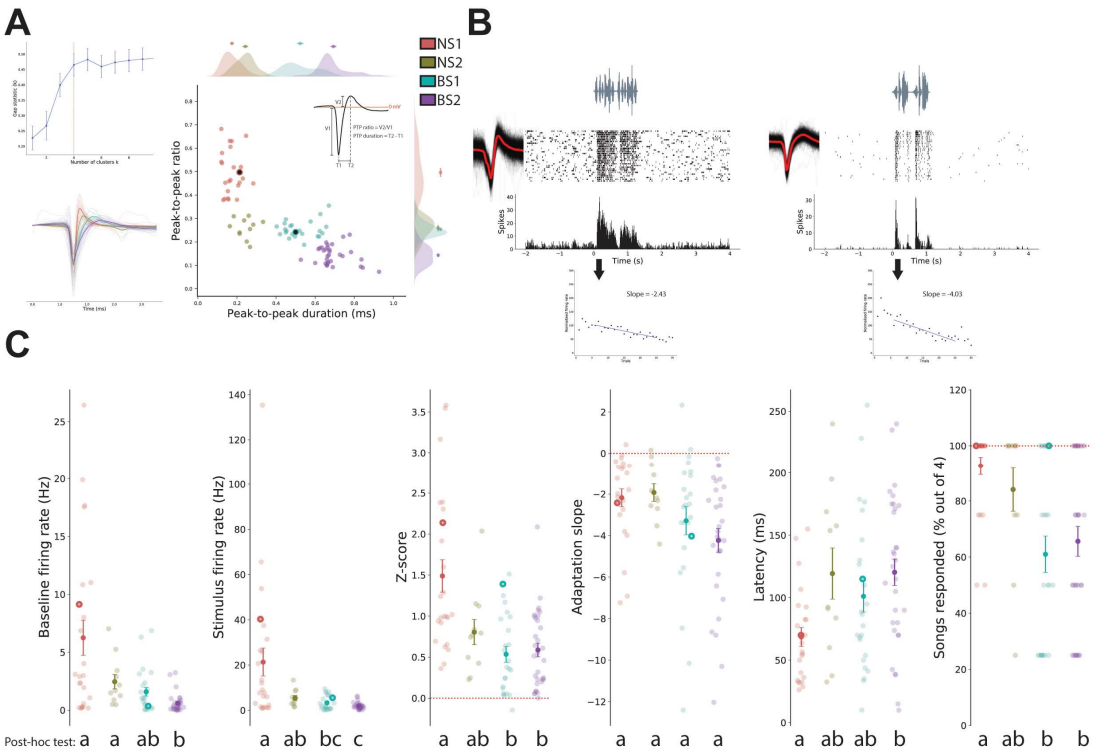

Figure 4.4: Cell type separation based on waveform measurements in *in vivo* recordings.
(A) Unsupervised hierarchical clustering results using peak-to-peak ratio vs duration optimally yielded 4 distinct clusters. Traces illustrate waveform averages by cluster type. The correlation plot shows the 4 distinct clusters and the kernel-density estimations of the distributions along single-axis with mean±SEM on top. (B) Representative PSTHs and adaptation slopes of NS1 (left) and BS1 (right) cell types. Representative examples in B are highlighted in all graphs according to cluster type. (C) Physiology parameters of the different cluster types. From left to right: baseline and stimulus firing rate, z-score, adaptation slope, latency to respond to song and percentage of songs responded to. Post-hoc test results are shown at the bottom. Significant differences ($p < 0.05$) are assigned different letters.

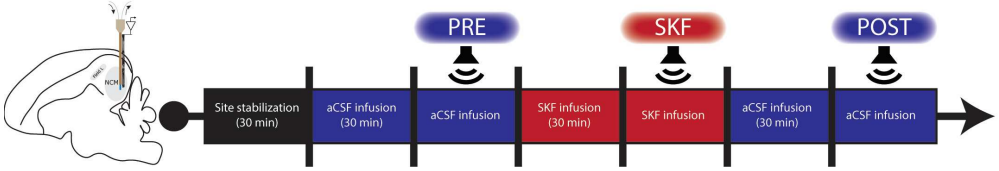

Figure 4.5: Timeline for drug infusions and playbacks starting after auditory site localization within NCM.

4.4.6 D1R activation reduces baseline and stimulus firing of NS1, but increases baseline firing of BS1 neurons *in vivo*

We next analyzed how SKF-38393 (SKF; 0.2 mM) affected single-unit responses to sound playback (timeline on Fig. 4.5).

Representative PSTHs for NS1 and BS1 cells are shown in Fig. 4.6A.

For baseline firing rate, GLM analyses followed by ANOVA comparing Treatment and Cell-Type show that SKF reduced the firing of NS1, while increased the firing of BS1 cells (Fig. 4.6B; Treatment: $F_{1,88}$=0.023, p=0.881; Cell-Type: $F_{3,88}$=7.132, p<0.001; Treatment*Cell-Type: $F_{3,88}$=4.397, p=0.006; Tukey's post-hoc test; PRE–SKF: NS1: t_{88}=2.893, p=0.005; NS2: t_{88}=–0.02, p=0.984; BS1: t_{88}=–2.139, p=0.035; BS2: t_{88}=–0.503, p=0.616).

For stimulus firing rate, GLM/ANOVA results showed that SKF decreased firing of NS1 cells (Fig. 4.6C; Treatment: $F_{1,88}$=2.399, p=0.125; Cell-Type: $F_{3,88}$=10.873, p<0.001; Treatment*Cell-Type: $F_{3,88}$=4.090, p=0.009; Tukey's post-hoc test; PRE–SKF: NS1: t_{88}=3.862, p<0.001; NS2: t_{88}=0.065, p=0.948; BS1: t_{88}=–0.506, p=0.614; BS2: t_{88}=–0.043, p=0.966).

To further illustrate the change in baseline vs stimulus firing due to SKF, we show a correlation between the %-change in baseline versus stimulus firing induced by SKF (Fig. 4.6D). Values above 0 in either axis indicate an increase in firing due to SKF. On average BS1 and BS2 data points are situated above 0 in both axes, while NS1 and NS2 data points are below 0. Changes in baseline vs stimulus firing are highly correlated (Pearson's r=0.876, p<0.001). Interestingly, the regression line slope's 95% confidence interval [0.693; 0.875] does not include and is lower than the slope of the identity line (slope=1), which suggests that baseline firing was more affected than stimulus firing.

For z-scores, GLM/ANOVA results showed that SKF decreased overall z-scores regardless of cell type (Fig. 4.6E; Treatment: $F_{1,88}$=6.701, p=0.011; Cell-Type: $F_{3,88}$=14.939, p<0.001; Treatment*Cell-Type: $F_{3,88}$=1.417, p=0.243).

Altogether, these data show that the D1R agonist SKF-38393 affects cell types differently, i.e. by reducing the firing of NS1 and increasing the firing of BS1. Moreover, regression analyses suggest that D1R activation is primarily mediating changes in baseline firing.

SKF-38393 treatment increased latency to respond (Fig. 4.6F; Treatment: $F_{1,86.488}$=5.250, p=0.024; Cell-Type: $F_{3,87.124}$=3.851, p=0.012; Treatment*Cell-Type: $F_{3,86.720}$=0.397, p=0.756).

Finally, SKF-38393 treatment decreased the % of songs units responded to (Fig. 4.6G; Treatment: $F_{1,88}$=7.350, p=0.008; Cell-Type: $F_{3,88}$=10.247, p<0.001; Treatment*Cell-Type: $F_{3,88}$=1.409, p=0.245) regardless of cell type.

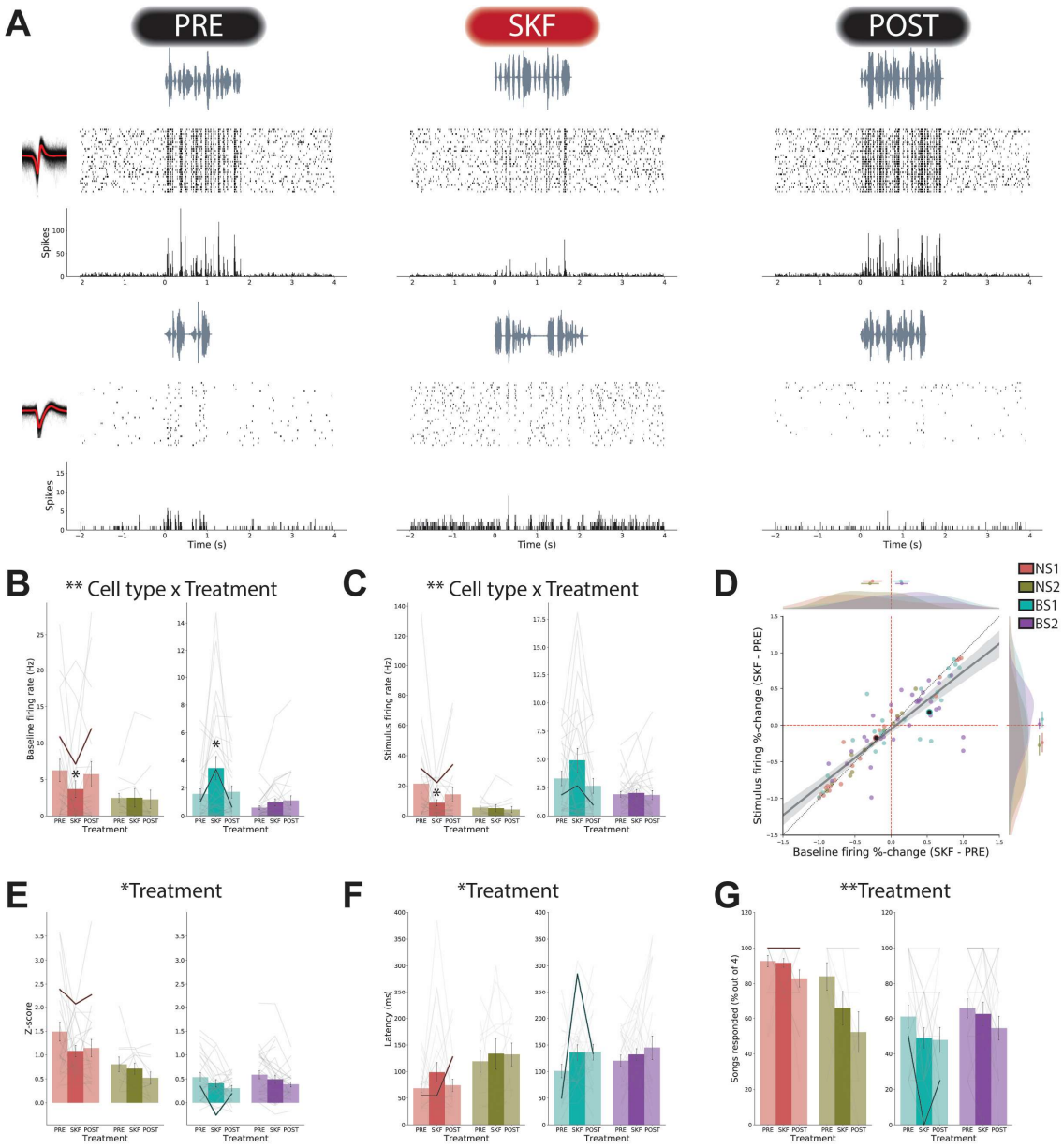

Figure 4.6: Effects of D1R activation *in vivo* on firing characteristics of NCM neurons.

(A) Representative PSTHs of a NS1 (top) and BS1 (bottom) cell in response to SKF. Note the change in stimulus and baseline firing in NS1 and BS1, respectively, due to SKF-38393 (0.2 mM). Representative cells are highlighted in all plots according to their cell type. (B) Baseline firing rate. D1R agonist reduced firing of NS1, but increased firing of BS1 cells. (C) Stimulus firing rate. D1R agonist reduced firing of NS1 cells. (D) Correlation between baseline and stimulus %-change during SKF infusion accompanied by kernel-density estimations of the distributions along single-axis with mean±SEM on top. Regression line across all datapoints is shown (solid line). Identity line is shown as a dotted line. The regression slope 95% confidence interval (shaded area) is less than and does not encompass 1, suggesting that baseline changes are higher than stimulus changes. (E) D1R agonist reduced Z-score, (F) increased latency and (G) decreased responsiveness

of NCM cells. Relevant statistical effects are highlighted on top of each plot. Post-hoc Tukey test results are displayed on the plots when interaction was significant. *p<0.05, **p<0.01.

4.4.7 D1R activation disrupts neuronal stimulus-specific adaptation *in vivo*

NCM neurons show stimulus-specific adaptation when birds are presented to repetitions of the same stimuli, which reflect short/medium-term memory formation [Chew et al., 1996; Lu and Vicario, 2017]. Therefore, we asked whether D1R activation would result in changes in adaptation to novel stimuli. Trial-by-trial stimulus firing rate were used for deriving adaptation slopes (see methods). Eight cells (3 BS1, 5 BS2) had to be removed from the analyses, because firing rate on the first trial used for the regression (trial 6; see methods) was 0 during either PRE or SKF playbacks. Representative rasters and corresponding slopes of an NS1 cell is shown in Fig. 4.7A. Fig. 4.7B depicts the slope through the normalized firing rate of trials 6-25 averaged by cell type. GLM/ANOVA analyses showed that SKF-38393 infusion reduced adaptation slopes, regardless of cell type (Fig. 4.7C; Treatment: $F_{1,80}$=8.794, p=0.004; Cell-Type: $F_{3,80}$=6.672, p<0.001; Treatment*Cell-Type: $F_{3,80}$=1.665, p=0.181). Note, however that this effect seems to be driven by all cell types but for BS2, which on average appears to remain unchanged by SKF. In fact, BS2 cells are the only group that retain non-zero slopes during SKF treatment (One-sample Wilcoxon signed-rank tests versus 0; NS1: p=0.308; NS2: p=0.549; BS1: p=0.052; BS2: p<0.001). Therefore, D1R activation disrupts stimulus-specific adaptation profiles of NCM neurons, which suggests adaptation and memory formation in NCM are modulated by local D1-receptors.

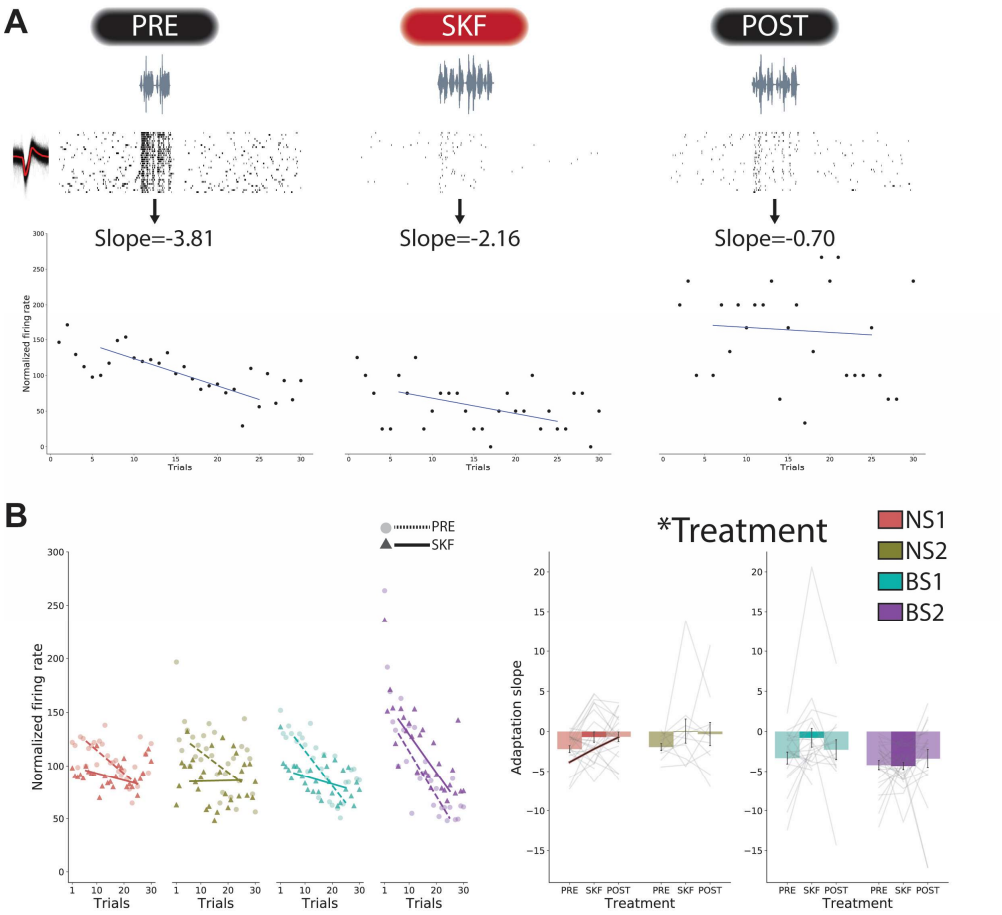

Figure 4.7: D1R activation disrupts neuronal stimulus-specific adaptation *in vivo*. (A) Representative PSTHs from NS1 highlighting a decrease in adaptation slope to a new stimulus due to SKF-38393 (0.2 mM) treatment. (B) The slope through the normalized firing rate of trials 6-25 averaged by cell type. (C) Adaptation slopes quantifications show a reduction in the slope due to SKF-38393. Relevant statistical effect is displayed on the top of the plot. *p<0.05.

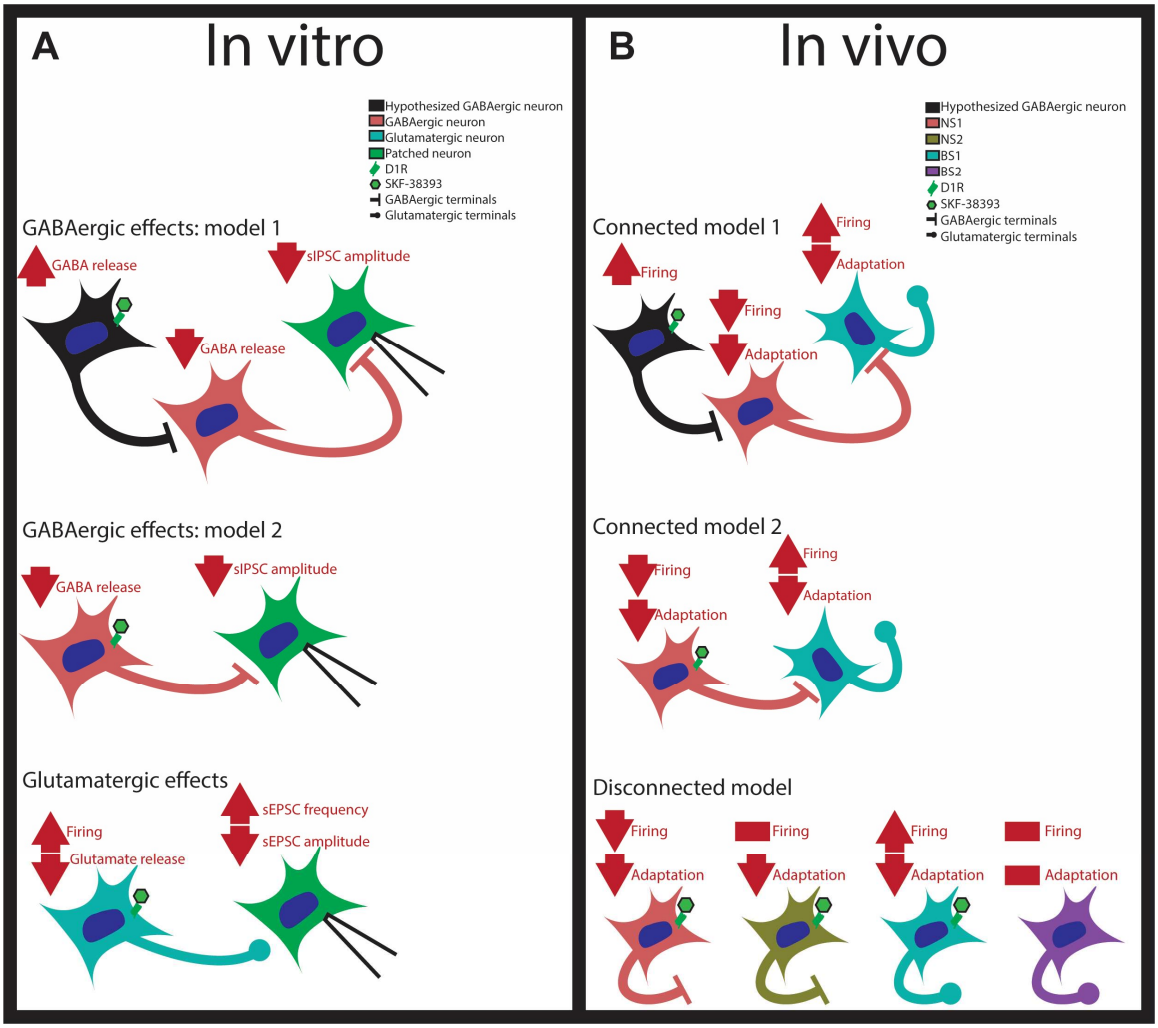

Figure 4.8: Network connectivity models hypothesized from present data. SKF-38393 effects are shown in red. (A) Models for *in vitro* results with sIPSCs (GABAergic) and sEPSCs (glutamatergic). GABAergic model 1 hypothesizes that D1R agonist binds preferentially to a GABAergic neuron upstream from the ones providing input to the recorded neuron. Binding of D1R agonist increases its GABA tone resulting in a reduction of GABA release immediately upstream of the recorded neuron. GABAergic model 2 suggests D1R effects directly cause a reduction in GABA release by the upstream neuron. Glutamatergic effects can be explained by a direct effect of D1R agonist on a glutamatergic neuron directly upstream from the recorded neuron, causing a reduction (perhaps a depletion) of glutamate release but an increase in firing rate. (B) Models for *in vivo* results. Connected models only depict cell types with changes in firing rate. Model one hypothesizes that D1R agonist binds to and excites a GABAergic neuron upstream of NS1 cells, which results in a reduction of NS1 firing and consequent disinhibition of BS1 cells. Model 2 offers a direct effect of D1R agonist on NS1 cells, resulting in a reduction in their firing, which results in disinhibition of BS1 cells. In the disconnected model, the effects observed here are due to D1R's binding to all cell types individually (except BS2, in which no effects were observed).

4.5 Discussion

In this study, we show that dopamine D1 receptors (D1R) mediate synaptic plasticity in the secondary association cortex (NCM) of a songbird. Specifically, we show that D1R protein is prevalent in NCM neurons, especially in aromatase-, GABA-, and parvalbumin-positive neurons. Activating D1R *in vitro* reduces the amplitude of GABAergic currents and decreases the amplitude but increases the frequency of glutamatergic currents. Activating D1R *in vivo* reduces firing of putative-inhibitory interneurons, while increases firing of putative-excitatory projection neurons. Finally, we show D1R activation disrupts stimulus-specific adaptation in NCM neurons, a phenomenon reflective of auditory memory formation.

A distinct feature of NCM among the auditory forebrain nuclei is the intense labeling for somatic aromatase, an enzyme that mediates conversion of testosterone into estradiol (E2) [Saldanha et al., 2000]. E2 production in NCM is elevated during social interactions and song playbacks [Remage-Healey et al., 2008; Remage-Healey et al., 2012], and extrinsic E2 application increases neuronal response to song playbacks [Remage-Healey et al., 2010]. We recently showed that blocking aromatase locally in NCM slows auditory association learning in an operant task [Macedo-Lima and Remage-Healey, 2020]. This finding led to the hypothesis that dopamine (DA) interacts with E2 signaling in NCM to support association learning. This line of reasoning is also supported by reports that DA in the auditory cortex mediates auditory association learning in gerbils [Schicknick et al., 2012], and that DA innervation and release are increased by steroid hormones in songbird NCM [Matragrano et al., 2011; Rodríguez-Saltos et al., 2018]. In the present study, we provide anatomical evidence to support this hypothesis, since ~33% of aromatase+ neurons coexpress D1R protein and these co-labeled neurons represent

~8% of all neurons in NCM (Fig. 4.1C). Interestingly, in striatum and preoptic area, E2 and DA systems cross-modulate [Becker, 1990; Lammers et al., 1999; Balthazart et al., 2002; Tozzi et al., 2015] and DA and E2 have been suggested to cross-activate each other's receptors [Olesen and Auger, 2008; Tozzi et al., 2015]. Our data suggests that in NCM E2 and DA signaling are acting in tandem to modulate learning and memory in the songbird auditory cortex. Thus, we hypothesize that this framework could apply to other vertebrates and brain circuits that contain aromatase and DA receptors, such as human auditory cortex [Yague et al., 2006].

Inhibition fundamentally controls neuronal responses to sounds [Wang et al., 2002; Razak and Fuzessery, 2009] and is required for associative learning in mammal auditory cortex [Letzkus et al., 2011]. DA receptors are prevalent in mammalian cortical inhibitory interneurons, especially in parvalbumin-expressing neurons [Le Moine and Gaspar, 1998]. Here, we show in the songbird NCM that D1R and inhibitory markers are frequently coexpressed by NCM neurons, representing more than half of the total GABA+ and D1R+ population, and about 24% of the total NCM population.

Parvalbumin-positive (PV+) cortical interneurons are generally characterized by their fast action potentials and high sustained firing frequency and play a central role in regulating microcircuits, mediating learning processes in hippocampus and cortex [Donato et al., 2013]. In the songbird song control circuit, PV+ neurons are recruited during singing, and PV+ neuron numbers correlate with critical period closure for song learning [Balmer et al., 2009; Yildiz and Woolley, 2017]. In the NCM, PV and calbindin seem to be expressed in different neuron subpopulations, such that PV+ but not calbindin+ neurons can express aromatase [Ikeda et al., 2017]. Here, we show that PV+

neurons represent ~8% of all NCM neurons, and ~11% of GABA+ neurons. Furthermore, 55% of the parvalbumin-expressing neurons in NCM also express D1R. We hypothesize that many of the effects we observed *in vivo* and *in vitro* can be attributed to this subpopulation. This hypothesis is also supported by the waveform phenotype of the NCM neurons we observe are sensitive to SKF-38393 (see below).

D1Rs are generally assumed to increase circuit excitability through $G\alpha_s$-protein coupling [Beaulieu and Gainetdinov, 2011]. In our *in vitro* experiments, D1R agonist SKF-38393 caused a seemingly counterintuitive reduction in the amplitude of GABAergic and glutamatergic currents. However, D1R-mediated depression is well documented in the mammalian nucleus accumbens for both GABAergic and glutamatergic (especially NMDA-mediated) synapses and is attributed to presynaptic plasticity [Pennartz et al., 1992; Zhang et al., 2014]. In contrast, in mammalian cortex, excitability-reducing effects are typically attributed to postsynaptic D2R-mediated effects, while D1Rs mediate excitability increases [Gonzalez-Islas and Hablitz, 2003; Darvish-Ghane et al., 2016]. In our *in vitro* proposed model (Fig. 4.8A), for the reduction of GABA release we suggest two scenarios: 1) D1Rs are predominantly mediating an increase in GABAergic tone by neurons upstream to those providing input to the recorded neuron, therefore reducing GABA release downstream and 2) D1Rs are acting directly on neurons providing input to the recorded neuron causing a direct reduction in GABA release. For the reduction of glutamatergic current amplitude, we suggest a presynaptic mechanism, perhaps explained by a depletion in presynaptic glutamate stores, resulting from the increased firing induced by D1R activation. Alternatively, SKF-38393 could be acting directly on the recorded neuron (postsynaptically) to result in amplitude

reductions. Further experiments with miniature events or with synaptic stimulation could help clarify these questions.

We are at the beginning of our understanding of how non-layered cortical microcircuits operate, including their component cell types. Most of the electrophysiology studies reporting *in vivo* waveform shape segregation in NCM has relied on a non-statistical split of peak-to-peak ratio at ~0.4 ms to divide recorded waveforms into the categories narrow- (NS) and broad-spiking (BS) units [Schneider and Woolley, 2013; Yanagihara and Yazaki-Sugiyama, 2016; Vahaba et al., 2017; Aurore et al., 2019]. One study using intracellular sharp-electrode recordings has reported 4 subtypes based on visual inspection of waveform shape and firing rates [Bottjer et al., 2019]. Our findings with unsurpevised hierarchical clustering using solely waveform shapes generally corroborates the latter finding, such that their two NS types displayed high and low firing rates, similar to our NS1 and NS2 respectively. Our two BS subtypes, however, are contained within the range of one of their BS subtype measurements. Their other BS subtype (termed double-trough) was not representative in our sample, possibly a limitation of extracellular recordings.

The firing characteristics of the cell types identified here led us to formulate hypotheses based on characteristics of mammalian cortical neurons. NS1 neurons are highly reminiscent of mammalian fast-spiking PV+ interneurons, exhibiting short and symmetrical action potentials, high firing, short latency to respond and low stimulus selectivity [Atallah et al., 2012; Tremblay et al., 2016]. The other cell types identified here do not exhibit statistically distinctive physiological characteristics among themselves, but BS1 and BS2 respond to fewer songs than NS1. Because of the narrower

waveform and lower firing rate, we suggest that NS2 resemble late-spiking interneurons, such as somatostatin+ or VIP+ interneurons [Tremblay et al., 2016]. Despite their distinct waveforms, we did not observe physiological features that distinguish BS2 vs BS1, other than the complete lack of response to SKF-38393 in BS2. We found ~33% of NCM neurons do not express either GABA or D1Rs (Fig. 4.2A). Therefore, it is possible that BS2 cells are part of a circuit in which D1R-signaling does not participate to produce effects on variables analyzed in this study.

Alternatively, some synaptic plasticity effects can require simultaneous D1- and D2-family receptor activation [Calabresi et al., 1992; Ichihara et al., 1992], and these receptors often heterodimerize and mutually regulate [Bordet et al., 2000; Marcellino et al., 2008]. D2-like receptors are not abundant in NCM, but their presence cannot be ruled out [Kubikova et al., 2010]. In fact, systemic D2 receptors have been shown to mediate song preference in adult female zebra finches [Day et al., 2019]. Therefore, simultaneous modulation of D1- and D2-family receptors, or of D2-family receptors alone, might be necessary to emulate DA effects in NCM.

We propose three models (Fig. 4.8B) for the effects we observed *in vivo*. If the cell types we recorded are part of the same microcircuit, it is plausible they are affecting each other's firing properties. Therefore, in our "connected model 1", we suggest that the D1R activation might be increasing the tonic firing of a GABAergic neuron upstream to NS1 cells, thus inhibiting them and disinhibiting BS1 cells. This model resembles a disinhibitory circuitry discovered in mammalian cortex for auditory associative learning, in which learning activates layer 1 inhibitory interneurons, which inhibit layer 2/3 PV+ interneurons, thus disinhibiting pyramidal neurons. These layer 1 neurons are activated

by cholinergic signaling [Letzkus et al., 2011], and are known to be 5HT3a+/VIP– interneurons [Tremblay et al., 2016]. Alternatively, our "connected model 2" depicts a single synapse and inhibitory effects of SKF-38393 on NS1 cells. Finally, our "disconnected model" summarizes our findings in each cell type and depicts isolated effects of the D1R agonist. Future experiments involving genetic targeting of specific neuronal subtypes could clarify these circuit properties.

Acetylcholine has been shown to affect SSA in mammalian auditory cortex and inferior colliculus [Metherato and Weinberger, 1989; Ayala and Malmierca, 2015]. However, to our knowledge, dopamine modulation of stimulus-specific adaptation (SSA) in vertebrate auditory cortex has not been explored. In mammalian auditory cortex, D1R-induced changes in microcircuit excitability have been shown to improve signal detection in an auditory avoidance task [Happel et al., 2014], and local D1R activation improves association learning [Schicknick et al., 2012]. In humans, systemic DAergic treatments have been shown to improve auditory language associative learning [Breitenstein et al., 2004; Knecht et al., 2004]. Since DA signaling in auditory cortex is involved in learning, it is plausible to hypothesize that DA could be affecting SSA. In songbird NCM, SSA has been shown to parallel familiarity with sounds, such that novel sounds will produce more negative slopes (i.e. higher SSA) than familiar, previously adapted sounds [Chew et al., 1996]. In fact, after successful behavioral association learning, learned sounds produce less SSA than novel sounds [Bell et al., 2015]. Here, we provide evidence that D1Rs are involved in this process, such that pharmacological D1R activation disrupts SSA in NCM neurons.

Our experiments were designed to test the prediction that blunt D1R activation would produce cellular plasticity in NCM. However, it is important to note that naturalistic DA signaling regulation is likely much more spatially and temporally targeted. Ventral tegmental area (VTA) neural activity reflects two signaling timescales, phasic and tonic firing, which produce differential DA release in terminals [Grace, 1991; Floresco et al., 2003]. More recently, DA release regulation at synaptic terminals in the rat nucleus accumbens was discovered to be independent of VTA source cell firing and signals reward expectation [Mohebi et al., 2019]. Therefore, it is likely that the effects observed here are a result of indiscriminate application of D1R agonist, when in reality, DA synaptic effects, as well as DA release are expected to be more nuanced spatially and in timing. With these aspects in mind, we hypothesize that indiscriminate D1R activation forces the NCM circuit into a "preadapted" state making it unable to adapt to subsequent presentation of novel sounds. Perhaps dopaminergic activation more precisely paired with sound stimuli would produce more specific changes. Therefore, future work should examine whether D1R activation in NCM paired with sounds would promote changes in SSA and association learning, including juvenile song learning.

We note that circuit origins of DA fibers to NCM are still an open question in the field. There are 8 major subpallial DAergic nuclei, which are fairly well conserved across vertebrates [Reiner et al., 1998]. One preliminary report suggests the caudal ventral tegmental area projects to NCM [Barr et al., 2019], which, if confirmed, would be an interesting avenue for studying auditory reward prediction learning. Other reports suggest that the locus coeruleus (LC) projects to NCM to provide norepinephrinergic (NErgic) input [Ikeda et al., 2015; Chen et al., 2016]. NE is a precursor to DA, and LC neurons are

known to release DA in addition to NE throughout the cortex [Devoto et al., 2005]. DA released by the LC onto dorsal hippocampus is involved in spatial learning and memory in rodents, independently of NE release [Kempadoo et al., 2016; Takeuchi et al., 2016]. Furthermore, future studies should clarify through neuronal tract tracing which specific nuclei provide DAergic inputs to NCM and whether the effects observed in this study can be mimicked by DA release from such nuclei.

In conclusion, we show that D1R signaling mediates key components of auditory circuitry, response and plasticity in the songbird auditory association cortex by shifting inhibitory-excitatory balance. Furthermore, we show that aromatase (estradiol-synthase) and D1R proteins are frequently found coexpressed in the same neurons. We propose that D1R effects in combination with E2 modulation could be mediating learning and memory in the sensory cortex.

CHAPTER 5

FINAL CONSIDERATIONS

In this dissertation, I examined the role of a high-order auditory cortical region in adult zebra finches by pharmacologically manipulating neuromodulatory signals. My experiments support the hypothesis that this brain region, the caudomedial nidopallium (NCM), is involved in binding sounds with outcomes in adult birds, which has been a matter of debate in the literature. Moreover, this dissertation provides insight into the role of neuroestradiol (E2) production within sensory cortical regions. In mammals, hippocampal E2 is a known modulator of spatial memory, but no conclusive data existed regarding similar phenomena in sensory cortical regions. I showed that in the NCM, inhibiting aromatase (E2 synthase) disrupted association learning in a novel operant task with social reinforcement, while it did not affect auditory retrieval/discrimination after the learning process was completed. I further showed that NCM activity itself was not required after the learning process was completed, such that inhibiting its neurons with GABA agonists after learning did not affect auditory performance. Together, these findings suggest that, after consolidation, auditory memories are not stored in or retrieved by NCM, and/or NCM is not needed for auditory discrimination. These findings led me to develop the hypothesis that local E2 modulates online associative learning signals in NCM. Specifically, E2-synthesizing cells could be themselves the target of learning signals (e.g., neuromodulation, ionic conductance changes), resulting in changes in E2 production. These, in turn could be affecting neuronal excitability, modulating afferent neurotransmitter release and/or perhaps even interacting with channels and receptors at the synapse. In mammals, plasticity in virtually all learning-dedicated brain regions is

dependent on dopamine (DA) regulation. Therefore, I hypothesized that E2 could be operating on DAergic signaling in NCM, which was reported to express D1 receptor (D1R) mRNA. Since there were no data on the cellular effects of activating these receptors (or even the cellular distribution of the proteins involved), I investigated whether DA signaling modulated synaptic plasticity in NCM. Specifically, I showed that D1R protein is prevalent in NCM neurons, especially in aromatase-, GABA-, and parvalbumin-positive neurons. Activating D1R *in vitro* reduced the amplitude of spontaneous GABAergic and glutamatergic currents and increased the frequency of the latter. Similarly, activating D1R *in vivo* reduced firing of putative-inhibitory interneurons, but increased firing of putative-excitatory projection neurons. Finally, I showed that D1R activation disrupted stimulus-specific adaptation in NCM neurons, a phenomenon reflective of active auditory memory formation. These data suggest that D1Rs are involved in inhibitory-excitatory balance in NCM at the anatomical, *in vitro* and *in vivo* levels. These findings led me to hypothesize putative network connectivity models for the NCM microcircuit. The most parsimonious scenario is that D1R activation preferentially increases GABAergic tone onto inhibitory interneurons, which reduces their firing and consequently increases the firing of downstream excitatory neurons (see Fig. 4.8).

A second aspect of the behavioral experiments reported in my second chapter led to a new set of concepts. Specifically, the findings suggest that the adult NCM and specifically aromatase activity within it play key roles in associating sounds with behaviorally relevant consequences. These findings corroborate previous reports that NCM neuronal activity reflects association learning in adults [Jarvis et al., 1995; Bell et

al., 2015] and song learning in juveniles [London and Clayton, 2008]. However, the novel task described in this dissertation does not allow for precisely dissecting post-learning performance into its components, namely sound memory retrieval and sound discrimination. After learning, to achieve high performance, birds need to both excel at discriminating among sounds and retrieving the memory about which sound is associated with reward. I showed that inhibiting NCM after learning does not affect performance, which suggests that NCM is not involved in either discrimination or retrieval. This is reminiscent of hippocampal function, which is required for encoding, consolidation and retrieval of early/detailed memories but not involved in the retrieval of sedimented memories [Wiltgen et al., 2010]. Interestingly, recent studies in the mammalian and avian hippocampus have also observed a role for estrogens in learning, both locally-produced and from the circulation [Bailey and Saldanha, 2015; Luine, 2016; Tuscher et al., 2016; Gervais et al., 2018; Paletta et al., 2018]. Moreover, in addition to neuromodulators, NCM is rich in NMDA receptors [Saldanha et al., 2004], which are classically regarded as key players in cellular memory formation processes, such as long-term potentiation and depression, in most mammalian excitatory synapses [Lüscher and Malenka, 2012]. Therefore, NCM possesses the major components of the molecular toolkit that together support hippocampal learning and memory, including machinery for E2 signaling, DA signaling and AMPA/NMDA receptors. A theoretical framework formulated by this dissertation is that NCM acts as an "auditory hippocampus", in which associations are formed then distributed to other brain regions for long term storage.

Another question raised here is whether E2 is acting through a genomic or membrane-mediated to affect association learning. Classically, steroid hormones are

referred to as “organizers” of nervous systems (particularly of sex differences) through genomic regulation, typically over extended timescales (days-months). More recently, great emphasis has been made on steroid actions at faster timescales (seconds-minutes), frequently through actions on membrane-bound receptors [Remage-Healey, 2014]. Results from this dissertation do not allow for direct conclusions about whether E2 acts rapidly or on membrane receptors to affect auditory learning. That said, I showed that NCM aromatase inhibition had an effect on reversal learning at the start of behavioral testing, suggesting a rapid effect, in line with a previous report that systemic aromatase inhibition rapidly reduced sound-induced immediate early gene expression in the NCM of male zebra finches [Krentzel et al., 2019]. In the future, application of specific antagonists for membrane-bound versus nuclear-acting receptors during auditory learning could help answer this question.

It is important to note that the behavioral experiments in my second chapter were performed in male songbirds. In females, fadrozole given orally did not impair immediate early gene EGR1 expression in NCM, but it did in males [Krentzel et al., 2019]. Similarly, unlike in males, NCM electrophysiological responses in females were unaffected by modulation of G-protein coupled E2 receptors [Krentzel et al., 2018]. It is possible that, since females also possess an ovarian source of E2, drugs that affect E2-signaling are needed in higher doses to produce an effect. Moreover, in males, NCM contains higher density of aromatase fibers than in females (although not of aromatase neurons), which suggests that local aromatization may be more important in males [Saldanha et al., 2000; Peterson et al., 2005]. This prediction can now be tested using the operant behavioral paradigm presented in Chapter 2. In preliminary behavioral

experiments, I found that females are also motivated to engage in the same behavioral task which provides an interesting avenue of future research, particularly for the exploration of sex differences in the role of aromatase in auditory learning.

The operant paradigm described in this dissertation was developed as a viable alternative tool to study goal-directed behavior in social animals. Many studies using operant conditioning paradigms for songbirds and other species have relied on food reinforcement [Cynx and Nottebohm, 1992; Benney and Braaten, 2000; Gentner and Margoliash, 2003; Gess et al., 2011; Schneider and Woolley, 2013; but see Tokarev and Tchernichovski, 2014; Chen and ten Cate, 2015], which requires not only food but social deprivation for hours before testing and thus possible distress for highly social species such as the zebra finch [Astheimer et al., 1991; Zann, 1996]. To mitigate these drawbacks, my dissertation describes a low-cost behavioral tool to assess auditory learning in zebra finches without the need for food deprivation or complete social isolation. In this task, birds learn to operate a switch to gain visual access to an individual in an adjacent cage. Future studies should explore, for example, if motivation to engage in the task is reflective of different social relationships (e.g. juveniles, novel vs familiar, pair-bonded individuals), and perhaps differing social systems in species that are not as socially-gregarious as zebra finches [Goodson and Kingsbury, 2011]. The brain regions and neurotransmitter systems that contribute to this phenomenon should also be explored.

An interesting question raised by this work is whether E2 production by the human auditory cortex [Yague et al., 2006] impacts language learning, or more broadly, auditory learning during development and/or adulthood. Interestingly, plasma E2 was found to be a strong positive predictor of language development in children of both sexes,

while testosterone was a strong negative predictor [Schaadt et al., 2015]. E2 levels also strongly correlated with melodious crying in infants, a predictor of language outcomes [Wermke et al., 2014]. Furthermore, aromatase gene mutations in humans were associated dyslexia and language processing and production [Anthoni et al., 2012]. These studies point towards an important role of E2 in human language development, but a causal relationship has not yet been established.

That said, in developing songbirds, E2 production inhibition during tutoring did not impair song learning, but resulted in increased neuronal firing to tutor song playback when the same animals became adults [Vahaba et al., 2020]. In adults, E2 production is increased during song playback [Remage-Healey et al., 2008], but is reduced in the juvenile NCM followed by an immediate increase after song playback [Chao et al., 2014]. Assuming that sound-outcome encoding is a general feature of NCM which extends to song-reinforcement learning in juveniles, my dissertation's data indicate that E2 production in NCM is important during adult auditory learning, predicting an opposite result in the juvenile NCM during a similar auditory learning task. Interestingly, some areas directly implicated in juvenile song learning and production also contain aromatase fibers and/or neurons, such as HVC and HVC shelf [Ikeda et al., 2017]. Future research should address whether E2 production/signaling directly in these cortical areas support song learning in juveniles, and what other roles they are playing in adults, in which these areas mostly support song production and not learning.

One curious finding of my second chapter is that inhibiting aromatase during learning resulted in a trend for an increase in response bias, i.e. indiscriminate responses to GO and NO-GO trials. One interpretation for this is that aromatase inhibition could

affect impulse control. Indeed, an involvement of aromatization/E2 in impulsive behavior has been suggested in humans [Smith et al., 2014] and rodents [Svensson, 2010; Bayless et al., 2013]. Future implementations of our behavioral task could help illuminate the comparative aspects of this proposed relationship between neuroestrogens and impulsive behavior in songbirds and other species. However, an alternative explanation for these findings is that zebra finches revert to response bias when performance is impaired. The task presented here consisted of two trial types (GO/NO-GO) and involved mild punishment (white noise burst). It is possible that learning impairments resulted in birds responding indiscriminately to trials, so they could still achieve high reward rates. To answer this question, future work could modulate punishment/reward saliency balance. I predict that increasing punishment intensity or decreasing reward quality/duration could result in a no-response bias in a learning impairment challenge.

To modulate goal-directed auditory learning, E2 could be acting in concert with and/or modulating reinforcement signals, such as midbrain dopamine (DA) release. In striatum and preoptic area of both birds and mammals, E2 and DA systems interact [Becker, 1990; Lammers et al., 1999; Balthazart et al., 2002; Tozzi et al., 2015] and DA and E2 can interact with the same receptors [Olesen and Auger, 2008; Tozzi et al., 2015]. This line of reasoning is also supported by reports that DA in the auditory cortex modulates auditory association learning in gerbils [Schicknick et al., 2012], and that DA innervation and release are increased by steroid hormones in songbird NCM [Matragrano et al., 2011; Rodríguez-Saltos et al., 2018]. Indeed, results in my fourth chapter show that 33% of aromatase-expressing neurons in NCM also express DA receptors and these double-labeled cells represent 8% of all NCM neurons. Therefore, studying E2 and DA

interactions in the auditory cortex could provide important insights about the network and cellular mechanisms behind the learning deficits induced by aromatase inhibition.

In my fourth chapter, I report that D1R activation impairs stimulus-specific adaptation (SSA) in NCM neurons. The neuromodulatory mechanisms underlying SSA are essentially unexplored. In mammalian auditory cortex and inferior colliculus acetylcholine has been shown to affect SSA [Metherato and Weinberger, 1989; Ayala and Malmierca, 2015], but DA modulation of stimulus-specific adaptation (SSA) in vertebrate auditory cortex has not been studied. In mammalian auditory cortex, D1R-induced changes in microcircuit excitability have been shown to improve signal detection in an auditory avoidance task [Happel et al., 2014], and local D1R activation improves association learning [Schicknick et al., 2012]. Interestingly, systemic DAergic treatments in humans have been shown to improve auditory language associative learning [Breitenstein et al., 2004; Knecht et al., 2004]. These reports show that DA signaling in auditory cortex is involved in auditory learning, but it remains to be tested whether DA is mediating learning through modulating SSA in mammals.

This dissertation provides evidence that D1Rs are involved in SSA, such that pharmacological D1R activation disrupts SSA in NCM neurons, which might seem counterintuitive. These experiments were designed as proof of the concept that blunt D1R activation would produce cellular plasticity in NCM. However, naturalistic DA signaling regulation is likely much more sophisticated. For example, ventral tegmental area (VTA) neurons have different firing regimens, which produce different DA outputs in target regions [Grace, 1991; Floresco et al., 2003]. DA release can also be regulated at the synapse, independently of VTA neuron firing [Mohebi et al., 2019]. Thus, I propose that

the effects observed in this dissertation resulted from indiscriminate D1R activation, when DA synaptic effects, as well as DA release are expected to be more nuanced in more physiological conditions.

In songbird NCM, SSA has been shown to parallel familiarity with sounds, such that novel sounds will produce more negative slopes (i.e. higher SSA) than familiar, previously adapted sounds [Chew et al., 1996]. In fact, after successful behavioral association learning, learned stimuli produce less SSA than novel [Bell et al., 2015]. Therefore, these studies raise the hypothesis that the indiscriminate D1R activation in my experiments forced the system into a "preadapted" state making it unable to adapt to subsequent presentation of novel sounds. Perhaps dopaminergic activation more precisely paired with sound stimuli would produce more specific changes. By extension, whether SSA modulation by D1Rs reflect changes in auditory learning in adults and song learning in developing songbirds are open questions.

This dissertation did not establish what the neural circuit origins of DA fibers to NCM are, and this is still an open question in the field. There are 8 major subpallial DAergic nuclei, which are fairly well conserved across vertebrates [Reiner et al., 1998]. One preliminary report suggests the caudal ventral tegmental area projects to NCM [Barr et al., 2019], which, if confirmed, would be an interesting avenue for studying auditory reward prediction learning. Other reports suggest that the locus coeruleus (LC) projects to NCM to provide norepinephrinergic (NErgic) input [Ikeda et al., 2015; Chen et al., 2016]. NE is a precursor to DA, and LC neurons are known to release DA in addition to NE throughout the cortex [Devoto et al., 2005]. DA released by the LC onto dorsal hippocampus is involved in spatial learning and memory in rodents, independently of NE

release [Kempadoo et al., 2016; Takeuchi et al., 2016]. Therefore, future work should clarify through neuronal tract tracing which specific nuclei provide DAergic inputs to NCM and whether the effects observed in this dissertation can be mimicked by inducing DA release from such nuclei.

In conclusion, this dissertation provides evidence that the NCM is a key region for online auditory association learning in adult songbirds. The findings here also suggest that E2 production within NCM – and NCM itself – plays a role in the pairing between sounds and behaviorally relevant consequences, and that this signal is likely distributed and stored in other brain regions after the initial association. This work also provides evidence that DAergic signaling is a likely target of E2 modulation, since a significant portion of aromatase-positive neurons contain D1Rs. My studies show that D1R signaling regulates key components of auditory circuitry, response and plasticity in NCM by shifting inhibitory-excitatory balance. I propose that D1R effects in combination with E2 modulation might be modulating learning and memory in the sensory cortex. Effects of neuro-E2 production had been previously demonstrated in mammalian and avian hippocampus for learning and memory [Bailey and Saldanha, 2015; Luine, 2016; Tuscher et al., 2016; Gervais et al., 2018; Paletta et al., 2018]. This dissertation builds on this literature by providing direct evidence that E2 production within the auditory cortex affects sensory learning potentially through tapping into the DAergic system, which itself modulates plasticity mechanisms associated with learning. This raises the hypothesis that these findings could apply to other vertebrates that contain aromatase and DA receptors in their auditory cortex, including humans.

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