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The Cellular Context of Estradiol Regulation in the Zebra Finch Auditory Forebrain

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THE CELLULAR CONTEXT OF ESTRADIOL REGULATION IN THE ZEBRA

FINCH AUDITORY FOREBRAIN

A Dissertation Presented

by

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ABSTRACT

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Estradiol, traditionally known as a hormone that communicates with distant cells in the body, is also synthesized locally in the brain to act as a neuromodulator. Neuromodulators differ from neurotransmitters in that they simultaneously affect a population of neurons and their actions are not limited to the synapse. One of the many effects of estradiol signaling is rapid modulation of auditory processing in response to external stimuli. The enzyme required for estradiol synthesis, aromatase, is highly expressed in the regions that are involved in higher-order processing of sounds in humans and songbirds. Since zebra finches, a type of songbird, are one of the few laboratory animals that communicate *via* complex learned vocalization, they are commonly used as a model for vocal learning and auditory processing. Although many aspects of the actions of estradiol in the zebra finch forebrain have been revealed, little is known regarding how estradiol levels are regulated *via* aromatase activity. First, this dissertation describes the procedure for *in vivo* microdialysis, a method that allows local estradiol detection in freely moving animals. Second, using *in vivo* microdialysis, we investigated whether another neuromodulator, norepinephrine, regulates global estradiol levels within a secondary auditory region, caudomedial nidopallium (NCM). The results showed no evidence that norepinephrine has a major role in controlling estradiol levels in the NCM. However, *in vivo* electrophysiological

recordings from the NCM revealed that norepinephrine has a similar role in auditory processing as estradiol but acts *via* a different mechanism. Finally, the dissertation examined the identities and organization of aromatase-expressing neurons and found that the heterogeneity of aromatase cells was different between different aromatase-positive regions. Aromatase-expressing cell bodies were found to be more prominent in regions with low expression of a transmembrane G-protein coupled estrogen receptor, GPER1, while high pre-synaptic aromatase-expressing regions expressed high amounts of GPER1. Moreover, aromatase-expressing cells were found in somato-somatic clusters. Preliminary data injecting dyes in clustered cells indicate that the neurons in clusters may be communicating with one another through gap junctions. Overall, this dissertation provides new knowledge for understanding the relationship between neuronal interactions and aromatase signaling in the context of auditory processing.

TABLE OF CONTENTS

LIST OF TABLES

LIST OF FIGURES

CHAPTER I

INTRODUCTION

Background

For many animals, altering behavior in response to external stimuli is critical for survival. One of the ways organisms adjust their behavior to the changes in the environment is *via* neuron-secreting substances called neuromodulators, produced during different physiological states. Accurately interpreting auditory information and its context is extremely important for social species that heavily rely on communication *via* vocalization for survival and reproduction. The context of auditory information is thought to be encoded by the actions of neuromodulators such as catecholamines and estradiol. Estradiol, a steroid traditionally considered to be a gonadal hormone, is produced within the brain and is known to have properties similar to classical neuromodulators (Reviewed in Remage-Healey, 2014). However, the specific mechanisms of how estradiol signaling is controlled remain to be elucidated. Studies have indicated that one of the mechanisms is *via* local regulation of estradiol synthesis by the enzyme aromatase which produces estradiol *via* conversion of testosterone. Although at least part of the molecular and cellular mechanisms that control aromatase activity has been revealed by previous studies, almost nothing is known about how neuronal connectivity across or within brain regions influence changes in aromatase activity or estradiol levels on a circuit level. This dissertation provides new insights into how estradiol actions in one auditory region could be either controlled by or interact with neuronal activity of other regions that project to or from the region.

This introduction is outlined as the following: (1) Background information on state-dependent modulation of auditory processing by two types of neuromodulators, estradiol and catecholamines from human and animal studies. (2) The mechanism of local estradiol regulation, the gaps in the literature and potential circuit mechanism of aromatase regulation. (3) The advantages of using songbirds, especially zebra finches, as a model for studying neuroestradiol actions in auditory processing. (4) Review of the literature on the role of the songbird secondary auditory region, the caudal medial nidopallium (NCM). (5) Estradiol and catecholaminergic actions in the NCM and potential interactions. (6) Hypotheses and questions addressed in this dissertation.

State-dependent modulation of auditory processing by estradiol and catecholamines: studies in humans and animal models

Many variables such as environmental changes, aging, mental disorders, and use of drugs can cause changes in auditory processing (Mccartney et al., 1994; Mendelson and Ricketts, 2001; Martin and Jerger, 2005; Liang et al., 2006; Winton-brown et al., 2011; Javitt and Sweet, 2015). These changes are mediated in part by the changes in chemicals that affect neuronal activity, such as hormones and neuromodulators. Neuromodulators are released by neurons and alter the activities of a population of neurons. Catecholamines and estradiol are examples of neuromodulators that are known to affect auditory processing (Examples of studies in different species: Anderer et al., 2004; Remage-Healey et al., 2010; Kudoh and Shibuki, 2006. Reviewed in Hurley et al., 2004; Caras, 2013; Krentzel and Remage-Healey, 2014; and Javitt and Sweet, 2015).

Catecholamines are a group of neuromodulators that are synthesized from the

amino acid tyrosine, and include dopamine, epinephrine, and norepinephrine. Some

disorders that affect the dopaminergic or noradrenergic system, such as autism,

depression and schizophrenia, are associated with disturbed auditory processing (Lam

et al., 2006; Tollkötter et al., 2006; Javitt and Sweet, 2015). Drugs such as

psychostimulants and depressants that act by interacting with the catecholaminergic

system also alter auditory processing (Hughes et al., 1988; Kössl and Vater, 1989; Tobey

et al., 2005; Dixit et al., 2006; Winton-brown et al., 2011). Norepinephrine is specifically

released by neuronal fibers whose cell bodies are located in the *locus coeruleus*, a

nucleus in the brain stem. In healthy individuals, norepinephrine is released in response

to stress and unexpected stimuli, regulates cortical arousal, and is involved in memory

retrieval and complex cognitive tasks (Sara and Bouret, 2012).

 In mammalian models, changes in norepinephrine levels induced either by environmental

stimuli or application of drugs or adrenergic agonists can alter neuronal activity in the auditory

cortex (Foote et al., 1975; Shinba et al., 1992; Edeline, 1995; Manunta and Edeline, 1997, 1999,

2004; Gaese and Ostwald, 2001; Ji and Suga, 2003; Syka et al., 2005; Salgado et al., 2011, 2012).

Moreover, as discussed in later sections, many studies in songbirds support the idea that

catecholamines may play a major role in auditory processing.

Estradiol is a type of estrogen that is produced in the brain and in other organs such as

the gonads. Although other neuromodulators including norepinephrine are also produced

peripherally, they do not cross the blood brain barrier (Reviewed in Edvinsson and MacKenzie,

1977). Since estradiol does cross the blood brain barrier, the neuronal changes due to estradiol

are due to changes both in circulating estradiol levels and in local estradiol synthesis. Estradiol

synthesis is mediated by the enzyme aromatase that converts testosterone to estradiol and

androstenedione to estrone, which in turn can be converted to estradiol. In the human and

monkey temporal cortex, where the auditory cortex is located, aromatase is expressed densely

in neurons (Yague and Muñoz, 2006; Yague et al., 2008). For women, menopause, hormonal

contraception, hormonal replacement therapy, and different phases of the menstrual cycle can

alter hearing and neuronal activity in auditory regions (Anderer et al., 2004; Walpurger et al.,

2004; Mordecai et al., 2008; Cowell et al., 2011). Although there are no studies that have

directly tested the effect of local estradiol synthesis on auditory processing in humans, systemic

blockade of estradiol synthesis or signaling with an aromatase inhibitor or estrogen receptor

blockers, respectively, are shown to alter auditory and speech processing (Wagner and Morrell,

1996; Jenkins et al., 2004; Bakoyiannis et al., 2016). Since aromatase expression is very low in

the auditory cortices in rodents (Sanghera et al., 1991; Wagner and Morrell, 1996), many studies

on local estradiol signaling on auditory processing has been done in songbirds, which have rich

aromatase expression in auditory regions (Shen et al., 1995; Metzdorf et al., 1999; Jacobs et al.,

2000; Saldanha et al., 2000). The role of neuromodulators in auditory processing in songbirds is

discussed further in later sections. Therefore, across species, both circulating and brain-

generated estradiol are shown to play a role in auditory processing.

Mechanism of local estradiol regulation: the gaps in the literature and potential circuit

mechanism of aromatase regulation

Estradiol has a broad range of actions often categorized loosely into two groups,

classical and non-classical actions (Vasudevan and Pfaff, 2008). Classical actions are mediated by

classic estrogen receptors that are located in or can translocate into the nucleus to act as

transcription factors. Non-classical actions are actions of estradiol that do not involve genomic

transcription and act through the activation of cellular signaling cascades *via* opening or

altering kinetics of ion channels, metabotropic receptors, receptors of other

neuromodulators (Boulware et al., 2005; Kumar et al., 2015; Peterson et al., 2016 etc.

Reviewed in Prossnitz et al., 2008; Kow and Pfaff, 2016). Many of the non-classical

actions are shown to be mediated by transmembrane estrogen receptors or estrogen

receptors associated with the plasma membrane *via* palmitoylation or other types of

protein modifications (Meitzen et al., 2013; Banerjee et al., 2014). Non-classical actions

are also called "rapid" actions because many of their effects can be observed within

seconds while the effects of classical actions take longer. While estradiol has both

classical and non-classical effects on neurotransmission and plasticity and both are likely

to be activated concurrently (Vasudevan and Pfaff, 2008), most neuromodulatory

effects that happen rapidly are considered to be mainly the consequences of non-

classical actions of estradiol (Compagnone and Mellon, 2000; Balthazart and Ball, 2006;

Remage-Healey et al., 2011b).

        While many published studies have focused on the downstream effects and

mechanisms of estradiol actions, one of the important questions that remains to be

answered is how estradiol signaling, itself, is controlled in the brain. Traditionally,

neuromodulator actions can be controlled by many mechanisms including its rate of

synthesis, rate of release, receptor expression, receptor location, ligand affinity states of

receptors, clearance, and degradation. Especially, as for other neuromodulators, the

amount of local estradiol and timescale is expected to be extremely important for the

specific and location-specific activation of downstream effects. However, as mentioned

above, estradiol is not only produced within the brain, but also can come from

peripheral sources through the bloodstream. In addition, unlike most neuromodulators, estradiol is lipophilic and is considered to be capable of diffusing through the plasma membrane and, therefore, not kept in vesicles (Charlier et al., 2013b). Therefore, local estradiol levels are most likely affected by estradiol clearance (including degradation), diffusion, and local synthesis

The only known mechanism of estradiol clearance is through metabolism. Estradiol can be converted either to estrone by 17 beta-hydroxysteroid dehydrogenase (17 β-HSD), or to various hydroxyestrodiols by the enzymes of the cytochrome p450 family (CYP) through oxidation (Zhu and Conney, 1998; Balthazart and Ball, 2006). To my knowledge, with the exception of aromatase activity, the only mechanisms of modulating of the activities of these enzymes that have been reported are *via* changes that involve translation, transcription, or degradation of the gene or protein. Although possible, no reports have been published, to date, on the rapid regulation of these enzymes except by aromatase. Even though the role of aromatase in clearance has been suggested in the field, no studies have tested this specifically (Charlier et al., 2015). Moreover, to rapidly control estradiol concentration, the enzymes are expected to be expressed close to the loci of action and near the plasma membrane. Although the subcellular localization of these enzymes has not been extensively studied, the currently available literature has suggested the presence of these enzymes only within the mitochondria (Miksys and Tyndale, 2002). Together, although clearance or metabolism of estradiol may contribute to the maintenance of global levels of estradiol, not enough evidence exists to suggest it is one of the main mechanisms of rapid and local regulation of estradiol signaling.

The presumed mechanism of regulating rapid estradiol signaling is *via* local estradiol synthesis. Aromatase activity in the brain changes rapidly in response to treatments that alters neuronal activity and application of neurotransmitters (Baillien and Balthazart, 1997; Balthazart et al., 2001, 2003a, 2011; Remage-Healey et al., 2008, 2009a, 2011a; Charlier et al., 2013b; Comito et al., 2016). Moreover, in some areas, aromatase is not only found in the cell bodies but also in the synaptic terminals which further supports the idea that estradiol actions are controlled by rapid changes in local aromatase activity (Roselli, 1995; Naftolin et al., 1996; Hojo et al., 2004; Peterson et al., 2005). *In vivo* fluctuations in estradiol within regions have been demonstrated using microdialysis (Remage-Healey et al., 2008). Moreover, local inhibition of aromatase activity with an aromatase inhibitor decreases local estradiol levels (Remage-Healey et al., 2008). These pieces of evidence suggest that, in addition to global and regional levels of estradiol, estradiol levels at the synaptic cleft are also important for the control of estradiol actions (Figure 1).
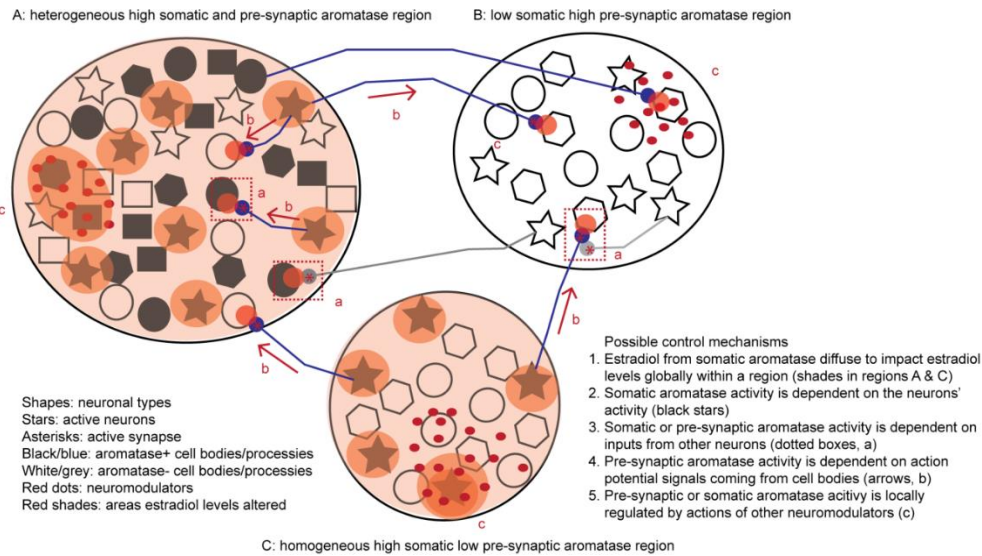
Figure 1: Possible role of circuits in the regulation of local estradiol levels

A schematic to illustrate the possible control mechanisms of aromatase activity and local estradiol levels when subpopulation of neurons (star shapes) are activated. Circles A, B, and C represents heterogenic regions with differing pattern of aromatase expression. Estradiol within in a region could be come from the blood stream (not shown) or synthesized from local somatic (black shapes) or pre-synaptic (blue lines) aromatase. In regions where aromatase is primarily found pre-synaptically (region B), aromatase+ terminals are likely to come from other regions that express somatic aromatase (regions A & C). In regions where aromatase is found both in the pre-synaptic boutons and cell bodies (A), aromatase+ terminals can come either from within the same region or a different region. In regions with heterogeneous aromatase+ neurons (region A), sub-regional aromatase concentration may be more important than in regions with homogeneous aromatase neuronal identity (region C). (1) Estradiol levels of the whole area can be upregulated by local aromatase in regions where aromatase is abundantly expressed (shades in regions A & C). (2) Sub-regional estradiol levels around aromatase+ neurons are up- or down- regulated *via* neuronal activity (shades around star shaped neurons). (3) Pre- or post- synaptic aromatase activity may depend on receiving inputs from other neurons. Signals that affect presynaptic aromatase activity can come from axoaxonic synapses (region C, dotted square a), while post-synaptic (or somatic) aromatase activity may depend on direct pre-synaptic inputs coming from either aromatase positive or negative terminals from neurons whose cell bodies reside inside (region A, top dotted square a) or outside (region A, bottom dotted square a) of the region. (4) Somatic or pre-synaptic aromatase activity is dependent on neuronal activity or action potentials (arrows b). The signals may come from inside (region A, arrows b) or outside of the region where the pre-synaptic aromatase terminals are. (5) Somatic or pre-synaptic aromatase activity can be affected by the actions of other neuromodulators (somatic, region A & B c; pre-synaptic, region C c). To avoid complication, possible circuit mechanisms of neuromodulators' actions on aromatase activity were excluded from this diagram (See Figure 2).

Estradiol levels could be rapidly regulated through the regulation of aromatase activity, in two ways: *i)* by changing the amount of aromatase protein and *ii)* by changing the enzymatic activity of aromatase. Since most rapid actions occur within seconds to minutes, the rapid increase in estradiol is mostly likely due to the change in aromatase activity and not to protein levels (Balthazart et al., 1990). Thus, only literature regarding rapid regulation of aromatase activity through activation of cellular signaling cascades will be reviewed. In different species, both *in vivo* and *in vitro*, evidence shows that aromatase activity is inhibited by phosphorylation-inducing environments such as the addition of magnesium (Mg2+), calcium (Ca2+), or ATP (Balthazart et al., 2001, 2003a; Remage-Healey et al., 2011a; Charlier et al., 2016; Comito et al., 2016). Although detailed comparisons of studies done in different species suggest slight species and regional differences in the mechanisms, in general, the results agree that aromatase is inhibited by kinase activities *via* phosphorylation and calmodulin binding to aromatase (Balthazart et al., 2005; Miller et al., 2008; Charlier et al., 2016; Comito et al., 2016). Moreover, conditions that induce increases in neuronal activity, such as the addition of potassium (K+), kainite, NMDA, or AMPA, decrease aromatase activity (Balthazart et al., 2001; Remage-Healey et al., 2008, 2011a). In the zebra finch secondary auditory region (which expresses high levels of pre-synaptic aromatase), local infusion of the pre-synaptic voltage calcium inhibitor conotoxin inhibited the K+-induced decrease in local estradiol levels in awake behaving birds (Remage-Healey et al., 2011a). Moreover, in supernatant-containing microsomes, synaptosomes, and mitochondria from this region, Ca2+ had a large inhibitory effect on aromatase activity while, in the supernatant from regions with known low pre-synaptic aromatase, Ca2+ had no effect on aromatase activity (Comito et al., 2016). This suggests that pre-synaptic aromatase and non-

presynaptic aromatase may be regulated through different mechanisms. For pre-synaptic

aromatase, increases in pre-synaptic calcium levels induced by neuronal activity may decrease

aromatase activity and, therefore, estradiol levels. Interestingly, this is in contrast to traditional

neuromodulators that are released in response to calcium influx induced by neuronal activity.

Moreover, dopamine, but not other catecholamines, inhibits aromatase activity in Japanese

quail brain homogenates (Baillien and Balthazart, 1997), suggesting that there could be multiple

pathways for regulating or modulating local aromatase activity (See Figure 1 & 2).
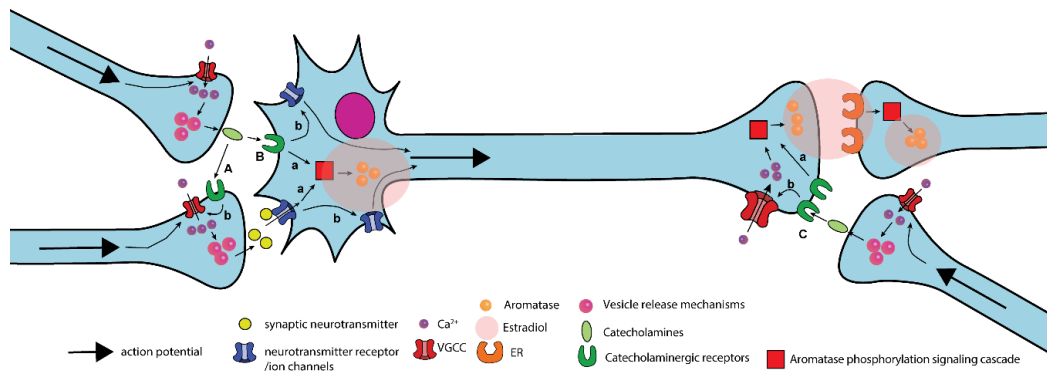
Figure 2: Potential mechanisms for how other neuromodulators may play a role in

regulating or modulating aromatase activity

Catecholaminergic receptors could potentially be acting at least at three different locations: (A)
Pre-synaptically to aromatase+ neurons, (B) Post-synaptically on aromatase+ neurons, or (C)
Pre-synaptically (*via* axo-axonic synapse) to pre-synaptic terminals. Catecholaminergic receptor
activation can change aromatase activity either by directly activating signaling pathways (a) or
indirectly *via* modulating channel openings (b). VGCC, voltage-gated calcium channel; ER,
estrogen receptor.

Advantages of using songbirds, especially zebra finches, as a model for studying

neuroestradiol actions in auditory processing

Studying the mechanisms of speech and language processing has been difficult due to

the lack of a good animal model. Only a small number of species other than humans

communicate with complex vocalizations that require auditory learning to appropriately respond to vocalizations. Although rodents and primates are closely related to humans, they are not perfect models as they produce unlearned vocalizations and their auditory systems are not as complex as humans (Petkov and Jarvis, 2012). In birdsongs, similarly to human speech, the volume, pitch, duration, syntax, and sequence of song elements convey information that is important for the receiver. Also, as mentioned above, songbirds have aromatase expression and activity in the cortical auditory regions that is high and comparable to human auditory cortex (Callard et al., 1978; Saldanha et al., 2000). In rodent cortex, although aromatase expression is moderate (Foidart et al., 1995; Tremere et al., 2011), aromatase activity is undetectable (Callard et al., 1978; Lephart, 1996). Therefore, songbirds, such as zebra finches, have become popular as a model for understanding complex vocal and auditory learning and for studying the role of estradiol in auditory processing. Although songbirds are distantly related to humans, expression patterns of some genes that are linked to speech learning are similar between humans and songbirds (Pfenning et al., 2014). The connectivity between and functions of avian auditory regions are also found to be analogous to those of humans (Brainard and Doupe, 2002; Jarvis, 2004; Castelino and Schmidt, 2010). For example, both the human secondary cortex and the songbird secondary auditory region, NCM, respond to visual information (Lewis et al., 2000; Kruse et al., 2004; Remage-Healey et al., 2012). Thus, despite the differences and lack of homology, the functionality and connections of different regions involved in vocalization and auditory memory are similar to that of humans.

Among many songbird species, zebra finches are one of the most popular in the laboratory. Zebra finches are not only easily kept and bred in captivity but also have a simple song usage and learning process. Zebra finches are socially monogamous and songs are sung by males to females during courtship. Since they are a social species, living in large groups in the wild, they are not as territorial as other species (Zann, 1996; Goodson et al., 1999). Therefore, songs are not often used in aggressive situations, and the primary purpose of songs is thought to be individual recognition, males attracting females, and tutoring the young (Clayton, 1987; Goodson et al., 1999). While many songbirds learn multiple songs over multiple seasons, zebra finches learn only one song throughout their lifetime and they learn it during an early, critical period in development. Generally, males learn their songs from their caregivers (called tutors), which are, in most cases, their fathers. Once they reach adulthood, their critical period for learning new song elements has closed. These features make zebra finches an attractive songbird species to conduct research on the basic mechanisms of song and auditory learning and to use them as a model for hormonal actions on auditory processing.

Although vocalizations can be used for many purposes, in zebra finches, one of the primary usages of vocalization is attracting mates during courtship. For females, the quality of songs is one of the important factors for choosing a mate (Forstmeier and Birkhead, 2004; Tomaszycki and Adkins-Regan, 2005; Holveck and Riebel, 2007). Females seem to acquire preferences for songs through development, and their experience impacts mate and song choice in adulthood (Collins, 1995; Riebel, 2000; Lauay et al., 2004; Riebel et al., 2009). While most regions in the auditory circuit show no gross differences in overall sizes and activity between females and males, detailed and direct comparisons have indicated differences in function and connectivity (Peterson et al., 2005; Pinaud et al., 2006; Rohmann et al., 2007;

12

Gobes et al., 2009; Remage-Healey et al., 2012; Krentzel and Remage-Healey, 2015 (review); Comito et al., 2016 etc.). Specifically, in males, some genetic and electrophysiological evidence suggest that tutor songs are represented in not only the motor regions but also in auditory regions (Bolhuis et al., 2000; Gobes and Bolhuis, 2007; Yanagihara and Yazaki-Sugiyama, 2016) suggesting that, unlike in females, in males auditory regions may be involved in song learning and production in addition to auditory learning and processing. Nevertheless, many studies of auditory regions have been conducted in males, resulting in difficulty interpreting the implications. Therefore, because of biological relevance to the behavior in the wild, to avoid potential confounding influences of singing and, depending on the purposes of the study, females may be preferable over males, for use as a model to study auditory processing.

Although the songbird brain is not homologous to that of mammals, there are many similarities in the functionalities of the regions and analogous connections between regions. As in mammals, in songbirds, auditory information from the cochlea is first processed in the cochlear nucleus, and then into the midbrain nucleus mesencephalicus lateralis, pars dorsalis (MLd; homologous to the inferior colliculus in mammals) and then through the thalamus (Ov) before arriving to the cortex (Karten H J, 1968; Kelley and Nottebohm, 1979; Brauth and McHale, 1988; Vates et al., 1996; Chirathivat et al., 2015). The first thalamorecipient is the primary auditory region field L, which is analogous to the primary auditory cortex in mammals. Field L neurons send projections to at least four secondary cortical regions: *i*) caudal mesopallium (CM); *ii*) ventral portion of the intermediate arcopallium (AIV); *iii*) HVC shelf (proper name), and *iv*) caudomedial nidopallium (NCM; Vates et al., 1996; Mandelblat-Cerf et al., 2014;

Chirathivat et al., 2015). Among these four regions, the NCM contains high expression of aromatase and has received the most attention with regard to estradiol signaling.

Zebra finches can discriminate songs of their own species (conspecific songs) from songs of other species (heterospecific songs) (Scharff et al., 1998; Braaten et al., 2007). In addition, they can recognize songs of their own (BOS, bird's own song), their tutors, and familiar individuals (Cynx and Nottebohm, 1992; Riebel et al., 2002; Braaten et al., 2007; Gobes and Bolhuis, 2007; Remage-Healey et al., 2010). The mechanism for how these auditory memories are stored has not been elucidated. In general, most types of memories are thought to be formed through similar complex processes that involve the following: processing or encoding of the experience and consolidation, storage, retrieval, and reinforcement of the memory (Nadel et al., 2012). Auditory memories in songbirds are thought to be formed in a similar manner. Nevertheless, importantly, motor memory that is required for vocalization and auditory processing that is necessary for correcting a bird's own singing performance, should not be confused with general auditory memory that is used in individual recognition or sexual preferences (Riebel et al., 2002; Gobes and Bolhuis, 2007). Since tutor song and BOS are extremely important for the birds' performances for singing, their memory is likely to be stored *via* a different mechanism than other auditory information.

Review of the literature on the role of the songbird secondary auditory region, the caudal medial nidopallium (NCM)

The role of the NCM in auditory processing or memory is unclear. Many hypotheses on the role of the NCM are derived from behavioral preference tests in lesioned birds and expression of a song-induced immediate-early gene, *egr-1*. Both females and males show

behavioral preference for familiar (including tutor, mate, and father's) songs over novel

songs (Miller, 1979; Riebel et al., 2002; Gobes and Bolhuis, 2007; Woolley and Doupe,

2008), and these results from innate preferences have been used in combination with

lesions and pharmacological manipulations to infer the role of the NCM (Gobes and

Bolhuis, 2007; London and Clayton, 2008; Remage-Healey et al., 2010; Canopoli et al.,

2016). Often, song preferences are used as evidence for sexual or social preference, or

the presence of memory or recognition of songs. In the NCM, exposing zebra finches to

songs leads to enhanced expression of immediate-early genes, such as *c-fos*, *arc*, and

egr-1 (Mello et al., 1992; Mello and Ribeiro, 1998; Bailey et al., 2002; Velho et al., 2005;

Tremere et al., 2009). All three of these immediate-early genes are inducible

transcription factors that are used as markers for neuronal activity and synaptic

plasticity (Reviewed in Alberini, 2009; Shepherd and Bear, 2011). Comparisons of

intensities of *egr-1* induction by different sound stimuli are commonly used to speculate

about the function of NCM. Although many studies have focused on the role of the

NCM, its function is still unclear due to difficulty in interpreting results of behavioral

preference to songs or the intensity of *egr-1* induction. Adding more complexity, there

seems to be a disagreement between studies in defining the boundaries of the NCM (

Terpstra et al., 2004). While some groups define the lateral boundary of the NCM to be

around 1mm medial to the midline (such as Mello and Clayton, 1994),other groups

include regions more lateral as part of NCM (such as Gobes and Bolhuis, 2007; for a

thorough survey of studies, refer to Terpstra et al., 2004). Some of the many hypotheses

for activities involving the NCM include the following: *i*) formation and maintenance of

auditory memory; *ii*) formation and maintenance of song memory used for vocalization

in males; and iii) Integration of auditory and other sensory information and encoding

context. In sections below, I will review existing literature on studies that led to the different

hypotheses on NCM function.

The evidence for the NCM being in involved in auditory memory comes from the

observations that both song-induced *egr-1* expression and electrophysiological responses of the

neurons are high in response to novel songs and habituate in response to repetition of the

same songs (Chew et al., 1995; Mello et al., 1995). This higher response to novel songs

compared to familiar songs is seen in both sexes (Chew et al., 1996; Woolley and Doupe, 2008;

Yoder et al., 2014), suggesting that the NCM is involved in general auditory memory. In

agreement with this hypothesis, in males, bilateral lesions of the NCM diminishes the birds'

preference for tutor songs (Gobes and Bolhuis, 2007). However, although this could be viewed

as an evidence for the NCM's involvement in familiar song memory or recognition, the results of

this study also support another hypothesis: that the NCM is important for tutor song learning in

males. In males, it is not clear whether the innate behavioral preference for tutor songs is due to

recognition of familiar individuals or innate affinity to tutor or songs similar to the bird's own

song. Male zebra finches naturally prefer their own song over other songs (Remage-Healey et

al., 2010). Since no equivalent studies can be performed in females, the implication of the lack

of tutor preference in the NCM lesioned birds is inconclusive. Altogether, although *egr-1*

expression and electrophysiological evidence suggest a role for the NCM in song recognition,

corresponding behavioral studies to fully support this hypothesis are lacking.

In addition to the role in song memory, in males, the NCM has been implicated in song

learning. Traditionally, as mentioned above, motor memory that is important for vocalization is

thought to be stored differently than auditory memory required for song recognition in different

nuclei. However, studies suggest that the NCM may play a role during the initial

formation of tutor song memory required for singing and song maintenance in

adulthood. During development in males, the basal expression of *egr-1* peaks around 30

days post-hatch (dph) when birds starts to vocalize and practice the tutor song (Jin and

Clayton, 1997). Moreover, infusing an extracellular signal-regulated kinase (ERK)

inhibitor, bilaterally, into the NCM in juveniles during access to their tutor impairs

eventual tutor song learning (London and Clayton, 2008). On the other hand, when the

NCM is lesioned at 40 dph after their first exposure to tutor songs, the animals are able

to sing songs similar to tutor songs as well as control animals, when they are older

(Canopoli et al., 2016). Studies in adults suggest that at least some molecular memory of

tutor songs are encoded in the NCM in adulthood. When males are exposed to their

tutor songs, *egr-1* induction is correlated with the birds' songs' similarities to their tutor

songs (Bolhuis et al., 2000). In addition, in adults, the NCM seems to be involved in

access to birds' own songs or tutor songs. When the NCM is bilaterally lesioned,

lesioned animals show no impairment in hearing, basic song recognition, or singing

performance (Gobes and Bolhuis, 2007; Canopoli et al., 2014). Lesioned birds also

perform as well as control animals in pitch shifting paradigms, where they are reinforced

to shift the pitch of one of the syllables in their songs to a higher frequency to avoid a

white noise punishment (Canopoli et al., 2014). This result implicates that NCM lesions

do not impair processing of the pitch of their own vocalizations. However, the lesioned

birds are not as precise as control animals in returning to the original pitch after the end

of the paradigm, which suggest that NCM lesions could instead impair access to the

template of their own songs (Canopoli et al., 2014). The above mentioned studies

suggest that the NCM plays some roles in song learning. However, this is unlikely to be

the only function of NCM since, although females do not sing, the overall size of the NCM is similar between females and males and the NCM in many studies show similar results when conducted both in females and males.

Studies on song-induced *egr-1* expression also provide evidence for NCM involvement in processing context information of auditory stimuli. Sound loudness, sound location, and visual information modify song-induced *egr-1* expression (Kruse et al., 2004; Avey et al., 2005). In the NCM of females but not males, *egr-1* expression is enhanced in response to female calls (Gobes et al., 2009). In females, *egr-1* expression is higher in response to female calls than to male calls. In males, while female calls cause no induction of *egr-1* expression when males are alone, the presence of other males enhances *egr-1* expression in response to female calls (Vignal et al., 2005). Moreover, physiological state also impacts song-induced *egr-1* expression levels (Park and Clayton, 2002). While in freely moving birds, conspecific songs induce higher expression of *egr-1* compared to generated noise, while in physically restrained birds, song-induced *egr-1* expression is similar in response to conspecific songs and noise (Park and Clayton, 2002). In a similar fashion to the *egr-1* response, electrophysiological studies also suggest that NCM activity is sensitive to context. In males, when animals are alone, the NCM neurons do not respond differently to familiar and unfamiliar female calls (Menardy et al., 2014). However, in the presence of other conspecific individuals, NCM neurons responds stronger to familiar females calls over unfamiliar female calls (Menardy et al., 2014). In contrast, in a similar study in females, NCM neurons responded more strongly to familiar male calls over unfamiliar male calls regardless of context (Menardy et al., 2012). In one study done in females, temporal bilateral lesioning of the NCM with lidocaine diminished females' preference to males with ordinary songs over males with distorted songs, suggesting that the NCM is involved in song processing

important for mate preference (Tomaszycki and Blaine, 2014). From this evidence, it appears that the NCM may play an important role in context processing for sounds, although more behavioral studies with physiological manipulations need to be performed to fully examine this hypothesis.

Estradiol and catecholaminergic actions in the NCM and potential interactions

The fact that environmental and social factors can alter NCM activity suggests that neuromodulators may play a role in modulating NCM activity. In the NCM, two of the most studied neuromodulator categories are neurosteroids and catecholamines. In the zebra finch NCM, a large amount of estradiol can be detected locally (Remage-Healey et al., 2008, 2012) and aromatase expression is high (Shen et al., 1995; Saldanha et al., 2000). Moreover, both tyrosine-hydroxylase (TH) and dopamine-beta-hydroxylase (DBH) fibers are found in the NCM (Bottjer, 1993; Mello et al., 1998). In the NCM of other songbird species, catecholamines and their metabolites have been detected from micro-punches (Sockman and Salvante, 2008; Matragrano et al., 2012). In the NCM, both estradiol and catcholamine synthesis has been shown to increase after song exposure. In the white-throated sparrow NCM, expression of phosphorylated TH was increased after 15 min of song exposure and the levels of dopamine metabolites, dihydroxyphenylacetic acid (DOPAC) and homovanillic acid (HVA), were enhanced in tissue from animals sacrificed after 30 min of song exposure (Matragrano et al., 2012). In zebra finches, when females (but not males) are exposed to conspecific song playback, the number of *egr-1* positive TH neurons increases in the locus coeruleus (Matragrano et al., 2012). Since locus coeruleus neurons are believed to be the principal site where the cell bodies of norepinephrine-synthesizing neurons localize, this result

indicates that norepinephrine levels may be enhanced in areas where DBH fibers are present, including the NCM. In both female and male NCM, when local estradiol levels are measured using *in vivo* microdialysis, estradiol levels are enhanced in response to song playback (Remage-Healey et al., 2008, 2012).

While evidence suggests that estradiol plays an important role in auditory processing, the exact signaling and mechanism controlling local synthesis is unclear. Blocking local estradiol synthesis acutely using an aromatase inhibitor, fadrozole, diminishes males' preferences for their own songs over conspecific songs (Remage-Healey et al., 2010). Also, in adult males, systemic fadrozole treatment for days prior to song exposure blocks NCM neuronal responses from habituating to the song (Yoder et al., 2012). In both females and males, NCM neurons expresses abundant aromatase in the cell bodies (Saldanha et al., 2000), and infusing exogenous estradiol directly into the NCM enhances NCM neuronal responses to auditory stimuli relative to the baseline (Remage-Healey et al., 2010, 2012). Although this suggests that estradiol has a common role in females and males, there is some evidence for sex differences. In males, estradiol levels in the NCM are not only enhanced with song stimuli but also enhanced with visual stimuli of a female or when they are interacting with females (Remage-Healey et al., 2008). In females, visual stimuli alone do not have effect on estradiol levels in the NCM (Remage-Healey et al., 2012). In females, fadrozole infusion to the NCM decreases relative electrophysiological auditory responsiveness and the effect persist after the drug infusion (Remage-Healey et al., 2012), while in males, fadrozole has a small decreasing effect on NCM auditory responsiveness during fadrozole infusion and a large rebound effect is seen after the fadrozole has been washed out (Remage-Healey et al., 2010). In addition, estradiol infusion into the male NCM enhances neuronal responses to BOS in the HVC, a sensory-motor nucleus for

vocalization (Remage-Healey and Joshi, 2012), suggesting that, at least in males,

estradiol signaling in the NCM is involved in processing or recognizing BOS. Moreover,

more aromatase is found pre-synaptically in males compared to females (Peterson et

al., 2005; Rohmann et al., 2007; Comito et al., 2016). These pieces of evidence together

with evidence for the involvement of the NCM in song learning and maintenance (see

previous section) suggest that sex differences in estradiol signaling may account for sex

differences in the function of the NCM. Therefore, both in females and males, estradiol

seems to be important for auditory processing while the mechanisms and the

functionality may be different between the sexes.

Evidence for the role of catecholamines in songbird behavior comes from

studies using N-(2-chloroethyl)-N-ethyl-2-bromobenzylamine hydrochloride (DSP-4), a

neurotoxin which selectively induces the degeneration of adrenergic neurons that

produce norepinephrine (Ross and Stenfors, 2015). However, most studies with DSP-4

are done on courtship and singing behavior and only a few studies specifically focus on

auditory processing or the role of NCM. In the female canary NCM, treatment with DSP-

4 decreases song-induced *egr-1* expression and diminishes the difference in the *egr-1*

responses between conspecific and heterospecific songs (Lynch and Ball, 2008). In zebra

finch males, when song-induced regional activity is measured by functional magnetic

resonance (fMRI), selectivity to BOS over conspecific songs and to heterospecific songs

over conspecific songs were reversed in DSP-4 treated birds (Poirier et al., 2009). In

seasonally-breeding songbirds, catecholaminergic signaling in the NCM are shown to

interact with circulating estradiol levels that are higher during the breeding season

compared to non-breeding seasons (LeBlanc et al., 2007; Matragrano et al., 2011;

21

Heimovics et al., 2012). Moreover, in other songbird species, catecholaminergic modulation alters behaviors that are associated with the NCM (Appeltants et al., 2002; Pawlisch et al., 2011). However, in zebra finches, the role of catecholamines in the NCM isless clear. In addition, the molecular or cellular mechanisms of how catecholamines affect auditory processing are not known for any species. Only one study has directly tested the role of norepinephrine on the functionality of the NCM. In this study, using adult female zebra finches, blocking adrenergic receptor subtypes with specific inhibitors, phentolamine and propranolol, shortly before song playback to the birds decreased song-induced *egr-1* and c-fos expression in the NCM (Velho et al., 2012). In the same study, when phentolamine was applied unilaterally to the NCM after birds were introduced to a song and the electrophysiological response to the same song or a new song was recorded shortly after, no difference was found in the habituation rate to the songs between phentolamine-treated and non-treated hemispheres. However, when phentolamine was applied before a song exposure and the response to the same song and to a new song was recorded about 20 hrs later, the relative habituation rate of the original song to the habituation rate of the novel song was smaller in the phentolamine-treated hemisphere compared to the control hemisphere. These results indicate that adrenergic signaling in the NCM during auditory experiences may be important for auditory memory formation.

In the NCM, the study of rapid neuromodulatory actions suggests that estradiol and some catecholamines, such as norepinephrine, may either have synergistic effects or otherwise interactive effects. As mentioned above, in the NCM, both estradiol and norepinephrine are detectable and the enzymes that are required for their syntheses are expressed. Estradiol and norepinephrine levels are also both elevated in response to songs and they are both important for song or auditory processing such as song preference and NCM electrophysiological

habituation to songs (Remage-Healey et al., 2008, 2010; Pawlisch et al., 2011; Matragrano et al., 2012; Velho et al., 2012; Vahaba et al., 2013; Yoder et al., 2014). Furthermore, the downstream effects on *egr-1* activation seem to be similar. Blocking estradiol synthesis or blocking adrenergic receptor activation reduces song-induced *egr-1* expression (Velho et al., 2012; Krentzel and Remage-Healey, 2014). In one study done in the NCM in canary males, aromatase-expressing cell bodies were shown to be surrounded densely by TH+ fibers (Appeltants et al., 2004), supporting the hypothesis that catecholamines, including norepinephrine, could be regulating aromatase activity through pre-synaptic inputs.

Hypotheses and questions addressed in this dissertation

In summary, in the zebra finch brain, while the importance both of catecholamines and estradiol in auditory processing has been examined, the precise mechanism of estradiol regulation and the extent of interaction with catecholaminergic signaling is unclear. As mentioned above, in the NCM, aromatase activity is enhanced when birds are exposed to auditory stimuli. However, whether this mechanism is due to or modulated by the change in neuronal activity of aromatase-expressing neurons or the activation of signaling cascades by the actions of other neuromodulators is not clear. Since there is strong evidence that the levels of both catecholamines and estradiol are enhanced during auditory experience, it is possible that the actions of one of the catecholaminergic neuromodulators alter aromatase activity. Even though studies in the NCM suggest that catecholamines can interact with estradiol, none of them compared their effects to estradiol or directly tested the possibility that catecholaminergic effects could interfere or interact with estradiol-dependent effects.

In addition, although we know that application of exogenous estradiol enhances auditory-evoked neuronal activity in majority of the neurons in the NCM, it is not clear *in vivo* how the estradiol levels are controlled at the level of circuits within the NCM. Both the presynaptic and somatic locations of aromatase are likely to have mechanistic importance in the higher order auditory processing in the NCM. At the same time, the activity-dependent regulation of aromatase activity, as well as the network connectivity of aromatase positive neurons are unknown but are important for understanding the mechanism of estradiol modulation of information processing. The organization, connection, and identity of aromatase neurons within cortical regions will provide clues to this mechanism.

Therefore, in this dissertation, I explore the central hypothesis that the network and chemical environment of aromatase cells is integral to the regulation of estradiol synthesis. More specifically, I *i)* Tested the role of norepinephrine in auditory processing and whether it plays a role in the regulation of aromatase activity and *ii)* Analyzed the identities and organization of aromatase positive cells in the NCM and other auditory regions.

CHAPTER II

IN VIVO DETECTION OF FLUCTUATING BRAIN STEROID LEVELS IN ZEBRA FINCHES

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Year: 2014.

Abstract/Introduction

This protocol describes a method for the *in vivo* measurement of steroid hormones in brain circuits of the zebra finch. A guide cannula is surgically implanted into the skull, microdialysate is collected through a microdialysis probe that is inserted into the cannula, and steroid concentrations in the microdialysate are determined using the enzyme-linked immunosorbent assay (ELISA). In some cases, the steroids measured are derived locally (e.g., neural estrogens in males), whereas in other cases, the steroids measured reflect systemic circulating levels and/or central conversion (e.g., the primary androgen testosterone and the primary glucocorticoid corticosterone). A reverse-microdialysis ("retrodialysis") method that can be used to deliver pharmacological agents into the brain to influence local steroid neurochemistry as well as behavior is also discussed.

Materials

It is essential that you consult the appropriate Material Safety Data Sheets and your institution's Environmental Health and Safety Office for proper handling of equipment and hazardous material used in this protocol.

Reagents

Artificial cerebrospinal fluid (aCSF) for zebra finch brain.

The bovine serum albumin (BSA) in the aCSF increases the solubility of lipophilic steroids (Remage-Healey et al. 2008, 2010); however, BSA causes interference with the ELISA for corticosterone. When measuring brain corticosterone levels, use BSA-free Dulbecco's phosphate-buffered saline (Sigma-Aldrich) instead of aCSF.

ELISA kit to detect the steroid of interest

Corticosterone EIA kit (Cayman Chemical; cat. no. 500655)

E2 EIA kit (Cayman Chemical; cat. no. 582251) for 17-ß-estradiol Testosterone ELISA kit (Enzo Life Sciences; cat. no. ADI-900-065)

Equithesin

We use an in-house formulation of this general anesthetic that contains 10.2 mg/mL Nembutal, 42.5 mg/mL chloral hydrate, 34.6% propylene glycol, 8.9% ethanol, and 21 mg/mL magnesium sulfate. Equithesin is administered intramuscularly at a dose of 3.2 mg/kg body weight. Isofluorane can be used as an alternative.

Ethanol (20% and 100%)

Lidocaine (2% in ethanol) or another local anesthetic Meloxicam

Zebra finches (males and females; age >120 d)

All finches used in our experiments are from our institutional breeding colony.

Equipment

Acoustic attenuation chamber (Audiometric Booth AB08; Eckel Industries)

The chamber has a one-way glass partition and a 10-mm portal for microdialysis inflow/outflow tubing.

Cotton-tipped applicators

Cyanoacrylate adhesive (e.g., Vetbond)

Dental acrylic (Perm Reline and Repair Resin; Hygenic; cat. no. H00358) Dissection microscope

(Zeiss OPMI 1)

Fluorinated ethylene propylene (FEP) tubing and adaptors (CMA Microdialysis)

Fraction collector (refrigerated) (CMA 470) (CMA Microdialysis)

This is optional, but it is very useful for the collection of large numbers of

samples.

Guide cannula (CMA 7) (CMA Microdialysis) Heating pad (FHC)

Kimwipes

Microdialysis cage

Microdialysis probe (CMA 7; 1 mm cuprophane probe membrane) (CMA Microdialysis)

The probe membrane has a 6-kDa cutoff, which allows the passive diffusion of

small molecules and pharmacological agents such as steroids and enzyme inhibitors

(~300 Da).

Microdialysis tubing adaptors (for connection to swivel) (Instech MC015)

Needles (22-, 26-, and 30-gauge)

Probe/guide clip (CMA)

Standard surgical tools

Stereotaxic apparatus (adapted for use with small birds)

We use a device that was custom-made by Herb Adams Engineering.

Swivel (dual-channel, quartz-lined) (Instech 375/D/22QM) Syringe, for injections (0.5-cc)

Syringe, for pump (1.0-cc; set to infuse at 2.0 µL/min) Syringe filters (0.22-µm)

Syringe pump (PHD 22/2000 Infusion from Harvard Apparatus)

Tether (a modified sleeve that connects the swivel to the cannula; see Fig. 3)

A counter-balanced lever arm can be obtained from Instech for microdialysis studies with mice.

Method

Surgery

The surgical procedure takes 30 min–1 h.

1. Weigh the zebra finch. Deprive the animal of food for ~40 min to allow the crop to empty and to prevent aspiration pneumonia during surgery.

2. Incubate the Equithesin in a warm water bath to dissolve any crystals in the solution.

3. Administer an intramuscular injection of 50 μL of Equithesin to the breast muscle of the finch.

For animals <100 d posthatch or <12 g in weight, inject 30–45 μL of Equithesin.

For Equithesin-induced anesthesia, breathing rates should be short and shallow. Wait 20 min until the bird is deeply anesthetized. If breathing is labored, terminate surgery immediately.

For isoflurane-induced anesthesia, use 2%–3% isoflurane with 0.4 L/min oxygen. Monitor the depth of anesthesia as described above; isoflurane typically takes effect within 5 min. Incrementally decrease the isoflurane throughout the procedure to prevent overdose while maintaining the depth of anesthesia.

4. Wrap the bird in a Kimwipe "tunic" and bind the body loosely with tape.

Animals are more comfortable during survival surgery if they are lightly restrained (Grandin 2007).

5. Remove the feathers from the head and around the ear canal using scissors. Alternatively, brush the head feathers back gently with a cotton-tipped applicator soaked in 20% ethanol.

6. Secure the bird on a stereotaxic apparatus on top of a heating pad set at ~34˚C.

Set the angle of the head to ensure the skull plane of the target area is perpendicular to the cannula entry point. For implantations targeting the caudal telencephalon, this head angle is typically 45˚.
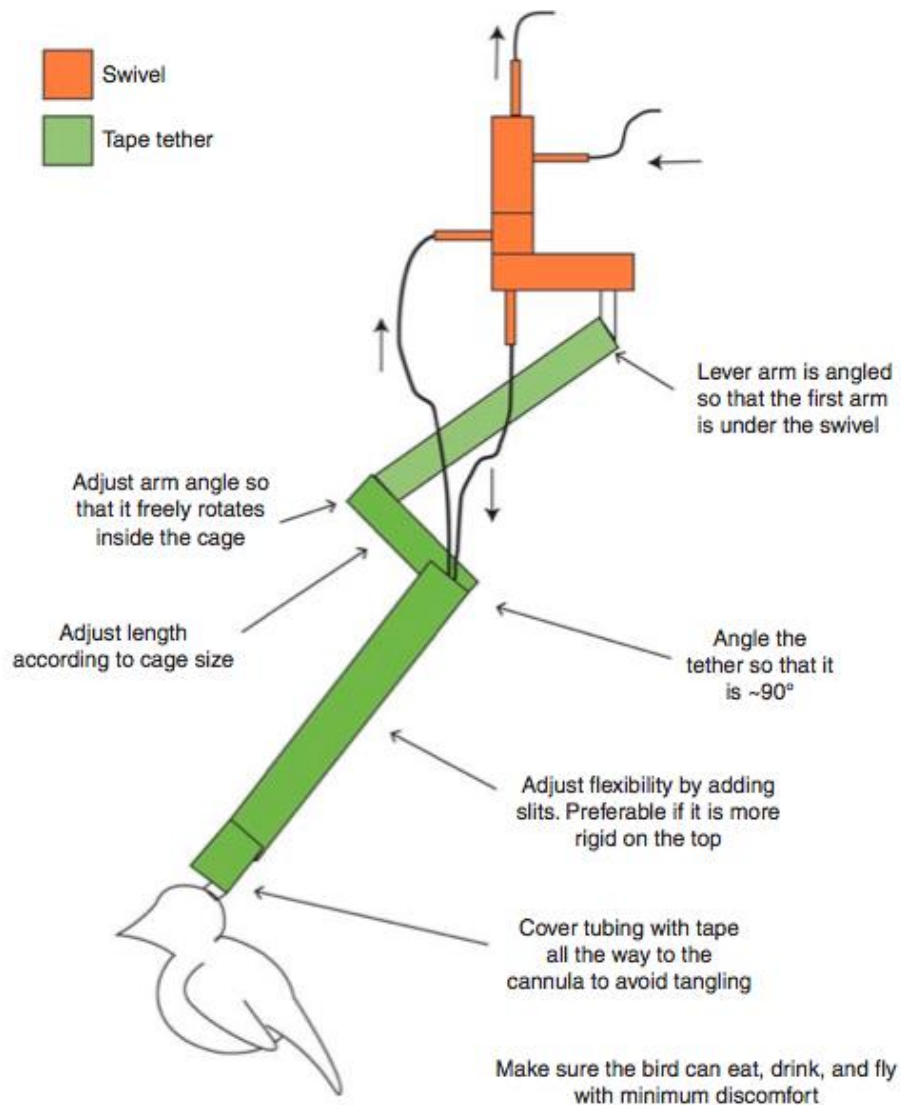
Figure 3: Schematic representation of the customizable tether design used in zebra finch *in vivo* microdialysis

At the top is the swivel connected to the microdialysis cage with inlet and outlet FEP tubing. The top of the tether (lever arm) contains either a thin wire or plastic straw (such as those that are shipped with CMA 7 probes to protect them) to enable lightweight rigidity, and it is then enshrouded with tape. The tether is angled to provide a lever arm to translate rotational torque from the bird to the swivel while also allowing full freedom of movement (including flight) inside the cage. Multiple tape component stages ensure flexibility in addition to an angled lever arm.

7. Inject 10–15 µL of 2% lidocaine subcutaneously into the scalp.

Smooth the lidocaine into the working area and confirm that the local anesthetic is effective by testing the scalp with light forceps.

8. Using iridectomy scissors, make incisions to the skin along the midline and expose the skull. Make lateral incisions from this medial incision at both rostral and caudal ends, forming an "I."

9. Locate the bifurcation of the midsagittal sinus (point-of-origin).

This can be best accomplished by making a small window incision (remove a 1 mm × 1 mm) in the upper leaflet of skull surrounding the sinus area. Leave the lower leaflet intact.

10. Secure a 26-gauge needle to a probe/guide clip attached to a micromanipulator and place the tip of the needle on top of the point-of-origin ("zero point") without piercing the lower leaflet. Note the coordinates on the micrometer scales for x and y dimensions on the stereotaxic apparatus.

11. Using stereotaxic coordinates create a small hole through both upper and lower leaflets of the skull using needle tips.

12. Carefully resect the dura mater inside the hole using a 30-gauge needle and expose the brain surface.

A shallow lateral resection is essential to avoid damaging underlying brain tissue.

13. Descend the guide cannula ventral to the target area using pre-established depth coordinates.

Leave the obdurator (the "dummy probe" supplied with the CMA cannula) inside the guide cannula.

14. Secure the guide cannula to the skull by applying a thin layer of cyanoacrylate adhesive around the opening, avoiding direct contact with the surface of the brain.

For subadults, use a #11 scalpel blade to etch small, shallow lines in the skull area roughening the skull surface and encouraging cyanoacrylate adhesion.

15. While the cyanoacrylate is drying, apply dental acrylic on top of it using cotton swabs. Layer additional cyanoacrylate as necessary.

Make sure that the dental acrylic completely covers the guide shaft of the cannula and at least one third of the cannula housing itself.

16. Reattach the scalp to all remaining exposed skull areas by applying cyanoacrylate under the skin and securing it to the skull and dental acrylic.

17. After recovery, monitor and treat the animal for perioperative pain as necessary with meloxicam (or another nonsteroidal anti-inflammatory drug).

Probe Implantation

Perform probe implantation 3–4 d after surgery. The procedure takes ~1 h.

18. Fill FEP tubing and the swivel assembled to the microdialysis cage with aCSF.

19. Prime the microdialysis probe by placing it in the probe clip and immersing it in 100% ethanol in a microcentrifuge tube. Keeping the probe immersed, pump water (for 20 min) and then aCSF (8 μL/min) into the probe through the inlet tube and attach a microdialysis tether to the probe using a small piece of tape. After flushing, remove the probe from the microcentrifuge tube and implant it in the guide cannula (see Step 20).

This transfer should be completed quickly (<1 min) so that the microdialysis probe does not dry out.

20. Grasp the bird in one hand, stabilize the head gently with fingertips and, using #5 forceps, remove the dummy probe from the guide cannula and replace it with the prefilled microdialysis probe.

Implantation can be completed under light isoflurane anesthesia to minimize

disturbance.

21. Apply small amount of cyanoacrylate adhesive to the exterior of the probe housing at the

top of the cannula to keep the probe in place.

Make sure the cyanoacrylate is external to the cannula channel to not impact

the CNS or probe, and double- check that aCSF is welling out from the outlet tubing after

the implantation.

22. Connect the inlet and outlet tubing to the microdialysis tubing inside the   chamber.

23. Attach the tether to the arm as shown in Figure 3. Adjust the tether height and lever arm,

and confirm that dialysate is flowing at the correct rate.

Most ELISAs require at least 50 µL of sample. A flow rate of 2 µL/min for 30 min

is sufficient to obtain such samples. See Troubleshooting.

Detection of Steroids

24. Begin *in vivo* experiments 8–12 h following implantation to allow implantation-induced

neuro- chemical responses to subside. Use a commercially available ELISA kit to detect steroids

in the dialysate.

In most cases, the dialysate can be run directly on the ELISA plate because our

aCSF with BSA is similar to the composition of the ELISA assay buffer. Always run four to

eight control wells for each ELISA with aCSF that has not been perfused through the

dialysate system to establish baseline comparisons.

Troubleshooting

Problem (Step 23): The aCSF dialysate is not flowing at the correct rate.

Solution: When the bird is removed from the system, flush ddH2O through the entire system (FEP tubing, swivel inlet, and outlet) for 60 min after every use at 8–10 µL/min. Continue flushing ddH2O at 0.5–1.0 µL/min until the next experiment. Filter the aCSF perfusate through a 0.22-µm syringe filter before pumping it through the microdialysis setup to prevent particulate buildup.

Problem (Step 23): The FEP tubing is clogged (i.e., there is no flow through a section of the dialysis setup).

Solution: Double-check that fluid is welling from the syringe tip at the desired flow rate. Run the system at 8 µL/min for ~1 min to try to flush bubbles or particulate matter from the tubing. Check that fluid is coming out of each connection of FEP tubing. Trim the ends of the FEP tubing at each connection to remove clogs.

Problem (Step 23): The clog is isolated to the bird (within the probe tubing).

Solution: Make sure that the FEP tubing in the tether is not tangled. Remove the tape around the FEP tubing and reapply to reduce possible rotational torque constriction.

Problem (Step 23): The clog is within the swivel.

Solution: Remove the microdialysis probe lead and run ddH2O through the swivel at 20 µL/min. If perfusate is welling from the swivel, wait 5 min at 8 µL/min. This will flush any particulate matter and/or bubbles in the swivel. Replace the swivel.

Problem (Step 23): The clog is within a section of FEP tubing.

Solution: Run at 20 µL/min through the isolated section of FEP for 5–10 min. Switch out the section of FEP tubing.

Discussion

The *in vivo* microdialysis procedure described here has been used successfully to detect fluctuating neurosteroids in the auditory forebrain (Remage-Healey et al. 2008, 2012; Ikeda et al. 2012) and in the hippocampus (Rensel et al. 2012; Rensel and Schlinger 2013) of behaving adult zebra finches. Similar methods have been developed to examine changing levels of catecholamines in behaving zebra finches (e.g., Sasaki et al. 2006).

This protocol can be adapted to reverse-microdialyze (retrodialyze) drugs and steroidogenic compounds (Remage-Healey et al. 2008). When selecting candidate retrodialysis compounds, it is important to consider molecular mass. Proteins and other large molecules >6 kDa are likely to be bound up in the microdialysis tubing, but see Ulrich et al. (2013) for further discussion. Typical retrodialysis experiments involve collecting several serial baseline samples with aCSF perfusion, switching to a new syringe prefilled with drug dissolved in aCSF for 30–60 min, and then a period of washout in aCSF. When retrodialysis experiments are performed inside a sound-attenuation chamber, syringe changes can be conducted outside the chamber, allowing experimental manipulation without disturbing the animal. When switching to a new solution allow 1–2 min of high rate of flow (8–10 µL/min) to confirm the perfusate is flowing and to eliminate air bubbles in the FEP tubing. Once flow is re-established the experiment can resume at the desired flow rate (i.e., 2 µL/min). Automated liquid switches (obtained from CMA Microdialysis) can also be used. It is important to account

for the "dead volume" of the microdialysis tubing and swivel, given the dimensions of FEP tubing

and the total volume of perfusate passing through the swivel. Calculations of dead volume,

factoring in the flow rate, allow for precise timing of sample collection during retrodialysis

intervals. The *in vivo* microdialysis methods described here can be used to study steroid

signaling in the brain for a variety of experimental purposes. Thus, the combined study of

neurochemistry and behavior in a vocal learning species now has a new set of powerful tools.

Recipe

Artificial Cerebrospinal Fluid (aCSF) for Zebra Finch Brain

Reagent	Final concentration
NaCl	199 mM
NaHCO3	26.2 mM
KCl	2.5 mM
MgSO4	1.0 mM
CaCl2	2.5 mM
Glucose	11.0 mM

Dissolve the reagents in ultrapure ddH2O (~18 Mfi) and adjust the pH to 7.4–7.6. Store

at 4˚C in the dark for a maximum of 4 wk. Add 1% bovine serum albumin (BSA; Sigma- Aldrich

A1470) to 50-mL aliquots and filter (with 0.2-μm syringe filters) to eliminate suspended particles

and reduce clogging of FEP tubing and probes.

Acknowledgements

NOREPINEPHRINE MODULATES CODING OF COMPLEX VOCALIZATIONS IN THE SONGBIRD AUDITORY CORTEX INDEPENDENT OF LOCAL NEUROESTROGEN SYNTHESIS

Authors: Ikeda MZ, Jeon SD, Cowell RA, Remage-Healey L.

Abstract

The catecholamine norepinephrine plays a significant role in auditory processing. Most studies to date have examined the effects of norepinephrine on the neuronal response to relatively simple stimuli, such as tones and calls. It is less clear how norepinephrine shapes the detection of complex syntactical sounds, as well as the coding properties of sensory neurons. Songbirds provide an opportunity to understand how auditory neurons encode complex, learned vocalizations, and the potential role of norepinephrine in modulating the neuronal computations for acoustic communication. Here, we infused norepinephrine into the zebra finch auditory cortex and per- formed extracellular recordings to study the modulation of song representations in single neurons. Consistent with its proposed role in enhancing signal detection, norepinephrine decreased spontaneous activity and firing during stimuli, yet it significantly enhanced the auditory signal-to-noise ratio. These effects were all mimicked by clonidine, an a-2 receptor agonist. Moreover, a pattern classifier analysis indicated that norepinephrine enhanced the ability of single neurons to accurately encode complex auditory stimuli. Because neuroestrogens are also known to enhance auditory processing in the songbird brain, we tested the hypothesis that norepinephrine actions depend on local estrogen synthesis.

Neither norepinephrine nor adrenergic receptor antagonist infusion into the auditory

cortex had detectable effects on local estradiol levels. Moreover, pretreatment with

fadrozole, a specific aromatase inhibitor, did not block norepinephrine's

neuromodulatory effects. Together, these findings indicate that norepinephrine

enhances signal detection and information encoding for complex auditory stimuli by

suppressing spontaneous "noise" activity and that these actions are independent of

local neuroestrogen synthesis.

Introduction

Neuromodulators are critical for state-dependent changes in neural circuit

activity and behavior. Norepinephrine in particular is important for both altering the

gain of sensory stimuli and for fast behavioral switching (Bouret and Sara, 2005). During

development, sensory plasticity of the visual and auditory cortex is dependent on

norepinephrine, indicating a key role in shaping early sensory experience (Kasamatsu et

al., 1979; Edeline et al., 2011; Shepard et al., 2015). Computational models and *in vitro*

recordings have generated predictions about the role of norepinephrine in shaping

neural circuit activity, *via* specific actions at adrenergic receptor (AR) subclasses

(Hasselmo et al., 1997; Carey and Regehr, 2009). In mammalian auditory cortex,

norepinephrine enhances auditory processing of pure tones and calls, by decreasing

spontaneous and auditory-evoked firing, altering ex- citatory/inhibitory balance, and

altering frequency tuning (Foote et al., 1975; Manunta and Edeline, 1997, 2004; Salgado

et al., 2011a, 2012). However, it is less clear what role norepinephrine plays in the

coding of complex sensory stimuli; that is, beyond the processing of simple stimuli, such

as tones and the tuning of receptive fields (Hurley et al., 2004).

Songbirds have become a premiere system for understanding how auditory neurons

encode complex, learned vocalizations (Woolley and Rubel, 2002; Elie and Theunissen, 2015).

Norepinephrine has been associated with auditory processing in the songbird brain (Sockman

and Salvante, 2008; Castelino and Schmidt, 2010), and norepinephrine can influence the activity

of songbird premotor neurons *via* actions at a-ARs (Cardin and Schmidt, 2004; Sizemore and

Perkel, 2008). Noradrenergic signaling is necessary for immediate-early gene induction in

response to song, as well as long-term adaptation of forebrain neurons to familiar stimuli (Lynch

et al., 2012; Velho et al., 2012). Songbirds therefore provide an exceptional opportunity to test

how norepinephrine modulates the neural coding of auditory stimuli, in particular the coding of

complex vocalizations important for social communication and learning.

Norepinephrine likely interacts with other neuromodulators in the control of audition.

In one forebrain region of songbirds, the caudomedial nidopallium (NCM), a region analogous to

mammalian secondary auditory cortex, local estradiol levels are elevated in response to hearing

songs (Remage-Healey et al., 2008, 2012). In the canary NCM, fibers expressing tyrosine

hydroxylase surround aromatase-positive neurons, suggesting that catecholamines regulate

local estradiol synthesis (Appeltants et al., 2004). Furthermore, inhibiting either catecholamine

or estradiol actions in the NCM disrupts auditory processing and immediate-early gene

induction (Lynch and Ball, 2008; Remage-Healey et al., 2010; Poirier and Van der Linden, 2011;

Velho et al., 2012; Vahaba et al., 2013). These studies suggest that catecholamines and

neuroestrogens interact to modulate auditory processing in songbirds, although this interaction

has not been tested in any system to date.

Here, we directly test the hypothesis that norepinephrine regulates the auditory coding

of complex vocalizations in the songbird forebrain. Furthermore, we examine whether

norepinephrine actions are dependent on local estradiol synthesis. We find that norepinephrine

enhances the coding accuracy of higher auditory cortical neurons for complex

vocalizations, an effect similar to neuroestrogen modulation, but without directly

impacting neuroestrogen synthesis.

Materials and Methods

A total of 74 female zebra finches were used in this study. The protocols for

animal care and use were approved by the Institutional Animal Care and Use Committee

at the University of Massachusetts (Amherst, Massachusetts). Adult female zebra

finches were housed in unisex cages un- der photoperiod of 14 h light (7:00 –21:00) and

10 h dark. We elected to perform these experiments in females because of the more

extensive prior literature on catecholamines in the auditory forebrain of female

songbirds (Sockman and Salvante, 2008; Matragrano et al., 2011, 2012; Pawlisch et al.,

2011). *In vivo* microdialysis procedures closely followed those as detailed in a recent

protocol paper (Ikeda et al., 2014).

Surgery. The protocol for surgeries were adapted from Remage-Healey et al.

(2008, 2010) and Remage-Healey and Joshi (2012) and Ikeda et al. (2014). Birds were

orally administered meloxicam (1 µl/g weight). After 20 min, birds were intramuscularly

injected with 45 µl of equithesin. Lidocane (20 µl, 4% in ethanol; Sigma-Aldrich) was

then injected subcutaneously under the scalp for local anesthesia, and an incision was

made to remove the skin to expose the skull. The bifurcation point of the midsagittal

sinus was used as point of origin. Using a stereotaxic frame with a micromanipulator,

the coordinates for cannula insertion and recording site were located.

For microdialysis experiments, a guide cannula with a dummy probe (CMA 7;

CMA Microdialysis AB) was inserted into the brain (1.1 mm lateral and 1.2 mm rostral

relative to the point of origin). The tip of the cannula was placed 1.4 mm ventral from the surface of the brain. The cannula was fixed to the skull with dental cement and cyanoacrylate. The birds were allowed to recover for at least 4 d following surgery before microdialysis began.

Retrodialysis. The day before starting experiments, the dummy probe was replaced with a microdialysis probe (CMA 7 with 2 mm membrane; Microdialysis Probe, CMA Microdialysis) prefilled with aCSF. The inlet and outlet tubing from the probe was connected to a microdialysis swivel (Instech Laboratories) and then to a syringe pump (PHD 2000 Harvard Apparatus) and a collection tube, respectively, with FEP tubing (0.12 mm inner diameter; CMA Microdialysis) (see Fig. 3.3*A*). Animals were placed inside a sound-attenuation chamber with a one-way glass partition (Eckel Industries) to allow undisturbed sample collection and manipulation of retrodialysis conditions. The probe was perfused at a flow rate of 0.5 µl/min with aCSF (199 mM NaCl, 26.2 mM NaHCO3, 2.5 mM KCl, 1 mM NaH2PO4, 1.3 mM MgSO4, 2.5 mM CaCl, 11 mM glucose, 1% BSA, pH 7.4 –7.6). Twenty-four hours after probe implantation, the flow rate was increased to 2 µl/min at least 30 min before the start of the experiment and kept at this rate until the end of the experiment.

Each experiment consisted of collecting consecutive samples twice before (Pre1 and Pre2), once during (Retro), and twice after (Post1 and Post2) retrodialysis treatments. All collection intervals were 30 min. Regular aCSF was pumped through the probe during the pre and post periods, while 0, 0.01, or 0.1 mM of norepinephrine-hydrocloride (Sigma-Aldrich) aCSF solution was infused through the dialysis probe during retrodialysis (Retro). Experiments were conducted twice a day in the morning (9:30 A.M.) and the afternoon (2:00 P.M.) for 3– 4 d with treatments randomized for each subject and period. The animals were monitored with a video camera during retrodialysis. In a subset of animals, experiments were performed at night to test for potential circadian effects of norepinephrine on estradiol levels in NCM. The night

experiments began at 9:30 P.M. relative time (i.e., 30 min after lights off). Estradiol

concentrations in the dialysate samples were measured by ELISA, previously validated with

GC/MS (Remage-Healey et al., 2008, 2012). All samples from each animal were assayed

in the same run to minimize interassay variation. For adrenergic antagonist retrodialysis

(reagents purchased from Sigma-Aldrich), samples were collected in the same manner

as norepinephrine retrodialysis, but only two samples were collected (once before and

once during antagonist treatment with 30 min collection intervals) because we did not

observe a difference between the two pre collection periods in the previous

retrodialysis experiment.

The videotaped trials were analyzed offline using JWatcher to assess whether

there were any changes in locomotor activity with treatment. Within 2 d after the last

experiment, birds were killed using an overdose of isoflurane. They were then either

rapidly decapitated or transcardially perfused with 0.1 M PBS followed by 10% formalin.

The brains were extracted and fixed in 20% sucrose formalin solution for at least 24 h at

4°C. The brains were then frozen and embedded in OCT at -80°C, sectioned by cryostat

(45 µm), and Nissl stained with thionin to confirm the location of the probe site. All

microdialysis experiments below are from birds confirmed to have probe sites restricted

to NCM.

Electrophysiology with retrodialysis. For electrophysiology experiments, the

skull dorsal to the NCM was marked with a #11 scalpel blade. Using cyanoacrylate and

dental acrylic, a custom-made head post was attached to the rostral skull. A silver wire

reference ground was inserted under the upper-leaflet of skull. Experiments were

conducted 1–2 d after surgery. On the day of the experiment, the bird was anesthetized

with three 30 µl intramuscular injections of 20% urethane spaced 30 min apart. After

the last injection, the bird was secured by the head post to an *in vivo* recording rig inside a

sound attenuation booth (Industrial Acoustics). Body temperature was maintained at ~37°C with

a heating pad (FHC Neurocraft). A small craniotomy exposed the brain surface dorsal to NCM. A

microdialysis probe (as above, filled with aCSF as above) and an extracellular electrode

(Carbostar-1, Kation Scientific) were inserted into the NCM (~1.4 mm ventral from the surface of

the brain) adjacent to each other (within 200 –500 μm). All recordings began after a mini- mum

20 min delay following implantation of the probe/electrode to allow implantation-induced

changes to subside. Each recording session collected NCM extracellular responses to

randomized 20 repetitions of four 2 s playback stimuli (CON1, conspecific zebra finch song 1;

CON2, conspecific zebra finch song 2; HET, heterospecific bengalese finch song; WN, white

noise; »70 dB). The interstimulus interval was at 10 s, and the total duration of each recording

was ~15 min. All recordings were amplified, bandpass filtered (300 –5000 Hz; A-M Systems), and

digitized at 20 kHz (Micro 1401, Spike 2 software; Cambridge Electronic Design).

To collect baseline responses to auditory stimuli, the first recording   was collected

following 30 min of aCSF perfusion (Pre). To test the effect of norepinephrine (N = 17),

recordings were conducted after 30 – 40 min infusion of 0.1 mM norepinephrine (in aCSF, NE),

and then followed by   30 min aCSF washout (Post). In a separate set of birds (N = 10), to test the

effect of norepinephrine in the presence an aromatase inhibitor, fadrozole (100 –500 μM)

(Wade et al., 1994), the NCM was perfused with fadrozole for 30 –50 min, before and during

and after 0.1 mM norepinephrine infusions. In a separate set of animals (N = 9), the effect of

adrenergic agonists (all from Sigma-Aldrich), cirazoline (N = 2), and clonidine (N = 6), and an α2

antagonist, idazoxan (N = 1), were tested at two different concentrations (0.5 mM, LOW; 5 mM,

HIGH). After the "Pre" recording, the sites were infused with low dose of the agonists, and then

with high dose. For the agonist experiments, we noticed that some single units (N = 6 of 13)

44

were not auditory for some stimuli at baseline (evoked firing — spontaneous firing < 0), but following agonist treatment they developed an auditory response pattern (evoked firing — spontaneous firing > 0), and these units were included in the analyses. Each infusion period was followed by recording sessions. For all experiments, recording sites were lesioned (10 µA for 10 s) to allow electrode site confirmation.

The dialysates during the infusions and recordings were collected and stored at -80°C and were later measured for estradiol levels with ELISA. Immediately after the electrophysiological recordings, animals were killed in the same way as the microdialysis experiment. The brains were extracted for anatomical confirmation of electrode and probe sites.

Double-label immunocytochemistry. A separate set of *n* = 2 animals were killed with an overdose of isoflurane and transcardially perfused with 0.1 M PBS followed by fresh ice-cold 4% PFA. Brains were extracted and postfixed in 4% PFA for 1 h. The brains were then cryoprotected in 30% sucrose PBS overnight. The brains were embedded in OCT and cryosectioned at 40 µm into 0.1 M PB. The free-floating sections were rinsed with PB and blocked and permeabilized with 10% normal goat serum in 0.3% PBS and Triton X-100 (PBT) for 1 h. The sections were incubated with anti-zebra finch aromatase rabbit antibody (1:2500 in 0.3% PBT; generous gift of Dr. Colin Saldanha) in room temperature for 1 h and in 4°C for 48 h. The sections were then washed with 0.1% PBT, incubated with goat anti-rabbit IgG (1:500 in 0.3% PBT; Vector Laboratories; 1 h), washed in 0.1% PBT, treated with avidin-biotin-peroxidase complex (Vectastain ABC Kit, Vector Laboratories; 90 min), washed again in 0.1% PBT, and developed in Vector NovaRED enzyme substrate (Vector Laboratories; 5–10 min). The sections were stained for DBH by repeating the procedure using anti-DBH rabbit (1:8000; Immunostar), goat

anti-rabbit IgG (1:500; Vector Laboratories), and Vector SG enzyme substrate (Vector Laboratories; 5–10 min). After development, the sections were mounted onto gelatin-coated slides. The slides were dried overnight and coverslipped with Permount (Fisher Scientific) and visualized under a light microscope (Zeiss).

Because the two primary antibodies were raised in the same species (rabbit), the immunostaining was analyzed with caution. However, generally, the two populations were clearly distinguishable from one another, as DBH was found in beaded fibers and aromatase was found in cell bodies and their processes. Sections exposed to both primary anti- bodies were compared with sections from the same animals in the same run exposed to one or the other primary antibody to assess reliability.

Data analysis. For dialysate samples, to normalize individual variability in levels of estradiol in the baseline samples (0.1–10 pg/ml), the raw data for collections Pre2, Retro, Post1, and Post2 were transformed according to the following formula:

(Raw pg/ml + 1 pg/ml)/ (Pre1 pg/ml + 1 pg/ml)

The effect of norepinephrine on the normalized estradiol levels across treatments was then tested using nonparametric Friedman ANOVA.

Electrophysiological recordings were analyzed using Spike2 (Cambridge Electronic Design). Baseline activity and stimulus-evoked activity were obtained from recordings 2 s before (baseline) and 2 s after (during stimuli) the beginning of the stimuli.

For multiunit activity, a threshold for each recording session was set such that spiking activity exceeded background noise by 1.5- to 3-fold. For each recording session, the threshold level was maintained for the entire set of recordings, so that all sampling periods per experiment (Pre, NE, Post) were analyzed with the same threshold (as in Remage-Healey and Joshi, 2012). The number of action potentials with amplitudes above threshold was counted for

each 10 ms interval (bin). Multiunit activity was analyzed to determine overall patterns

(see, e.g., Fig. 3.1*C*); all results reported below are for sorted single units.

To determine whether norepinephrine induced changes in NCM auditory-

evoked activity at the level of single neurons, recordings were sorted for single-unit

analysis (as in Remage-Healey et al., 2010; Remage- Healey and Joshi, 2012). Large-

amplitude single units were identified *via* waveform sorting using principal component

analysis (see Fig. 3.1*B*). Spikes with defined waveforms were matched to a sorting

template with an accuracy range of 60%–100%, and the spike *z*-score values were

calculated for each identified single unit. Playback peristimulus time histograms were

generated for each single unit to verify auditory responsiveness. Only units with

refractory periods longer than 1 ms were included in the analysis. The normalized

auditory response ("auditory *z*-score") values were calculated by taking the difference

between the mean number of spikes per bin during the stimulus (2 s from the onset)

and the mean number of spikes per bin during the baseline period (2 s before stimulus

onset) divided by the SD of the difference between the stimulus and baseline periods

(Remage-Healey and Joshi, 2012). Bursts were defined as a series of three or more

spikes with interspike interval < 10 mS. The signal-to-noise ratio was also computed by

calculating the ratio between the number of spikes per second during the stimulus and

the number of spikes per second during baseline (Manunta and Edeline, 1999).

To assess whether norepinephrine influences auditory encoding, a pattern

classifier was used to test how an individual cell's temporal patterning of response to

each stimulus was distinguishable from its response to other stimuli in the presence or

absence of norepinephrine. A customized MATLAB (version 8.1) script was written using

the built-in function *classify*. Spike trains were binned in 160 –2000 ms intervals, and all

spikes in each bin were summed to yield a representation of the firing pattern in terms of the

number of spikes/bin. We used a hold-one-out cross-validation technique to train and test the

classifier. Because there were a total of 20 trials in each recording, the classifier was trained

using the spiking pattern (spikes/bin) from a subset of 19 trials to classify those 19 trials into the

four different stimulus categories (CON1, CON2, HET, WN). The classifier then assigned the

remaining spiking pattern (the test pattern) to one of the four stimulus categories, based on its

similarity to the learned categories. This was repeated 20 times, such that each of the 20

recorded trials served as the test pattern on one occasion. Classification accuracy (% correct)

was calculated based on the success rate of the classifier for correctly identifying the category of

the stimulus that elicited the held-out (test) spiking pattern.

For the electrophysiological recordings, the changes in different parameters across

different treatments were analyzed using two-way repeated-measures (TW-RM) ANOVA, where

sound stimuli and treatments were used as factors. When sphericity was violated, Greenhouse-

Geisser correction was used for adjustment. *Post hoc* comparisons were performed with

nonparametric paired Wilcoxon signed-rank tests (pW-SRT).

Results

Norepinephrine enhances the auditory-evoked activity and signal-to-noise ratio for NCM auditory neurons

In vivo retrodialysis was coupled with extracellular electrophysiological recording to test

whether norepinephrine (0.1 mM) has an effect on auditory-evoked activity in the NCM (Fig.

3.1*A*; *N* = 17 animals). An example multiunit response from one animal is shown in Figure 4*C*.

Upon inspection of histological sections, we noted that retrodialysis probes were positioned in

the rostral auditory lobule (NCM/CMM boundary along the mesopallial lamina) in 5

experiments, whereas the remaining (*N* = 12) experiments had probes correctly placed in the

caudal NCM. We therefore restricted our analyses to the experiments in which the probes were correctly positioned within caudal NCM (N = 12 birds). Single units from the caudal NCM were sorted using principal component analysis (Fig. 3.1B). For N = 18 auditory- responsive single units (stimulus-evoked firing — spontaneous (prestimulus) firing > 0), norepinephrine significantly increased the auditory z-score for all stimuli (Fig. 3.1D; TW-RM ANOVA, main effect treatment: $F(2,30)$ = 13 p = 0.00011; main effect stimuli: $F(1.2,18)$ = 13 p = 0.0013; treatment × stimulus interaction: $F(6,90)$ = 6.8 p = 0.0000054; pW-SRT: PRE vs NE, CON1, p = 0.0041; CON2, p = 0.021; HET, p = 0.0012; WN, p = 0.044). The z-score values returned back to baseline levels after washout (pW-SRT: NE vs POST, CON1, p = 0.0018; CON2, p = 0.00048; HET, p = 0.0090; WN, p = 0.012), indicating that the effect of norepinephrine was reversible. We also calculated the signal-to- noise ratio, which has traditionally been used to measure how sensory neurons are modulated by norepinephrine to enhance signal detection (Foote et al., 1975; Manunta and Edeline, 1997). As with z-scores, signal-to-noise ratios significantly increased with norepinephrine infusion for all stimuli (Fig. 3.1E; TW-RM ANOVA, main effect treatment, $F(2,34)$ = 19, p = 0.0033; main effect stimuli, $F(1.1,18)$ = 20 p = 0.00021; treatment × stimulus interaction, $F(2.0,52)$ = p = 0.053; pW-SRT: PRE vs NE, CON1, p = 0.00025; CON2, p = 0.0015; HET, p = 0.00021; WN, p = 0.00058). The signal-to-noise values returned back to baseline levels after washout (pW-SRT: NE vs POST, CON1, p = 0.0069; CON2, p = 0.00049; HET, p = 0.036; WN, p = 0.0030). As in many sensory systems, the signal-to-noise ratio was highly correlated with other measures of auditory processing. The z-score values and signal-to-noise ratio during the PRE period were significantly correlated (Pearson correlation, r = 0.76, p = 0.00021), and the

response strength values (firing activity during stimulus — prestimulus firing) were also highly

correlated with signal- to-noise ratio ($r = 0.74$, $p = 0.00066$).

Norepinephrine suppresses spontaneous firing

The changes in signal-to-noise ratio may be due to changes in spontaneous activity,

auditory-evoked activity, or both (Foote et al., 1975; Manunta and Edeline, 1997). In the rodent

auditory cortex, norepinephrine causes increases in signal-to-noise ratio in cells whose

spontaneous activity is concurrently suppressed (Manunta and Edeline, 1997). Thus, in the NCM

neurons recorded here, we tested whether the norepinephrine- induced enhancement in signal-

to-noise ratio was due to a decrease in spontaneous activity (Fig. 3.1). In the same units isolated

above ($N = 18$), we observed that norepinephrine caused a significant decrease in the number of

spikes and bursts during spontaneous firing (i.e., activity before auditory stimulus presentation)

while having no significant effects on auditory- evoked firing during stimuli (Fig. 3.2 *A*, *C*; pW-

SRT: PRE vs NE, number of spikes during spontaneous firing [$p = 0.014$] and during presentation

of stimuli [$p = 1.00$]; number of bursts during spontaneous firing [$p = 0.0069$] and during

presentation of stimuli [$p = 0.90$], data not illustrated). Furthermore, the variance in the number

of spikes was significantly reduced for spontaneous firing, but it did not change for firing during

stimuli (data not illustrated; pW-SRT: PRE vs NE, base- line, $p = 0.012$; during stimuli, $p = 0.43$).

The percentages of spikes found within bursts was decreased following norepinephrine infusion

for spontaneous firing but not during the presentation of stimuli (data not illustrated, pW-SRT:

PRE vs NE, baseline, $p = 0.0090$; during stimuli, $p = 0.32$). In mammalian auditory cortex,

norepinephrine is known to alter the firing frequency both before stimulus onset and during the

stimulus, and the degree of change in spontaneous firing is related to the change in auditory-

evoked firing (Foote et al., 1975; Manunta and Edeline, 1997, 1999). To determine whether this

relationship holds true in avian auditory cortex, we examined correlations between auditory-

evoked and spontaneous activity. During the pre-NE period, there was a significant

correlation across our population of single units between the spontaneous firing rate

and firing rate during stimulus presentations (data not illustrated; Pearson correlation, r

= 0.79, p = 0.000098). Then, taking into account the changes during norepinephrine

treatment, the change in spontaneous firing frequency upon norepinephrine

retrodialysis positively correlated with the NE- induced changes in firing frequency

evoked by auditory stimuli (averaged across all four auditory stimuli; Fig. 3.2*A*; Pearson

correlation, r = 0.84, p = 0.000011) and the change in the number of bursts for

spontaneous firing positively correlated with the change in the number of bursts for

firing driven by auditory stimuli (also averaged across all four auditory stimuli; data not

illustrated, Pearson correlation, r = 0.88, p = 0.0000015). Therefore, for our population

of single NCM neurons, the degree of enhancement of evoked firing enabled by

norepinephrine was directly related to the degree of concurrent suppression of

spontaneous firing activity.
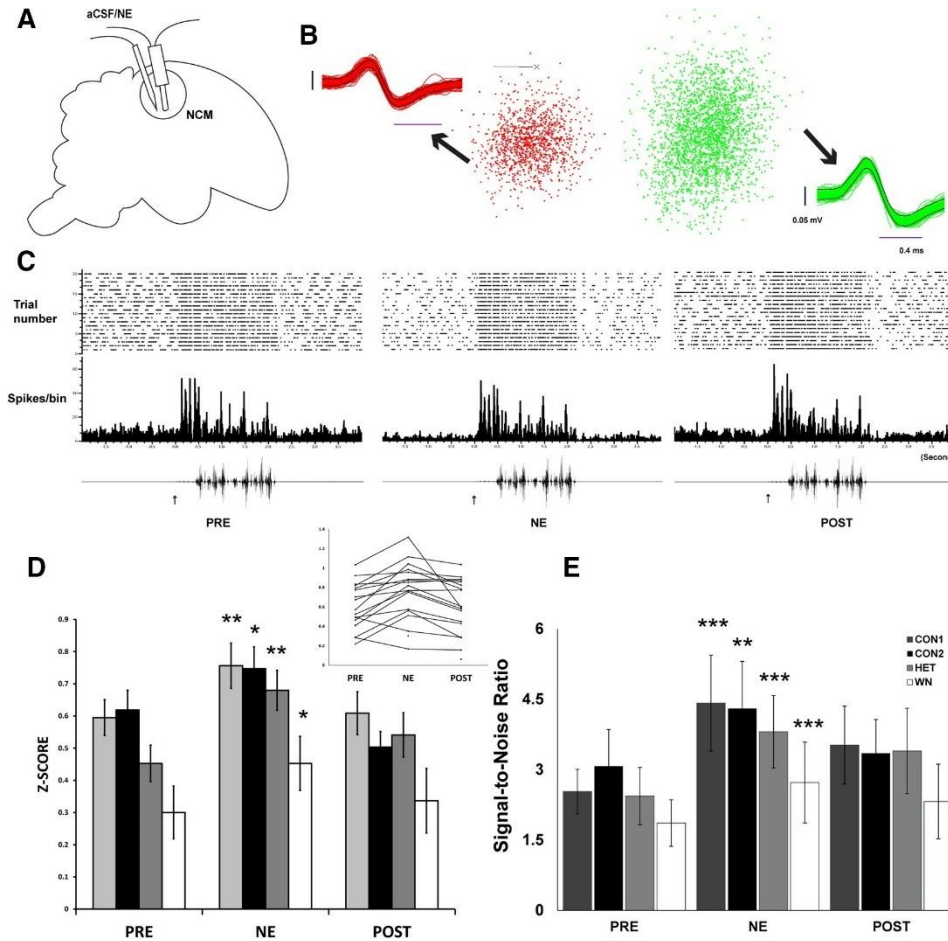
Figure 4: Norepinephrine modulates responses to auditory stimuli in caudal

NCM

A, Experimental setup. In urethane-anesthetized animals, a microdialysis probe was inserted into the NCM. An electrode was inserted caudal to the probe. Norepinephrine dissolved in aCSF (0.1 mM) was administered through the probe. ***B***, A representative plot of single-unit clusters isolated using principal component analysis. Insets, 100 sequential traces overlaid from identified neurons. ***C***, A typical multiunit response to a conspecific song (CON1) from one animal. Raster plots of spikes incorporated in *z*-score analysis (top) and peristimulus time histograms (100 ms bins, middle) relative to the onset to the stimulus (arrows, oscillograms at bottom). ***D***, ***E***, Norepinephrine enhances the auditory *z*-score (***D***) and signal-to-noise ratio (***E***) similarly in NCM single units. Data are mean ± SEM (*N* = 18). Inset, Within cell comparison of *z*-score for the CON1 stimulus. CON, Conspecific zebra finch songs; HET, heterospecific Bengalese finch song; WN, white noise *$p < 0.05$ (vs PRE). **$p < 0.01$ (vs PRE). ***$p < 0.001$ (vs PRE).
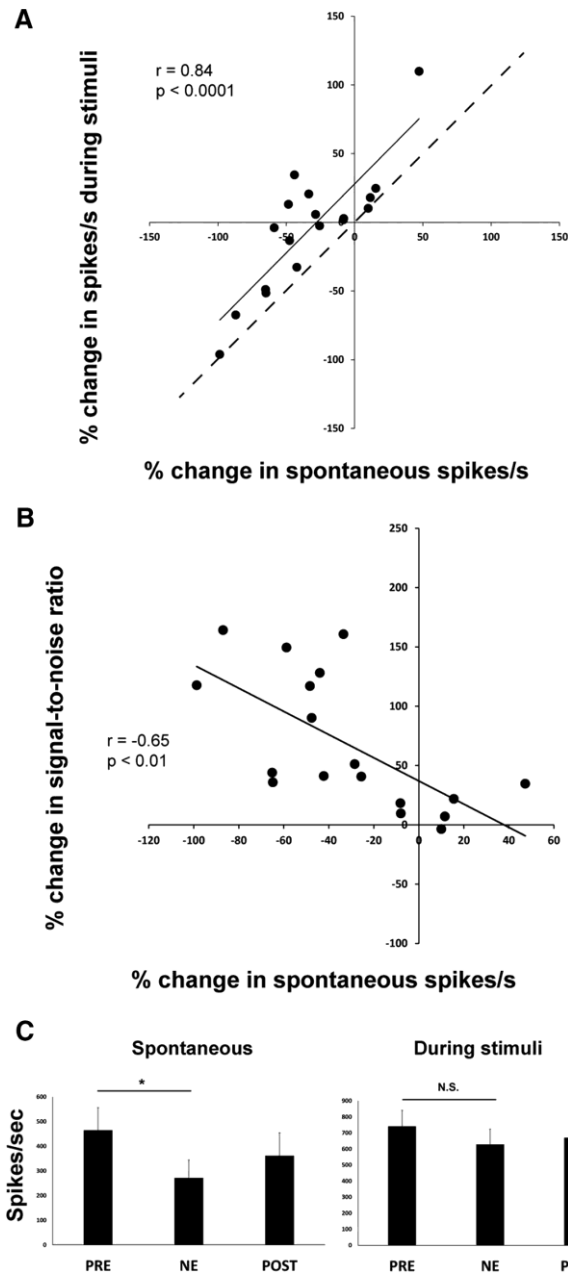
Figure 5: Norepinephrine induces changes in spontaneous activity and firing during stimuli, which result in the enhancement of the auditory signal-to-noise ratio

Spontaneous activity was measured 2s before the beginning of stimuli; spiking activity during auditory stimuli was extracted for2s from the beginning of the stimuli. A significant correlation between the percentage change in the number of spontaneous spikes and the percentage change in signal-to-noise ratio for CON1 (**A**) and percentage changes in number of spikes during stimuli (averaged across all four stimuli) (**B**). *r* and *p* values are derived from Pearson correlations. **C**, Norepinephrine suppresses spontaneous firing (left) but does not change the

firing rate during stimuli (averaged across all stimuli, right). Dashed line indicates a unity line. Data points above this line indicate an increase in signal-to-noise ratio (mean ± SEM). N.S., Not significant, $p > 0.05$. *$p < 0.05$ (vs PRE).
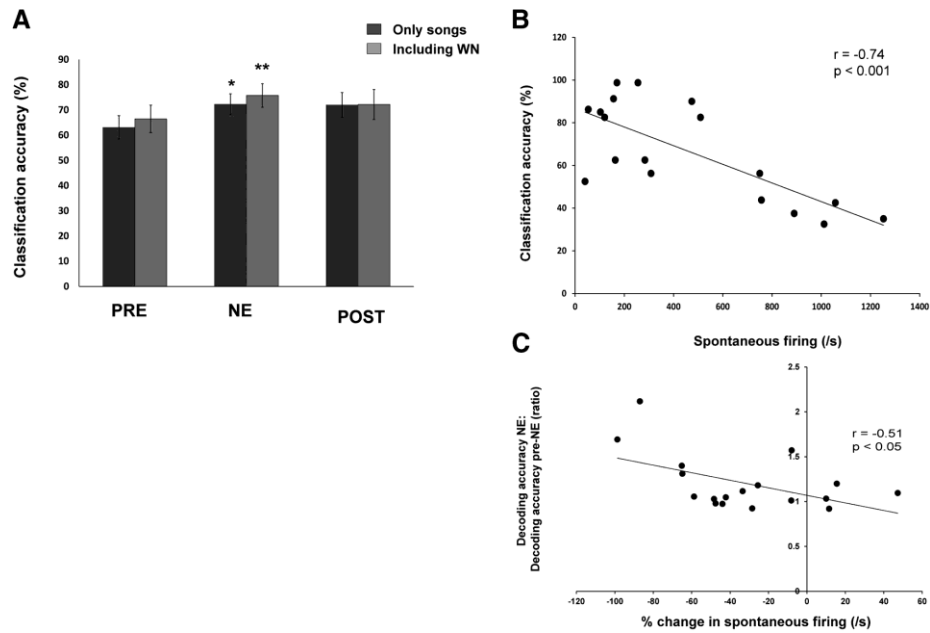
Figure 6: Norepinephrine enhances neural coding for complex vocalizations

(i.e., song)

A, Classification accuracy (mean ± SEM) among four stimuli, including white noise (with WN, bin size 160 ms) and among songs (no WN, bin size 400 ms). *$p < 0.05$ (within-group comparison vs PRE). **$p < 0.01$ (within-group comparison vs PRE). **B**, A significant correlation between spontaneous firing and classification accuracy in the absence of treatment (top). **C**, A significant correlation between percentage change in spontaneous firing induced by NE and relative classification accuracy during NE infusion (bottom).

In rodent auditory cortex, Manunta and Edeline (1999) reported that the change in spontaneous firing frequency by nor- epinephrine is correlated with an enhancement in signal-to-noise ratio. During the pre-NE period, there was a significant correlation between spontaneous firing and signal-to-noise ratio for songs but not for white noise (data not illustrated; Pearson correlations; CON1: $r =$ —0.71, $p = 0.0011$; CON2: $r =$ —0.72, $p = 0.00076$; HET: $r =$ —0.60, $p = 0.0091$; WN: $r =$ —0.28, $p = 0.26$).

With norepinephrine, the change in spontaneous firing negatively correlated significantly with the change in signal-to-noise ratio to birdsongs but not white noise (Fig. 3.2B; CON1, $r =$ —0.65, $p = 0.0032$; CON2, $r =$ —0.73, $p = 0.00052$; HET, $r =$ —0.60, $p = 0.0083$; WN, $r =$ —0.36, $p = 0.14$; data not illustrated for CON2, HET, and WN). Together, norepinephrine in the avian auditory NCM appears to have a similar neuromodulatory function for the processing of complex sounds, as in mammalian auditory cortex for tone processing. The observations that norepinephrine enhances the signal-to-noise ratio while also decreasing spontaneous activity then raised the question whether norepinephrine effectively changes the ability of NCM neurons to encode complex auditory stimuli.

Norepinephrine significantly enhances auditory coding of complex stimuli (i.e., song)

Norepinephrine may not only enhance the detection of stimuli but also enhance the brain's ability to accurately encode complex sounds, such as song. Using the same set of recordings ($N = 18$ units), a classifier was trained to discriminate the firing patterns produced in response to the four stimuli presented during the experiments (see Materials and Methods). The classification ac- curacy was tested with 8 different bin sizes between 160 and 2000 ms. The data plotted in Figure 6A are from the smallest bin sizes for which the classifier could be run, using data including songs and white noise (with WN) and data including songs but no white noise (without WN). The classifier we used requires at least one spike in each bin for all stimuli,

to be able to find a solution. We observed that, with smaller bin sizes, it became more likely that there was at least one bin with no spikes, which sometimes made it not possible to run the classifier. In the "with WN" condition, there is one more stimulus category (i.e., the white noise category) than in the "without WN" condition. This reduces the probability that there will exist a bin with no spikes for any stimulus and therefore allows the classifier to perform at smaller bin sizes for the "with WN" condition than in the "without WN" condition. Norepinephrine significantly increased classification accuracy for all bin sizes tested (pW-SRT: PRE vs NE, 2000 ms, $p = 0.0052$; 1000 ms, $p = 0.023$; 667 ms, $p = 0.015$; 500 ms, $p = 0.0029$; 400 ms, $p = 0.00071$; 267 ms, $p = 0.0021$; 200 ms, $p = 0.0022$; 160 ms, $p = 0.0074$; "with WN"; Fig. 3.3*A*). Therefore, norepinephrine appears to have an effective role in enhancing the coding accuracy of auditory stimuli in NCM neurons, across a variety of time windows.

Because there was also a significant decrease in spontaneous firing with norepinephrine (see above), we speculated that a component of spontaneous firing may be acting as background "noise" and interfering with classification accuracy. To assess whether there is a simple relationship between a neuron's spontaneous firing rate and its stimulus decoding accuracy in the absence of norepinephrine, correlations between these two parameters were calculated. Within the pre-NE period, the decoding accuracy of the classifier was significantly inversely correlated with spontaneous firing across the population of NCM neurons (Fig. 3.3*B*; Pearson correlation, $r = -0.74$, $p = 0.00043$). That is, the lower the spontaneous firing rate of a neuron measured be- fore stimulus onset, the higher the decoding accuracy achieved by the classifier for the firing patterns elicited in that neuron during auditory stimulus presentation. In contrast, decoding accuracy for firing patterns elicited by auditory stimuli was not correlated with firing

rate during those same stimuli (data not illustrated; Pearson correlation, $r = -0.41$, $p = 0.090$).

Therefore, low spontaneous firing is associated with more precise stimulus encoding by NCM

neurons, which is consistent with the hypothesis that a component of spontaneous firing is

likely to be contributing "noise" to the ongoing auditory encoding of NCM neurons. This

interpretation is in line with the observation noted earlier that NE decreased the spontaneous

firing rate, but not the firing rate during stimuli (Fig. 3.2*C*). In further agreement with this

interpretation, the ratio of the decoding accuracy during NE to decoding accuracy pre-NE was

significantly negatively correlated with both the percentage change in spontaneous firing (Fig.

3.3*C*; Pearson correlation, $r = 0.51$, $p = 0.032$) and the percentage change in firing during stimuli

(data not illustrated; Pearson correlation, $r = -0.61$, $p = 0.0076$). In summary, our findings

suggest that norepinephrine modulates songbird auditory neurons by enhancing signal

detection and decoding accuracy *via* reducing background noise firing.

An α2-adrenergic receptor (AR α2) agonist mimics the effects of norepinephrine

In rodent auditory cortex, the NE-induced decrease in spontaneous activity is similar to

GABA-induced decreases in spontaneous activity (Manunta and Edeline, 1997), and NE-induced

increases in inhibitory currents are mediated by an AR α2 mechanism (Salgado et al., 2011b).

Therefore, we predicted in the NCM that activation of AR α2 by an agonist, clonidine, would

mimic the effects of norepinephrine in the NCM. The effect of clonidine was tested on a new set

of birds ($N = 6$ animals, $N = 13$ units) with stereotaxic coordinates directed at the caudal NCM.

We used two different concentrations (0.5 and 5 mM) derived from dose- dependent effects of

clonidine reported in other regions in the zebra finch brain (Cardin and Schmidt, 2004). Like

norepinephrine, clonidine significantly enhanced NCM signal-to-noise ratio (Fig. 3.4*A*, top; pW-

SRT: PRE vs HIGH, CON1, $p = 0.011$; CON2, $p = 0.0016$; HET, $p = 0.0051$; WN, $p = 0.030$),

decreased the number of spikes during auditory presentations (Fig. 3.4*A*, middle; pW-SRT: PRE

vs HIGH, CON1, *p* = 0.017; CON2, *p* = 0.030; HET, *p* = 0.021; WN, *p* = 0.069), and

decreased spontaneous spiking (Fig. 3.4*A*, bottom; pW-SRT: PRE vs HIGH, *p* = 0.011).

This effect was dose-dependent, as the low dose of clonidine had mixed effects (Fig.

3.4*A*; pW-SRT, PRE vs LOW, signal-to-noise ratio: CON1, *p* = 0.44; CON2, *p* = 0.0041; HET,

p = 0.042; WN, *p* = 0.36; spikes during stimuli: CON1, *p* = 0.37; CON2, *p* = 0.83; HET, *p* =

0.49; WN, *p* = 0.24; spontaneous firing: *p* = 0.14). Moreover, like norepinephrine, the

clonidine-induced change in spontaneous firing was correlated with the clonidine-

induced change in firing during stimuli (Fig. 3.4*C*; Pearson correlations, all stimuli

combined, LOW, *r* = 0.90, *p* = 0.000022, data not illustrated; HIGH, *r* = 0.93, *p* =

0.0000030), and the clonidine- induced change in spontaneous firing was correlated

with the clonidine-induced change in signal-to-noise ratio (Fig. 3.4*D*; Pearson

correlations, all stimuli combined, LOW, *r* = —0.77, *p* = 0.0019, data not illustrated;

HIGH, *r* = —0.57, *p* = 0.038). We also tested the effect of cirazoline, which is a AR α1

agonist and AR α2 antagonist (a new set of *N* = 2 birds, *N* = 4 units) (Ruffolo and

Waddell, 1982). In all cells tested, cirazoline caused a mean increase in spontaneous

activity and the firing during auditory stimuli, while having no discernible influence on

the signal-to- noise ratio (Fig. 3.4*B*), although these changes did not reach statistical

significance (pW-SRT, PRE vs LOW, signal-to-noise ratio: CON1, *p* = 0.58; CON2, *p* = 1.0;

HET, *p* = 0.58; WN, *p* = 1.0; spikes during stimuli: CON1, *p* = 0.10; CON2, *p* = 0.10; HET, *p*

= 0.10; WN, *p* = 0.10; spontaneous firing: *p* = 0.10; PRE vs HIGH, signal-to-noise ratio:

CON1, *p* = 0.20; CON2, *p* = 0.36; HET, *p* = 0.10; WN, *p* = 0.58; spikes during stimuli:

CON1, *p* = 0.10; CON2, *p* = 0.10; HET, *p* = 0.10; WN, *p* = 0.10; spontaneous firing: *p* =

0.10). It is important to note that some of the effects of cirazoline could be due to an α2

antagonism, although a separate α2 antagonist we tested, idazoxan, did not influence

any of the tested measures (N = 2 units, data not illustrated). In summary, the major actions of norepinephrine on NCM auditory signal detection were mimicked by the AR α2 agonist, clonidine.

Norepinephrine does not affect estradiol levels in the NCM

The NCM region of the songbird is enriched with aromatase (Saldanha et al., 2000; Peterson et al., 2005); therefore, estradiol may act as an intermediary mechanism for the actions of norepinephrine described above. Estradiol was shown in single NCM units to increase the *z*-score values to multiple stimuli (Remage-Healey and Joshi, 2012) in a similar fashion as described here for norepinephrine. Therefore, we tested the hypothesis that norepinephrine regulates auditory processing *via* the regulation of local estradiol levels.

To test the direct effect of norepinephrine on estradiol levels, *in vivo* retrodialysis was used in a new set of awake behaving animals (N = 18) to measure the changes in local estradiol levels in NCM in response to norepinephrine infusions (0, 0.01, and 0.1 mM; Fig. 3.5*A*). There were no significant changes in NCM estradiol levels in response to norepinephrine infusions (Fig. 3.5 *B*, *C*; Friedman ANOVA:0 mM, y 2 = 2.7, N = 17, df = 3, p = 0.44; 0.01 mM NE, y 2 = 4.2, N = 14, df = 3, p = 0.24; 0.1 mM NE, y 2 = 3.4, N = 15, df = 3, p = 0.33). The result was also not dependent on the time of the experiment (Fig. 3.5*C*): all p > 0.05 for morning (N = 13 experiments), afternoon (N = 15 experiments), and night (N = 8 experiments). We also scored the birds' activity levels during the norepinephrine infusions in the daytime. There was no effect of norepinephrine retrodialysis into NCM on locomotor activity (number of hops/min: control: 2.1 ± 0.67, N = 9; 0.01 mM NE: 7.21 ± 3.6, N = 11; 0.1 mM NE: 5.5 ± 2.2, N = 10 experiments), indicating that birds were equivalently behaviorally responsive during the retrodialysis treatments. To further evaluate this hypothesis, in a new set of animals, we tested the effects of

adrenergic antagonists on estradiol levels to determine whether blockade of

endogenous norepinephrine signaling would influence local NCM estradiol levels (N =

20). Because all AR subtypes are expressed in the NCM (Velho et al., 2012), prazosin,

RX821001, or propanolol was infused to block α1, α2, and þ receptors, respectively (all

doses = 0.1 mM). None of the adrenergic antagonists had detectable effects on local

estradiol levels within NCM (mean estradiol levels relative to baseline during antagonist

infusions, vehicle: 1.1 ± 0.15, N = 18 experiments; prazosin: 1.2 ± 0.10, N = 16

experiments; propanolol: 1.7 ± 0.61, N = 11 experiments; RX821001: 2.0 ± 0.55, N = 14

experiments) (pW-SRT: Pre vs during antagonist, vehicle, p = 0.28; prazosin, p = 0.62;

propanolol, p = 1.0; RX821001, p = 0.19). Together, these results indicate that

norepinephrine signaling does not directly impact local forebrain estradiol levels, and

raises the possibility that the effects of norepinephrine on the electrophysiology of NCM

neurons are independent of local estradiol synthesis.

The effects of norepinephrine on NCM neuronal activity are not dependent on local estradiol synthesis

To directly test whether or not the effects of norepinephrine on auditory-

evoked responses are dependent on local estrogen synthesis within NCM, estradiol

production was blocked using retrodialysis of the specific aromatase inhibitor, fadrozole

(100 –500 μM) (Wade et al., 1994) during norepinephrine retrodialysis. In a separate set

of animals (N = 10), for N = 20 single units in NCM, the z-score response to auditory

stimuli was recorded before and during fadrozole infusion and then during

norepinephrine and fadrozole coinfusion. Norepinephrine (0.1 mM) significantly

enhanced auditory-evoked responses even in the presence of fadrozole (Fig. 3.6*A*; TW-

RM ANOVA: main effect stimuli: $F(1.3,25)$ = 6.8, p = 0.0096; main effect treatment:

$F(2.2,42) = 4.0$, $p = 0.022$; treatment × stimulus interaction: $F(4.7,88) = 2.7$, $p = 0.027$; pW-SRT: PREFAD vs FAD+NE, CON1, $p = 0.00032$; CON2, $p = 0.0014$; HET, $p = 0.00032$; WN, $p = 0.046$).

Moreover, the degree of change in *z*-score values with NE infusion were not significantly different from the same measure collected in the first experiment we conducted with norepinephrine in the absence of fadrozole treatment (Student's *t* test: percentage change from PRE to NE from first experiment, $N = 18$; vs percentage change from PREFAD vs FAD+NE, $N = 20$; $p = 0.56$, data not illustrated). Importantly, the changes in auditory-evoked responses during norepinephrine retrodialysis were not accompanied by changes in local estradiol levels in NCM (Fig. 3.4*B*; Pearson correlation: $r = 0.022$, $p = 0.95$), further indicating that nor- epinephrine alters NCM neuronal response properties but does not drive elevations in local estradiol concentrations. Together, these results indicate that the actions of norepinephrine on NCM neurons are not dependent on local estradiol synthesis in NCM.
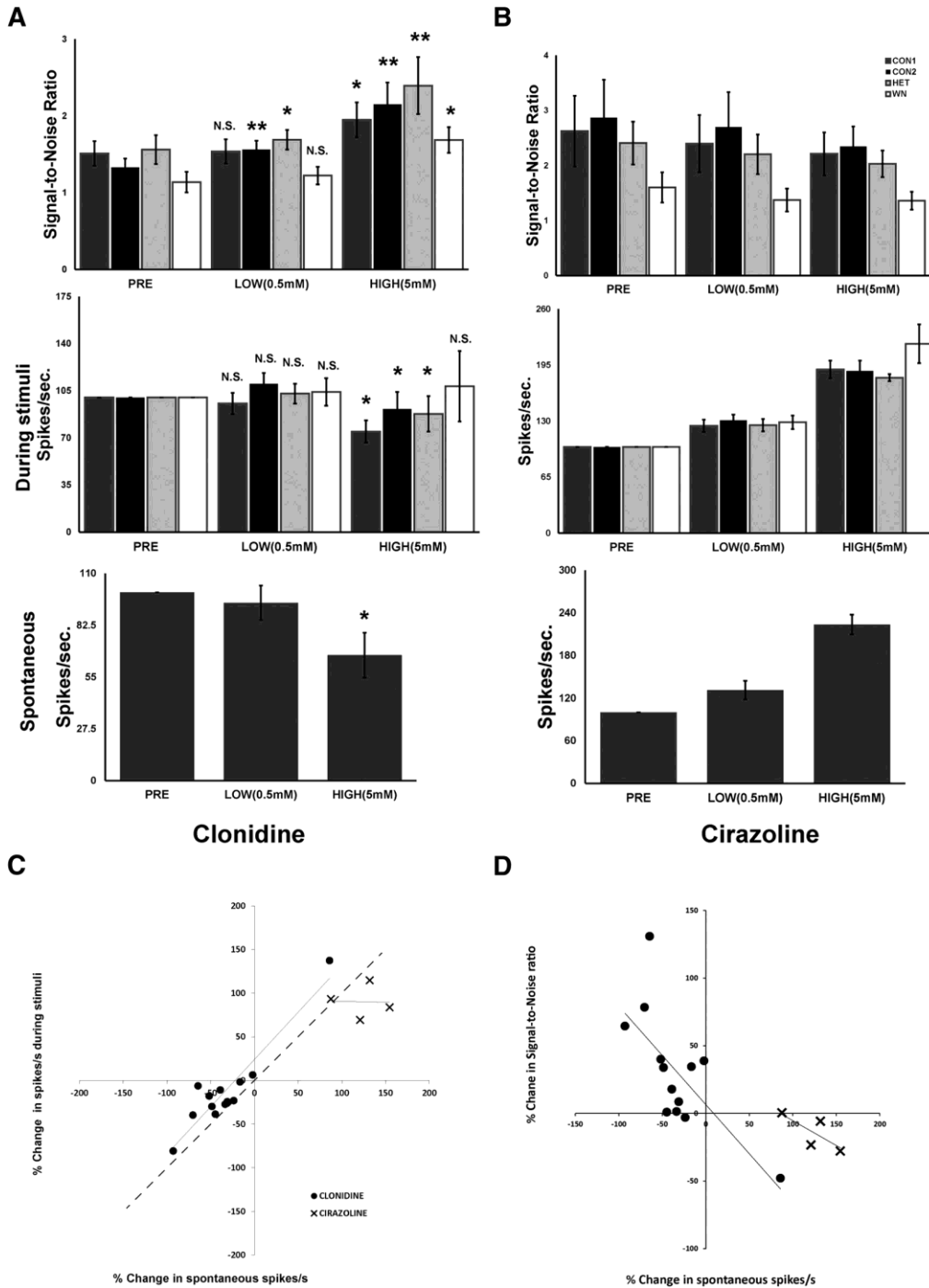
Figure 7: The ARα2 agonist clonidine mimics the effects of norepinephrine in the

NCM

A, *B*, Signal-to-noise ratio is enhanced by clonidine at both low and high dose (mean ± SEM, top). Both spiking activity during stimuli (middle) and spontaneous spikes (bottom) were suppressed by clonidine. There was a mean increase for cirazoline but was not statistically

significant (spikes during stimuli normalized to "PRE" values; mean ± SEM). The number of spontaneous spikes; data are mean ± SEM. N.S., Not significant, $p > 0.05$. *$p < 0.05$ (vs PRE). **$p < 0.01$ (vs PRE). *C*, A significant correlation between percentage changes in spontaneous spikes and the percentage changes in the number of spikes during stimuli for clonidine ($r = 0.93$, $p = 0.0000030$; for similar findings with norepinephrine, compare with Fig. 3.2*A*). There was no relationship for cirazoline ($r = $ —0.031, $p = 0.96$). *D*, A significant correlation between percentage changes in number of spontaneous spikes and percentage changes signal-to-noise ratio to CON1 for clonidine ($r = $ —0.70, $p = 0.0067$) but not for cirazoline ($r = $ —0.75, $p = 0.24$).
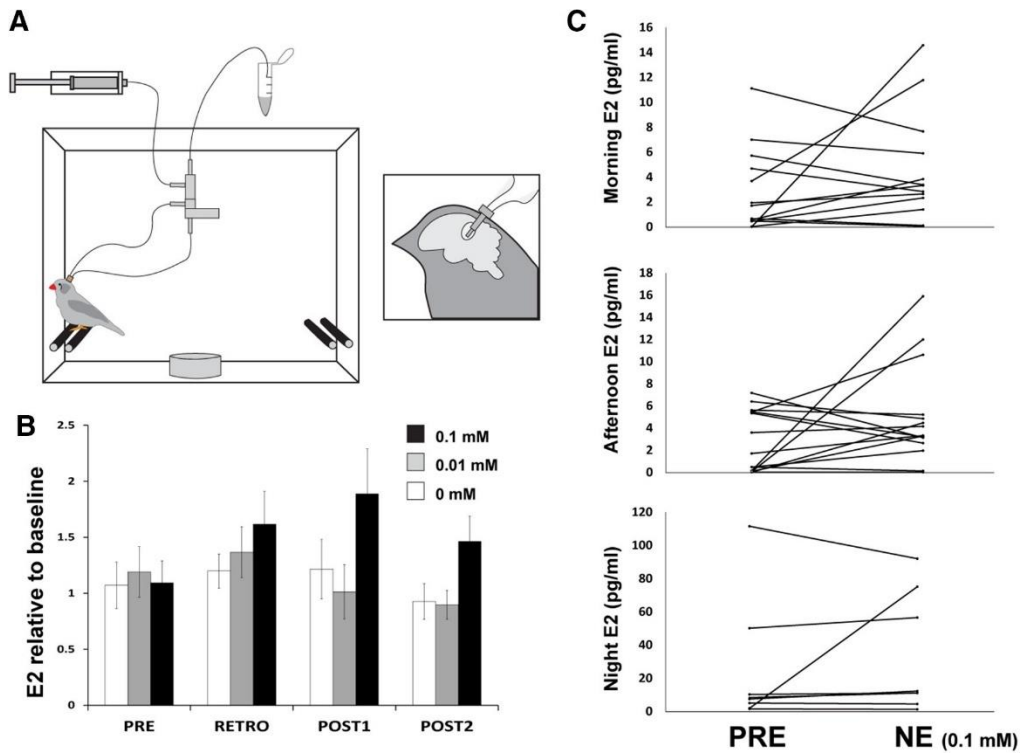
Figure 8: Norepinephrine has no effect on local estradiol levels in NCM

A, Experimental setup for *in vivo* reverse microdialysis. A microdialysis probe was inserted into the caudal NCM through a guide cannula. The solutions were delivered through tubing and a microdialysis swivel, and all manipulations were external to a sound isolation chamber to minimize disturbance. *B*, Estradiol (E2) levels relative to baseline (mean ± SEM) before (PRE), during (RETRO), and after (POST1 and POST2) norepinephrine infusions. Dialysates were collected every 30 min. 0 mM NE, $N = 17$; 0.01 mM NE, $N = 14$; 0.1 mM NE, $N = 15$. *C*, Estradiol levels in individual birds before (PRE) and during (0.1 mM NE) norepinephrine infusions during experiments conducted in the morning (9:30 A.M., $N = 13$, top), afternoon (2 P.M., $N = 15$, middle), and night (9:30 P.M., $N = 8$, bottom).

Norepinephrine synthesizing fibers are largely dissociated from estradiol synthesizing neurons

The microdialysis and electrophysiology results above together indicate that norepinephrine enhances auditory processing and auditory coding using mechanisms that are not dependent on local estradiol synthesis in NCM. To examine the anatomical relationship between norepinephrine- and estradiol-producing neurons in NCM, DBH and aromatase enzymes were stained with double-label immunocytochemistry. In addition to a lack of double-labeled processes, we observed markedly few occurrences of direct physical interactions between aromatase-ir neurons and DBH-ir fibers in the NCM (Fig. 3.6*C*). This anatomical dissociation within the NCM is inconsistent with a principal role of norepinephrine to regulate aromatase-expressing neurons in the NCM.

Discussion

This study demonstrates the following: (1) norepinephrine enhances auditory detection in the songbird auditory cortex; (2) norepinephrine also enhances the coding accuracy of individual neurons for complex sounds, *via* a reduction in concurrent spontaneous firing; (3) norepinephrine's effects are mimicked by an α2 agonist; and (4) norepinephrine achieves similar effects as estradiol in modulating forebrain auditory processing in the songbird, but these effects are not directly dependent on neuroestrogen synthesis.

We show that norepinephrine similarly enhances auditory- evoked responses, signal-to-noise ratios, and auditory encoding, and does so by decreasing spontaneous activity. The amount of spontaneous firing positively correlates with firing rates during stimuli presentation, and norepinephrine decreases the degree of noise in the spontaneous activity. Furthermore, spontaneous firing rate inversely correlated with classification accuracy and signal-to-noise ratio for songs, the NE-induced change in spontaneous activity was correlated with the change in

firing during stimuli, and the NE-induced change in both the spontaneous firing and firing during stimuli inversely correlated with the change in classification accuracy and signal-to-noise ratio. Thus, we propose that spontaneous activity irrelevant to auditory stimuli ("noise") is maintained throughout the stimulus-evoked response in the songbird auditory cortex and that norepinephrine increases neuronal signal detection and coding accuracy by suppressing this constitutive noise (Fig. 3.6*D*).
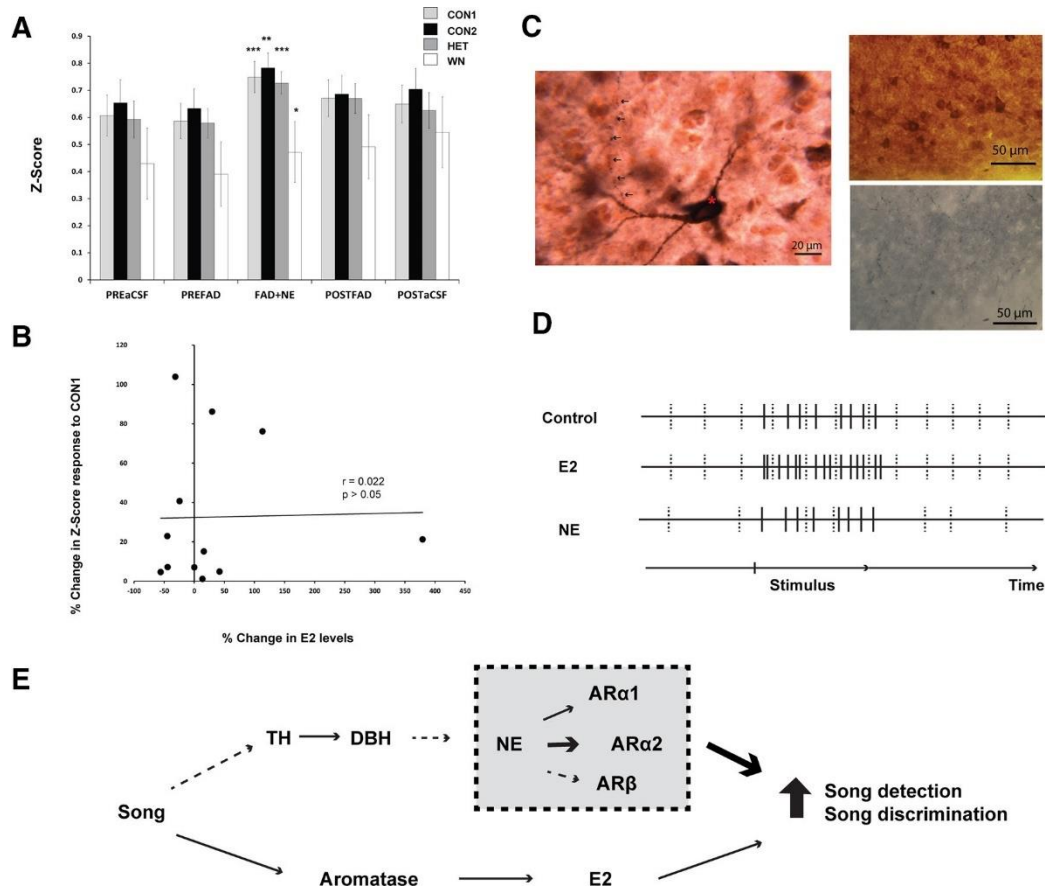
Figure 9: Norepinephrine enhances auditory-evoked responses independent of

aromatase activity

A, *z*-score (mean ± SEM). PREaCSF, Before drug treatment; PREFAD, fadrozole pretreatment; FAD+NE, fadrozole and norepinephrine; POSTFAD, washout with fadrozole solution; POSTaCSF, washout with aCSF. *$p < 0.05$, versus PREFAD. **$p < 0.01$, versus PREFAD. ***$p < 0.001$, versus PREFAD. *B*, There was no significant correlation between percentage change in estradiol (E2) levels and percentage change in *z*-score values in response to CON1. *C*, Double immunostaning of DBH fibers (arrows) and aromatase neurons (red asterisk). Photomicrograph is representative of the occurrences in which DBH-ir fibers and aromatase-ir cell bodies and processes exhibited limited direct physical interactions in NCM (left). Single-label aromatase (top) and DBH (bottom) immunoreactive staining in NCM (right). *D*, Schematic illustration of the effects of norepinephrine and estradiol. Estradiol enhances auditory-evoked firing while norepinephrine decreases background noise, resulting in less spontaneous firing (dashed lines). *E*, Summary of what is known from previous findings (thin arrows) and the current study (thick arrows) in NCM. Solid arrows, tested in zebra finch; dashed arrows, demonstrated in non-zebra finch songbirds.

68

Our findings with zebra finch NCM build upon findings in primate and rodent auditory cortex, in which norepinephrine decreases spontaneous firing frequency and enhances the signal- to-noise ratio for tones and calls (Foote et al., 1975; Manunta and Edeline, 1997, 1999), and the change in signal-to-noise ratio correlates with the change in baseline firing activity (Manunta and Edeline, 1997, 1999). To our knowledge, in the songbird auditory system, the direct effects of norepinephrine on neuronal firing frequency have been examined in only one other study (Cardin and Schmidt, 2004). Similar to our findings here for NCM, the administration of norepinephrine to nucleus interface, a premotor region essential song production, enhances stimulus selectivity in HVC, a sensorimotor region. Our results now indicate that norepinephrine enhances the signal-to-noise ratio and auditory coding in the songbird auditory forebrain, which likely enhances the neural discrimination of sounds that must be extracted as signals from noisy environments (Schneider and Woolley, 2013). We find it particularly interesting that norepinephrine induces a change in sensory coding accuracy *via* a concurrent suppression of spontaneous activity in the songbird because it provides a test- able prediction for a similar mechanism for sensory coding in mammalian auditory cortex.

We report that the AR α2 agonist, clonidine, mimics the effects of norepinephrine on electrophysiological auditory responses, whereas the AR α1 agonist, cirazoline, does not. This is consistent with findings in mammalian sensory cortex, in which AR α2 and α1 agonists typically have differential effects on sensory processing (Sato et al., 1989; Nai et al., 2009; Salgado et al., 2011b). Our findings identifying the AR α2 mechanism are particularly interesting because α2-mediated noradrenergic actions are key for associative plasticity *in vitro* in mammalian cerebellum (Carey and Regehr,

2009) and involve the suppression of the rhythmic-generating Ih current (Robbins and Arnsten,

2009). Therefore, the suppression of concurrent spontaneous firing *via* an AR α2 mechanism

identified here may be important for associative plasticity and memory functions in the songbird

NCM for complex song, during both juvenile and adult periods of song learning.

In rodent auditory cortex, norepinephrine's actions involve modulation of inhibitory

neurotransmission to achieve an enhancement of signal-to-noise ratio (Foote et al., 1975;

Manunta and Edeline, 1997; Salgado et al., 2011a). Differential effects of norepinephrine on

spontaneous versus evoked activity in auditory cortex are thought to be achieved *via*

differential, layer- specific actions on inhibitory interneurons (Hasselmo et al., 1997; Salgado et

al., 2011b). Similarly, auditory processing and encoding depend on inhibitory neurotransmission

in the song- bird NCM (Pinaud and Mello, 2007; Pinaud et al., 2008; Tremere et al., 2009; Jeong

et al., 2011; Tremere and Pinaud, 2011), although the layered cortical organization typical of

mammals is not as evident in songbirds (Wang et al., 2010). Therefore, despite the apparently

divergent neuronal architecture in the songbird auditory cortex, there appears to be a striking

conservation of modulatory mechanism by norepinephrine shared between birds and mammals.

Identifying specific cell types and receptor types, along with computational modeling, will

further dissect how complex vocalizations are processed and modulated in higher-order circuits

by norepinephrine.

Although our study confirms that neurons in NCM respond significantly to AR α2

agonists, it does not provide concrete evidence for the role of endogenous norepinephrine in

behaving animals. Our electrophysiology experiments were conducted on urethane-

anesthetized birds, which may have precluded our ability to detect endogenous actions of

norepinephrine, which are highly sensitive to anesthetic state (Vazey and Aston-Jones, 2014). In

this and other songbird species, however, blocking endogenous norepinephrine actions with

specific receptor antagonists in awake subjects can affect auditory-dependent

behavioral and immediate-early gene responses (Pawlisch et al., 2011; Velho et al.,

2012). In other sensory systems, norepinephrine exerts powerful effects on behavioral

discrimination of stimuli (e.g., Escanilla et al., 2010). Future experiments on songbird

audition can be conducted in this species with awake recordings or in combination with

locus coeruleus stimulation.

The current study provides several lines of evidence that nor- epinephrine does

not act *via* changing aromatase activity in female zebra finch NCM. Norepinephrine did

not affect estradiol levels in NCM, and its actions on the firing properties of NCM

neurons were not dependent on aromatase activity. In addition, aromatase-positive

NCM neurons were largely dissociated from DBH-expressing fibers. It remains to be

determined whether this pattern of modulation is similar in males because the

distribution of aromatase-positive cells and fibers is reported to be sexually dimorphic in

the NCM (Saldanha et al., 2000; Peterson et al., 2005). In previous studies, estradiol

enhanced baseline- normalized auditory evoked responses in the NCM of both sexes

(Tremere et al., 2009; Remage-Healey et al., 2010, 2012; Remage-Healey and Joshi,

2012) and estradiol enhanced auditory encoding of NCM neurons (Tremere and Pinaud,

2011). We show that norepinephrine similarly enhances auditory-evoked responses and

enhances auditory encoding in a similar timescale as estradiol. Importantly, estradiol

enhanced spiking activity during auditory stimuli, but it had no effect on spontaneous

spiking activity (Remage-Healey et al., 2010), whereas here norepinephrine decreases

spontaneous activity (Fig. 3.6*D*).

The work presented here raises the possibility that norepinephrine and estradiol

signaling independently regulate sensory-dependent behaviors. In songbirds, estradiol

and catecholamine levels are elevated in response to song presentation in a similar timescale

(Remage-Healey et al., 2008, 2012; Matragrano et al., 2012). Among vertebrates,

norepinephrine release in the cortex is generally associated with arousal and attention (Aston-

Jones and Bloom, 1981a, b; Aston-Jones et al., 1999). By contrast, the changes in NCM estradiol

levels seem to be specific to social interactions and auditory playbacks (Remage-Healey et al.,

2008, 2012). As these studies suggest, depending on the environmental context, both

norepinephrine and estradiol may be released concurrently to modulate NCM sensory

processing.

Norepinephrine and estradiol signaling are both required for song-induced immediate

early gene ZENK (*egr-1*) induction in the NCM, and song discrimination and preferences (Lynch

and Ball, 2008; Remage-Healey et al., 2008; Pawlisch et al., 2011; Tremere and Pinaud, 2011;

Velho et al., 2012; Vahaba et al., 2013). Both aromatase and AR α1 mRNA are found in neurons

that express inducible zenk mRNA in response to auditory stimuli in the NCM (Jeong et al., 2011;

Velho et al., 2012). As shown here with norepinephrine, the change in firing rate during song

stimuli with estradiol was shown to enhance the ability of NCM neurons to encode songs

(Tremere and Pinaud, 2011). Together, these studies suggest that, although the upstream

mechanisms appear to be different, they may activate common downstream intracellular

pathways that are not currently well understood. Norepinephrine and estradiol may have

differential roles in different parts of the NCM or in different cell types, and future experiments

should elucidate their divergent mechanisms of action.

As in songbirds, human language processing is likely to be dependent on both

neuroestrogen and catecholamine signaling. Polymorphisms in the brain-specific promoter for

the aromatase gene in humans have been associated with specific language impairments

(Anthoni et al., 2012), and auditory processing can vary with fluctuating estrogen levels in

women (Tillman, 2010). Similarly, many mental disorders, such as schizophrenia, post-traumatic stress disorder, attention deficit hyperactivity disorder, and autism, are associated with adrenergic dysfunction as well as impairment in speech processing and dysregulation of the auditory cortex (Corbett and Stanczak, 1999; Newport and Nemeroff, 2000; Rapin and Dunn, 2003; Calhoun et al., 2004; Sweet et al., 2009). Studying the role of neuromodulators and their interactions in cortical circuits that encode and decode complex sounds may provide insight into treatments of neurological and sensory- processing disorders.

CHAPTER IV

CLUSTERED ORGANIZATION AND REGION-SPECIFIC IDENTITIES OF ESTROGEN-PRODUCING NEURONS IN THE ZEBRA FINCH FOREBRAIN

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Abstract

The neuromodulatory role of estradiol has been reported in many regions of the brain. However, regional differences in cellular distributions of aromatase suggest the control mechanism for estradiol levels could be different between brain regions and even within regions at the micro-circuit level. The underlying differences in estradiol synthesis between neurons may be associated with the identities of the aromatase-expressing neurons. Since neuronal activity is thought to drive aromatase activity, aromatase-expressing neurons with different neuronal identities might be enhancing aromatase activity in a time-specific manner by firing at different timings. Fast-spiking interneurons expressing the calcium binding protein parvalbumin are known to fire at specific timings relative to certain behaviors (Wild et al., 2005; Schneider et al., 2014). Here, we investigated the regional differences in the identity and connections of aromatase-expressing neurons using a specific aromatase antibody together with the interneuron markers, parvalbumin and calbindin. Aromatase is co-expressed with parvalbumin in the NCM and HVC shelf but not in the NCL or hippocampus. A different calcium binding protein, calbindin, was not expressed with aromatase in any regions. Notably, aromatase-expressing neurons formed somato-somatic clusters. In preliminary experiments injecting small tracers, po-pro-1 or neurobiotin, into the clustered cells suggest that at least some of the clustered neurons may communicate *via* gap junctions. Moreover, aromatase expression was

compared with G-protein Coupled Estrogen Receptor 1 (GPER1) expression patterns to see if the amount of estradiol-synthesizing cells is related to the amount of estradiol-sensitive cells. We found higher GPER1 expression in regions with low expression of somatic aromatase, but included regions with known high pre-synaptic aromatase such as the HVC and RA, compared to the NCM and NCL. This suggest that GPER1 may be involved mainly in rapid actions of estradiol synthesized from synaptic terminals on sensorimotor function. Overall, this study provides new insights on how interneuronal communication within and across regions may play a role in aromatase synthesis and estrogen actions.

Introduction

Songbirds, especially zebra finches, are one of the popular models used to study rapid estradiol signaling in complex auditory processing (Remage-Healey, 2014). In many ways the songbird model has an advantage over other model systems, and one of which is its similarity to humans in the abundance of aromatase, the enzyme required for estradiol synthesis, in the cortex (Yague and Muñoz, 2006). In zebra finches, high expression of aromatase is found in the caudomedial nidopallium (NCM), a region analogous to the mammalian secondary auditory cortex (Shen et al., 1995; Saldanha et al., 2000; Peterson et al., 2005). In addition, in the zebra finch brain, many other regions involved in auditory processing and song control are found to express aromatase to different degrees (Vockel et al., 1990; Saldanha et al., 2000). Although many molecular, cellular and physiological studies have elucidated key aspects of the mechanism of controlling estradiol levels in the brain, many of those studies were done using brain homogenates or using *in vivo* microdialysis which disrupt at least some circuits and

75

neuronal structures, and are unable to resolve the precise importance of connectivity,

organization, and identities of the neurons that may be important. (Baillien and

Balthazart, 1997; Balthazart et al., 2001, 2003a, 2005, 2006, 2011; Remage-Healey et al., 2008,

2009b, 2010, 2011a; Jeong et al., 2011; Remage-Healey and Joshi, 2012; Charlier et al., 2013b,

2016; Comito et al., 2016). Moreover, in the brains of living animals, there may be meaningful

differences in estradiol signaling at the level of microcircuits or even at the level of cellular

compartment that cannot be detected by methods that only allow detection of global changes

of estradiol in a region (Remage-Healey et al., 2008; Ikeda et al., 2014).

In the NCM, aromatase is highly expressed in cell bodies (Saldanha et al., 2000), and

exposing birds to certain sensory stimuli leads to an increase in estradiol levels within this region

(Remage-Healey et al., 2008, 2009b, 2012). Further, changes in estradiol levels lead to changes

in neuronal activity (Remage-Healey et al., 2010, 2012) and neuronal activity leads to changes in

estradiol levels (Balthazart et al., 2001, 2006; Remage-Healey et al., 2011; Reviwed in Charlier et

al., 2015). In addition, in different species, aromatase is expressed in pre-synaptic boutons

(Naftolin et al., 1996; Peterson et al., 2005; Rohmann et al., 2007), allowing the possibility that

local levels of estradiol within regions are finely controlled at the synapse (Remage-Healey et al.,

2011a; Cornil et al., 2012). Therefore, both the neuronal activity of aromatase-expressing

neurons themselves, as well as the neuronal activity of afferent inputs to aromatase-expressing

neurons likely govern aromatase activity. However, the specific afferents or efferents of

aromatase neurons are unclear.

The neurotransmitter profiles of aromatase-expressing neurons and afferent inputs to

aromatase-expressing neurons in the NCM have been examined in only a few studies. Using *in*

situ hybridization, aromatase mRNA was shown to be expressed by cells that also express either inhibitory or excitatory neuronal markers, glutamic acid decarboxylase (GAD65) or vesicular glutamate transporter 2 (VGlut2), respectively (Jeong et al., 2011). In the cortex of humans and monkeys, aromatase is expressed in pyramidal neurons and in interneurons co-expressing the inhibitory neuronal markers parvalbumin or calbindin (Yague and Muñoz, 2006; Yague et al., 2008). In zebra finches, however; the regions that express calbindin overlap with those of aromatase (Pinaud et al., 2006), whether or not aromatase-expressing neurons also express calbindin is not known. If aromatase and estradiol play similar roles and are regulated in a similar fashion across species, it is likely that, as in humans and monkeys, aromatase-expressing cells in the zebra finch consist of multiple types of interneurons.

Moreover, the majority of aromatase-expressing neurons in the hippocampus (HP) and a low percentage of aromatase-expressing neurons in *the* NCM co-expresses N-Methyl-D-Aspartate (NMDA) receptors (Saldanha et al., 2004), suggesting that the amount of glutamatergic inputs into aromatase-expressing cells are region-specific, and may be important in regulating aromatase activity. In the NCM, the low numbers of aromatase neurons co-expressing NMDA-R may be due to aromatase-expressing neurons receiving non-glutamatergic inputs. Nevertheless, how aromatase activity is modulated by afferent inputs is unclear. To be able to design future studies that examine how estradiol levels are precisely regulated at macro- and micro-circuit levels, it is important to know the neuronal identities of aromatase-expressing neurons and how they differ between regions.

Furthermore, the localization of estradiol receptors relative to where aromatase is expressed is important for understanding estradiol signaling in neural circuits. The G-protein coupled estrogen receptor (GPER1) is a non-classical transmembrane estrogen receptor thought to be involved in rapid actions of estradiol (GPER1 in CNS, reviewed by Alexander et al., 2016). In the NCM, while classical estrogen receptors are expressed (Metzdorf et al., 1999; Saldanha and Coomaralingam, 2005; Jeong et al., 2011) the expression of GPER1 is relatively modest (Acharya and Veney, 2011). Song-control regions and some auditory regions that previously were shown to express low amounts of aromatase, express higher levels of GPER1 compared to auditory regions that express high levels of aromatase (Acharya and Veney, 2011). Evidence so far supports the idea that GPER1 expression is higher in regions with high pre-synaptic aromatase inputs to primarily respond to rapid pre-synaptic changes in estradiol. However, the expression of GPER1 and aromatase have never been compared directly, and it is possible that GPER1 expression is proportional to somatic aromatase expression, when compared in the same animals. Thus, the cellular identity and organization of the estradiol-producing and estradiol-responding neurons are not well known.

This study tests the hypothesis that the neuronal makeup of aromatase-expressing cells are interneurons, region specific, and are closely associated with GPER1-expressing neurons. This will be tested using antibodies against the zebra finch aromatase protein together with different neuronal markers. We predicted that: i) as in humans and monkeys, zebra finch aromatase-expressing neurons are a mixture of parvalbumin-(PV) and calbindin-(CB) expressing neurons (Yague and Muñoz, 2006; Yague et al., 2008); ii) aromatase-expressing neurons are organized to interact with interneurons or even other aromatase neurons, and iii) GPER1 expression is proportional to aromatase expression.

<center>**Methods**</center>

Animals

Adult (> 110 days post-hatch) male (N=6) and female (N=6) zebra finches were taken from the aviary. Most of these birds (N=10) were isolated in sound attenuating chambers overnight and then were exposed to conspecific songs for 30 min. After the playbacks, the birds were left in the dark for 30 min. These procedures were performed because tissues from some of the animals were used for pilot studies involving visualization of song-induced immediate early gene expression. The conditions were kept the same for the rest of the animals to control for potential variability in aromatase expression caused by the environment. Two additional animals did not go through the playback procedure but their tissues were stained in the same manner as animals that were isolated and presented with song playback. Because we did not observe differences in staining in tissue from these animals as compared to animals that received playbacks, their tissue is included with the rest of the results in this study.

The birds were euthanized by isoflurane overdose, transcardially perfused with 0.1M PBS followed by 4% paraformaldehyde. The brains were extracted, post-fixed in 4% paraformaldehyde at room temperature for 2hrs, and submerged in 30% sucrose PB in 4 °C. The brains were then molded in OCT compound and sectioned sagittally at 35μm using a cryostat (Leica, Germany). The sections were kept in -20 °C in cryoprotectant at 4 °C in PB until use.

Immunocytochemistry

<center>79</center>

Brain sections were washed thoroughly with PB, permeabilized and blocked with 10% normal goat serum in 0.3% phosphate buffer triton X (PBT) for 2hrs at room temperature, and incubated with primary antibodies diluted in 0.3% (PBT) (see table for dilutions) at room temperature for 1hr and then at 4 °C for two nights (<48hrs). After the primary incubation, the sections were washed with 0.1% PBT three times for 15 min each and were incubated in secondary antibodies (anti-rabbit, anti-mouse, and/or anti-guinea pig [either Alexa 488-or Alex 594-conjugated, raised in goat, Thermo Fisher Scientific Inc., MA, USA; 1/200) for 1hr at room temperature. The sections were washed three times for 15 min each in 0.1% PBT and once in PB. The sections were mounted onto gelatin-coated slides and coverslipped using ProLong Diamond Antifade Mounting Medium (Thermo Fisher).

For non-fluorescent images, the sections were instead incubated with biotinylated secondary antibodies (1/500) for 1hr in room temperature and then with 1:100 of solutions A and B provided in VECTASTAIN kit (PK-6100, Vector laboratories CA, USA) in 0.3% PBT for 90 min. The sections were washed again three times with 0.1% PBT for 10 min each and then the stains were visualized using peroxidase substrate kits (Vector SG, Sk4700 or Vector NovaRed Sk-4800; Vector laboratories).

The sections were then mounted onto gelatin-coated slides, dried at room temperature, dehydrated in increasing concentrations of ethanol, and then coverslipped using permount media (Thermo Fisher).

Table 1: Primary antibodies and concentrations

Antibody	Protein discription	Host species	Company and catalog number	Dilution	Citations (antibody specificity)
anti-aromatase	enzyme required for estradiol synthesis	rabbit	gift from Dr. Saldanha	1/2000	Saldanha et al., 2000
anti-calbindin	calcium binding protein	mouse	Sigma Aldrich C9848	1/2000	Pinaud et al., 2006
anti-parvalbumin	calcium binding protein, a marker for fast-spiking interneurons	mouse	Millipore MAB1572	1/10,000	Li et al., 2013
anti-PSD 95	post-synaptic density protein, a marker for synapse	mouse	Millipore MAB1596	1/1000	Chaudhury et al., 2010
anti-NeuN	a neruonal marker	mouse	Millipore MAB377	1/2000-1/5000	Mullen et al., 1992
anti-GPER1	membrane bound G-protein coupled estrogen receptor	rabbit	MBL International LS-A4268	1/2000	Acharya & Veney, 2012

Confocal imaging

Fluorescently labeled sections were imaged using a confocal microscope (NA1, Nikon, Tokyo, Japan) with NIS-Elements imaging software (Ar). The laser strength and gain were determined separately for each section since the intensity of the staining was not consistent across sections; however, the imaging settings were kept consistent across regions within each section (See Figure 11 for the regions of interest). Pictures of the NCM (ventral and dorsal), CMM, and HP were taken from sections 0.2-1mm lateral to the midline and pictures of HVC, HVC shelf, NCL, RA were taken from sections 1.7-2.5mm lateral to the midline. Since the boundaries between the NCL and NCM and between the arcopallium and NCL are not clearly defined in the literature, the medial-most aspect of nucleus taeniea (Tn) was used as a landmark for the lateral boundary of the NCM, and pictures of the NCL were taken from sections that were in-plane with RA. For each section, stitch images using 10x magnification were taken and used to observe the overall pattern of the staining in the sections and as a reference for higher magnification images (60x, oil). Once regions were identified, 60x images were taken using z-stack settings (1μm z-steps for 9-15μm). Zoomed-in images of individual neurons or clusters were taken using z-step sizes (0.1μm-0.5μm).

Image analysis

The number of immunostained cells and the total number of cells (DAPI-positive nuclei)
in each z-stack image were counted by blinded experimenters using FIJI software (National
Institutes of Health, USA). For each region, the numbers of aromatase-positive cells were
divided by the number of DAPI+ stained nuclei to calculate the percentage of aromatase positive
cells per total number of cells (percentage of aromatase+ cells). Co-expression of two antigens
was unambiguously confirmed by comparing both maximal projection images and individual z-
slice images (z-slice images ranged from 9 μm to 15μm in terms of thickness). To avoid including
potential neurons that were negative for aromatase but were densely innervated by aromatase-
positive terminals, cells with low fluorescent signals ("ghost cells") were excluded from analysis
for all cell counts in this study. Examples of "ghost cells" and "aromatase-expressing" cells are
shown in (Figure 10). Previously, Saldanha et al. (2000) showed that only terminal aromatase is
found in the arcopallium while somatic aromatase cells are found in the NCL. In our sections, we
found high intensity cells in the NCL and ghost cells in the nearby arcopallium within the same
image. When sections were double-labeled with the post synaptic marker postsynaptic density
protein 95 (PSD-95) and with aromatase, ghost cells showed higher signals for PSD-95 than
"aromatase-expressing" cells (Figure 10). When zoomed-in, many of the PSD-95 signals co-
localized with aromatase signals (Figure 20). Although this is not concrete evidence, ghost cells
are likely to be cells receiving aromatase+ afferents and not expressing intracellular aromatase
protein. In HVC and arcopallium, although there are no aromatase+ somatas, the presence of
pre-synaptic aromatase terminals (Saldanha et al., 2000; Peterson et al., 2005) and aromatase
activity (Vockel et al., 1990; Rohmann et al., 2007) has been reported in these regions   We
carried out a separate analysis measuring the optical density of randomly chosen cells (N=15

somatic and N=24 ghost cells from 11 pictures taken in 5 regions), confirming that cells

that were included in the analysis as aromatase+ cells had significantly higher optical

density than ghost cells (unpaired T-test, p < 0.0001; OD: somatic 2.13+/- 0.24; ghost

0.87 +/- 0.11).

Clusters were defined as a group of two or more cells forming somato-somatic

contacts (i.e. zero distance between the cell bodies at 60x magnification). The number

of cells within each cluster was counted manually by scrolling through z-stack images in

FIJI.
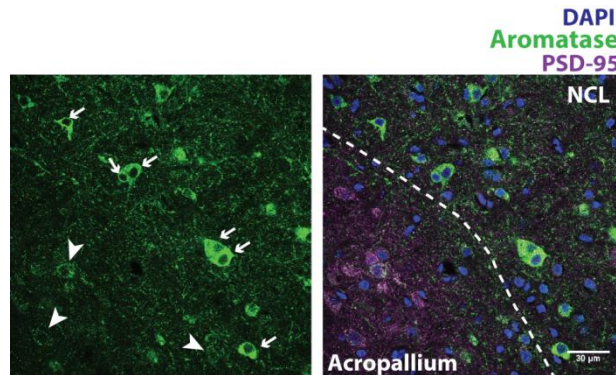
Figure 10: Aromatase+ cells included in analysis and "ghost cells."

60x single z-image at the border between arcopallium and NCL from tissue stained against PSD-95 and aromatase. High-intensity fluorescent cells (arrows, "aromatase+ cells") are found in the NCL while low-intensity fluorescent cells (arrow heads, "ghost cells") overlapping with PSD-95 signals are found primarily in the arcopallium. Ghost cells are likely to reflect inputs from pre-synaptic aromatase terminals, as described by (Saldanha et al., 2000).

Dye injections

To test whether gap junctions exist between clustered neurons, a small dye (Po-pro-1,

Thermo Fisher Scientific, MA) was injected into a clustered cell. The injections were made into

cells on 300 µm thick slices prepared for whole-cell intracellular recording for a separate study.

Po-pro-1 (in DMSO 10 %) or neurobiotin (10%) was mixed with Alexa 488 (10 %) and internal

solution (K-gluconate, KCl, CaCl2, HEPES, EGTA, MgATP, NaGTP, Na2-Phosphocreatine) and

injected with a glass pipet while applying current steps (~20 min). After dye injections, the slices

were fixed at 4 % paraformaldehyde in 4 °C. Two days later, the slices were mounted on slides,

coverslipped using ProlongGold (Thermo Fisher), and imaged with confocal microscopy.

Data analysis

Statistical analyses were performed using statistical software R. One-way ANOVA

(ANOVA), two-way ANOVA (TW-ANOVA), or post hoc *t*-tests were conducted when appropriate.

Results

The neuronal composition of aromatase positive nuclei is region-dependent

To test whether the heterogeneity of estradiol-producing cells in the zebra finch brain

were different across regions, zebra finch brains were immunostained with antibodies against

the zebra finch aromatase, and co-labeled with other neuronal markers (Figure 11). In

agreement with previous findings, the percentages of aromatase+ cells were high in the NCM

and HVC shelf (Saldanha et al., 2000). No aromatase+ cells were found in the ventral CMM

(Figure 12), although a few sparse and isolated aromatase+ cells were found in the dorsal CMM

close to the ventricle (Metzdorf et al., 1999, Figure 4.8A). No difference in the percentages of

aromatase+ cells was found between ventral and dorsal NCM. In contrast to Saldanha et al.

(2000), the percentage of aromatase+ cells was significantly higher in the hippocampus (HP)

than in other regions (Figure 11, 12 & 17B; ANOVA for all regions: $F_{(7, 67)} = 7.46$; post-hoc t-

tests: HP vs any region, $p < 0.05$; CMM, N= 10; NCMv, N= 11; NCMd, N= 11; HP, N= 11; NCL, N=

8; HVC shelf A, N= 8; HVC shelf V, N= 8; HVC shelf P, N= 8). Also, notably, the total number of

aromatase-positive cells in the NCL were comparable to that of the NCM (Figure 12), suggesting

the importance of rapid estradiol signaling in this region. There were no sex differences in the total number of aromatase-positive cells in the NCM (F, N=6; M, N=5. TW-ANOVA: sex, $F_{(1, 18)}= 0.79$; region, $F_{(1,18)}= 0.50$; sex*region, $F_{(1,18)}= 0.23$, p= 0.64), while there was a significant sex difference observed in the HVC shelf (F, N=3; M, N=5. TW-ANOVA: sex, $F_{(1,18)}= 6.8$, p= 0.02; region, $F_{(2,18)}= 1.0$, p= 0.36; sex* region, $F_{(2, 18)}= 1.3$, p= 0.28). In the HVC shelf, more aromatase positive cells are found in females than males. This is interesting considering the HVC is larger in volume and plays an important role in singing in males (Gahr, 1996)
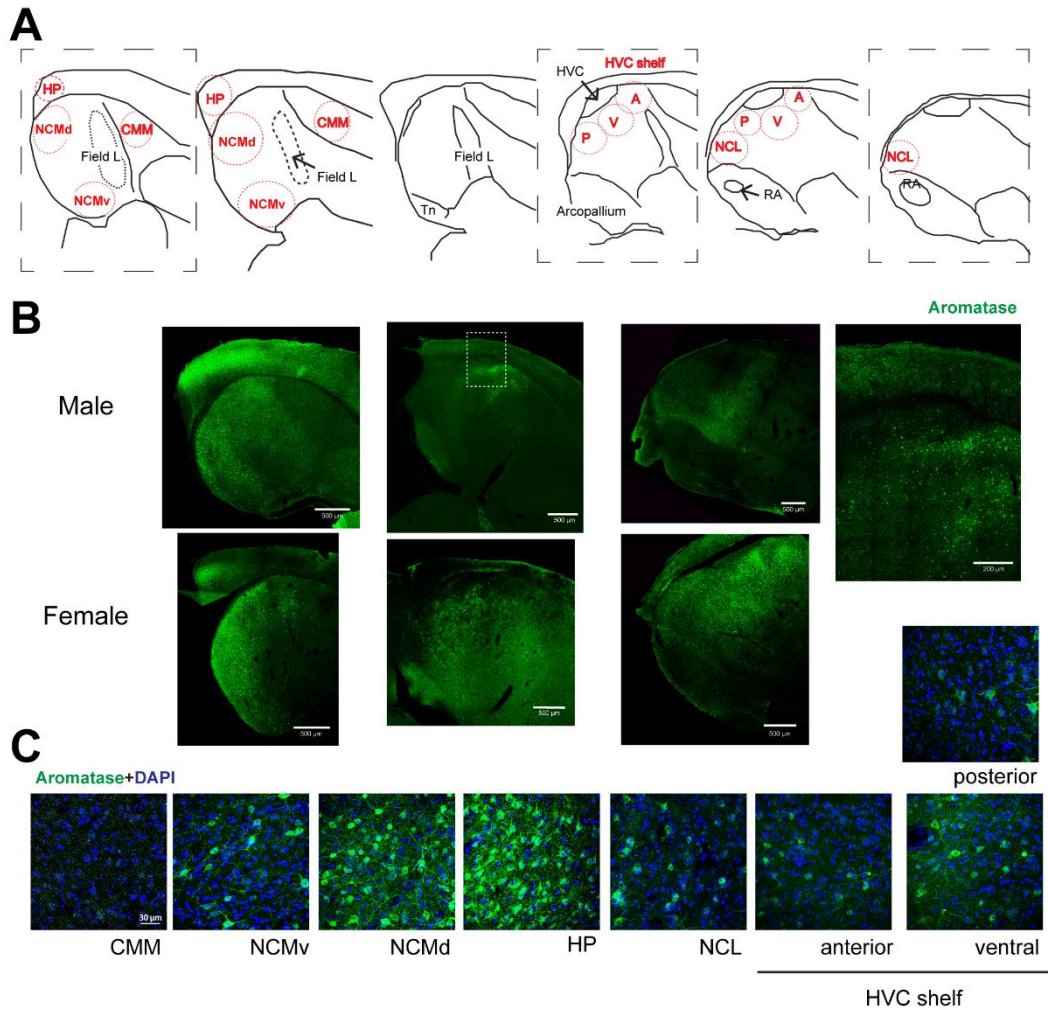
Figure 11: Regions of interest and the aromatase expression pattern

Female and male zebra finch brains were stained with aromatase antibody and imaged using a confocal microscope. (**A**) Illustration of sagittal sections medial (left) to lateral (right) (~0.5 – 2.2 mm from midline, ~0.3 mm apart). Red circles are approximate areas where 60x images were taken for counting aromatase+ cells. Dashed boxes represent areas from which pictures in **B** were taken. A-anterior; V-ventral; P-posterior. Modified illustrations from ZEBrA: A Zebra Finch Expression Atlas, RRID: nif-0000–24345; http://www.zebrafinchatlas.org. (**B**) Stitched images taken with 10x objective. *Right*, zoomed in stich image using 60x objective from area within dashed lines (C) Projection images from regions of interest using a 60x objective.
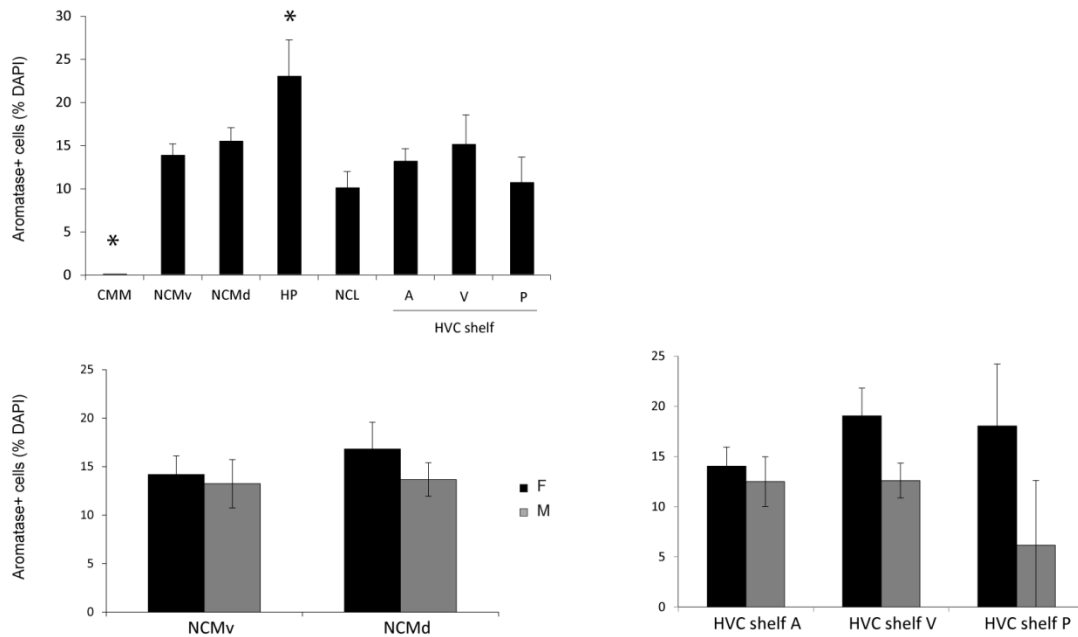
Figure 12: Quantification of aromatase expression.

The number of aromatase and DAPI expressing cell bodies were counted in 60x z-stack images. Top, Percentage of aromatase-expressing cells among DAPI+ cells in the regions of interest. HP and CMM are significantly different from other regions (Mean ± SEM, post-hoc t-test vs other regions: *, $p < 0.05$). CMM, N= 10; NCMv, N= 11; NCMd, N= 11; HP, N= 11; NCL, N= 8; HVC shelf A, N= 8; HVC shelf V, N= 8; HVC shelf P, N= 8. *Bottom*, No sex differences were found in NCM while there was a significant sex difference in HVC shelf.

Double-labeling immunocytochemistry with antibodies against NeuN showed that > 99% of aromatase cells were neurons (Figure 14). This is in agreement with a previous study showing that most aromatase+ cells in the uninjured zebra finch brain are non-glial (Peterson et al., 2004). Furthermore, double-staining with traditional interneuron markers parvalbumin and calbindin showed that none of the aromatase positive neurons expresses calbindin in any regions investigated (Figure 13B). By contrast, 5 % of ventral and 15% of dorsal NCM aromatase+ neurons were positive for

parvalbumin (Figure 13C). The percentages of parvalbumin+ aromatase neurons were region-specific (Figure 13C; TW-ANOVA: sex, $F_{(1, 47)}= 0.75$, $p= 0.39$; region, $F_{(7, 47)}= 7.3$, $p< 0.001$; sex*region, $F_{(7,47)}= 0.14$, $p= 0.99$. CMM, N= 9 (F, N=5; M, N=4); NCM, N=10; HP, N= 9 (F, N=6; M, N=3): NCL, N= 5 (F, N=3; M, N=2): HVC shelf A, N= 7 (F, N=2; M, N=5); HVC shelf V, N= 6 (F, N=3; M, N=3); HVC shelf P, N= 7). Although the percentage of aromatase+ cells was high in the HP and NCL, only a few of these cells (<1%) co-expressed parvalbumin (Figure 13C). Therefore, NCM aromatase+ neurons are a mixture of a small number of parvalbumin+ neurons and many of other types of neurons (>85%), and the contrast in the proportion of parvalbumin positive, aromatase+ cells between the NCM and NCL supports the idea that estradiol signaling and the role of estradiol are different between these regions. Overall, the difference in the density and identity in the aromatase+ cells are region-specific.
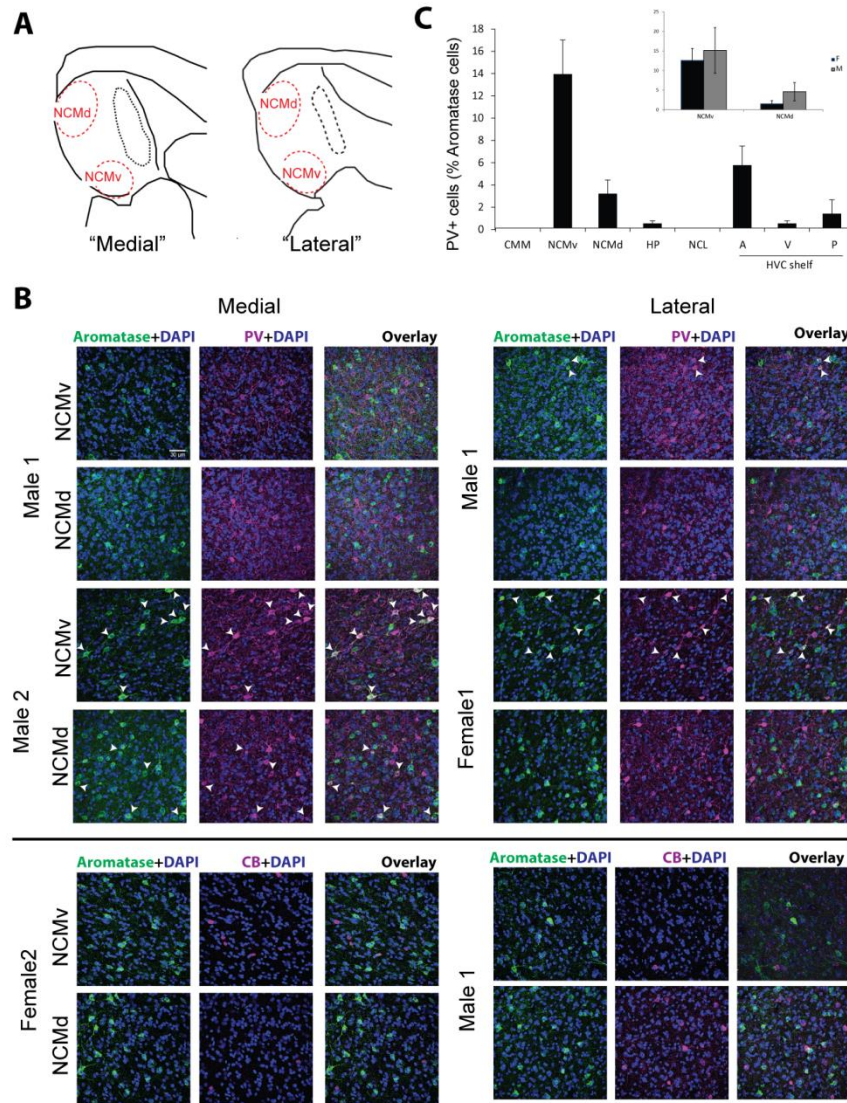
Figure 13: Aromatase+ cells co-express PV and the fraction of co-expressing cells differs

between individuals

Aromatase was double-labeled either with PV or CB. Individual variability was seen in PV and aromatase co-expressing cells in the NCM but was not due to the location where images were taken. (**A**) Schematics of lateral and medial sections to illustrate where sample images from medial and lateral NCM images in **B** were taken. (**B**) 60x projection images from medial and lateral NCM sections double-labeled either for aromatase and PV (*top*, three subjects) or aromatase and CB (*bottom*, two subjects). (**C**) Quantification of PV+ aromatase+ cells. Inset, data from NCM separated by sex. (Mean ± SEM,).
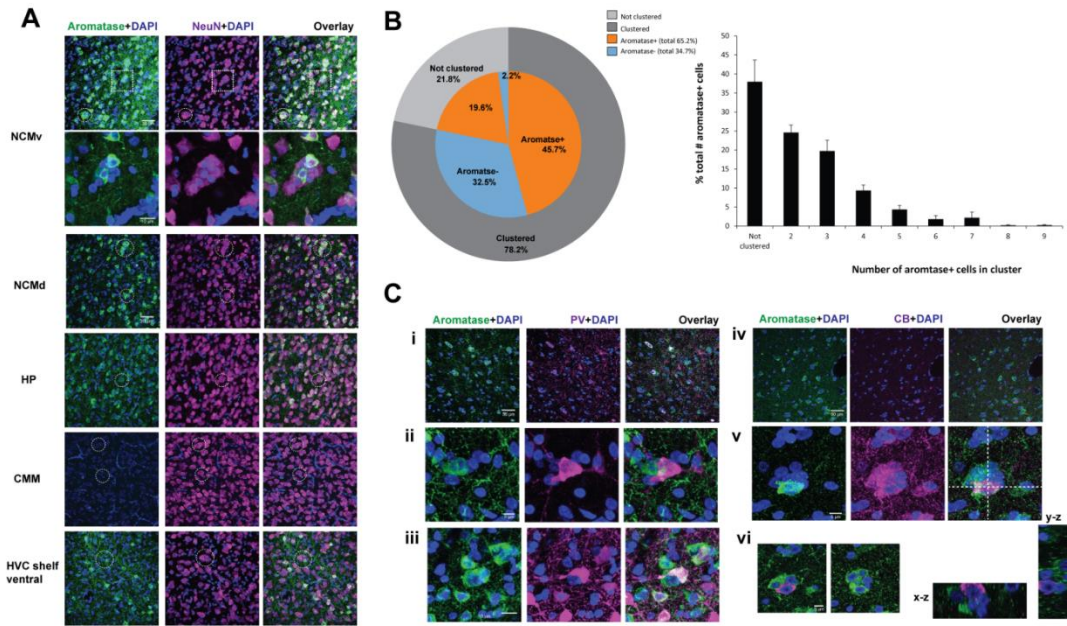
Figure 14: The majority of aromatase+ cells are neurons and are found in clusters

(**A**) Maximum projection images of 60x z-stack images taken from NeuN and aromatase double-labeled sections. Exemplars of NeuN clusters are circled with dotted lines. Images in the second row are zoomed-in images of the cluster in the dotted square in the first row (NCMv). (**B**) *Left*, Percentages of clustered, not clustered, aromatase+, and aromatase- neurons in the image from NCMv in **A**. *Right*, Percentages of aromatase+ cells found in clusters with different numbers of other aromatase+ cells (Mean ± SEM, N=11). (**C**) Aromatase+ cell bodies clustered with CB+ and PV+ cells. *i* & *iv*, Single 60x z-images in NCMv. *ii, iii, & v*, Maximum projection of zoomed-in 60x z-stack images of exemplars of aromatase clusters with PV+Aromatase- cells (ii), PV+Aromatase+ cells (iii), and CB+Aromatase- cells (v). *vi*, *left,* two single z-images from cluster shown in *v* to illustrate that the CB+ cell in *v* is not co-expressing aromatase. *Right*, Orthogonal depth-profile images (y-z and x-z images) of the maximum projection image in *v*.
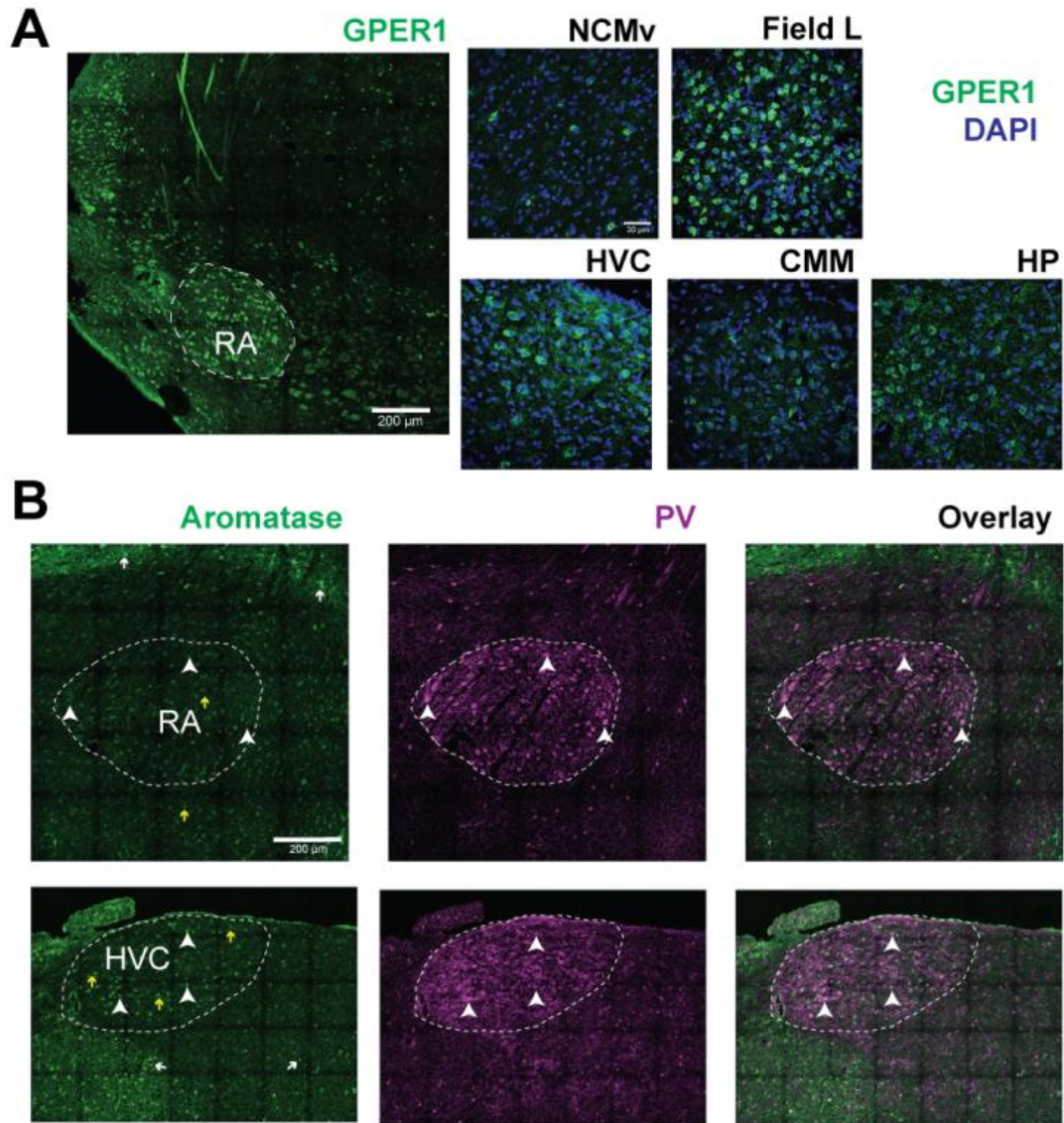
Figure 15: GPER1 expression is elevated especially in regions where somal aromatase

expression is low

Sagittal plane sections were stained with GPER1 or aromatase antibody. (A) *Left,* 60x stiched z-stack image of arcopallium. *Right*, 60x z-stack images in different regions. GPER1 expression is highest in field L, HVC, and RA, and low in NCM. Intermediate levels of expression are found in arcopallium outside of RA and in CMM. (B) 60x stich z-stacked image of arcopallium (*top*) and a region including HVC (*bottom*) in sections stained against aromatase. *White arrows* point to somatic aromatase and *yellow arrows* point to examples of ghost cells. *White arrow heads* point to ghost cells that are positive for PV.

Aromatase-expressing neurons form clusters with other neurons in NCM

How cells are organized gives clues to how they communicate with one another. A striking characteristic of the neurons visualized with NeuN is that they form somatic clusters with other neurons (Figure 14). Notably, the clusters in the HP were less compact and discrete than clusters in other regions that were analyzed. Detailed analysis of a picture from a double-label staining for aromatase and NeuN in the ventral NCM revealed that NCMv neurons are predominantly found in clusters (78.2%) and were positive for aromatase (65.2%, Figure 14B). Analyses of pictures from all animals shows that the majority of aromatase+ neurons are in contact with at least one other aromatase+ neuron (65.2± 5.76 %) and many are found in large clusters composed of more than three neurons (Figure 14B). Both parvalbumin+ and calbindin+ cells were found in clusters with aromatase+ cells (Figure 14C), suggesting that a variety of neurons cluster with aromatase cells. Similar to aromatase, many GPER1 expressing neurons were also found in clusters in the HVC, CMM, NCL, and NCM (Figure 15). Therefore, the organization of aromatase+ neurons are region-specific and many aromatase+ neurons in the NCM cluster with different cell types. A preliminary experiment was performed to test the diffusion of gap junction-permeable dyes into nearby clustered cells. When a small fluorescent dye, po-pro-1, or neurobiotin was injected into a clustered cell, the dye was observable in an adjacent cell (po-pro-1, Figure 19; neurobiotin, not shown), suggesting that gap junctions may exist between the clustered somato-somatic connections between neurons (Lima MM, Ikeda MZ, Scarpa GB, and Remage-Healey L., unpublished).

Aromatase and GPER1 expression pattern suggest the importance of rapid estradiol signaling in sensorimotor regions

By taking advantage of the fact that we were able to detect aromatase signals in regions reported to be negative for somatic aromatase, we compared this distribution to that of the membrane estrogen receptor, GPER1. Many neurons were outlined by low intensity signals when stained with the aromatase antibody ("ghost cells", Figure 10), possibly representing aromatase+ synaptic terminals. Many ghost cells were present in the HVC, CMM, field L and arcopallium (including RA) (Figure 15 & 17, ghost cells in field L is not shown). Since ghost cells were low in fluorescence, they were generally not visible unless relatively high laser gain settings were used to obtain the images (Figure 15). When sections were stained with GPER1 antibody, as shown previously, high signals were observed in the HVC, HP, and RA (Acharya and Veney, 2011; Figure 15). In the NCM, GPER1+ cells were found sporadically. Although not as high in the HVC, GPER1 expression in the CMM and arcopallium outside of the RA was relatively high. Unexpectedly, GPER1 signal was very high in field L. Therefore, low somatic aromatase areas such as the HVC, field L, arcopallium, and CMM showed relatively high expression of GPER1 and large numbers of aromatase ghost cells. In addition, in the RA and HVC, some ghost cells were positive for PV, suggesting that PV+ neurons are responsive to estradiol signaling. This result provides additional evidence that estradiol plays important roles in auditory processing and vocalization in regions with low numbers of somatic aromatase but with high levels of presynaptic aromatase.
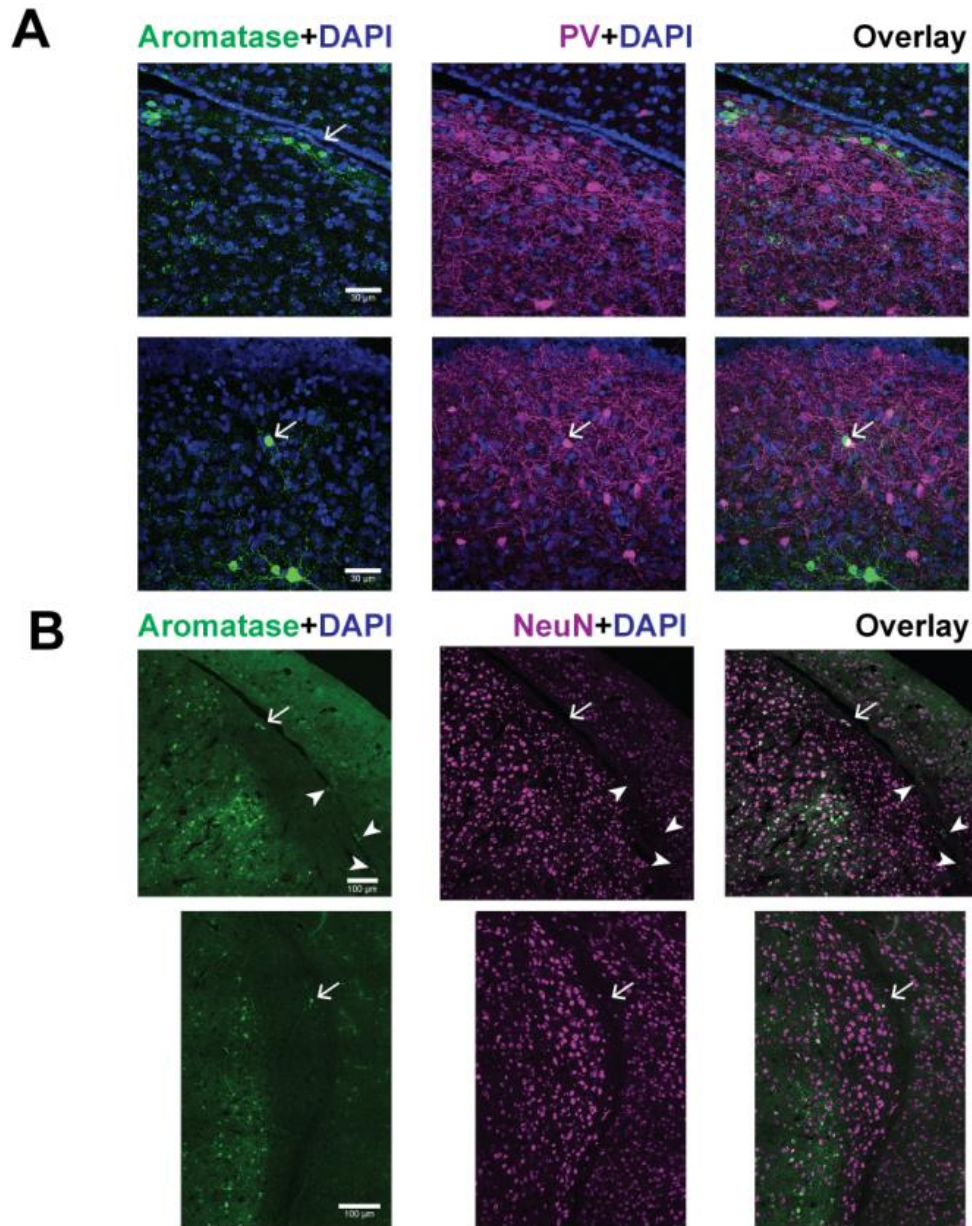
Figure 16: Aromatase cells positive and negative for NeuN in the HVC.

Exemplars of aromatase+ neurons in the HVC. (**A**) Maximum projections of 60x z-stack images of sagittal sections stained for aromatase and PV. *Top*, aromatase+ cells close to the ventricle. *Bottom*, aromatase+ cell in the middle of the HVC away from the HVC shelf and ventricle. (B) Maximum projections of 60x stiched z-stack images of sagittal sections stained for aromatase and NeuN. Aromatase+ cells in the HVC away from the ventricle are NeuN+ (arrows), while aromatase+ cells at the edge of the tissue are negative for NeuN.
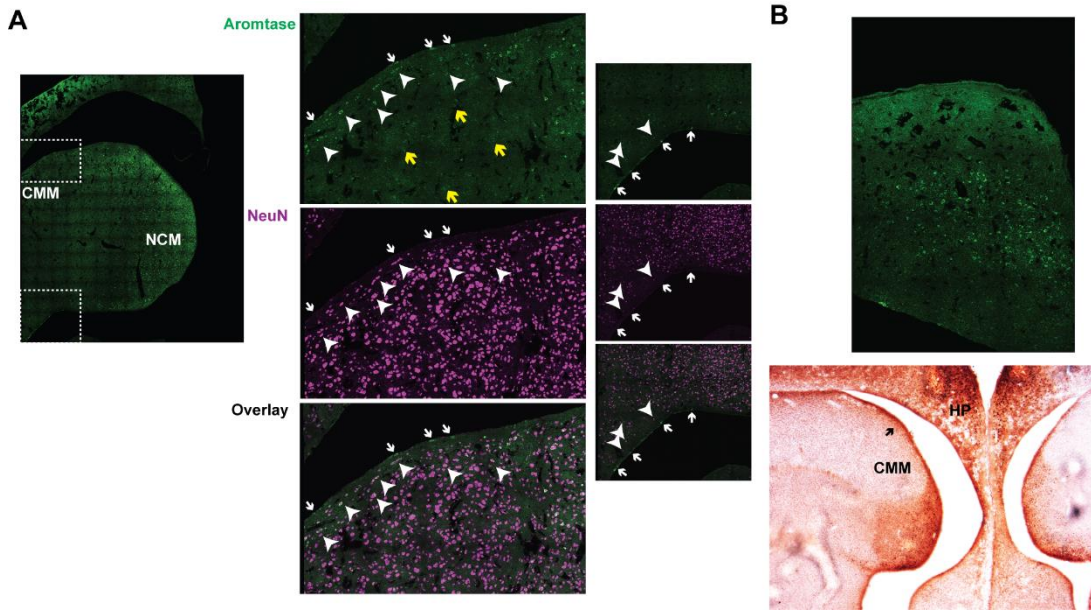
Figure 17: Aromatase+ cells in the CMM and HP

(A) *Left*, 60x stiched single z-image of a sagittal section from the caudal medial telencephalon stained for aromatase and NeuN (only the fluorescent signal for aromatase is shown). *Right*, cropped, zoomed-in images of areas surrounded by white dashed lines in left image. *White arrows* show examples of aromatase+ cells that are negative for NeuN at the edge of the tissue. *White arrow* heads point to aromatase+ neurons positive for NeuN. *Yellow arrows* point to examples of ghost cells in the CMM. (B) *Top*, 60x single z-image of a hippocampus sectioned in the sagittal plane stained for aromatase. *Bottom*, bright field 10x image of a caudal section of a brain sectioned in the coronal plane and stained for aromatase. *Black arrow* indicates neurons aligning the lateral ventricle.
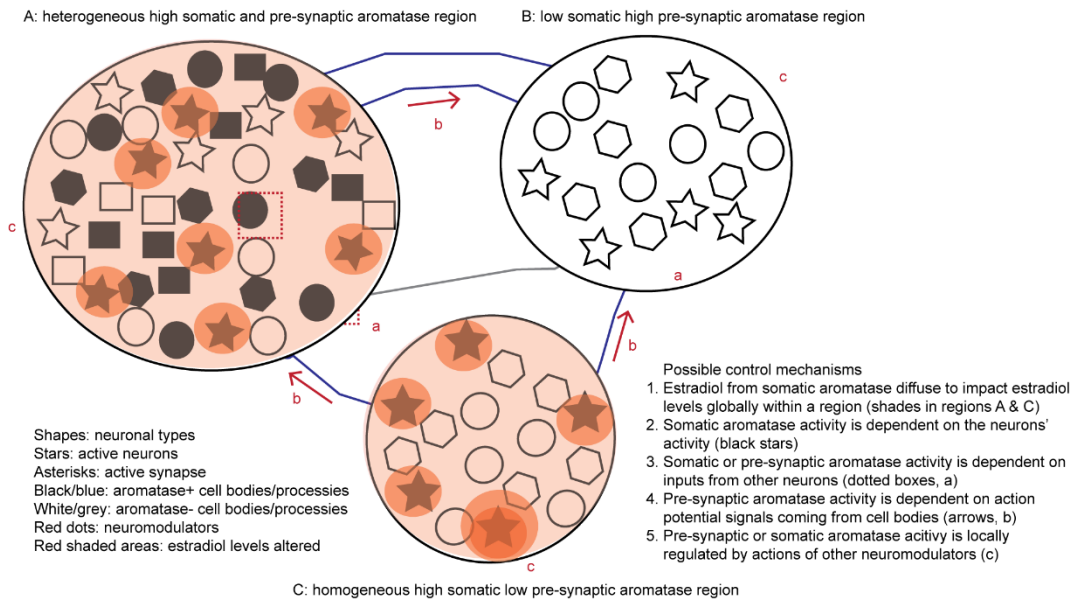
Figure 18: Model for the control mechanism of aromatase activity and regulation of local estradiol levels

A schematic to illustrate putative control mechanisms of aromatase activity and local estradiol levels when subpopulations of neurons (star shapes) are activated. Circles A, B, and C represent heterogenous regions with differing pattern of aromatase expression. Estradiol within in a region could be come from the blood stream (not shown) or synthesized from local somatic (black shapes) or pre-synaptic (blue lines) aromatase. In regions where aromatase is primarily found pre-synaptically (region B), aromatase+ terminals are likely to come from other regions that express somatic aromatase (regions A & C). In regions where aromatase is found both in the pre-synaptic boutons and cell bodies (A), aromatase+ terminals can come from either from the same region or from a different region. In regions with heterogeneous aromatase+ neurons (region A), sub-regional aromatase concentrations may be more important than in regions with homogeneous aromatase neuronal identity (region C). (1) Estradiol levels of the whole area can be upregulated by local aromatase in regions in which aromatase is abundantly expressed (shades in regions A & C). (2) Subregional estradiol levels around aromatase+ neurons are up- or down-regulated *via* neuronal activity (shades around star-shaped neurons). (3) Pre- or post-synaptic aromatase activity may depend on receiving inputs from other neurons. Signals that affect presynaptic aromatase activity can come from axoaxonic synapses (region C, dotted square a), while post-synaptic (or somatic) aromatase activity may depend on direct pre-synaptic inputs coming either from aromatase positive or negative terminals from neurons whose cell bodies reside inside (region A, top dotted square a) or outside (region A, bottom dotted square a) of the region. (4) Somatic or pre-synaptic aromatase activity is dependent on neuronal activity or action potentials (arrows b). The signals may come from inside (region A, arrows b) or outside of the region where the pre-synaptic aromatase terminals are. (5) Somatic or pre-synaptic aromatase activity can be affected by the actions of other neuromodulators (somatic, region A & B c; pre-synaptic, region C c).
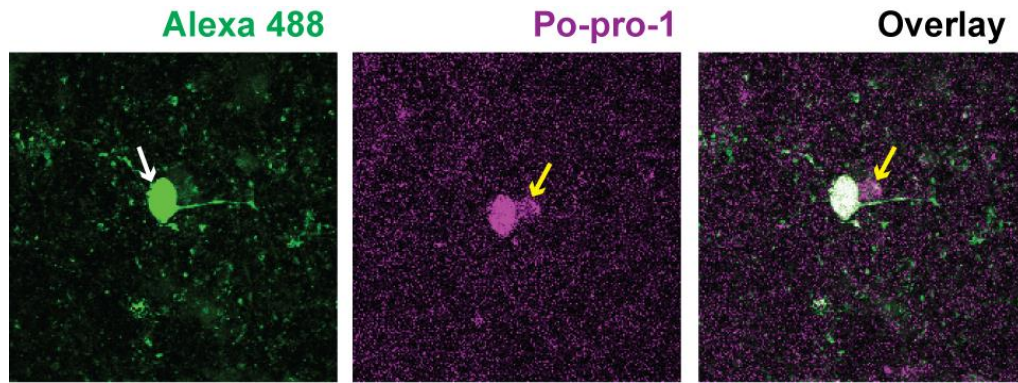
Figure 19: Gap junction-permeable dye diffuses into adjacent clustered cell

A small fluorescent dye permeable to most gap junctions, po-pro-1 (Thermo Fisher, molecular mass 579 Da), mixed with Alexa 488 was injected into a cell clustered with another cell in the NCM. The dyes were injected *via* applying current steps in an intracellular whole-cell recording setup. *White arrow* points to the injected cell. *Yellow arrow* points to po-pro-1 diffused into cell adjacent to the injected cell.
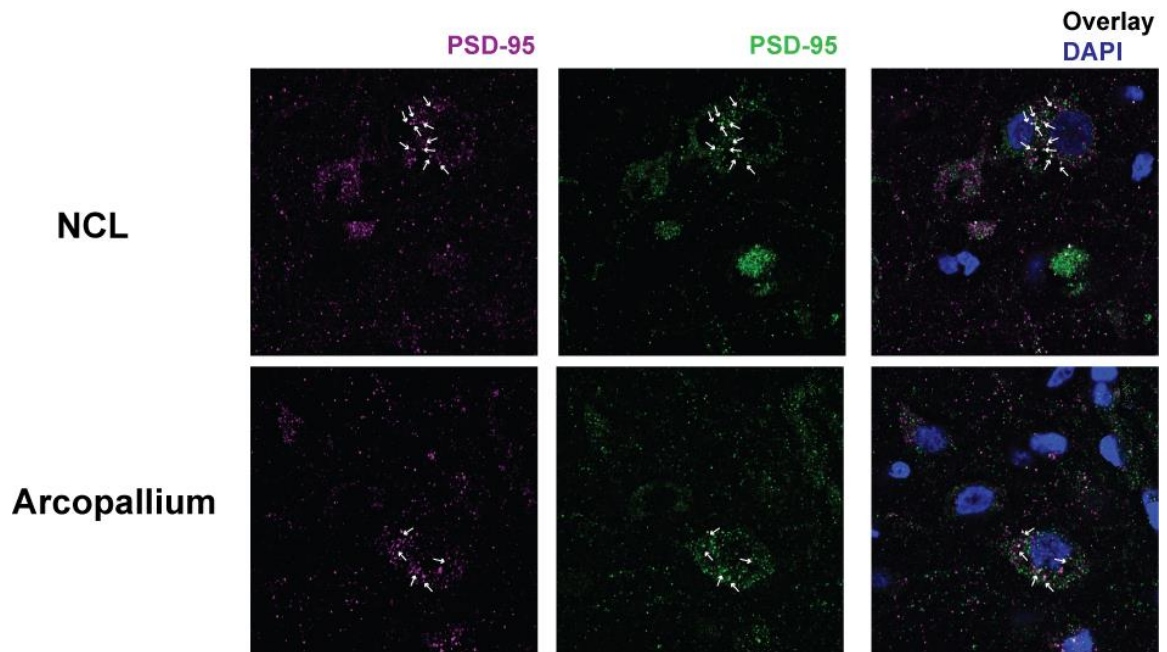
Figure 20: PSD-95 and aromatase puncta are localized close to each other

Sections were stained with antibodies specific for aromatase and PSD-95, and were imaged with a confocal microscope. The *arrows* show co-localizing puncta that may be evidence for pre-synaptic aromatase+ terminals forming synapses.

Discussion

The current study examined the regional differences in distribution, organization, and identities of aromatase expression in the zebra finch brain. The anatomical observations described here provide evidence for complexity in the regulation of estradiol signaling. First, we report that although many brain regions express aromatase, the identities of the neurons expressing aromatase are distinct between regions. More specifically, the interneuron marker parvalbumin was expressed in a portion of aromatase+ neurons in the NCM, while, in the HP and NCL, where the percentage of aromatase positive neurons were equivalent to or greater than that of the NCM, parvalbumin was not co-expressed with aromatase. This study is, to our knowledge, the first to provide direct anatomical evidence for the differences in neuronal makeup between the NCL and NCM, and to report that the amount of aromatase expressed in caudal NCL is equivalent to that of the NCM. Moreover, the fact that aromatase+ neurons in the NCM were a mixture of parvalbumin positive and negative neurons and that the percentage of co-expression was different between ventral and dorsal NCM suggest that neuronal estrogen synthesis is differentially regulated within and between subregions of the NCM. Since aromatase activity has been suggested to be sensitive to neuronal activity (Balthazart et al., 2001, 2006; Remage-Healey et al., 2009b), our study shows that estrogen synthesis could be under exceedingly localized control in different classes of neurons within and between brain regions (Figure 18). In addition, the fact that aromatase+ cells in the human temporal cortex consists of glia and neurons, some of which expresses calbindin or parvalbumin, should be taken into account when comparing the two species (Yague and Muñoz, 2006; Yague et al., 2008). How different cell types interconnect and how different inputs into aromatase cells act to regulate aromatase activity may be different between the zebra finch and humans. In the HP, we found more aromatase+ somatas than in any other regions we analyzed, while in a previous study,

there were less cell bodies expressing aromatase in the HP compared to the NCM

(Saldanha et al., 2000). This could be due to overestimating aromatase-expressing cells

by falsely including aromatase negative cells that are densely innervated by aromatase+

terminals. However, if the discrepancy between our study and the previous study is not

methodological in origin, it may be due to the difference hormonal conditions of the

animals, since the HP aromatase levels are sensitive to circulating estradiol levels

(Saldanha et al., 2000)

Second, we discovered that neurons are found in discrete clusters in the NCM,

and that aromatase-expressing neurons are densely clustered. In songbirds, cell clusters

in the brain have been described only in a few studies (Fortune and Margoliash, 1992;

Gahr and Garcia-Segura, 1996; Kirn et al., 1999; Medina et al., 2013). In zebra finches,

cell clusters have been reported in the nidopallium, mesopallium, and hyperpallium

(Fortune and Margoliash, 1992; Medina et al., 2013). Medina et al. (2013), suggested

that the clusters in the mesopallium are composed of one neuron and multiple glial

cells, based on Nissl stain. Our use of NeuN staining in the NCM and other areas

indicates that the majority of the clusters are composed of neurons in all regions we

examined including the CMM. In the ventral NCM, the many of aromatase+ neurons

were found in clusters, which suggest the possible role of electric coupling in the

regulation of aromatase activity. Not much is known about their function except that

the neuronal clusters in canary HVC are coupled with gap junctions (Gahr and Garcia-

Segura, 1996). Preliminary data suggest that clusters found in NCM are at least coupled

with pores that are permeable to compounds that diffuse through gap junctions in other

systems.

Third, the comparison between aromatase and GPER1 expression suggest the importance of rapid estradiol signaling through terminal-expressed aromatase in many regions, including those that have not been previously reported in the literature, such as the CMM and RA. In some regions, such as the NCM, NCL, and HP, the expression levels between GPER1 and aromatase were similar to each other while in other regions, such as the CMM, field L, HVC and arcopallium, GPER1 expression was high, even though only ghost cells (which are speculated to be synaptic terminals) showed the aromatase staining. Importantly, this study is the first to report the extensive expression of GPER1 in field L. It is possible that in the regions where both aromatase and estrogen receptors are abundant, estradiol is operating through volume transmission, affecting many neurons in the region, while, in other regions where only aromatase is pre-synaptically expressed, estradiol fluctuations are limited locally to the synapses (Figure 18). In regions where somatic aromatase is rich, local neuronal activity is likely to be regulating aromatase activity. On the other hand, in regions with only pre-synaptic aromatase expression, aromatase activity may be dependent both on local activity and on the activity of the nuclei where their cell bodies reside. It is possible that recently-found projections from the HVC shelf and NCL to the ventral intermediate arcopallium (AIV) and the known projections from the NCM to CMM could be the source of ghost cells the arcopallium and CMM respectively (Vates et al., 1996; Mandelblat-Cerf et al., 2014). In this study, GPER1 and aromatase was not double-labeled due to lack of an optimal antibody. However, in the future, we will test the hypothesis that aromatase-expressing terminals are innervating GPER1-expressing neurons. Experiments directly identifying the source the aromatase+ synaptic terminals are necessary to further our understanding of the role and mechanisms of estradiol signaling in these regions.

Fourth, we occasionally found neurons with intense aromatase signals in the HVC, CMM, and areas close to the edge of the telencephalon. In canaries, aromatase mRNA has been reported along the lateral ventricle (Metzdorf et al., 1999). Using injured zebra finches, a previous report showed that ventricular aromatase+ cells were morphologically similar to radial glia marker-expressing cells as well as the expression pattern (Peterson et al., 2004). We found that although many aromatase+ cells that were at the lateral edge of the tissue were negative for NeuN, the aromatase+ cells that were further from the edge of the tissue were positive for NeuN. In the CNS, neurons and radial glia are born in the ventricular zone, and the radial glia give rise to migrating neurons (Alvarez-Buylla et al., 1990; Noctor et al., 2001). The cells at the edges of the tissue were morphologically similar to the radial glia-like cells found in the ventricular zone in canaries (Alvarez-Buylla et al., 1990). Therefore, we suspect that the aromatase+ cells at the edge of the tissue are radial glia associated with migrating aromatase+ neurons. Although the functions and the identities of the neurons were not pursued in the current study, whether or not these aromatase+ neurons are migrating neurons that on their way to a different destination, or actively involved in HVC function, together with why they express aromatase should be tested in future studies.

The current study provides compelling anatomical evidence for regional differences in estradiol regulations and strong motivation for taking regional differences, neuronal circuits within and across regions, cell types, and cellular organization into account when studying estradiol regulation and mechanisms in the future.

CHAPTER V

DISCUSSION

The current dissertation examined the role of norepinephrine in estradiol synthesis and identities of aromatase-expressing cells. The results not only gave answers to initial questions but also provided new insights that established grounds for future studies on the mechanisms controlling estradiol signaling. Specifically, the first chapter describes a method, measurement of neurosteroids using *in vivo* microdialysis, which allowed quantifying estradiol levels in the brain in living, behaving animals. The second chapter showed that norepinephrine is not involved in controlling estradiol levels, despite the fact that it has similar effects as estradiol on sound-induced neuronal activity relative to pre-stimulus activity. In the third chapter, the identities of estradiol-producing cells were shown to be heterogeneous and that they are found in clusters.

In chapter 1, we report the protocol for *in vivo* microdialysis, a method that allows measuring neurosteroids locally in the brain in freely moving animals within a relatively small time frame. Traditionally, neurosteroid levels in the brain have been measured from extracted brain tissue. Although neurosteroid extraction from brain tissue allows estimation of absolute steroid levels, it does not allow within-subject comparison at different time points. *In vivo* microdialysis allows multiple collections from the same animals and estradiol levels in the dialysates can be directly measured using enzyme-immunoassays (EIAs) without additional steps. Also microdialysis probes can be used to deliver drugs locally (retrodialysis). However, *in vivo* microdialysis

103

disrupts the cytoarchitecture of the neurons and induces many biological responses due to

injury. Also, even the smallest microdialysis probes are still too large to detect differences in

estradiol levels within a region smaller than 1mm in diameter. Technological advancement in

visualization of aromatase activity or estradiol using reporter molecules in slices or in whole

brains would advance the field.

In chapter 2, we showed strong evidence that norepinephrine does not play a role in

controlling or modulating estradiol levels in the NCM. Using retrodialysis, we directly infused

norepinephrine into the NCM and saw no effect on local estradiol levels. Prior to this research,

little information was available as to how actions of other neuromodulators may affect estradiol

levels or interfere with actions of estradiol *in vivo*. Although results from previous studies

implicated the possibility of catecholamines affecting estradiol levels, in the songbird NCM, no

studies had tested this directly *in vivo*. In quail brain homogenates, when the homogenates

were treated with dopamine or dopamine receptor agonists, aromatase activity decreased

(Baillien and Balthazart, 1997). However, because this study was done in homogenates, it did

not take into account the cellular localization of dopamine receptors and aromatase, and other

factors in the intracellular environment that may have prevented the interactions between

dopamine receptors and aromatase. Therefore, we were the first to directly test the rapid effect

of neuromodulators on estradiol synthesis *in vivo*. Although this study clearly shows that

norepinephrine does not rapidly affect global estradiol level within NCM, it is still possible that

norepinephrine has some indirect, long-term or minor effects on estradiol levels. Since

norepinephrine alters neuronal activity in the NCM (Ikeda et al., 2015) and estradiol levels are

sensitive to neuronal activity (Remage-Healey et al., 2011a), the changes in neuronal activity

may indirectly affect estradiol levels. While much is known about what kinds of experiences

elevate norepinephrine concentrations in the brain (Aston-Jones and Bloom, 1981a,

1981b; Cardin and Schmidt, 2004; Lynch et al., 2012), little is known about how

experience changes estradiol levels in specific areas. Studies suggest that both estradiol

and catecholamines, including norepinephrine, levels are elevated in response to songs

(Remage-Healey et al., 2008, 2012; Lynch et al., 2012; Matragrano et al., 2012).

However, whether or not there are subtle differences in timing or stimuli they respond

to are not known. Due to technical difficulties, we failed to test whether both

norepinephrine and estradiol levels are elevated simultaneously in response to song

playback. If norepinephrine and estradiol are elevated at approximately the same time

after song playback, the mechanisms of how that happens and whether their actions on

auditory processing interfere with one another would be interesting to test in the

future.

Moreover, in the same chapter, we show that, in the NCM, norepinephrine

enhances normalized auditory-evoked activity similarly to estradiol but independent of

estradiol synthesis. Previous to this research, the effect of norepinephrine on neuronal

activity and how it compared to estradiol was unknown, even though studies

manipulating the catecholaminergic and estradiol synthesis or levels have shown similar

outcomes, and suggesting they have similar effects (Remage-Healey et al., 2008, 2010,

2012; Pawlisch et al., 2011; Matragrano et al., 2012; Velho et al., 2012; Vahaba et al.,

2013; Krentzel and Remage-Healey, 2014). Norepinephrine had been shown to enhance

the signal-to-noise ratio of sound-induced neuronal activity in the neurons of rodent

and monkey auditory cortices (Foote et al., 1975; Manunta and Edeline, 1997). Similarly,

in the zebra finch NCM, estradiol has been shown to enhance auditory evoked neuronal

activity (Remage-Healey et al., 2010). Although our results suggest that estradiol and norepinephrine independently enhance the signal-to-noise ratio of neuronal activity in response to auditory stimuli, we did not show how this is accomplished at the circuit level. Estradiol and norepinephrine may be modulating the same or different populations of neurons (e.g., Figs. 1-2). While most of the neurons examined responded to norepinephrine, our results could be biased due to how single units were sorted (and excluding non-auditory units). For a few neurons norepinephrine strongly enhanced auditory-evoked activity in a fashion similar to that of estradiol. Testing whether the same neurons are responsive both to norepinephrine and estradiol may elucidate some mechanism as to how NCM activity is modulated by multiple neuromodulators. Whether norepinephrine- and estradiol- producing terminals are innervating the same sets of cells or whether adrenergic receptors and estrogen receptors are expressed in proximity to each other are unknown. Moreover, even though this study shows that norepinephrine and estradiol seem to affect many neurons *via* different mechanisms, at the end, they are likely to activate the same cellular pathways that lead to the induction of *egr-1* and auditory processing. Previous studies have found that blocking estradiol signaling or adrenergic receptors actions inhibits song-induced *egr-1* expression (Tremere et al., 2009; Velho et al., 2012; Krentzel and Remage-Healey, 2014). Moreover, either blocking estradiol synthesis or blocking adrenergic receptors is sufficient to suppress behavioral preferences for songs (Remage-Healey et al., 2010; Pawlisch et al., 2011). Future studies should investigate how adrenergic and estrogen actions are both necessary for both *egr-1* induction and song preference while they seem to have independent electrophysiological effects.

In chapter 3, we examined how aromatase-expressing neurons are heterogeneous and organized in different regions, which would provide us with clues as to how estradiol may be

regulated at the macro- and microcircuit levels. We found that, similarly to monkey and

human cortices, in the NCM, some aromatase-expressing neurons co-expresses an

inhibitory neuronal marker, parvalbumin (Yague and Muñoz, 2006; Yague et al., 2008).

Since parvalbumin neuron numbers in the NCM have been linked to male song learning

(Asik and Kirn, 2015), future experiments should test whether aromatase-expressing

parvalbumin neurons have specific roles in auditory learning. At the same time, unlike

humans and monkeys, aromatase was not co-expressed with calbindin. However, the

differences between zebra finches and humans does not necessarily make zebra finches

a model system inferior to monkeys for studying estradiol signaling, since human and

monkey cortices are also different from one another. While in monkeys, aromatase

positive cells are mostly neurons and some co-express calretinin, in humans, many

aromatase-expressing cells are glial and none were co-expressing calretinin (Yague and

Muñoz, 2006; Yague et al., 2008). Similar to monkeys, but unlike humans, we found that

aromatase-expressing cells in the zebra finch were mostly neurons. Therefore, although

monkeys are relatively evolutionarily closer to humans than zebra finches, zebra finches

and monkeys are equally different from humans with respect to the profiles of

aromatase+ cells. This suggests that estradiol signaling in humans is distinct from other

animals and there are no perfect animal models that would model exactly what

estradiol does in the cortex. Moreover, the heterogeneity of aromatase-expressing cells

suggests that, even within regions, aromatase activity may be uniquely regulated in cell-

type or input-specific manners.

We also found that, in the zebra finch brain, many neurons, including

aromatase-expressing neurons, form somato-somatic clusters. Aromatase-expressing

neurons were found to cluster with both aromatase negative and other aromatase positive

neurons. Previously, in the HVC, gap junctions were found between neurons forming clusters

(Gahr and Garcia-Segura, 1996). Since the presence of gap junctions in clusters may provide us

with clues about how aromatase activity is modulated, we injected a small fluorescent dye, po-

pro-1, or neurobiotin into one of the clustered neurons to see if they would diffuse to other

neurons in the same cluster. In fact, in our preliminary dataset, we found instances where po-

pro-1 or neurobiotin filled other neurons that were not injected. Therefore, we suspect that

aromatase-expressing neurons are connected *via* gap junctions and aromatase in those neurons

may not only be regulated pre-synaptically or *via* synaptic inputs into the cell body, but also *via*

signals coming in through gap junctions. Since the control of aromatase activity involves calcium

signaling and actions of protein kinases (Balthazart et al., 2001, 2003b, 2005; Remage-Healey et

al., 2011a; Charlier et al., 2013a; Comito et al., 2016), diffusions of those signaling molecules or

electric signals *via* the gap junctions are likely to have effects on aromatase phosphorylation and

hence aromatase activity. On the other hand, GPER1-expressing neurons were also found in

clusters in some regions. Estradiol is known to modulate gap junction expression in the ovary

(Burghardt and Anderson, 1981), but whether estradiol has rapid modulatory effect on gap

junction permeability is not known. Gap junction permeability can be modulated by dopamine

and other neuromodulators and are sensitive to calcium, cyclic AMP, and PKA-dependent

actions (Hampson et al., 1992; Perez-Velazquez et al., 1994; Burghardt et al., 1995; Reviewed in

Pereda, 2014). Since GPER1 actions affect cAMP-dependent pathways (Filardo et al., 2002),

GPER1-mediated estradiol actions may modulate gap junction permeability. In the HVC, in a

study in which dual recordings were performed to identify synaptically-coupled neurons, the

authors reported that they did not observe any electrically-coupled neurons (Mooney and

Prather, 2005). However, among all the paired neurons they recorded from, the cells were next

to each other in only two cases, and whether or not they had somato-somatic contacts were not examined. In our study, aromatase positive neurons were found clustered with parvalbumin positive neurons and calbindin positive neurons, suggesting that aromatase activity could depend on inputs or signaling from multiple types of neurons. If neurons in clusters are communicating *via* gap junctions, it would be interesting to examine the distribution patterns of connexins and how they have a role in affecting aromatase activity. Previous to this study, cell clustering has been reported in field L, mesopallium, and hyperpallium, but whether or not they consisted of only neurons and whether clustering had functional importance has never been tested (Fortune and Margoliash, 1992; Medina et al., 2013). Our study now suggests that somato-somatic communication *via* gap-junction coupling may be important for aromatase activity and estrogen signaling in the cortex.

Also in chapter 3, we show evidence that aromatase-expressing terminals play a role in estradiol signaling in regions that do not express many somatic aromatase, such as motor regions involved in vocalization including the HVC and RA. When zebra finch brain sections were stained with a specific aromatase antibody, faint signals were found in some neurons (ghost cells) in regions where, previously, aromatase was found only pre-synaptically. Assuming ghost cells could be cells that were heavily innervated by aromatase positive synaptic terminals, we examined their distribution. In the HVC and RA, ghost cells were found on parvalbumin expressing neurons, suggesting that synaptic estradiol signaling onto parvalbimin expressing interneurons is important in these regions. Moreover, when sections from the same animals were stained for aromatase and GPER1, we found high expression of GPER1 in regions that were low in aromatase

expression, such as field L, HVC, CMM, and arcopallium (including RA). In regions with high

expression of somatic aromatase, such as the NCM and NCL, we found a moderate amount of

GPER1-expressing neurons. This suggests that, in regions with low numbers of aromatase-

expressing neurons, GPER1-expressing neurons may be terminated by aromatase positive

terminal projections coming from high somatic aromatase regions. Importantly, prior to this

study, the role of estradiol or GPER1 signaling in regions that belong to the song production

pathway has received little attention. In future experiments, the role of GPER1 in these song

production regions and the relationship between GPER1 and aromatase positive synaptic

terminals should be explored in greater detail. Therefore, we provided new evidence for why

estradiol signaling should be studied in regions outside of the auditory pathway and the

importance of interregional connections *via* aromatase-expressing terminals.

In summary, in chapter 3, we show anatomical evidence for potential interneuronal

interactions playing roles in estradiol levels at different scales. Previous to this study, most

studies on aromatase activity were done using brain homogenates or *in vivo* microdialysis which

disrupts circuits and neuronal structures, and the results that came out of these studies did not

resolve the connectivity, organization, and identities of the neurons that express aromatase.

Moreover, in zebra finches, the neurotransmitter profiles of aromatase-expressing neurons

were not well known. In one study in the NCM, in situ hybridization against aromatase and

inhibitory or excitatory neuronal markers, glutamic acid decarboxylase (GAD 65) or vesicular

glutamate transporter 2 (VGlut2) respectively, showed that aromatase is co-expressed with

these neuronal markers (Jeong et al., 2011). However, this study did not distinguish between

different inhibitory neurons or focus on cytoarchitecture of the aromatase neurons. Since

neuronal activity has been implicated in modulating aromatase activity, our report on profiles of

aromatase-expressing neurons and inter-neuronal connections that aromatase neurons

have with other neurons should be beneficial for designing or interpreting future

results. Knowing the identity of aromatase-expressing cells, cell type specific control of

estradiol synthesis using viral vectors can be used in combination with behavioral assays

and electrophysiological recordings to specifically test the role of estradiol synthesis by

different neuronal types. However, in order to understand the mechanism of controlling

aromatase activity, examining the receptor profiles of aromatase positive neurons

express may be more beneficial since inputs from other neurons are likely to have a

major effect on aromatase activity. Whether or not aromatase positive neurons have

common inputs from specific types or populations of neurons is now an active area of

research.

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