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ACUTE ESTROGEN SYNTHESIS AND ACTION IN THE AUDITORY CORTEX OF DEVELOPING MALE ZEBRA FINCHES (TAENIOPYGIA GUTTATA)

Daniel Vahaba

ACUTE ESTROGEN SYNTHESIS AND ACTION IN THE AUDITORY CORTEX OF DEVELOPING MALE ZEBRA FINCHES (*TAENIOPYGIA GUTTATA*)

A Dissertation Presented

by

DANIEL M. VAHABA

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ABSTRACT

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Birdsong, as with human speech, is learned during an age- and experience-dependent sensitive period early in life. Songbirds must first memorize their parents' song during a sensory phase, then refine their own burgeoning vocalizations to match the auditory memory of their parents' song during a sensorimotor phase. While the error-correction aspect of the sensorimotor phase of song learning is comparatively well understood, it is largely unknown how auditory memories are formed and how auditory processing may change across development to facilitate song memorization. The songbird caudomedial nidopallium (NCM) is a brain region that encodes complex communication signals like song and is rich in aromatase (enzyme necessary for converting precursor androgens to estrogens) and estrogen receptors. In adults, acute estrogen signaling enhances auditory encoding, suggesting that one role for 17β-estradiol (E2) in NCM during development may be to enhance auditory processing and facilitate auditory memorization. Moreover, in the hippocampus of rodents, birds, and nonhuman primates, local E2 acts to enhance post-training memory consolidation. As such, I set out to determine whether this role for E2 in

auditory processing and memorization occurs within the auditory cortex of juvenile songbirds. I tested this hypothesis across several experiments: I first tested how local E2 administration in NCM modulated auditory processing in developing songbirds. Next, I explored how changes in developing neural architecture and aromatase expression are aligned with distinct song learning phases. I then tested how global and local aromatase inhibition following song learning sessions impacted motor production, vocal learning, and neurophysiology in developing songbirds. Finally, using a stimulus-specific adaptation paradigm, I determined whether findings in juvenile songbirds extended to adults. Specifically, I locally blocked local E2 synthesis in NCM immediately following song exposure and subsequently measured neural recognition of the exposed song. My results showed that sensory coding is substantially enhanced in the NCM of sensory-aged birds compared to song-producing (sensorimotor-aged) juvenile birds, and that E2 exerts an age- and hemisphere-dependent effect on modulation of auditory processing. I also found that cell density in NCM peaks in sensory-aged birds, and is overall higher in dorsal vs. ventral NCM, but that aromatase and parvalbumin expression remain high and constant across development; no hemispheric differences for cell density or expression were found. Further, I found that neither circulating nor locally-derived E2 are required for tutor song memorization in development and adulthood; however, estrogen synthesis blockade can impair song production in developing birds and can also transform the lasting neural representations of autogenous and tutor song in adulthood. Taken together, this

dissertation provides new insights into the pleiotropic effects of rapid steroid signaling and synthesis within the auditory cortex of developing male songbirds with implications for communication processing and sensorimotor learning.

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CHAPTER I

NEUROESTROGENS RAPIDLY SHAPE AUDITORY CIRCUITS TO SUPPORT COMMUNICATION LEARNING AND PERCEPTION: EVIDENCE FROM SONGBIRDS

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Abstract

Steroid hormones, such as estrogens, were once thought to be exclusively synthesized in the ovaries and enact transcriptional changes through intracellular nuclear receptors over the course of hours to days. However, estrogens are also locally synthesized within neural circuits, wherein they rapidly (within minutes) modulate a range of behaviors, including spatial cognition and communication. Here, we review the role of brain-derived estrogens (neuroestrogens) as modulators within sensory circuits in songbirds. We first present songbirds as an attractive model to explore how neuroestrogens in sensory cortex modulate vocal communication processing and learning. Further, we examine how estrogens may enhance vocal learning and auditory memory consolidation in sensory cortex via mechanisms similar to those found in the hippocampus of rodents and birds. Finally, we propose future directions for investigation, including: 1) the extent of developmental and hemispheric shifts in aromatase and membrane estrogen receptor expression in auditory circuits; 2) how neuroestrogens may impact inhibitory interneurons to regulate audition and critical period plasticity; and, 3) dendritic spine plasticity as a candidate mechanism mediating estrogen-

dependent effects on vocal learning. Together, this perspective of estrogens as neuromodulators in the vertebrate brain has opened new avenues in understanding sensory plasticity, including how hormones can act on communication circuits to influence behaviors in other vocal learning species, such as in language acquisition and speech processing in humans.

Introduction

In nature, animals confront an overwhelming number of sensory cues. Processing this stream of sensory information is necessary to evaluate potential mates, mediate territory disputes, recognize kin, identify neighbors, and detect predators. Production and perception of air-borne cues manifest across multiple modalities, such as visual displays, tactile/vibrational signals, chemical cues, as well as auditory signals (Smotherman and Narins, 2000; Ota et al., 2015; Mangiamele et al., 2016; Shamble et al., 2016; Ai et al., 2017; Endevelt-Shapira et al., 2018). While most species integrate multimodal information, many rely primarily on acoustic cues for intraspecies communication, i.e., vocal communication.

Vocal communication is widespread among vertebrates. Humans specialize in spoken language. Rodents emit ultrasonic vocalizations (USVs) across many contexts, ranging from mother-pup interactions (Portfors, 2007), courtship and mating (Holy and Guo, 2005), and social play (Knutson et al., 1998). The vast majority of teleosts, such as toadfish and midshipman fish, produce underwater calls (Bass, 2008). But for most acoustically-communicating vertebrates, these vocalizations are innate. Experience-dependent vocal learning

is only found in a handful of animals, including songbirds and humans (Petkov and Jarvis, 2012). Thus, hearing serves a unique dual function in songbirds: to both detect and learn their species-specific vocal communication signals.

Here, we review the neural circuits and neuromodulation of auditory processing in a well-studied songbird species, the Australian zebra finch (*Taeniopygia guttata*). We suggest that songbirds in general, and zebra finches in particular, offer a unique opportunity to investigate how rapid estrogen signaling in sensory cortex enables both the processing and learning of vocal communication cues across development and in adulthood. Further, we provide suggestions for areas of future research on this topic, and suggest possible clinical implications of this research for understanding human cognition and language.

Neuromodulators that tune neural circuits

For intra-species communication to have adaptive value, an organism must integrate external and internal cues – such as energy reserves, social standing, and reproductive status – and adjust ongoing communication encounters. Such flexibility allows for context (both current and previous) to guide communication for both sender and receiver. In the vocal communication domain, the neural circuits that underlie vocal production as well as hearing must therefore be sensitive to context, by way of neuromodulation.

The recent scientific fascination with neural ‘connectomics’ has produced detailed neural circuit diagrams for a number of organisms. But it has also revealed that a wiring diagram is a useful predictor of behavior only when the

dynamic ‘functional connectivity’ of that diagram is taken into account (Bargmann, 2012; Bargmann and Newsome, 2014; Marder et al., 2014). Neuromodulators such as biogenic amines, neurotransmitters, neuropeptides, and even gases like nitric oxide all are produced within neural circuits to exert modulatory effects (Katz and Lillvis, 2014; Nusbaum et al., 2017; Petersen and Hurley, 2017). To momentarily alter the wiring diagram, that is, to shift the functional connectivity of a neural circuit, modulators can influence the efficacy and even the sign (excitation vs. inhibition) of synaptic connections on a minute-by-minute timescale, enabling extraordinary circuit- and behavioral flexibility. There is now growing appreciation that steroid hormones can act as neuromodulators via local synthesis and action in neural circuits (Balthazart and Ball, 2006; Woolley, 2007; Remage-Healey, 2014; Rudolph et al., 2016; Kelly and Vitousek, 2017). The emergent perspective that steroids may be genuine neuromodulators of neural circuits and behavior has been useful in guiding the exploration of neuroestrogen synthesis and action in the songbird auditory forebrain, as we describe in detail below.

Estrogens can be rapidly synthesized within sensory circuits to act as neuromodulators

Estrogens were classically thought to be secreted exclusively from the gonads. However, it is now clear that estrogens and other steroid hormones are also synthesized within the brain (London, 2016; Balthazart et al., 2018). Initial evidence for brain-derived estrogens (neuroestrogens) came about from the discovery of brain aromatase expression in multiple vertebrate species.

Aromatase, the enzyme necessary for converting precursor androgens into subsequent estrogens, was previously described solely in peripheral tissue. In the 1970s, the first direct evidence for the capacity of central estrogen production came about through a number of landmark studies describing neural aromatase in both humans (Naftolin et al., 1971; Naftolin et al., 1975a; Naftolin et al., 1975b), and across a diverse range of vertebrate taxa, including reptiles, fish, amphibians, and birds (Callard et al., 1978b; Callard et al., 1978a). Follow-up work in songbirds demonstrated that the brain is the primary source of both local and circulating estrogens (Schlinger and Arnold, 1992), which suggested a novel role for central estrogen synthesis to locally target neural circuits.

We now understand that brain-derived estrogens can also rapidly tune neural circuits and impact a diverse range of behaviors. Initial evidence for rapid effects of estrogens on synaptic physiology came from single-neuron recordings in the preoptic area (POA) of female rats, in which 17β-estradiol (**estradiol**) altered firing rates within seconds (Kelly et al., 1976). Since then, acute effects of estrogens on neuronal activity states and cellular events have been reported for the hypothalamus, hippocampus, striatum, amygdala, brainstem, and more recently auditory cortex (Dufy et al., 1979; Nabekura et al., 1986; Mermelstein et al., 1996; Chaban et al., 2003; Abraham et al., 2004; Remage-Healey and Bass, 2004; Bryant et al., 2005; Woolley, 2007; Vasudevan and Pfaff, 2008; Remage-Healey et al., 2010b). Functionally, estradiol's impact on circuit physiology is exceptionally diverse in terms of behavioral actions, timing, and species. In mice, aromatization is key to organize the medial amygdala early in life to selectively

respond to opposite-sex olfactory cues (Bergan et al., 2014). At a more acute timescale, testosterone rapidly increases visually-guided responses to a female stimulus in male goldfish, likely through estrogens/rapid aromatization (Lord et al., 2009; Mangiamele et al., 2017). Even nociception within the dorsal horn of Japanese quail is rapidly modulated by acute estrogen actions (Evrard and Balthazart, 2004). Therefore, estrogen synthesis in the brain is important for many behaviors, neural circuits, and species, at a range of timescales.

Classically, steroid hormones like estradiol were thought to exclusively target intracellular nuclear receptors and affect transcriptional changes over the course of hours to days. However, estrogen receptors found on dendritic and axonal processes in guinea pig hypothalamic neurons provided the first evidence of a non-nuclear site for the neural actions of estrogens (Blaustein et al., 1992). Since then, evidence has emerged that estrogens can rapidly influence neuronal activity through membrane-docked estrogen receptors (both ERα and ERβ) that are associated with metabotropic-glutamate receptors (Micevych and Mermelstein, 2008; Mermelstein, 2009). More recently, rapid actions of estrogens have also been found to act through a G-protein coupled estrogen receptor, GPER1 (formerly the orphaned ‘GPR30’) (Srivastava et al., 2013; Rudolph et al., 2016; Barton et al., 2017). The emergent understanding of these many mechanisms for steroid actions were presented in a recent review previewing this special issue (Balthazart et al., 2018). Below, we describe the contribution of recent work in songbirds testing the role of rapid neuroestrogen signaling in shaping sensory processing, and place this work in a broader context.

The songbird auditory circuit as a model to explore rapid estrogen actions on vocal communication processing and learning

Songbirds are a powerful system to explore local estrogen actions in sensory circuits across the lifespan. First, the forebrain circuits that guide auditory-dependent behaviors are enriched with estrogen receptors and estrogen synthase (aromatase), especially as compared to rodent sensory cortices (as reviewed in Vahaba and Remage-Healey, 2015). In agreement with high aromatase concentrations, the brain is the primary site of estradiol synthesis in male zebra finches (Schlinger and Arnold, 1992), so much so that circulating estrogen levels persist in castrated males (Adkins-Regan et al., 1990). The abundance of estrogen production and signaling in the songbird auditory forebrain makes it an attractive system to measure and manipulate neuroestrogen content and evaluate its effects on audition and learning. Below, we review how neuroestrogens are generated in the songbird brain, and their rapid effects on physiology and related behaviors in both adult and developing songbirds.

The organization of auditory circuits is relatively conserved across the class Aves. As in other vertebrates, birds perceive acoustic signals beginning at peripheral hair cells in the ear, and these auditory signals reach central cortical regions in the auditory forebrain (reviewed in Jarvis, 2004). As shown in **Figure 1**, brainstem and midbrain auditory signals are initially relayed from the thalamic nucleus oviodalis (Ov) to the avian auditory telencephalic homologue of primary auditory cortex (Field L complex; Field L2), which sends afferent projections to secondary auditory cortex, including the caudal mesopallium (CM) and the

caudomedial nidopallium (NCM) (Vates et al., 1996; Jarvis, 2004; Wang et al., 2010).

Interestingly, while this auditory pathway is conserved across birds (Bonke et al., 1979a; Wild et al., 1993; Vates et al., 1996; Wang et al., 2010), a high concentration of aromatase and estrogen receptors in avian forebrain

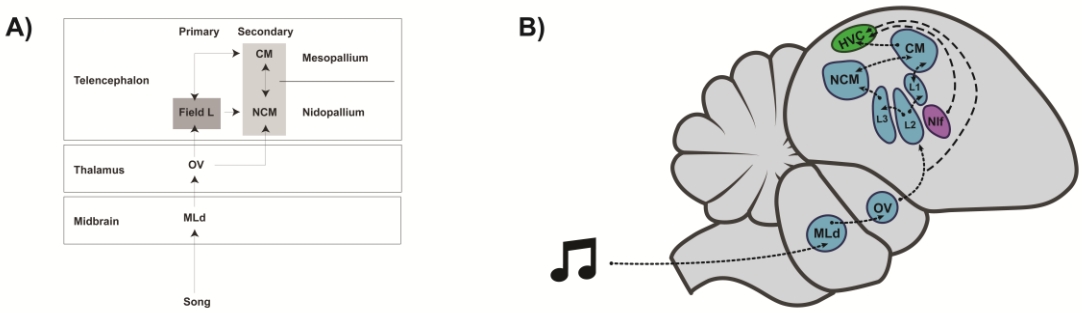

Figure 1: Two schematized songbird auditory circuits.

A-B) Auditory stimuli, such as song, arrives from the brainstem and cochlear nuclei (not shown) into the midbrain MLd and into the thalamic OV (ovoidalis). The primary thalamic recipient of auditory information in the cortex is the Field L complex (L1, L2, and L3), which projects to the caudomedial nidopallium (NCM) and caudal mesopallium (CM), which are themselves reciprocally connected. Auditory information reaches HVC (used as a proper name) by way of the nucleus interface (NIf), which itself receives projections from CM (not illustrated for clarity). Not depicted are the forebrain basal ganglia and song motor circuit pathways that are essential for song learning and production. Adapted from Brenowitz and Remage-Healey (2016); Vahaba et al. (2017).

distinguishes vocal learning birds from other species (Metzdorf et al., 1999; Silverin et al., 2000; Yoder and Vicario, 2012), especially within NCM (Caras and Remage-Healey, 2016). Within the auditory forebrain, aromatase is almost exclusively found in NCM, whereas little to no aromatase has been described in Field L or CM of zebra finches (Saldanha et al., 2000; Peterson et al., 2005; Pinaud et al., 2006; Ikeda et al., 2017). Therefore, NCM provides the predominant source of estrogens to the auditory forebrain circuitry in male and

female zebra finches. In addition to being regionally concentrated in NCM, aromatase is also co-expressed in specific cell types, namely parvalbumin-positive interneurons (Ikeda et al., 2017). The exceptional capacity for estrogen synthesis in the songbird brain has led to investigations of its functional significance for cognition and sensory processing.

Acutely synthesized estrogens within songbird auditory forebrain rapidly alter physiology. In zebra finches, estrogens are produced through both *de novo* steroidogenesis and through aromatization of circulating androgens (London et al., 2006; London et al., 2009; Remage-Healey et al., 2010a). Social interactions and song playbacks rapidly increase neuroestrogen production in the NCM of both adult male and female zebra finches (Remage-Healey et al., 2008; Remage-Healey et al., 2012). Functionally, local increases in neuroestrogen within NCM directly enhance auditory function. In anesthetized zebra finches, perfusing estradiol in NCM rapidly increased auditory-evoked firing rates and bursting in NCM, consistent with a neuroestrogen-dependent enhanced representation of communication signals (Remage-Healey et al., 2010b; Remage-Healey, 2012). Similarly, acute peripheral estrogen treatment results in a stronger song-evoked fMRI BOLD response bilaterally in the auditory lobule (which includes Field L, CMM, and NCM) of male European starlings (De Groof et al., 2017). Interestingly, global suppression of estrogen synthesis in these same animals specifically reduced auditory responsiveness in the left but not right hemisphere auditory lobule (De Groof et al., 2017). In addition to direct effects in NCM, estradiol in NCM also increases stimulus-selectivity and auditory

responsiveness in downstream regions, including the key sensorimotor nuclei HVC and NIf (Remage-Healey and Joshi, 2012; Pawlisch and Remage-Healey, 2015). Taken together, estradiol in NCM enhances central auditory processing; however, it remains to be determined whether enhanced neural representations translate into improved audition as assessed by psychophysic or behavioral measures.

So far, we have limited clues about how neuroestrogen signaling in NCM regulates behavior. Inhibiting local estrogen synthesis in the NCM of male zebra finches rapidly suppressed behavioral preferences for the birds' own song when presented in the left, but not right hemisphere (Remage-Healey et al., 2010b). Studies using peripheral administration of aromatase inhibitors also support the general idea of estrogen synthesis and auditory function in songbirds (Yoder et al., 2012; Alward et al., 2016b). One intriguing possibly is that in addition to rapidly guiding auditory encoding, local neuroestrogen production in NCM may also facilitate auditory memory consolidation of recent experiences in adults, as we discuss below.

Neuroestrogen provision may help consolidate recent auditory experiences

In addition to facilitating audition, elevated neuroestrogens in NCM may also rapidly enhance the consolidation of recent experiences. While this idea has been explored to a lesser extent in auditory circuits, accumulating evidence indicates that estrogens enhance cognition in another estrogen-sensitive brain region: the hippocampus (HP). Since the 1990s, exogenous estrogens were known to have mnemonic-enhancing properties in spatial memory tests (Luine

and Rodriguez, 1994). Early studies by Packard & Teather provided the first behavioral description of memory-enhancement from *post-training* peripheral and intra-hippocampal presentations of estradiol in rodents (Packard and Teather, 1997b, a; Packard, 1998). These studies built on the emerging idea that estrogens mediate ovarian-cycle dependent changes in dendritic spine plasticity in the hippocampus (Woolley and McEwen, 1992), and provided a behavioral/functional consequence of this plasticity. Since then, rapid estrogen synthesis and action in hippocampus has become an active area of investigation (see reviews by Choleris, Frick, Luine, and Korol in this same Special Issue), and a more detailed understanding of its mechanism has emerged.

Generally, estrogens acting in HP enhance spatial memory and object recognition (Srivastava et al., 2013; Galea et al., 2017). In rodents, estradiol's ability to enhance memory consolidation is limited to a time-sensitive window immediately after learning: subsequent recall is unaffected by estradiol treatments if presented >2 hours after initial training (Fernandez et al., 2008). As such, the relatively acute impact on memory consolidation is likely mediated by rapid neuroestrogen signaling (Tuscher et al., 2016b). One puzzle associated with these findings is the limited, indirect evidence for aromatase in the rodent HP (Wu et al., 2009; Tabatadze et al., 2014; Sato and Woolley, 2016; Tuscher et al., 2016b). By contrast, the songbird hippocampus is highly enriched with synaptic and axonal aromatase protein, suggesting it is well positioned to facilitate rapid, non-classical steroid signaling (Saldanha et al., 2000; Saldanha et al., 2004; Peterson et al., 2005; Rohmann et al., 2007; Remage-Healey et al.,

2011; Ikeda et al., 2017). In agreement with this, hippocampal estradiol typically facilitates spatial cognition in zebra finches (Oberlander et al., 2004; Rensel et al., 2013; Bailey and Saldanha, 2015; Bailey et al., 2017), and blocking GPER1 in HP completely prevents learning a food caching task (Bailey et al., 2017). With these recent findings in mind, neuroestrogens may play a similar role in sensory learning in songbirds, including the processing and consolidation of recent auditory experiences (Vahaba and Remage-Healey, 2015).

In addition to providing a source of estrogens to the auditory system, NCM is also implicated in auditory learning and recognition memory in adult songbirds (Chew et al., 1995; Mello et al., 1995; Bolhuis and Gahr, 2006; Gobes and Bolhuis, 2007; Hahnloser and Kotowicz, 2010). NCM exhibits a seasonal enlargement during breeding photoperiods in European starlings, who are open-ended song learners (De Groof et al., 2009). In adult zebra finches, NCM is considered a focal region for storing recent auditory representations (Chew et al., 1995; Kruse et al., 2000; Stripling et al., 2001; Dong and Clayton, 2008, 2009; Smulders and Jarvis, 2013; Soyman and Vicario, 2017). Single, brief exposures (40 mins) to a song results in a short-term memory for the trained song and subsequent recognition in NCM (Dong and Clayton, 2009). While NCM appears to be required for adult auditory memory consolidation and recognition, the molecular mechanisms supporting this are only recently becoming clearer (London and Clayton, 2008; Ahmadiantehrani and London, 2017) and may involve rapid estrogen signaling.

Neuromodulators act within central auditory circuits to enable post-training memory consolidation. Like estrogens, local noradrenergic modulation of NCM is required for both auditory processing (Ikeda et al., 2015; Lee et al., 2017) and memorization (Velho et al., 2012). Moreover, estradiol levels increase in adult NCM during social and song exposure, which may facilitate changes necessary for auditory memory formation (Remage-Healey et al., 2008; Remage-Healey et al., 2012). Auditory memory consolidation in NCM involves epigenetic modifications (Phan et al., 2017), which is also a route by which estradiol mediates spatial learning in rodents (Zhao et al., 2010). In adult songbirds, inhibiting global estrogen synthesis impairs short-term auditory memorization and recognition in NCM. While the specific role for neuroestrogens in sensory learning has yet to be directly tested in adult songbirds, local estradiol in the olfactory bulb of mice improves odor memory consolidation (Dillon et al., 2013), providing an intriguing parallel. In the following section, we consider how a similar mechanism may exist for consolidating sensory (tutor) memories across the song learning critical period in juvenile songbirds.

Evidence that estrogens are involved in auditory processing necessary during developmental song learning

Both male and female developing songbirds form an auditory memory of their tutor's song that is necessary for accurate vocal (song) learning and imitation. In closed-ended learners, song models are acquired across a critical period early in development classically described as occurring across two phases: 1) tutor song memorization ("sensory phase"), and 2) motor rehearsal

("sensorimotor phase") (London, 2017). During the ***sensory phase*** (tutor song memorization), pre-vocalizing songbirds begin encoding/memorizing their father's or older sibling's song beginning around 25 days post-hatch (**dph**) (Immelmann, 1969; Roper and Zann, 2006; Deregnaucourt and Gahr, 2013), or possibly earlier since some embryonic birds are selectivity responsive to adult conspecific song (Colombelli-Negrel et al., 2012; Spencer and Minderman, 2018). Once a tutor song 'template' memory is formed, birds begin to evaluate their burgeoning vocal imitations compared to the tutor memory during the ***sensorimotor phase***. The ***sensorimotor phase*** (motor rehearsal) is akin to early infant babbling (Doupe and Kuhl, 1999; Aronov et al., 2008; Lipkind et al., 2013; Prather et al., 2017) and begins with emergent vocalizations, followed by song refinement, and eventual song crystallization that coincides with sexual maturation. In the case of zebra finches, the sensorimotor phase ends with a single highly stereotyped song produced throughout adulthood. While the behavioral study of song learning has intrigued scientists as far back as Aristotle, the neural mechanisms enabling song learning has a relatively more recent history beginning around the 1960s.

Other recent reviews have provided excellent coverage of the role of motor and cortical-basal ganglia pathways in sensorimotor learning (Mooney, 2009; Brainard and Doupe, 2013), and here we restrict our discussion on neuroestrogens and song learning by focusing on tutor memorization during the sensory phase and the contributions of auditory forebrain circuits, namely NCM (Bolhuis and Gahr, 2006; Bolhuis et al., 2010). While other auditory forebrain regions are likely involved in auditory memory acquisition for learned song (e.g.

CMM, Terpstra et al., 2006; Jeanne et al., 2011) , as well as other auditory-responsive regions (Adret et al., 2012; Mandelblat-Cerf et al., 2014; Piristine et al., 2016; Roberts et al., 2017), these areas are largely devoid of aromatase in cell bodies as well as neurites, compared to the high expression found in NCM (Saldanha et al., 2000; Ikeda et al., 2017), and thus direct roles for neuroestrogens are unlikely.

NCM is considered a primary site required for tutor song memorization and representation (Bolhuis and Gahr, 2006; Clayton, 2013; Bolhuis and Moorman, 2015) but see (Canopoli et al., 2016, 2017). Compared to sensorimotor-aged males, auditory-evoked firing rates and the coding accuracy of single neurons for individual song stimuli in NCM are both elevated in pre-singing, sensory-aged zebra finches that are beginning to form auditory memories of their tutor song (Vahaba et al., 2017). In developing songbirds, tutor song playback evokes higher immediate-early gene expression in NCM than does a novel male's song (Gobes et al., 2010). Innate preference for tutor song in adults is abolished when NCM is bilaterally lesioned (Gobes and Bolhuis, 2007). Further, like adult songbirds, habituation to specific vocalizations occurs in NCM early in development, suggesting a role in encoding recent/familiar auditory experience (Stripling et al., 2001; Miller-Sims and Bottjer, 2014). Transcript levels for the plasticity-related immediate early gene (IEG) *egr-1* (also known as *zenk*) peak in male NCM during the onset of sensory learning/opening of the song learning critical period (Jin and Clayton, 1997). In parallel with this, blocking plasticity-related MAPK signaling pathway in the auditory lobule (including both

NCM and CMM) specifically during developmental tutoring prevents accurate tutor song imitation in adulthood (London and Clayton, 2008). Furthermore, tutoring naïve juvenile songbirds rapidly biases a subpopulation of single NCM neurons towards selectivity for the tutor's song (Yanagihara and Yazaki-Sugiyama, 2016). These findings together suggest that NCM is required for accurate tutor song encoding, memorization and imitation, yet the molecular mechanisms enabling putative NCM-dependent auditory memory consolidation are less well known (Moorman et al., 2011). Since neuronal cell density is adult-like by 20 dph in NCM (Stripling et al., 2001), and as auditory responsiveness is markedly enhanced in sensory-aged songbirds compared to sensorimotor-aged males (Vahaba et al., 2017), it may be that age-dependent changes in steroid hormones and their cognate receptors across the song learning critical period in development may partially explain NCM's role in song learning/tutor song memorization.

Steroid hormones can limit song learning critical period plasticity during development, such as androgens which, like estrogens, also exert fast-actions on neural circuits (Wu et al., 2001; Bass and Remage-Healey, 2008; Foradori et al., 2008; Kelley and Bass, 2010). In developing songbirds, administering androgens, such as testosterone (T), to closed-ended learners before adult-like song is achieved leads to premature song and circuit crystallization (Korsia and Bottjer, 1991; Whaling et al., 1995; Bottjer and Johnson, 1997) but see (Templeton et al., 2012). One idea explaining this is that as sexual maturation approaches alongside song maturation, testosterone acts to crystallize a song circuit and

enable adult-like courtship. Accordingly, circulating T peaks towards the tail-end of the song learning/sexual maturation period, potentially signifying the 'closure' of the critical period for song learning (Marler et al., 1987). In addition to prematurely crystallizing plastic song production, androgen implants early in development also lead to parallel premature 'adult'-like physiology in the song motor pathway (Livingston and Mooney, 2001). Therefore, androgens impede motor variability by blocking vocal exploration, leading to stereotyped/crystallized song during development. In keeping with this model, androgens continue to exert profound influence over song motor circuits in adulthood (Alward et al., 2013; Alward et al., 2014; Alward et al., 2016a; Alward et al., 2017).

Song learning experiments based on circulating levels and peripheral hormone manipulations are confounded by the fact that brain is the main source of circulating steroids in songbirds (Schlinger and Arnold, 1992). For example, if testosterone acts as a cue to end song learning plasticity once adequate song is achieved, one would expect that peripheral T levels correspond to song learning fidelity. However, peripheral T levels measured at 100 dph in male zebra finches do not correlate with the degree of tutor song imitation (Deregnaucourt et al., 2013). Moreover, circulating T levels do not change in male zebra finches between the sensorimotor phase (50-60 dph) and the closing of the song learning critical period (105 – 130 dph), suggesting peripheral androgen levels are stable across development (Mori and Wada, 2015). Unlike androgens, circulating estradiol levels during the sensory phase of song learning are a more reliable predictor of eventual song similarity in adulthood (Marler et al., 1988). As

such, estrogens are a candidate neuromodulator of tutor song encoding and memorization.

Estrogens are well-positioned to regulate song learning due to the unique distribution of aromatase and estrogen receptors in vocal learners, as well as the role of estrogen in masculinizing vocal circuits in females. Although NCM is a highly conserved auditory forebrain region across *Aves* (Wild et al., 1993; Wang et al., 2010), as mentioned above there is a unique abundance of estrogen receptors and aromatase distribution in the avian forebrain of vocal learners, including NCM, compared to innately vocalizing birds (Metzdorf et al., 1999; Silverin et al., 2000; Yoder and Vicario, 2012). It is interesting to note that unlike songbirds, innately vocalizing male ruffed grouses have somatic aromatase protein expression in the Field L complex (Corfield et al., 2013). One role for local estradiol may be to establish song learning neural circuits. Exogeneous estradiol exposure in female zebra finch chicks, who do not normally sing in adulthood, masculinizes the neural song circuit by enlarging song nuclei, and enables male-like vocal learning and production (Gurney and Konishi, 1980). Follow-up studies demonstrated that brain-derived estrogens could account for the masculinization of the song motor pathway in zebra finches (Holloway and Clayton, 2001). Taken together, these studies suggest that neuroestrogens are required for vocal learning (motor) circuits in songbirds. Therefore, estradiol may be important across development for song learning, and perhaps specifically within the sensory phase, given that estradiol enhances auditory processing in adult songbirds.

Peripheral levels of estrogens and cortical membrane estrogen receptors peak during the sensory phase of song learning, suggesting local neuroestrogens in NCM may influence tutor song memory consolidation. Sparrows, zebra finches, and canaries all have elevated levels of circulating estradiol exclusively during the sensory phase of song learning, a period critical for encoding and consolidating the model song (Pröve, 1983; Weichel et al., 1986; Marler et al., 1987; Marler et al., 1988), however see (Adkins-Regan et al., 1990). In swamp sparrows, this sensory phase estradiol peak is a reliable predictor for eventual tutor song imitation in adulthood (Marler et al., 1987). Alongside changes in local and global estradiol, GPER1 transcript levels peak at 30 dph in male telencephalon (which includes NCM) and are 5-times higher at that age than in adult males (Acharya and Veney, 2011). As GPER1 is one putative mechanism by which neuroestrogens rapidly enhance auditory processing (Remage-Healey et al., 2013; Krentzel et al., 2018), a coincident peak in circulating estradiol levels and cortical GPER1 expression suggests a role for estradiol in auditory memory consolidation in NCM.

Neuroestrogens in NCM may be important during development for modulating online auditory processing to guide tutor song memory consolidation. In contrast with adults with increased neuroestrogen production during song exposure, juvenile zebra finches have reduced estradiol levels in NCM during social tutoring, and this is followed by a sharp rise one hour post-training (Chao et al., 2015). The functional role of these dynamics during tutoring is unclear. One hypothesis is that acute changes in neuroestrogens within NCM modulates

online auditory processing, as in adults which may be important for tutor song memory consolidation. Recently, it was revealed that locally presented estradiol within NCM rapidly transforms auditory encoding in a lateralized, and age-dependent fashion in developing male zebra finches (Vahaba et al., 2017). Therefore, since both adult and developing NCM is left-lateralized for auditory processing and memory consolidation (reviewed above for adults; Moorman et al., 2012; Chirathivat et al., 2015; Moorman et al., 2015), neuroestrogens in NCM may guide tutor song memorization by impacting sensory coding in a hemisphere-specific manner. The extent of interactions between neuroestrogens and established cell-signaling and molecular mechanisms enabling auditory processing and memory consolidation in songbirds remain to be tested (London and Clayton, 2008; Moorman et al., 2011; Ahmadiantehrani and London, 2017). In the sections that follow, we suggest future research directions to elucidate our understanding of how neuroestrogens mediate cognitive and sensory processes (**Figure 2**).

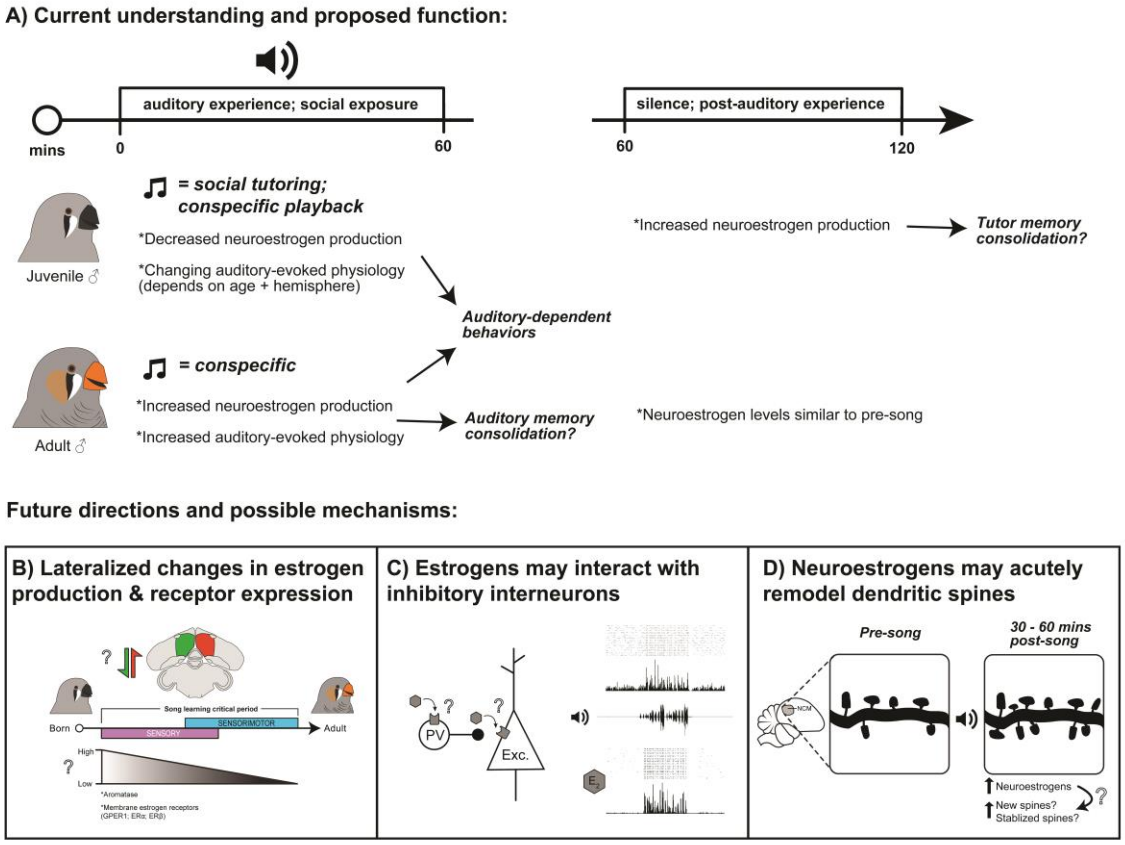

Figure 2: Current understanding and proposed function/mechanisms of rapid estrogen signaling in song auditory forebrain.
A) Top: Experimental timeline from previous studies on awake or anesthetized songbirds. “Auditory experience” is often conspecific song playback through speakers, whereas social exposure is always a live, adult conspecific (male tutor, or male/female) presentation. Bottom: The physiological effects and temporal fluctuation of neuroestrogen production depends on age. In juvenile male zebra finches, song tutoring leads to an immediate decline in local estrogen content, whereas sixty minutes after the offset of tutoring, there is a rapid elevation in estrogen production that may be important for consolidating tutor song memories. When estrogens are presented in NCM, there are age- and hemisphere-dependent effects on spontaneous and auditory-evoked physiology. In adults, song playbacks and social presentations both elicit immediate elevations in neuroestrogen production, which rapidly enhance auditory responses both physiologically and behaviorally. We propose that the rapid production and actions of neuroestrogens B) change across the critical period for song learning, depending on age and hemisphere; C) impact local inhibitory circuits to modulate auditory signal detection; and D) regulate dendritic spine plasticity necessary for sensory memory consolidation. E2 = 17-beta-estradiol; Exc. = excitatory projection neuron; PV = parvalbumin-expressing interneuron. Portions of this figure adapted from (Vahaba et al., 2017).

Outlook & future directions

1) How does the expression of aromatase and estrogen receptors change across development?

Presently, much of what is known about estrogen receptor and aromatase expression in the songbird brain is from studies on adult songbirds. Thus, relatively little is known about how the actions or production of estrogens may change in the songbird brain across development. Previous work describing songbird aromatase expression in the brain has been limited by age (pre-CP aged subjects, Saldanha et al., 2000), inference of protein expression via mRNA measurement , neuroanatomical spatial resolution and antibody specificity (Palkovits punches: (Schumacher and Balthazart, 1987; Balthazart et al., 1990; Vockel et al., 1990), focusing on non-sensory cortices (Vockel et al., 1988), or limited point-sampling during vocal learning (typically ~45 dph only, Saldanha et al., 1999). While estrogen production gradually increases in NCM across development, it will be important to verify and expand on this by quantifying bilateral aromatase protein expression, as peripheral hormone changes may also impact these findings (Chao et al., 2015). Further, as neuronal cell density is adult-like by 20 dph in NCM, developmental changes in intrinsic synaptic physiology, and auditory-evoked extracellular activity (Jin and Clayton, 1997; Kudo and Yazaki-Sugiyama, 2017; Vahaba et al., 2017) may in part be explained by changing estrogen production across ontogeny (Chao et al., 2015). Acute estrogen action and/or synthesis may explain developmental changes in auditory properties during development, as GPER1 transcript levels are 5-times higher in sensory-aged male telencephalon compared to adults (Acharya and Veney,

2011). Therefore, as recently suggested by physiological recordings (Vahaba et al., 2017) hemispheric- and age-dependent changes in sensory coding may be accounted for by the expression and/or activity of neuroestrogen-related signaling molecules like aromatase.

2) Do neuroestrogens interact with inhibitory neuronal networks to shape developmental song learning?

Sensory circuits primarily consist of interconnected excitatory and inhibitory neurons. Excitatory neurons receive and transmit signals within and across brain regions, whereas local inhibitory interneurons shape the gain, coding, selectivity, and modulation state of local cortical networks (Pi et al., 2013; Vallentin et al., 2016; Natan et al., 2017). Inhibitory neurons therefore shape specific auditory response states, making them primary regulators of processing and plasticity (Blackwell and Geffen, 2017). There is a diverse set of cortical GABAergic interneurons involved in auditory encoding (Tremblay et al., 2016; Wood et al., 2017), including the widely studied parvalbumin (PV) expressing neurons.

Parvalbumin is a protein directly important for calcium buffering and is a reliable marker for a subtype of inhibitory cortical interneurons (reviewed in Aizenberg et al., 2015). In mammalian cortex, parvalbumin-positive neurons are the primary inhibitory cell type, including auditory cortex (Xu et al., 2010). Specifically found within layers 2-6 of mammalian auditory cortex, parvalbumin-positive (PV+) neurons are required for encoding amplitude, frequency tuning, and sensorimotor integration, as well as auditory discrimination and adaption

(Cruikshank et al., 2001; Moore and Wehr, 2013; Schneider et al., 2014; Aizenberg et al., 2015; Natan et al., 2017). As such, sharper frequency tuning is associated with recruitment of PV+ cells in auditory cortex (Li et al., 2014).

Tuning by PV+ cells in auditory brain regions may be regulated in part by rapid estrogen synthesis and signaling. In mammals, estrogen receptors are exclusively and highly expressed in PV+ interneurons (≥80%) (Blurton-Jones and Tuszynski, 2002; Higaki et al., 2012). Moreover, peripheral estradiol administration increases PV+ neurons in the arcuate nucleus of adult female rats (Sotonyi et al., 2010), a hypothalamic brain region in which estradiol acts rapidly via membrane-bound estrogen receptors (Roepke et al., 2009). In addition to rapid estrogen actions targeting PV cells, aromatase itself is highly and consistently co-expressed in PV+ neurons within human and nonhuman primate temporal cortex (Yague et al., 2006; Yague et al., 2008; Yague et al., 2010; Azcoitia et al., 2011). Taken together, PV+ neurons are critical for sensory coding in mammalian auditory circuits, and rapid estrogen actions on and synthesis within PV+ cells likely participate in the integration of auditory signals.

In songbirds, inhibitory neurons in NCM may contribute to auditory learning and processing. Nearly half of all neurons In NCM are GABAergic, which are activated by song presentations (Pinaud et al., 2004; Pinaud et al., 2008), and are necessary for shaping auditory processing, selectivity, and memorization (Pinaud et al., 2008; Yanagihara and Yazaki-Sugiyama, 2016). By rapidly tuning inhibitory neurotransmission necessary for auditory-evoked neural activity, neuroestrogens may modulate auditory physiology in NCM. As with human and

nonhuman primate temporal cortex (Yague et al., 2006; Yague et al., 2008), PV and aromatase are co-expressed in neurons within adult songbird auditory brain regions, including NCM (Ikeda et al., 2017). Song learning during development also provides a unique opportunity to explore how estrogens and inhibitory circuits in NCM may regulate critical period plasticity. Like aromatase and estrogen receptors, parvalbumin is uniquely expressed in forebrain song nuclei of avian vocal learners (Hara et al., 2012), and higher activation of PV cells corresponds to 'better' visual learning in an avian association cortex-like brain region (Ambalavanar et al., 1999). Songbirds thus offer a powerful model to explore natural mechanisms gating critical period plasticity for learned complex vocal signals in auditory forebrain (London, 2017), as well as testing the role for rapid estrogen actions in PV cells on auditory encoding.

3) Do neuroestrogens acutely remodel dendritic spines in NCM to facilitate auditory plasticity?

Estrogens enhance cognition via fast-actions on dendritic spines (Luine and Frankfurt, 2012; Srivastava, 2012). Peripheral estrogen treatment improves learning and memory, and rapidly (within 30 – 40 mins) increases hippocampal synaptogenesis and dendritic spine density (MacLusky et al., 2005; Phan et al., 2012; Jacome et al., 2016). Supporting the role of local and fast actions of estradiol mediating synaptic plasticity, estradiol rapidly (after 30 mins) increases dendritic spine densities in cortical neurons via nongenomic mechanisms, (Srivastava et al., 2008), and blocking *in vivo* estrogen synthesis centrally, within HP, prevents estradiol-dependent circuit plasticity (Vierk et al., 2015). Together,

estrogens quickly modify dendritic spine dynamics that are functionally and behaviorally necessary for improved memory raising the prospect of similar mechanisms for auditory memory consolidation in songbirds.

In adult male zebra finches, dendritic spine densities in NCM rapidly double soon after brief (30 mins) exposures to novel song, an effect which is suppressed when endocannabinoid signaling is blocked (Gilbert and Soderstrom, 2013; Holland and Soderstrom, 2017). Intriguingly, acute estrogen treatment rapidly suppresses inhibitory synaptic transmission in rodent HP via an interaction with the cannabinoid receptor type 1 (CB1) (Huang and Woolley, 2012). As NCM is thought to integrate auditory information in adult songbirds by modulating inhibitory activity, rapid estrogen signaling in NCM may help encode and consolidate auditory experience by increasing dendritic spine density.

Developing songbirds may also undergo similar estradiol-dependent spine remodeling for tutor song memorization. In developing zebra finches, experience- and age-dependent changes in dynamic spine stabilization are critical for song learning and HVC circuit development (Roberts et al., 2010). As estradiol rapidly modulates spine dynamics in mammalian neural circuits, post-tutor neuroestrogen elevations in NCM may be important for consolidating recent tutor experience (tutor song) through acute dendritic spine alterations in developing auditory forebrain. Interestingly, both extracellular signal-regulated kinase (ERK) and mammalian target of rapamycin (mTOR) signaling cascades are required in the auditory forebrain of developing male songbirds for tutor memorization and imitation (London and Clayton, 2008; Ahmadiantehrani and London, 2017), which

are two intracellular routes of action required for estradiol-induced memory consolidation and related synaptic plasticity modifications in adult rodents (Fortress et al., 2013; Tuscher et al., 2016a).

Does work on neuroestrogens in songbirds and other species have clinical implications for human cognition and communication?

In humans, an association between circulating hormones and hearing has been established most convincingly for women across the menstrual cycle and during pregnancy. There is an abundance of studies showing that hormonal cycles can shift the behavioral threshold to detect sounds, verbal memory (Fernandez et al., 2003; Zimmerman et al., 2011), as well as the otoacoustic emissions detected from women (Al-Mana et al., 2010; Caras, 2013). There is now increasing interest in the role of estrogens in mediating the pathophysiology of auditory dysfunction as well, and the role of hormone-replacement therapy (HRT) in changing auditory function (Frisina and Frisina, 2016). Clearly, however, more work is therefore needed in non-human animal models to understand the basic mechanisms of how hormones like neuroestrogens can impact vocal communication processing and memory.

There is also evidence that hormones are important for speech perception and language learning during development in humans. As with songbirds (Marler et al., 1987), elevations in circulating estradiol during development are a positive predictor of future language success in children (Wermke et al., 2014; Schaadt et al., 2015). Children with social and sensory processing difficulties, such as autism, have difficulties with voice processing and recognition, as well as

underconnected auditory circuits (Gervais et al., 2004; Abrams et al., 2013). Autism and related speech language disorders may be due in-part to estrogen abnormalities, such as aromatase gene mutations (Anthoni et al., 2012). Therefore, work in animal models such as songbirds will help elucidate how estrogens transform auditory circuits in development, especially as it relates to learned vocal communication.

Conclusions

Studies on songbirds have provided critical progress toward understanding the rapid, nongenomic effects of neuroestrogens on physiological, molecular, and behavioral responses in vertebrates. Our current perspective that locally produced estradiol in songbird auditory forebrain occurs in social situations and enhances auditory processing should now direct future studies to address the functional significance more broadly. As such, songbirds will continue to serve as a valuable animal model to further reveal how brain-generated estrogens interact with sensory circuits to enable natural vocal communication perception and learning across the lifespan. Going forward, it will be difficult to disentangle whether estrogens improve auditory learning due to improved hearing, or whether neuroestrogens enhance both hearing and learning via independent mechanisms. Accordingly, studies of songbirds can allow us to disentangle these two actions in future studies (Vahaba et al., 2017). As songbirds and humans share the rare and remarkable suite of traits for learned vocalizations (Chakraborty and Jarvis, 2015; Prather et al., 2017), future studies may reveal more direct parallels for neuroestrogens in central auditory circuits

necessary for communication learning and processing, potentially leading to important translational discoveries.

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CHAPTER II

CENTRAL HYPOTHESIS

The central hypothesis that has guided my experiments is that rapid E2 synthesis within NCM modulates hearing-related neural activity and auditory memory consolidation across development in male zebra finches (*Taeniopygia guttata*). First, I identified neuroestrogens' impact on cortical physiology by manipulating E2 signaling in the auditory cortex across the critical period for vocal learning and measured resultant auditory-evoked neural activity to assess how neuroestrogens shape sensory representations. Next, I first determined whether cortical E2 is required for consolidation of a recent auditory experience by blocking E2 production in NCM immediately after an auditory learning experience in adult songbirds, and measuring neural recognition in NCM using a habituation paradigm. Then, I evaluated how E2 regulates vocal imitation and neural representation of a social model in developing songbirds by inhibiting systemic and cortical E2 production immediately after vocal learning sessions, and measured how well pupils imitated their social model (tutor) in adulthood. I followed up bioacoustic analyses on song learning subjects with neural recordings in NCM and HVC in adulthood and determined how representations of autogenous and tutor song were impacted from early-life hormone manipulations. Finally, I quantified changes in aromatase, parvalbumin, and neuronal density in the auditory forebrain across development by measuring somatic aromatase expression in the auditory cortex across the critical period, as well as parvalbumin expression, and neuronal density. These histological experiments

determined how changes in E2 content correspond to changes in estrogen synthase, cortical interneurons, and overall changes in NCM morphology across development. Taken together, these aims help clarify how nongenomic steroid signaling and production operate within a developing and developed auditory forebrain within the context of complex acoustic communication encoding and consolidation.

CHAPTER III

SENSORY CODING AND SENSITIVITY TO LOCAL ESTROGENS SHIFT DURING CRITICAL PERIOD MILESTONES IN THE AUDITORY CORTEX OF MALE SONGBIRDS.

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Abstract

Vocal learning occurs during an experience-dependent, age-limited critical period early in development. In songbirds, vocal learning begins when pre-singing birds acquire an auditory memory of their tutor's song (sensory phase) followed by the onset of vocal production and refinement (sensorimotor phase). Hearing is necessary throughout the vocal-learning critical period. One key brain region for songbird auditory processing is the caudomedial nidopallium (NCM), a telencephalic region analogous to mammalian auditory cortex. Despite NCM's established role in auditory processing, it is unclear how the response properties of NCM neurons may shift across development. Moreover, communication processing in NCM is rapidly enhanced by local E_2 administration in adult songbirds; however, the function of dynamically fluctuating E_2 in NCM during development is unknown. We collected bilateral extracellular recordings in NCM coupled with reverse microdialysis delivery in juvenile male zebra finches (*Taeniopygia guttata*) across the vocal learning critical period. We found that auditory-evoked activity and coding accuracy were substantially higher in the NCM of sensory-aged animals compared to sensorimotor-aged animals. Further,

we observed both age-dependent and lateralized effects of local E_2 administration on sensory processing. In sensory-aged subjects, E_2 decreased auditory responsiveness across both hemispheres; however, a similar trend was observed in age-matched control subjects. In sensorimotor-aged subjects, E_2 dampened auditory responsiveness in left NCM, but enhanced auditory responsiveness in right NCM. Our results reveal an age-dependent physiological shift in auditory processing and lateralized E_2 sensitivity that each precisely track a key neural “switch point” from purely sensory (pre-singing) to sensorimotor (singing) in developing songbirds.

Significance Statement

Vocal communication, such as language and birdsong, is learned during an age-limited critical period early in development. Initially, infants and songbirds exclusively listen to memorize their native tongue before producing nascent vocalizations. We show that the transition from pre-singing to vocalizing in developing songbirds is accompanied by a large shift in auditory gain and coding in cortical neurons. Further, while estrogens generally improve hearing in adulthood, we found that brain estrogens either enhanced or diminished auditory responsiveness depending on both critical period phase and cerebral hemisphere. Our findings therefore highlight a neural transition in auditory processing and lateralized hormone sensitivity at a key stage in development, and similar mechanisms could be relevant for speech processing and language acquisition in humans.

Introduction

Critical periods are windows of heightened experience-dependent neuroplasticity in which early sensory input shapes neural circuits and behaviors. Critical period research has historically focused on how sensory exposure or deprivation drive cortical and behavioral shifts in development (Lorenz, 1937; Wiesel and Hubel, 1963; Bolhuis, 1991; Hensch, 2005). Some critical periods for learned behaviors, such as vocal communication, shift from being purely sensory (auditory) to an active sensorimotor phase (vocal production, exploration, and refinement) (Kuhl, 2010). Such behavioral transitions are likely accompanied by neural changes in sensory processing. Relatively little is known about factors that change during vocal communication learning, however, as experience-dependent learned vocal communication ('vocal learning') is found in only a handful of animal species, including humans and songbirds (Petkov and Jarvis, 2012).

In some songbird species, such as zebra finches (*Taeniopygia guttata*), males are the exclusive vocal learners (Immelmann, 1969). Males learn song during two developmental phases (**Fig. 3*A***). In the sensory phase, birds acquire an auditory memory of their tutor's song, and then slowly refine their burgeoning vocalizations to approximate this tutor memory during the sensorimotor phase (Mooney, 2009). Research on the neural circuitry of vocal learning has largely explored song production premotor and cortico-basal ganglia circuits (Roberts et al., 2012; Brainard and Doupe, 2013). While auditory processing is necessary for song learning (Thorpe, 1954; Konishi, 1965), far less is known about the contribution of the auditory cortex during song learning in early development.

The caudomedial nidopallium (NCM**; Fig. 3*B***) is key for auditory processing. NCM receives projections from primary cortical thalmo-recipient Field L, and is considered the avian analogue of the mammalian secondary auditory cortex (Vates et al., 1996; Wang et al., 2010). NCM is important for both processing species-specific vocal communication (Mello et al., 1992; Theunissen et al., 2004), as well as auditory memory consolidation (Chew et al., 1995; London and Clayton, 2008; but see Canopoli et al., 2014). Further, much like the neural circuits for human language processing, NCM's role in auditory memory encoding and processing appears to be lateralized (Avey et al., 2005; Moorman et al., 2012, 2015; De Groof et al., 2013). Despite this clear role in auditory function, it is unclear how NCM's response properties shift across the vocal learning critical period.

In zebra finches, auditory behavioral perception and discrimination are adult-like as early as ~30 days post-hatching (dph; Braaten et al., 2006). Studies on developmental changes in NCM neurophysiology have focused on the putative opening and closing of the sensory phase (20 and ~30-35 dph, respectively; Böhner, 1990), but not beyond (Stripling et al., 2001; Miller-Sims and Bottjer, 2014). While there are subtle differences between juvenile age groups for song selectivity, auditory preferences and response magnitude at 35 dph are comparable to adults. Similarly, Jin and Clayton (1997) found that NCM neuronal cell density is also similar to adults at 20 and 30 dph. To date, changes in communication processing in auditory forebrain outside of the sensory phase has been limited to immediate-early gene studies on 45 dph zebra finches

(Bailey and Wade, 2003, 2005), and physiology studies on tutor song selectivity at ~22 or ~60 dph (Adret et al., 2012; Yanagihara and Yazaki-Sugiyama, 2016, respectively).

Circulating estrogens fluctuate across the critical period in several songbird species (Pröve, 1983; Weichel et al., 1986; Marler et al., 1988; but see Adkins-Regan et al., 1990), and predict vocal learning success (Marler et al., 1987), as in humans (Wermke et al., 2014). Estrogen levels in NCM gradually increase over the critical period, and also acutely in response to single tutoring bouts in juvenile male zebra finches (Chao et al., 2015). In adult songbirds, both circulating (Maney et al., 2006; Caras et al., 2012), and brain-derived estrogens (neuroestrogens; namely 17β-estradiol [E_2]) (Remage-Healey et al., 2010b; Remage-Healey and Joshi, 2012) generally enhance complex communication encoding within telencephalic auditory brain regions, including NCM. Unlike other avian auditory forebrain nuclei that are devoid of estrogen synthase (Field L and CMM; **Fig. 1*B***), NCM is highly enriched with aromatase (Saldanha et al., 2000; Peterson et al., 2005). Moreover, while ascending auditory circuits are conserved across Aves, aromatase is uniquely found within the NCM of vocal learners (Metzdorf et al., 1999; Silverin et al., 2000). Together, these observations suggest that fluctuating neuroestrogens in NCM may dynamically influence auditory processing in development.

We tested two hypotheses, that: 1) auditory responsiveness to natural communication signals in NCM changes across the critical period for vocal

learning; and 2) NCM auditory responsiveness and coding are rapidly modulated by changes in local estrogens.

Materials & Methods

Subjects

All animal procedures were performed in accordance with the Institutional Animal Care and Use Committee at the University of Massachusetts Amherst. Male zebra finches (*N* = 31 birds; *n* = 26 for estradiol experiments; *n* = 5 for control recordings) were obtained from our breeding colonies, ranging in age from 25 – 95 days post-hatch (dph). Hemisphere was considered the unit of replication, as NCM is a bilateral structure with no direct reciprocal connections between hemispheres (Vates et al., 1996). Subjects' were initially binned by age reflecting the different critical period phases for song learning (**Fig. 3*A***): sensory, 25 – 34 dph (left = 4; right = 5); sensory/sensorimotor: 40 – 64 dph (left = 13; right = 8); and sensorimotor: 65 – 95 dph (left = 5; right = 3). Zebra finches begin displaying overt sexually dimorphic plumage at around 40 dph. For subjects <40 dph, or that did not have male features (black striations, brown badge feathers, orange cheeks, etc.), DNA was extracted from whole blood, and a PCR was run to determine their sex (*see below*). Subjects were raised in mixed-sex breeding colonies following a 14:10 light:dark cycle. Once selected for the experiment, subjects were housed in an acoustic isolation chamber with a nonrelated adult companion female. For pre-singing 25 – 34 dph subjects, the experiment was either carried out the same day as the surgery, or subjects were isolated with a

companion female for 1 day prior to the experiment. For 40 - 95 dph birds, subjects were co-housed with a companion female for 2 to 7 days prior to the experiment in order to capture birds' own song (BOS), which was recorded using Sound Analysis Pro (Tchernichovski et al., 2000) via an omni-directional microphone (Countryman; Menlo Park, CA, USA) inside a sound-attenuation chamber (Eckel Acoustics; Cambridge, MA, USA).

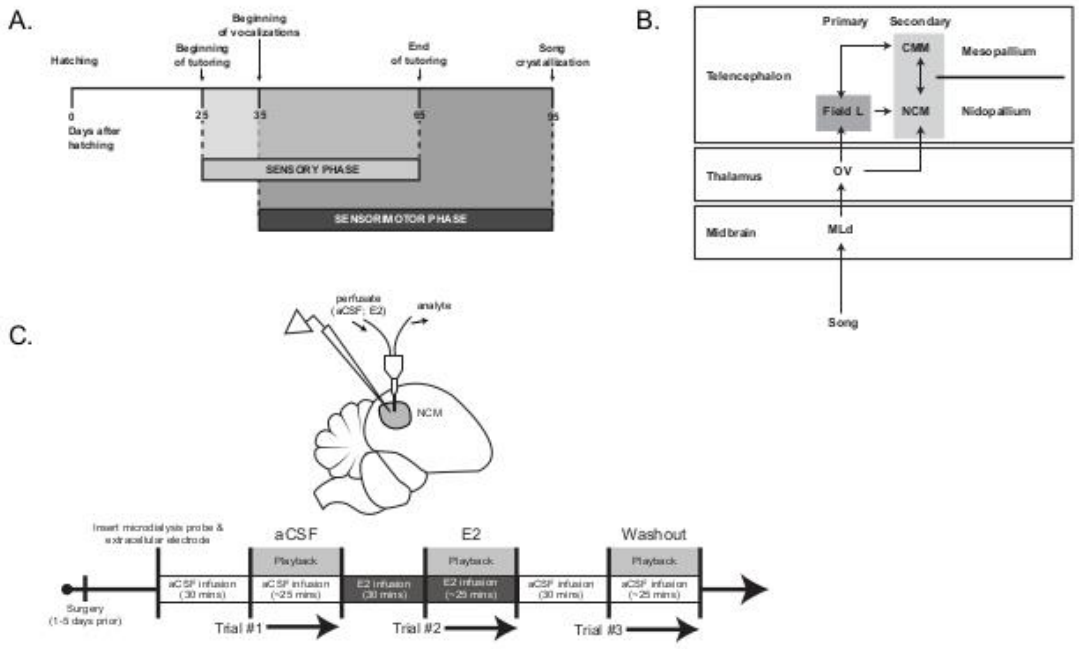

Figure 3: Critical period timeline, avian auditory circuit, and experimental paradigm.

A, The critical period for song learning unfolds across a 3 timespan. While some songbird species begin song learning and recognition at embryonic stages of development (Colombelli-Negrel et al., 2012), zebra finch sensory learning begins at 25 days post-hatch (Clayton, 2013). Autogenous song production can occur as early as 35 dph (typically closer to 40 dph; personal observation), and initially overlaps with the sensory learning phase, until 65 dph when sensorimotor-only learning continues as birds begin to refine their developing subsong until eventual song crystallization (~100 dph). Timeline adapted after Clayton (2013). *B*, Schematic of the avian ascending auditory neural circuit. After sounds are first processed in upstream peripheral and brainstem auditory regions, communication is encoded within the midbrain nucleus MLd (dorsal part of the lateral mesencephalic nucleus), which sends projections to the thalamic nucleus ovoidalis (Ov). Ov sends projections primarily to Field L, comparable to mammalian primary auditory cortex, as well as to NCM (Vates et al., 1996). Secondary auditory cortex regions NCM (caudomedial nidopallium) and CMM (caudomedial mesopallium) are reciprocally connected and receive afferent projections from Field L. *C*, Experimental setup and paradigm. *Top*: *in vivo* microdialysis and extracellular electrophysiology schematic. A microdialysis cannula was first descended into NCM (apx. 1.10 mm ventral; light gray circular region). Afterwards, a carbon-fiber electrode was placed within the proximate region of perfusate diffusion. *Bottom*: experimental timeline. DPH, days post-hatch day; MLd, dorsal part of the lateral mesencephalic nucleus; nucleus ovoidalis, Ov; NCM, caudomedial nidopallium; CMM, caudomedial mesopallium; aCSF, artificial cerebrospinal fluid; E_2, 17β-estradiol.

Sex Determination PCR

For juvenile birds without discernable male features (<35 dph), whole blood was obtained from the ulnar vein, and DNA was subsequently extracted using a commercially-available kit (QIAmp DNA Mini Kit; Qiagen #51304). Purified DNA was subsequently used for PCR using a set of degenerate primers linked to the Z- and W-chromosomes (Griffiths et al., 1998). Amplified PCR product was then visualized alongside a negative control (water) and both adult male and female positive controls on a 2% agarose gel using electrophoresis. Subjects with two bands separated by 36 bp were excluded from the study (indicating presence of W chromosome; thus females), and subjects showing a single band (indicating no W chromosome) were retained for the experiment.

Surgery

Surgery was performed one to five days prior to the experiment for most subjects (**Figure 3*C***; surgery was conducted the day of recordings in 2 birds). Animals were food deprived for 30 minutes prior to an intramuscular injection of Equithesin (30 - 40 µL). Twenty minutes after Equithesin, birds were wrapped in a cloth jacket, and secured to a custom designed surgical stereotaxic apparatus (45° head angle; Herb Adams Engineering) with a heating pad underneath them (36° C). Afterwards, scalp feathers were removed, and a 20 µL subcutaneous injection of lidocaine (2% in ethanol; Sigma-Aldrich) was administered under the scalp. The scalp was then resected, and a positioning-needle was placed just posterior to the midsagittal sinus bifurcation (MSB) and used as a 0-point anatomical reference. The skull was then marked at the anterior-most extent of

NCM: rostral = -1.20 mm, and lateral/medial = 0.90 mm, relative to the MSB. This marking provided a site for microdialysis probe implantation on the day of recording (see below) alongside recording electrodes immediately adjacent (caudal) into NCM. A silver wire was implanted between skull leaflets over the cerebellum to serve as a reference ground. A head-post was then affixed to the bird using cyanoacrylate and dental cement. Following surgery, birds were placed in a recovery cage on a heating pad (36° C) with available food and water until they awoke from the anesthetic. After recovery, birds were given an oral administration of Meloxicam (1 µL/g weight; 0.1 mg/mL), and returned to their acoustic isolation chamber in a separate cage from the companion female.

Anesthetized Extracellular Electrophysiology & Acute Estradiol Treatment

On the day of the experiment, subjects were food deprived for 30 minutes prior to initial anesthetic injections. After 30 minutes of food deprivation, 90 - 100 µL of 20% urethane was evenly administered across three injections separated by 45 minutes each. Once the subject was anesthetized, subjects were brought to the recording room, and affixed to a custom head-post stereotaxic apparatus (45° head angle; Herb Adam Engineering). A small fenestra was made over one hemisphere of NCM and the dura was resected. A microdialysis probe (CMA-7; Harvard Apparatus) was first inserted just anterior to the intersecting point of NCM (as marked by the prior surgery; apx. 1.10 mm ventral; **Fig. 3*C***) and artificial cerebrospinal fluid (aCSF) was perfused at 2 µL/minute using a syringe pump (PHD 2000; Harvard Apparatus). Implanting microdialysis probes creates an acute injury in the brain, which includes local increases in glial aromatase

after 24 hours in male zebra finches (Saldanha et al., 2013). Here, microdialysis probes were implanted for no longer than 4 hours, so it is unlikely that injury-induced glial aromatase influenced NCM properties within the time course of the current experiments.

After the probe was inserted, a carbon fiber electrode (CarboStar-1 [Kation]; Minneapolis, MN) was placed within the proximity of the microdialysis probe and a recording site was found using search stimuli (**Fig. 3*C***). A recording site was determined as being within NCM based on its: 1) anatomical coordinates (0.80 – 1.40 mm ventral) and 2) spontaneous and stimulus-evoked activity using a set of non-experimental stimuli (*search stimuli*, see below).

After at least 30 minutes of aCSF infusion had elapsed, the first of three trials began (**Fig. 3*C***). Each trial included 20 repeats of each stimulus with an inter-stimulus interval of 10 ± 2 s (*experimental stimuli*, see below), lasting approximately 25 minutes. Following the end of the first playback trial, 17-β-estradiol (E_2; 30 µg/mL [110 µM]; dose based on similar studies (Remage-Healey et al., 2010b; 2012; Remage-Healey and Joshi, 2012; Pawlisch and Remage-Healey, 2015)) was retrodialyzed for 30 minutes, and afterwards, a new playback period (using the same stimuli as in trial 1) was presented while E_2 was continuously infused. The same steps for E_2 were repeated with aCSF alone for trial 3 as a washout period. At the end of the recording session, electrolytic lesions were performed at the recording site for later anatomical confirmation. The infusion/playback regiment in trials 1 – 3 were repeated when possible in the contralateral NCM (*n* = 12 of 26 subjects).

At the end of the experiment, birds were killed via rapid decapitation. Brains were removed and placed in a 20% sucrose-formalin solution at 4° C to allow for tissue fixation. Once fixed, brains were frozen in an embedding medium (O.C.T. compound; Tissue-Plus; Fisher HealthCare) and stored at -80° C until they were subsequently sectioned at 45 µm and Nissl-stained for histological verification of probe and electrode placement.

Auditory Stimuli & Playback

Five unique conspecific songs and one white noise (WN) stimulus were used to initially identify auditory responsive recording sites typical of NCM (*search stimuli*). For playback trials, a unique set of *experimental stimuli* were used and included two novel conspecific male songs (CON1 and CON2; different from *search stimuli* CON), heterospecific song (Bengalese finch; HET), and WN. Bird's own song (BOS) and temporally-reversed BOS (REV-BOS) was used when available for 40 - 95 dph animals. If BOS was unavailable for a 40 - 95 dph subject (n = 4), an age-matched juvenile male conspecific song (JUV CON) and temporally-reversed JUV CON (REV-JUV CON) was used instead. For all sensory-aged subjects, a 40 dph JUV CON and REV-JUV CON was presented in place of BOS and REV-BOS. All stimuli were ~2 s in duration (two motif renditions of directed song with introductory notes; ~1.7 – 2.4 s total duration), normalized to ~70 dB (A-weighted), and bandpass filtered at 0.3 – 15 kHz using Adobe Audition. Each playback trial randomly presented 20 repetitions of each stimulus (15 repetitions initially for the first 3 subjects) with a randomly

determined inter-stimulus interval of 10 ± 2 s between each stimulus. The average playback trial duration was ~25 minutes.

Data Analysis

Multi-unit electrophysiological recordings were analyzed offline using Spike2 (ver. 7.04, Cambridge Electronic Design, Cambridge, UK). For each unique subject's multi-unit analysis, a voltage threshold to distinguish signal from noise was initially set based on Trial #1, and maintained across all subsequent trials. Thresholds were set at least 2-fold above the noise-band of a given recording. Recordings were then analyzed by suprathreshold activity aligned to the playback of auditory stimuli. ***Stimulus-evoked firing frequency*** was defined as the total number of spikes (threshold crossings) 2 s post-auditory stimulus onset divided by the number of trials (stimulus repeats), whereas ***spontaneous firing frequency*** was defined as the number of threshold crossings 2 s period prior to the onset of an auditory stimulus divided by the total number of trials. To account for firing variability across subjects, auditory responses were normalized using *Z*-score transformations using the following equation:

$$Z\text{-}score = \frac{\bar{S} - \bar{B}}{\sqrt{Var(S) + Var(B) - 2Covar(S,B)}}$$

Where *S* is the number of spikes during stimulus response (2 s, beginning at stimulus onset), and *B* is the number of spikes during baseline (2 s prior to stimulus onset). $\bar{S}$ and $\bar{B}$ represent the means of these values across all stimulus presentations for a given playback trial.

Single-unit spike sorting

While multi-unit physiological recordings provide information about population responses, we also isolated single neurons to investigate auditory responsiveness for cells with high signal-to-noise ratios. Isolating single-units provide an increased sample size, reducing animal usage numbers and allowing us to track the response properties of single units (1-2 units per recording site) over time in response to estrogen modulation. To identify putative single-neurons for analysis, Trial #1 multi-unit recordings were sorted for large-amplitude single-unit templates based on waveform using default settings in Spike2 (n = 53 single-units). Sorted single units were retained for analysis if they were distinctly clustered from noise or other units in a principal components analysis space, and had an interspike interval (ISI) > 1 ms (i.e., zero ISIs were within the 1 ms bin for all units; **Fig. 5*A***). Following sorting, each single unit was confirmed to be auditory responsive using visual inspection of peristimulus time histograms, as well as by paired *t*-tests comparing each unit's spontaneous and stimulus-evoked firing rates. Units that were statistically responsive ($p < 0.05$) to at least one auditory stimulus during Trial #1 were included. On average, each multi-unit recording site yielded 1 – 2 distinct and auditory-responsive single-units. Peak-to-trough waveform durations were measured to initially distinguish broad- vs. narrow-spiking neurons (as in Schneider and Woolley, 2013; Yanagihara and Yazaki-Sugiyama, 2016); however, we did not observe cell type-specific descriptive effects. Due also to inferential statistical power limitations, we opted to group all single units in our analyses and disregard waveform classifications.

Pattern Classifier

A custom pattern classifier was developed in Python to assess reliability and discriminability of neuronal responses to different stimuli (similar to Caras et al., 2015; as in Lee et al., 2017). For each single-unit recording, the stimulus-evoked firing responses to the 6 different stimuli were compared iteratively. At the start of each run of the classifier, one trial of each stimulus was pseudorandomly selected as the template (6 templates). All remaining 19 trials for each stimulus (114 trials total) were compared one at a time to the templates using a similarity measure. This procedure was repeated 1000 times to generate a confusion matrix, which represents data in terms of actual versus predicted stimulus classification (**Fig. 5*F***).

Before comparison, each response to a stimulus iteration was Gaussian-filtered. The standard deviation (σ) of the filter was employed as a variable for each cell, i.e. the classifier was run with varying σ values of 1, 2, 4, 8, 16, 32, 64, 128 and 256 ms (1000 simulations for each). The filter that yielded the highest accuracy score was used for that cell. Templates and trials were correlated by using the R_{corr} method (Schreiber et al., 2003; Caras et al., 2015):

$$R_{corr} = \frac{\vec{s}_{trial} \cdot \vec{s}_{template}}{|\vec{s}_{trial}| \times |\vec{s}_{template}|}$$

Where $\vec{s}$ represents the vectors of the trial and the template responses after filtering, which are dot-multiplied then divided by the product of their lengths. This calculation returns a value between 0 and 1, which represent total dissimilarity or total similarity, respectively. The stimulus type of the template that provided the

highest R_{corr}(trial, template) value was considered the predicted stimulus for the trial in analysis. Therefore, percent accuracy scores were generated by how well each neuron's firing pattern was predictive of the auditory stimulus.

The classifier output for each neuron was assessed statistically via a trial shuffling approach (Caras et al., 2015). Trials were stripped of stimulus labels, pseudorandomly shuffled and relabeled, essentially generating random responses to the stimuli. The pattern classifier was then run with this shuffled dataset. The distribution of the accuracies (means of diagonals in the confusion matrices) generated in each run of the original dataset was compared with the shuffled dataset via Cohen's *d*. Cohen's *d* was > 0.2 for all single-units included in our analysis, which is considered a modest effect size (Cohen, 1988). As there were 6 stimuli presented to each bird, the trial shuffling accuracy yields distributions centered at 16.67% (i.e. "chance" graphed for visual reference; e.g. dashed-line in **Fig. 5*F***). In contrast to the Z-score, which measures how much the stimulus response is relative to baseline across all trials, R_{corr} is a correlation-based metric that takes into account spike-timing variability phenomena such as jitter, missing spikes and noise in a trial-by-trial basis (Schreiber et al., 2003).

Code Accessibility

The Python code developed for the pattern classifier can be made available upon request.

Statistical Analyses

All statistical analyses were performed using IBM SPSS Statistics for Windows (version 23; Armonk, NY). To test for developmental shifts in multi-unit activity, we conducted three-way ANOVAs (phase * hemisphere * stimulus) separately on trial #1 data (aCSF: *Z*-score, firing rates, and classification accuracy). Similar methods were used for testing development changes in single-unit activity. To determine effects of E_2 on auditory responsiveness, we performed a mixed-effects ANOVA (ME-ANOVA; within-subject factor: treatment; between-subject factors: hemisphere, stimulus). Separate ME-ANOVAs were run for <35 dph vs. 40+ dph subject (see *Results*). For ME-ANOVAs, we restricted our statistical analyses to aCSF and E_2 trials (#1 and #2, respectively) as we were interested in estrogenic effects on auditory processing; however, we present washout data (trial #3) in all relevant figures to provide a visual comparison. If a significant interaction was found in the ME-ANOVA model (e.g. significant hemisphere * trial interaction), separate follow-up ME analyses were run for each factor level (e.g. separate analysis for left vs. right NCM * trial). All post-hoc comparisons were performed using Tukey's HSD. All statistical tests with $p < 0.05$ were considered significant. See **Table 1** for all statistical tests employed for each figure illustrated.

Results

Distribution of ages * hemisphere

We recorded from 26 unique juvenile male subjects. Of the initial 26 subjects, we obtained 12 successful bilateral recordings. NCM is a bilateral structure with no direct reciprocal connections between hemispheres (Vates et al., 1996), so drug infusions administered to the initial hemisphere are unlikely to directly impact physiology in the contralateral hemisphere. NCM recordings from adult males (195+ dph) were obtained from a separate set of experiments using identical methods without microdialysis probe (*n* = 4 subjects) to serve as a visual comparison (e.g. **Fig. 4*B***).

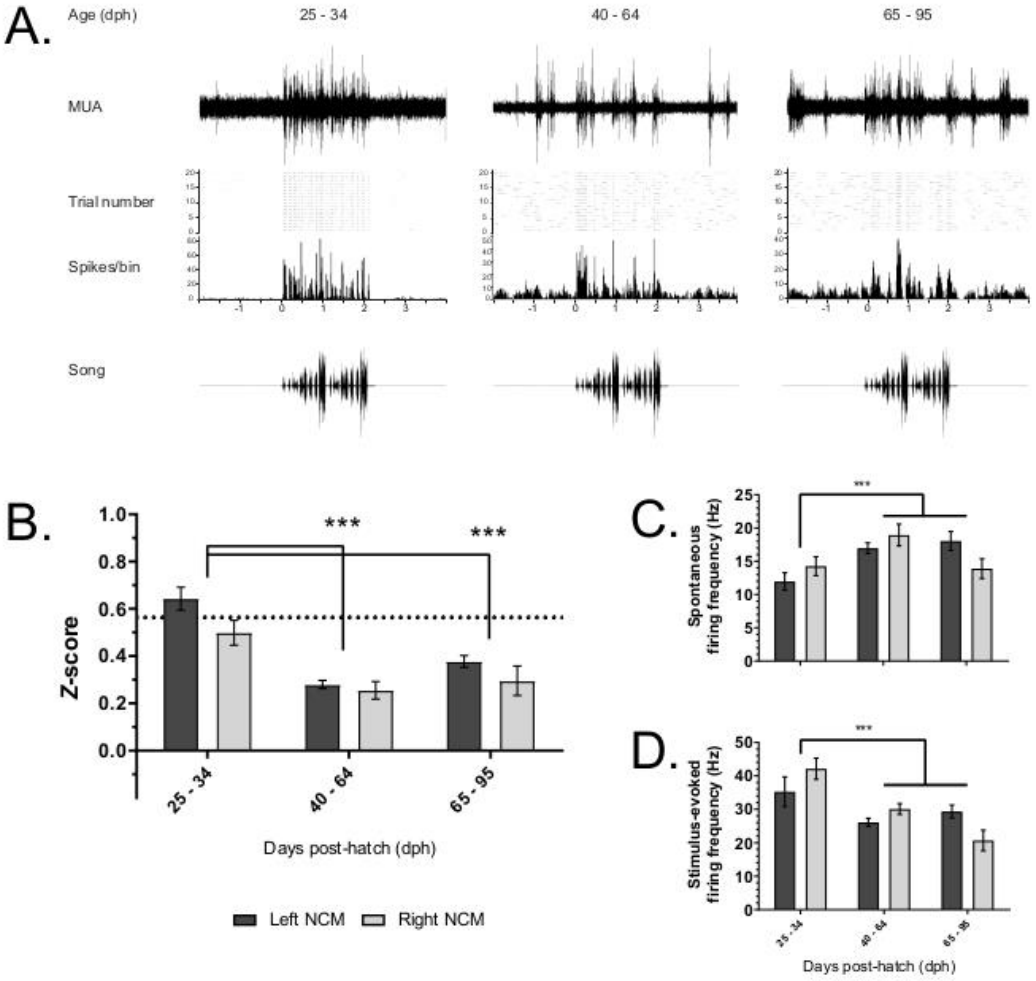

Figure 4: Multi-unit shifts in NCM auditory responsiveness across development.

A, Representative multi-unit recordings from a 25, 47, and 95 dph subject (right, left, and left hemisphere, respectively). *Top*: Representative response to a single presentation of conspecific song (CON2) from a multi-unit recording during Trial #1 (aCSF); *Middle*: raster plot and corresponding peri-stimulus time histogram (6 seconds duration) across all CON2 presentations during Trial #1 (aCSF); *Bottom*: CON2 sonogram. *B*, 25 – 34 dph subjects have higher normalized auditory response than both 40 – 64 and 65 – 95 dph birds. Dotted-line in *B,* is average CON *Z*-score from adult male NCM recordings from a separate study (graphed for visual comparison; $n = 4$ birds [195 – 360 dph; average age = 267.7 dph]). *C, D,* Based on *Z*-score results, we analyzed birds based on critical period phase (sensory [25 – 34 dph] vs sensorimotor [40 – 95 dph]), and found that sensory-aged birds' NCM have C, lower spontaneous firing rates, and *D,* elevated stimulus-evoked firing rates compared to sensorimotor-aged subjects. *** $p < 0.001$ (Z-score: 25 – 34 dph vs. 40 – 64 dph, and 25 – 34 dph vs 65 – 95 dph; spontaneous and stimulus-evoked firing: sensory-aged vs. sensorimotor-aged). MUA, multi-unit activity; CON2, conspecific song 2.

Developmental shifts in NCM auditory physiology and encoding

As we were interested in developmental differences in auditory responses, we initially divided our data into three conventional age groups based on their phase in the critical period for song learning (**Fig. 3*A***): 1) 25 – 34 dph (sensory-aged; n = 5); 2) 40 – 64 dph (sensory/sensorimotor-aged; n = 13); and 3) 65 – 95 dph (sensorimotor-aged; n = 8); as in Livingston and Mooney (2001).

We first analyzed multi-unit recordings to assess whether auditory encoding during baseline conditions (Trial #1; aCSF) differed across subjects depending on the developmental phase and hemisphere (**Fig. 4*A***). Multi-unit auditory Z-scores in the left NCM were significantly higher than in the right NCM across development (left: 0.368 ± 0.019; right: 0.340 ± 0.029; mean ± SEM, $F_{(1, 220)} = 6.663$, $p = 0.010$, $\eta^2 = 0.035$). Further, there was a significant age-dependent effect on auditory responsiveness (**Fig. 4*B***; $F_{(2, 220)} = 37.156$, $p < 0.001$, $\eta^2 = 0.275$), such that 25 – 34 dph phase subjects demonstrated significantly higher auditory Z-scores (0.563 ± 0.037) compared to both 40 – 64 dph (0.271 ± 0.018; $p < 0.001$) and 65 – 95 dph subjects (0.349 ± 0.027; $p < 0.001$); there were no significant differences between 40 – 64 dph and 65 – 95 dph subjects ($p = 0.059$). There were no significant hemisphere * age interactions for trial #1 Z-scores, $F_{(2, 220)} = 1.464$, $p = 0.233$, $\eta^2 = 0.012$. Further, multi-unit classification accuracy showed a similar effect of age ($F_{(2, 240)} = 6.257$, $p = 0.002$, $\eta^2 = 0.059$), whereby 25 – 34 dph subjects had higher accuracies (72.31 ± 2.64%) compared to both 40 – 64 dph (54.20 ± 2.86%; $p < 0.001$), and 65 – 95 dph subjects (58.46 ± 4.05%; $p = 0.001$); 40 – 64 and 65 – 95 subjects were

statistically similar (p = 0.936). No effect of hemisphere on accuracy was observed ($F_{(1, 240)}$ = 3.254, p = 0.073, η^2 = 0.016).

As there were no overall age * hemisphere interactions for trial #1 normalized auditory responses and classification accuracy, and because 40 – 64 dph and 65 – 95 dph subjects were statistically similar, we divided subjects into two juvenile age groups for all subsequent analyses: 1) **sensory-aged** (25 - 34 dph), and 2) **sensorimotor-aged** (40 - 95 dph). This division closely matches a major developmental transition for young male zebra finches, namely before (sensory phase) and after (sensorimotor phase) autogenous singing begins (Clayton, 2013).

Developmental differences in Z-score can be the result of elevated stimulus-evoked firing rates, reduced spontaneous firing rates, or a combination of both. Therefore, we assessed whether differences in multi-unit spontaneous and/or stimulus-evoked firing frequency in NCM explained elevated *Z*-scores in sensory-aged subjects (**Fig. 4*C, D***). Sensory-aged subjects had both significantly reduced spontaneous firing (13.246 ± 0.977 Hz) and higher stimulus-evoked firing (39.087 ± 0.2.646 Hz) compared to sensorimotor-aged subjects (*spontaneous*: 17.432 ± 0.653 Hz, $F_{(2, 222)}$ = 11.136, p = 0.001, η^2 = 0.037; *stimulus-evoked*: 27.295 ± 0.864 Hz, $F_{(2, 222)}$ = 11.136, p = 0.001, η^2 = 0.067). The effect of age on spontaneous firing rates was independent of hemisphere (hemisphere: $F_{(1, 222)}$ = 1.064, p = 0.303, η^2 = 0.005; hemisphere * age: $F_{(1, 222)}$ = 0.509, p = 0.477, η^2 = 0.001). Similarly, no hemisphere * age interactions ($F_{(1, 222)}$

= 2.032, $p = 0.155$, $\eta^2 = 0.005$) nor an overall effect of hemisphere were found for stimulus-evoked firing ($F_{(1, 222)} = 3.092$, $p = 0.080$, $\eta^2 = 0.017$).

Developmental shifts in single-unit activity

While examining multi-unit activity provides information about how population of neurons respond to auditory stimuli, we also analyzed isolated single neurons using waveform template matching (**Fig. 5*A, B***; see *Methods*) to investigate whether developmental changes in auditory responsiveness could be explained by the activity of single neurons. Spontaneous firing rates were lower in sensory-aged subjects (3.34 ± 0.28 Hz) compared to sensorimotor-aged subjects (4.91 ± 0.25 Hz; $F_{(1, 292)} = 8.204$, $p = 0.004$, $\eta^2 = 0.027$; **Fig. 5*C***). No other significant interactions or main effects were found for spontaneous firing. Stimulus-evoked firing was statistically similar in sensory-aged and sensorimotor-aged juveniles; $p = 0.315$; $\eta^2 = 0.003$; **Fig. 5*D***), and there was no effect of hemisphere ($F_{(1, 292)} = 0.293$, $p = 0.589$, $\eta^2 = 0.001$), nor a hemisphere * age interaction ($F_{(1, 292)} = 0.239$, $p = 0.626$, $\eta^2 = 0.001$). As with the multi-unit findings, single-units from sensorimotor-aged males had significantly lower Z-scores (0.310 ± 0.012) compared to units from sensory-aged males (0.461 ± 0.026; $F_{(1, 292)} = 25.561$ $p < 0.001$, $\eta^2 = 0.080$; **Fig. 5*E***). There was no effect of hemisphere ($F_{(1, 292)} = 0.065$, $p = 0.798$, $\eta^2 < 0.001$), nor a hemisphere * age interaction ($F_{(1, 292)} = 0.469$, $p = 0.494$, $\eta^2 = 0.002$) for single-unit Z-scores.

To evaluate whether developmental changes in communication processing affected auditory encoding, we analyzed the physiology data using a pattern classifier (see *Methods*). Irrespective of hemisphere, sensory-aged

subjects demonstrated higher accuracy rates (53.86 ± 2.50%) compared to sensorimotor-aged subjects (40.38 ± 1.57%; $F_{(1, 262)} = 11.321$, $p = 0.001$, $\eta^2 = 0.041$; **Fig. 5*F***). In summary, our findings indicate that auditory neurons in NCM track critical period phase transitions leading to higher auditory responsiveness and coding in sensory-aged, pre-signing birds.

Effects of estradiol on NCM physiology and encoding are hemisphere- and age-dependent

Estradiol enhances stimulus-evoked activity in the NCM of adult male and female songbirds (Remage-Healey et al., 2010b; Remage-Healey et al., 2012; Remage-Healey and Joshi, 2012). Further, E_2 production is rapidly enhanced in NCM during social interactions and song playbacks (Remage-Healey et al., 2008). While there are dynamic changes in neuroestrogen synthesis in the NCM of developing songbirds during and following song tutoring (Chao et al., 2015), it is unknown whether E_2 locally modulates stimulus-evoked activity as in adults. Since we observed clear developmental differences in auditory responsiveness and coding, we elected to analyze subjects separately by age groups for E_2's effect on auditory responsiveness.

Estradiol reduces overall NCM firing in sensory-aged subjects

Estradiol significantly decreased *Z*-scores in sensory subjects (*aCSF*: 0.461 ± 0.026; *E2*: 0.406 ± 0.035; $F_{(1, 72)} = 9.659$, $p = 0.003$; $\eta^2 = 0.118$; **Fig. 6*A***), independent of hemisphere or stimulus ($p > 0.292$). As with normalized auditory responses, E_2 also reduced spontaneous and stimulus-evoked firing rates

(spontaneous: $F_{(1, 72)} = 23.085$, $p < 0.001$; $\eta^2 = 0.243$; stimulus-evoked: $F_{(1, 72)} = 14.151$, $p < 0.001$, $\eta^2 = 0.164$; **Fig. 6*C, D***), independent of hemisphere or hemisphere * trial interactions ($p > 0.05$). Further, E_2 treatment reduced classification accuracy across both hemispheres; $F_{(1, 54)} = 7.68$, $p = 0.003$, $\eta^2 = 0.153$ (*aCSF*: 51.18% ± 3.35; *E_2*: 38.87% ± 1.95; **Fig. 6*B***). However, the descriptive data suggest E_2's overall effect on accuracy was influenced by effects in right NCM (**Fig. 6*B***; a main effect of hemisphere was non-significant, $p = 0.067$). All other main effects and interactions for stimulus and hemisphere were non-significant across all physiological and classification measurements for sensory-aged subjects ($p > 0.80$).

We noted a general trend for attenuated firing rates and Z-scores across trials for sensory-aged subjects (e.g., compare 'washout' periods to 'pre' periods in **Fig. 6**). Therefore, in a separate set of sensory-aged birds ($n = 5$ birds; 6 single units), we tested whether observed decreases in neural activity also occurred in the absence of E_2 treatment. To this end, aCSF was administered across all 3 trials in place of E_2 and a washout trial (trials #2 and #3, respectively), and resulting activity was compared between trials #1 and #2. Normalized auditory responses decreased across trials (**Fig. 6*A****, inset*), but this was not statistically significant ($F_{(1, 30)} = 3.542$, $p = 0.070$; $\eta^2 = 0.106$; trial 1 aCSF = 0.41 ± 0.03; trial 2 aCSF = 0.34 ± 0.04), nor were changes in spontaneous firing rates ($F_{(1, 30)} = 0.473$, $p = 0.497$; $\eta^2 = 0.016$; trial 1 aCSF = 2.55 ± 0.15 Hz; trial 2 aCSF = 2.37 ± 0.26 Hz). However, there was an overall significant decrease in stimulus-evoked firing ($F_{(1, 30)} = 5.095$, $p = 0.031$; $\eta^2 = 0.145$; trial 1

aCSF = 7.44 ± 0.56 Hz; trial 2 aCSF = 5.92 ± 0.78 Hz), and classification accuracy ($F_{(1, 30)}$ = 17.075, p < 0.001; η^2 = 0.363; trial 1 aCSF = 47.92 ± 3.21%; trial 2 aCSF = 36.55 ± 2.56%) across trials 1 and 2. There were no significant stimulus * trial interactions, nor any overall effects of stimulus (p > 0.10). Together, results from sensory-aged birds suggest that while E_2 may dampen auditory responsiveness in NCM, this pattern is difficult to disentangle from overall decreases in neuronal firing and classification accuracy in rundown trials with aCSF only.

Estradiol imparts hemisphere-dependent changes in sensorimotor-aged subjects

For sensorimotor-aged subjects, there was a significant trial * hemisphere interaction for Z-score ($F_{(1, 202)}$ = 4.435, p = 0.036; η^2 = 0.021; **Fig. 7*A***), such that E_2 significantly reduced Z-scores in the left ($F_{(1, 112)}$ = 4.845, p = 0.030; η^2 = 0.041), but not in the right hemisphere ($F_{(1, 90)}$ = 2.131, p = 0.148; η^2 = 0.023). Further, E_2 imparted a hemisphere-dependent effect on firing rates in sensorimotor-aged subjects (spontaneous: $F_{(1, 202)}$ = 6.594, p = 0.011; η^2 = 0.032; stimulus-evoked: $F_{(1, 202)}$ = 9.426, p = 0.002, η^2 = 0.045; **Fig. 7*C, D***). Specifically, E_2 significantly decreased both spontaneous and stimulus-evoked firing in left NCM (spontaneous: p = 0.023; η^2 = 0.045; stimulus: $F_{(1, 112)}$ = 8.066, p = 0.005; η^2 = 0.067), whereas overall firing rates in right NCM were significantly increased (spontaneous: p = 0.011; η^2 = 0.069; stimulus-evoked: $F_{(1, 90)}$ = 7.226, p = 0.009, η^2 = 0.074). Classification accuracy was statistically unaffected by E_2 treatment ($F_{(1, 202)}$ = 3.369, p = 0.068, η^2 = 0.016; **Fig. 7*B***). In summary, these data suggest

that acute modulation of NCM auditory responsiveness by E_2 is lateralized, and that E_2 in the right hemisphere of NCM enhances overall neural firing, independent of changes in stimulus coding in sensorimotor-aged birds, whereas the opposite is observed in left NCM.

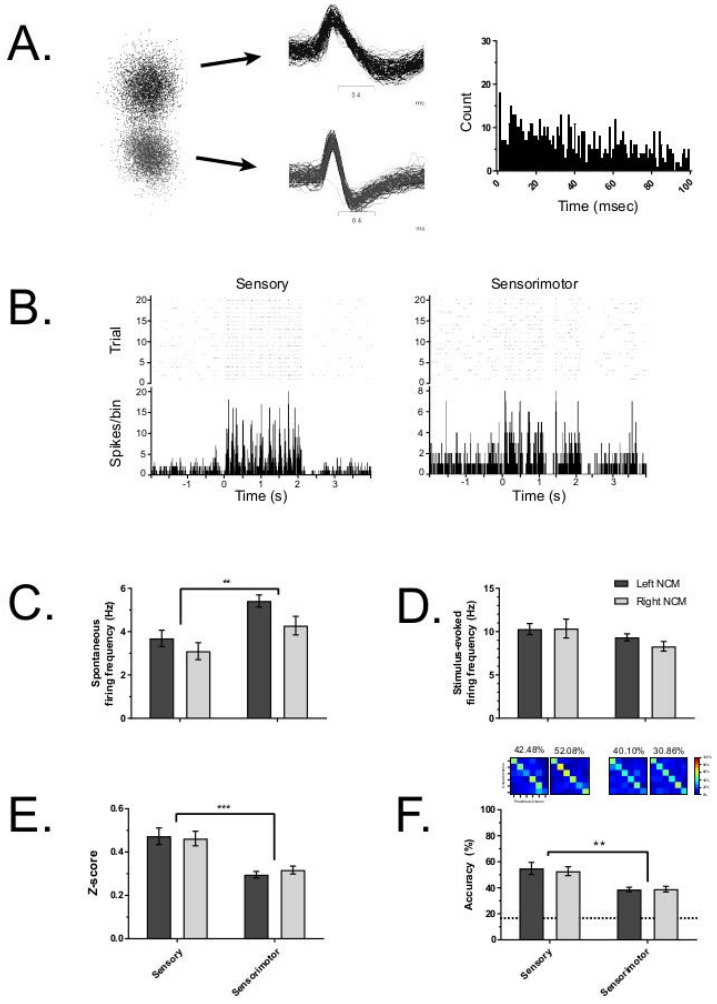

Figure 5: Single-unit auditory response and encoding in NCM is elevated during sensory phase.

A, Representative single neurons. *Left*: two sorted single units distinctly clustered in principal components space; *Middle*: 100 sequential iterations from two separate single neurons overlaying their respective waveform template. *Right*: inter-stimulus interval plots for top single unit. Each bin = 1 ms. Units derived from Trial #1 (aCSF) recording from a sensory-aged subject (30 dph; left NCM). *B*, Raster plot and peri-stimulus time histogram from representative single-units from a sensory-aged and sensorimotor-aged bird (33 [right NCM], and 71 dph [left NCM], respectively). *C, D*, Spontaneous firing rates are lower in sensory-aged subjects irrespective of hemisphere; however, *D,* there are no age-dependent differences in single-unit stimulus-evoked firing rates. *E*, *F*, Across hemispheres, *E,* single-unit auditory *Z*-scores and *F*, classification accuracy are significantly higher in sensory-aged birds. Dotted-line in *F,* is chance-level prediction for classifier (1 in 6 chance for accurately classifying a given stimulus = 16.67%). *** $p < 0.001$; ** $p < 0.01$ (sensory-aged vs. sensorimotor-aged).

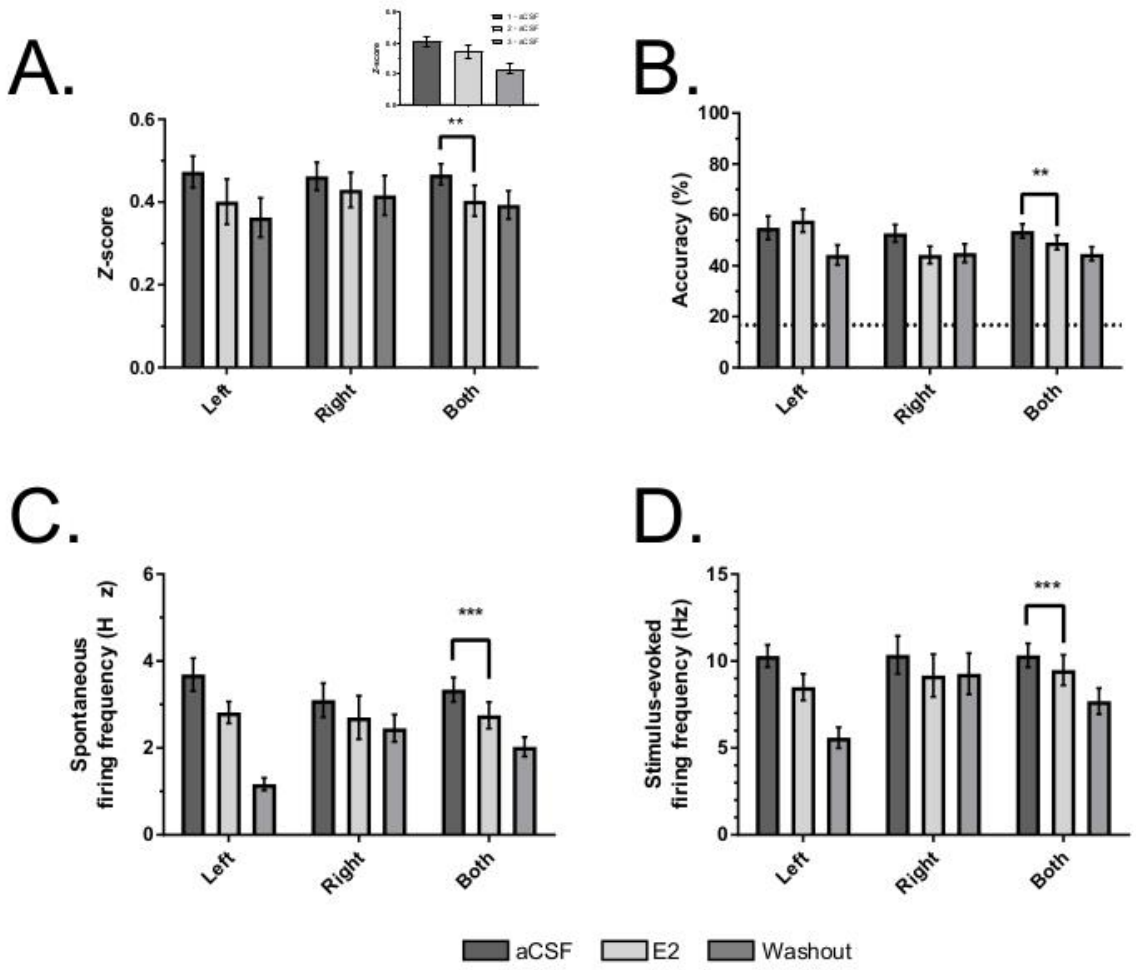

Figure 6: Estradiol (E_2) dampens auditory responsiveness in NCM.
A, B, C, D, Relative to aCSF (Trial #1), E_2 treatment decreased *A,* Z-scores, *B,* classification accuracy, *C,* spontaneous, and *D,* stimulus-evoked firing rates in the NCM of sensory-aged subjects. Hemisphere-specific averages are depicted for visual comparison and consistency, but there was no trial * hemisphere effect. Averaged measurements across hemispheres are depicted in the last set of columns (*Both*); ** $p < 0.01$ (effect of trial; Trial #1 vs. Trial #2). Dotted-line in *B,* is chance-level prediction for classifier (1 in 6 chance for accurately classifying a given stimulus = 16.67%). *Inset* in *A,* average z-score across trials in aCSF rundown experiment ($p = 0.07$; Trial #1 vs. Trial #2; $n = 5$ sensory-aged birds; 6 single-units).

Naturalistic sounds elicit higher single-unit auditory responses in NCM across age

In addition to developmental and E_2 effects on NCM auditory physiology, we compared stimulus-dependent effects on single-unit auditory responsiveness.

As work on physiological preference for natural sounds over synthetic tones in telencephalic auditory forebrain nuclei has been previously reported in several oscine species (Leppelsack and Vogt, 1976; Bonke et al., 1979b), including zebra finches (Theunissen et al., 2004; Hauber et al., 2007), we report all the main effects of stimulus in **Table *2*** for concision. In short, we found that NCM is typically more responsive to naturalistic auditory stimuli (song) compared to a synthetic sound (white noise).

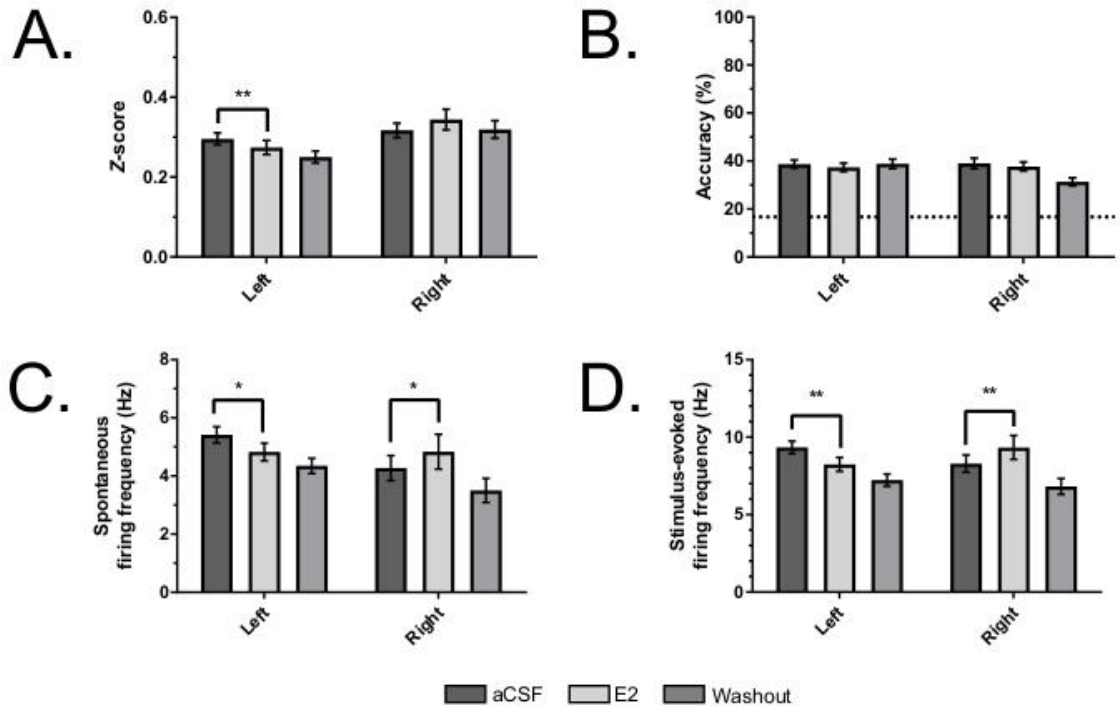

Figure 7: The effects of estradiol (E_2) on auditory responsiveness in the NCM of sensorimotor-aged birds are lateralized.
A, B, Depending on hemisphere, E_2 treatment either increases (right NCM) or decreases (left NCM) auditory *Z*-scores relative to aCSF (Trial #1) in sensorimotor subjects. However, *B,* classification accuracy remains unaffected. *C, D,* Similar to *Z*-scores, both *C,* spontaneous and *D,* stimulus-evoked firing rates decrease or increase in response to E_2 depending on hemisphere (left or right NCM, respectively). Dotted-line in *B,* is chance-level prediction for classifier (1 in 6 chance for accurately classifying a given stimulus = 16.67%). * $p < 0.05$ (left/right: Trial #1 vs. Trial #2); ** $p < 0.01$ (left/right: Trial #1 vs. Trial #2).

Table 1. Statistical table

Results	Data structure	Type of Test	Observed power (α = 0.05)
Fig. 2B, Z-score	Assumed normal distribution; age (25 - 34; 40 - 64; 65 - 95 dph) * hemisphere (left NCM; right NCM)	Three-way ANOVA	hemisphere = 0.728; age = 1.00; hemisphere * age = 0.251
Fig. 2C, spontaneous firing rate	Assumed normal distribution; phase (sensory; sensorimotor) * hemisphere (left NCM; right NCM)	Three-way ANOVA	hemisphere = 0.058; phase = 0.738; hemisphere * phase = 0.266
Fig. 2D, stimulus-evoked firing rate	Assumed normal distribution; phase (sensory; sensorimotor) * hemisphere (left NCM; right NCM)	Three-way ANOVA	hemisphere = 0.092; phase = 0.918; hemisphere * phase = 0.626
Fig. 3C, Z-score	Assumed normal distribution; phase (sensory; sensorimotor) * hemisphere (left NCM; right NCM)	Three-way ANOVA	hemisphere = 0.057; phase = 0.999; hemisphere * phase = 0.105
Fig. 3D, classification accuracy	Assumed normal distribution; phase (sensory; sensorimotor) * hemisphere (left NCM; right NCM)	Three-way ANOVA	hemisphere = 0.051; phase = 0.918; hemisphere * phase = 0.070
Fig. 3E, spontaneous firing rate	Assumed normal distribution; phase (sensory; sensorimotor) * hemisphere (left NCM; right NCM)	Three-way ANOVA	hemisphere = 0.482; phase = 0.815; hemisphere * phase = 0.069
Fig. 3F, stimulus-evoked firing rate	Assumed normal distribution; phase (sensory; sensorimotor) * hemisphere (left NCM; right NCM)	Three-way ANOVA	hemisphere = 0.084; phase = 0.171; hemisphere * phase = 0.078
Fig. 4A, Z-score	Assumed normal distribution; trial (aCSF; E2) * hemisphere (left NCM; right NCM)	Mixed effects ANOVA	trial = 0.866; hemisphere = 0.119; trial * hemisphere = 0.182
Fig. 4A, *inset;* Z-score (rundown)	Assumed normal distribution; trial (trial #1 - aCSF; trial #2 - aCSF)	Mixed effects ANOVA	trial = 0.445
Fig. 4B, classification accuracy	Assumed normal distribution; trial (aCSF; E2) * hemisphere (left NCM; right NCM)	Mixed effects ANOVA	trial = 0.866; hemisphere = 0.450; trial * hemisphere = 0.369
Fig. 4C, spontaneous firing rate	Assumed normal distribution; trial (aCSF; E2) * hemisphere (left NCM; right NCM)	Mixed effects ANOVA	trial = 0.997; hemisphere = 0.050; trial * hemisphere = 0.104
Fig. 4D, stimulus-evoked firing rate	Assumed normal distribution; trial (aCSF; E2) * hemisphere (left NCM; right NCM)	Mixed effects ANOVA	trial = 0.960; hemisphere = 0.185; trial * hemisphere = 0.363
Fig. 5A, Z-score	Assumed normal distribution; trial (aCSF; E2) - separate analyses by hemisphere (left vs. right)	Two-way repeated measures ANOVA	left NCM = 0.588; right NCM = 0.303
Fig. 5B, classification accuracy	Assumed normal distribution; trial (aCSF; E2) - separate analyses by hemisphere (left vs. right)	Two-way repeated measures ANOVA	left NCM = 0.293; right NCM = 0.196
Fig. 5C, spontaneous firing rate	Assumed normal distribution; trial (aCSF; E2) - separate analyses by hemisphere (left vs. right)	Two-way repeated measures ANOVA	left NCM = 0.629; right NCM = 0.725
Fig. 5D, stimulus-evoked firing rate	Assumed normal distribution; trial (aCSF; E2) - separate analyses by hemisphere (left vs. right)	Two-way repeated measures ANOVA	left NCM = 0.804; right NCM = 0.758

Table 1: Statistical table.

Table 2. Stimulus-specific effects on NCM single-unit auditory responsiveness

Dependent variable (single-unit data)	Model	Statistical tests	*F*-values & degrees of freedom	*p*-value	Effect size (partial η^2)	Post-hoc results
Development (aCSF; Trial #1 only)						
Z-score	phase * hemisphere * stimulus	Three-way ANOVA; Tukey's HSD	$F_{(7, 292)} = 4.682$	< 0.001	0.101	WN < CON1, CON2, HET, JUV CON, and JUV REV CON ($p <$ 0.003)
Stimulus-evoked firing	phase * hemisphere * stimulus	Three-way ANOVA; Tukey's HSD	$F_{(7, 292)} = 2.400$	0.022	0.054	WN < CON1 and HET ($p <$ 0.022)
Classification accuracy	phase * hemisphere * stimulus	Three-way ANOVA; Tukey's HSD	$F_{(7, 262)} = 2.529$	0.016	0.063	WN < JUV CON ($p =$ 0.023)
Effect of E2 (aCSF vs. E2)						
Sensory						
Z-score	trial * hemisphere * stimulus	Three-way ANOVA	$F_{(5, 72)} = 2.062$	0.080	0.125	n/a
Stimulus-evoked firing	trial * hemisphere * stimulus	Three-way ANOVA	$F_{(5, 72)} = 1.495$	0.202	0.094	n/a
Classification accuracy	trial * hemisphere * stimulus	Three-way ANOVA	$F_{(1, 54)} = 1.298$	0.278	0.107	n/a
Sensorimotor						
Z-score						
Left NCM	trial * stimulus	Two-way ANOVA; Tukey's HSD	$F_{(7, 112)} = 3.097$	0.005	0.162	WN < BOS, CON1, CON2, and HET ($p <$ 0.038)
Right NCM	trial * stimulus	Two-way ANOVA	$F_{(5, 90)} = 2.275$	0.054	0.112	n/a
Stimulus-evoked firing						
Left NCM	trial * stimulus	Two-way ANOVA	$F_{(7, 112)} = 1.365$	0.227	0.079	n/a
Right NCM	trial * stimulus	Two-way ANOVA	$F_{(5, 90)} = 0.558$	0.732	0.030	n/a
Classification accuracy						
Left NCM	trial * stimulus	Two-way ANOVA; Tukey's HSD	$F_{(7, 112)} = 2.415$	0.024	0.131	WN < JUV CON ($p =$ 0.048)
Right NCM	trial * stimulus	Two-way ANOVA	$F_{(5, 90)} = 0.880$	0.498	0.047	n/a

Table 2: Stimulus-specific effects on single-unit NCM auditory responsiveness.

Discussion

Here, we demonstrate that auditory neurons in pre-singing, sensory-aged male zebra finches have higher auditory responses to natural communication vocalizations compared to older juvenile males. Moreover, sensitivities to E_2 signaling in auditory cortex change with age: while sensory-aged birds showed an overall decrease in auditory response when treated with E_2, sensorimotor-aged birds showed a divergent response to E_2 depending on hemisphere (either overall increase or decrease). Taken together, this study is the first to our knowledge to consider developmental and hemispheric effects on sensory coding and rapid steroid modulation of auditory processing.

Ontogenetic shifts in vocal communication encoding

During the critical period phase for auditory memory formation, pre-singing (sensory-aged) juvenile songbirds encode communication signals with higher fidelity than juveniles beginning autogenous song production (sensorimotor-aged). As such, elevated auditory-evoked responses in sensory-aged birds suggest the transition from purely auditory encoding (sensory phase) to song production with gradual modification through error-correction (sensorimotor phase) learning may track these perceptual developmental shifts. To our knowledge, this is one of the first studies to document neurophysiological changes in the NCM of pre-singing and sensorimotor learning in juvenile male songbirds. Prior studies have described developmental shifts in the auditory forebrain, but have mainly compared 20 vs 35 dph songbirds (all sensory-aged). Amin et al. (2007) described adult-like auditory responses in the brainstem in 20

and 35 dph zebra finches, and stimulus-dependent auditory selectivity in the CMM of 35 dph birds. In awake recordings of NCM, electrophysiological auditory responses are comparable in 20 and 30-35 dph (Stripling et al., 2001; Miller-Sims and Bottjer, 2014). Our results build upon these findings by expanding the span of time considered during the critical period. These findings inform how learning-dependent transitions during maturation shift auditory processing within NCM.

The elevated auditory processing we observe during in sensory-aged subjects may be related to the coincident formation of a tutor auditory memory during this critical period of development. While auditory input is necessary during the song refinement and error-correction phase in sensorimotor-aged birds (e.g. Mandelblat-Cerf et al., 2014); initially, birds must solely listen before they sing. Perhaps enhanced auditory activity and encoding in NCM during early development ensures a high-fidelity tutor song memory acquisition for young males to subsequently imitate. As NCM is one of the putative loci for tutor song memory (Bolhuis and Gahr, 2006; London and Clayton, 2008; Gobes et al., 2010), elevated auditory responsiveness may be important for early tutor memory consolidation. Alternatively, an increasing amount of tutor experience may facilitate neural transitions from a more broadly tuned auditory circuit (sensory-aged; higher auditory neural activity) to a more selectively tuned circuit (sensorimotor-aged; relatively dampened auditory response). Yanagihara and Yazaki-Sugiyama (2016) found that a relatively short period of tutoring (10 days) radically shifted a sub-population of single neurons' auditory selectivity in the NCM of juvenile males, and biased neuronal responses toward primarily the tutor

and/or birds' own song. If tutoring experience itself shapes auditory selectivity, then perhaps less experience with tutor or exposure to adult song in general in sensory-aged subjects (9 days relative to onset of critical period opening) compared to older juveniles (15 – 70 days) explains heightened auditory responsiveness in NCM. However, our finding that stimulus classification accuracy is higher in sensory-aged subjects suggests that rather than NCM being broadly tuned to any sound, young juvenile songbirds can accurately distinguish naturalistic communication signals with higher fidelity than sensorimotor-aged birds.

One caveat to our interpretation that there is a neural "switch point" in auditory processing that precisely tracks behavioral transitions during vocal learning (sensory/pre-singing → sensorimotor/singing) is the ability to dissociate true developmental effects from E_2-dependent effects. In adult songbirds, song presentation elicits an increase in E_2 levels in NCM, while in juveniles, tutoring leads to decreased E_2 in NCM and increased levels afterward (Remage-Healey et al., 2008; Remage-Healey et al., 2012; Chao et al., 2015). As such, auditory presentations alone may elicit changes in local E_2 availability that may be age-dependent. However, it remains to be tested whether song presentations to anesthetized songbirds, such as in our study, drive local changes in E_2 production as with awake, behaving songbirds. Thus, future experiments should clarify whether local E_2 synthesis in NCM is state-dependent, and should also explore whether local infusion of an aromatase inhibitor during song presentation

blocks and/or unmasks age-dependent and estradiol-dependent regulation of auditory responsiveness in NCM.

Future experiments should also consider these identified developmental milestones in the NCM of juvenile females, who also learn song early post-hatching for eventual mate selection in adulthood (Miller, 1979; Riebel, 2000; Terpstra et al., 2006). The extent that elevated auditory responses in NCM of sensory-aged juveniles are similar between males and females will contribute information about its underlying mechanism.

Acute effects of estrogens on sensory-aged songbirds

Sensory-aged male zebra finches begin forming auditory memories of their tutor's song before attempting their own vocalizations (Mooney, 2009). As such, we predicted that E_2 would enhance auditory tuning as it does in adults (Remage-Healey et al., 2010b; Pinaud and Tremere, 2012; Remage-Healey et al., 2012; Remage-Healey and Joshi, 2012; but see Lattin et al., 2017). However, E_2 treatments led to significant decrements in auditory processing irrespective of hemisphere. One explanation may be that E_2 dynamics change during development. Chao et al. (2015) observed acute decreases in E_2 levels during tutoring in the NCM of developing male zebra finches, but also that NCM E_2 levels increase immediately after a tutoring session. As such, acute neuroestrogen production may impair auditory memory acquisition during a learning session in sensory-aged songbirds (Korol and Pisani, 2015; Rensel et al., 2015), whereas post-training E_2 increases may facilitate memory consolidation (Srivastava et al., 2013; Frick, 2015; Vahaba and Remage-Healey,

2015). Further, the expression of telencephalic GPER1 (G-protein coupled estrogen receptor 1 that can mediate rapid neuroestrogen signaling [Rudolph et al., 2016]) is five-fold higher in sensory-aged zebra finches (Acharya and Veney, 2011). Therefore, NCM may be particularly sensitive to low concentrations of E_2 in sensory-aged animals. This work thus suggests that dynamic changes in estrogen receptor and aromatase protein expression in NCM across development may explain an initial suppressive effect of E_2 signaling on auditory processing in sensory-aged male songbirds.

One important caveat to these results is that in a separate set of sensory-aged birds with aCSF retrodialyzed across all three trials (run-down experiment), we observed decreased classification accuracy and stimulus-evoked firing rates, as well as a trend for reduced normalized auditory responsiveness. These results make it more difficult to disentangle the effects of E_2 on decreases in NCM responsiveness and encoding in sensory-aged subjects from purely time-dependent effects. Nonetheless, E_2 reduced spontaneous firing in sensory-aged birds, which was not observed in aCSF-only trials, and may reflect a true dampening of auditory responsiveness. Moreover, the run-down experiment emphasizes how our observations of increased firing during E_2 treatment, as seen in the right NCM of sensorimotor-aged subjects, are likely counteracting this overall steady run-down effect in juvenile males.

Acute, lateralized effects of estrogens on sensorimotor-aged songbirds

The lateralization of E_2 actions on auditory encoding and firing rate in NCM differ across development. In sensorimotor-aged birds, E_2 imparts a

hemisphere-dependent effect. In left NCM, E_2 led to decreased normalized auditory response, as well as spontaneous and stimulus-evoked firing rates, without affecting classification accuracy. In contrast, E_2 administration in the right NCM increased stimulus and spontaneous-evoked firing rates, without impacting normalized auditory responses or classification accuracy. These data add to a growing literature on the lateralized neuromodulation of hearing by brain hormones. For example, oxytocin receptors are preferentially upregulated the in left auditory cortex of maternal female rats, which enhances pup call saliency/encoding (Marlin et al., 2015). In male European starlings, inhibiting aromatase suppresses vocal communication responses in the left, but not right hemisphere of the auditory forebrain (De Groof et al., 2017). Similarly, blocking E_2 synthesis in left but not right NCM extinguishes male songbirds' behavioral preference for their own song (Remage-Healey et al., 2010b). Therefore, our findings add further evidence for hemisphere-dependent hormone neuromodulation of communication processing in auditory cortex, and expands this concept to include developing animals.

Prior work on developmental neuromodulation has not addressed how sensitivities to E_2 may differ by hemisphere, and whether estrogen synthase or estrogen receptor expression is similarly lateralized. Chao et al. (2015) found decreased E_2 in NCM during tutor song exposure in developing male subjects; however, E_2 was only measured within the left NCM. Therefore, our current results suggest that E_2 fluctuations in right NCM may increase or remain unchanged during tutoring. Future experiments should also clarify changes in

aromatase and estrogen receptors (both nuclear [ERα and ERβ] and membrane-bound [GPER1; mGluR1/ERα] across development and between hemispheres, as these factors may also account for divergent effects of E_2 on auditory physiology in NCM across the critical period. Alternatively, the auditory cortex of juvenile male zebra finches may mature at different rates depending on hemisphere. Our data suggest that the right NCM matures faster than the left, as E_2 enhancement of auditory responsiveness is more adult-like in the right vs left NCM of sensorimotor-aged subjects (Remage-Healey et al., 2010b). Future experiments exploring developmental changes should also identify whether NCM is lateralized in neuronal development across the critical period, as well, since there are no reported differences in NCM cell density between developing vs. adult male NCM (Stripling et al., 2001), nor any published quantifications of left vs right neuronal density in NCM at any age.

These findings contribute to a broader point of interest on how steroid hormones may participate in learning. Accumulating evidence demonstrate that rapid, local E_2 synthesis and signaling is critically linked to neural plasticity in the hippocampus and amygdala (Zhao et al., 2010; Srivastava et al., 2013; Bailey et al., 2017; Bender et al., 2017). Less is known about rapid E_2 signaling and plasticity in sensory cortices, such as the auditory cortex. In adult zebra finches, blocking global E_2 synthesis impairs neural adaptation to familiar songs in NCM, a proxy for auditory memory formation (Yoder et al., 2012). In juvenile songbirds, circulating E_2 predicts tutor imitation accuracy (Marler et al., 1987); however, the majority of studies on hormones and song learning in development have focused

on androgens. Administering testosterone or dihydrotestosterone to juvenile songbirds prematurely crystallizes song (Korsia and Bottjer, 1991; Bottjer and Hewer, 1992; Whaling et al., 1995; Livingston and Mooney, 2001; however, see Templeton et al., 2012). Therefore, it remains to be tested how neuroestrogen synthesis in the auditory forebrain is involved in vocal learning. Our results suggest that local E_2 may interfere with auditory encoding in sensory-aged birds and within the left NCM of sensorimotor-aged birds, whereas E_2 presented to the right NCM in sensorimotor-aged animals may aid in encoding song. These possibilities await future experimental tests to determine potential functional roles for E_2 in song learning.

Conclusion

Here, we demonstrate that robust shifts in sensory processing in the auditory cortex precisely track experience-dependent critical period milestones, and extend our understanding of estrogen-dependent neuromodulation of auditory responsiveness across development. Our findings indicate that age and hemisphere are critical factors to consider when evaluating sensory physiology in development and in response to neuromodulators. Further, these data provide insight into a broader understanding of how estrogen signaling, and audition may change across the lifespan, and in relation to hemisphere and communication learning. In humans, estrogens generally enhance hearing in adulthood, and appear to have a lateralized effect on listening (Tillman, 2010) and verbal memory (Fernandez et al., 2003). Interestingly, Wild et al. (2017) found adult-like neural responses to speech in the auditory cortex of 3- and 9-month old infants, a

time during which circulating estrogen levels predict future language success (Wermke et al., 2014; Quast et al., 2016). As such, future research should consider both hormonal state and hemisphere when studying hearing-evoked neural changes in auditory cortex.

CHAPTER IV

BRAIN ESTROGEN PRODUCTION AND THE ENCODING OF RECENT EXPERIENCE

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Abstract

The vertebrate central nervous system integrates cognition and behavior, and it also acts as both a source and target for steroid hormones like estrogens. Recent exploration of brain estrogen production in the context of learning and memory has revealed several common themes. First, across vertebrates, the enzyme that synthesizes estrogens is expressed in brain regions that are characterized by elevated neural plasticity and is also integral to the acquisition, consolidation, and retrieval of recent experiences. Second, measurement and manipulation of estrogens reveal that the period following recent sensory experience is linked to estrogenic signaling in brain circuits underlying both spatial and vocal learning. Local brain estrogen production within cognitive circuits may therefore be important for the acquisition and/or consolidation of memories, and new directions testing these ideas will be discussed.

Introduction

Historically, steroid hormones were thought to be produced exclusively in peripheral endocrine glands and to influence vertebrate behavior through long-term (hours to days) regulation of gene expression. In the case of estrogens,

these ‘classical’ effects are mediated in the brain via the nuclear steroid receptors, estrogen receptor α (ERα) and ERβ. It is now clear that the brain itself is also a key site of steroid hormone synthesis and action (Corpechot et al., 1981). Brain-derived steroids provide a local source of neuromodulators that can act upon neural circuits at rapid timescales akin to classical neurotransmitters (seconds to minutes) (Remage-Healey, 2014). While the rapid effects of steroid hormones are often studied in the context of sexual behavior (Cornil et al., 2013), the role of neurosteroids in behaviors and neural systems beyond reproduction has only recently received attention. One area in particular has been understanding how estrogen signaling may enhance or otherwise alter cognition on momentary timescales. While there are a host of hormones that modulate learning and memory (Orr et al., 2009; Rabinowitz et al., 2014), the potent endogenous estrogen 17β-estradiol (E2) has a clear influence on cognition and neural plasticity (Srivastava et al., 2013; Luine, 2014; Bailey and Saldanha, 2015). As such, this review will concentrate on the role of locally-synthesized brain E2 in learning and memory.

Focusing on recent findings, we evaluate three fundamental aspects of E2 and cognition: 1) the expression of estrogen synthase (aromatase) in brain regions critical for memory consolidation; 2) how measurement and manipulation of relatively rapid E2 synthesis relates to encoding recent experience; and 3) whether learning and post-learning epochs are associated with periods of E2 production and/or suppression. For the purposes of this review, we define the following terms:

Learning: active process of acquiring new information through experience.

Memory: stored information and/or consolidation of new information from a learning experience/event

Cognition: an active, sensory-dependent process that encompasses both a learning event (e.g. training) and the subsequent consolidation of the memory about that event (e.g. post-training), which can be recruited in future contexts.

Recent experience: a discrete window of time including both a potential learning event and the ~2-hour period that follows immediately after the learning event.

Encoding: the active process of memory consolidation of a recent learning event.

Does the role of E2 in brain regions associated with cognition depend on the local availability of aromatase, as well as membrane estrogen receptors, within these same regions?

Estradiol appears to influence learning and memory across a diverse group of species, including: nematodes (Sugi et al., 2011), songbirds (Bailey et al., 2013), rodents (Luine, 2014), and nonhuman (Lacreuse et al., 2014) and human (Sherwin, 2012) primates. One interesting observation supporting the proposed role of acute neuroestrogen signaling in cognition is the presence of aromatase (estrogen synthase) in brain regions critical for memory encoding, consolidation, and recall among vertebrates. Aromatase expression is conserved across several functionally homologous neural structures in vertebrates (Callard et al., 1978b). ***Figure 8*** presents for the first time a cross-species comparison of

aromatase expression in three brain regions that facilitate distinct types of memory: 1) fear memory consolidation and social recognition (***amygdala*** (Bergan et al., 2014)); 2) spatial navigation and novel object recognition (***hippocampus*** (Boulware et al., 2013; Bailey and Saldanha, 2015)); and 3) vocal communication learning, and language acquisition (***auditory cortex/forebrain*** (Bailey and Saldanha, 2015)). Neuronal aromatase is enriched in these canonical 'memory' regions in mammals and their functionally similar regions in nonmammalian species; we present representatives showing this in human (*Homo sapiens*) and nonhuman primates (*Maca mulatta*), rodents (*Mus musculus*), birds (*Taeniopygia guttata*), reptiles (*Aspidoscelis uniparens*), and fish (*Porichthys notatus*). While aromatase is found in the brain of amphibians (Nakagawa and Iwabuchi, 2012; Iwabuchi et al., 2013; Coumailleau and Kah, 2014), the spatial resolution and region specificity are less clear and difficult to resolve for present purposes. Of note, at present, there is a paucity of direct evidence for the presence of aromatase in mouse hippocampus (Wu et al., 2009; Stanic et al., 2014), which may be explained by the promoter used to identify its presence. A recent finding in *Xenopus* provides intriguing evidence that there may be multiple splice variants for brain-specific aromatase (Nakagawa and Iwabuchi, 2012). Therefore, the absence of evidence for aromatase in mouse hippocampus (as well as the auditory cortex) may be due to antibody specificity. In contrast to mice, aromatase is reliably found in rat dorsal hippocampus (Tabatadze et al., 2014).

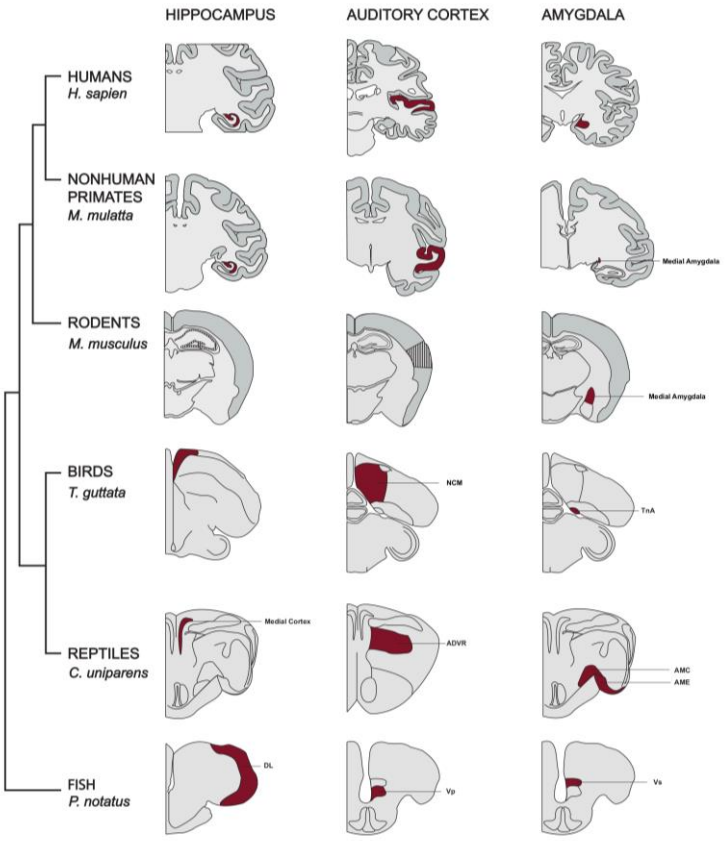

Figure 8: Aromatase is typically expressed in brain regions crucial for cognition among vertebrates.

Aromatase expression is abundantly expressed within the hippocampus, auditory cortex/forebrain, and amygdala of several representative species across a wide range of classes. Black stripped-filled brain regions indicate no reported presence of aromatase, whereas maroon-filled brain regions indicate detectable presence of aromatase as assessed through various techniques. Briefly, 1) hippocampus - humans: (Yague et al., 2010; Azcoitia et al., 2011); rhesus macaques: (Yague et al., 2008); mice: not seen in hippocampus (Wu et al., 2009; Stanic et al., 2014); but see (Ivanova and Beyer, 2000); birds: (Saldanha et al., 2000; Peterson et al., 2005); reptiles (medial cortex): (Krohmer et al., 2002; Dias et al., 2009); fish (dorsolateral telencephalon): (Forlano et al., 2001; Menuet et al., 2003); 2) auditory cortex/forebrain – humans: (Stoffel-Wagner et al., 1998; Yague et al., 2006); rhesus macaques: (Yague et al., 2008); birds (caudomedial nidopallium; NCM (Butler et al., 2011)): (Saldanha et al., 2000; Peterson et al., 2005); reptiles (anterior dorsal ventricular ridge; ADVR (Butler et al., 2011)): (Dias et al., 2009); fish (posterior portion of the ventral telencephalon; Vp): (Forlano et al., 2001; Forlano et al., 2005; Fergus and Bass, 2013); 3) amygdala - humans: (Biegon et al., 2015); rhesus macaques: (Takahashi et al., 2014); mice: (Wu et al., 2009); birds (nucleus taenia; TnA): (Saldanha et al., 2000); reptiles: (Krohmer et al., 2002; Dias et al., 2009; Cohen and Wade, 2011, 2012); fish (supracommissural nucleus of the ventral telencephalon; Vs (Northcutt, 1995; Bass et al., 2000)): (Forlano et al., 2001; Forlano et al., 2005).

While the presence of aromatase demonstrates the capability for local E2 synthesis, acute changes in neurophysiology and behavior typically depend on membrane-bound ERs present within these same aromatase-expressing brain regions. In addition to membrane-trafficked versions of the classical nuclear ERs (ERα and ERβ), there are also several membrane-bound estrogen receptors (mERs) that rapidly modulate E2-dependent behaviors (Seredynski et al., 2015) and neurophysiology (Woolley, 2007), including: mERs associated with a membrane glutamate receptor (mGluR), Gq-coupled mER (Gq-ER), GPER1 (formerly GPR30), and ER-X (as reviewed in Frick, 2015). These cognate mERs are typically co-expressed in aromatase-enriched brain regions associated with the encoding of recent experience. For example, both aromatase and GPER1 are found in the hippocampus, nucleus taeniae of the amygdala (TnA), and the

caudomedial nidopallium (NCM; functionally homologous to mammalian secondary auditory cortex) of adult and developing male songbirds (Acharya and Veney, 2011). Regions such as NCM and hippocampus are necessary for auditory and spatial memory consolidation, respectively, across the lifespan (London and Clayton, 2008; Yoder et al., 2012; Rensel et al., 2013).

In sum, the molecular machinery necessary to both synthesize and respond to local E2 fluctuations are found within neural structures critical for memory consolidation and encoding. It is therefore important to consider the functional significance of aromatase expression and its relationship to learning.

What is the relationship between fluctuating brain E2 levels and the acquisition vs. consolidation of recent experience?

In addition to the strong overlap of aromatase expression in functionally homologous brain regions across diverse taxa, there is ample evidence to suggest that acute neuroestrogen synthesis actively influences learning and memory. Local E2 production is implicated in learning and memory across a broad range of species, including humans, non-human primates, songbirds, rodents, and nematodes (Sugi et al., 2011; Luine, 2014; Bailey and Saldanha, 2015; Frick, 2015). Research has primarily focused on hippocampal-dependent memory and E2, and mounting evidence indicates that exogenous E2 enhances hippocampal-dependent memory consolidation (which may reflect endogenous fluctuations during and after learning). For example, E2 infused into the dorsal hippocampus of adult female mice within a critical 2 hour window following a training event caused an enhancement in subsequent recognition memory

performance (Frick, 2015). In addition to an E2-dependent enhancement, systemic and local inhibition of aromatase activity impairs spatial and auditory memory consolidation in songbirds, as well as long-term potentiation (LTP) in rodents (Vierk et al., 2012; Yoder et al., 2012; Bailey et al., 2013). Therefore, exogenous manipulation of E2 availability impacts the encoding of recent experience in spatial memory tasks. However, it is less clear if pharmacologically induced changes in local E2 levels reflect physiological changes of neuroestrogen production in non-manipulated animals.

Understanding the molecular mechanisms of learning and memory has been dominated by approaches that manipulate the neurochemistry and activity of cognitive circuits. Recent approaches now allow the measurement of the on-line activity and neurochemical state of cognitive circuits. Relevant to the current topic, *in vivo* central E2 measurements have provided direct information about physiological changes in local steroid environments, and have been successfully adapted for songbirds (Chao et al., 2015), quail (Ubuka et al., 2014), rats (Sato and Woolley, November 2014 (Washington D.C., USA)), and nonhuman primates (Kenealy et al., 2013). Studies using *in vivo* microdialysis, as well as brain content assays of macroarea homogenates, have revealed that E2 synthesis is elevated following recent learning events (Chao et al., 2015; Tuscher et al., November 2013 (San Diego, CA, USA)). Specifically, E2 levels are elevated within 60 mins subsequent to spatial navigation and vocal communication training (Chao et al., 2015; Tuscher et al., November 2013 (San Diego, CA, USA)). This timeframe parallels the critical window for pharmacological effects on

enhancing or impairing memory consolidation by administering E2 or inhibiting aromatase, respectively (Bailey et al., 2013; Bailey and Saldanha, 2015; Frick, 2015). One functional consequence of post-learning elevations in brain E2 may be the rapid enhancement of synaptogenesis in critical cognitive structures such the hippocampus and prefrontal cortex (Inagaki et al., 2012). Thus, E2 appears to be dynamically upregulated immediately after learning events, and these increases are likely important for dendritic spine alterations and modulations of synaptic strength. In this way, modifying the strength of functional synaptic connections between neurons is a key candidate mechanism for E2 altering higher cognitive function, such as learning and memory.

A competing hypothesis – is the enhanced memory consolidation mediated by the suppression of E2 synthesis during a learning event vs. a rebound increase in E2 after training?

Work in rodents and songbirds has led to the idea that rapid post-training E2 elevations are cognitively enhancing. However, recent findings in rodents and songbirds highlight the intriguing possibility that dynamic ***suppression*** of E2 synthesis during a learning event may be a critical component of memory formation/consolidation (Korol and Pisani, 2015). In adult rats, systemic treatment with an aromatase inhibitor prior to and during a spatial learning task actually improves working memory in subsequent tests (Alejandre-Gomez et al., 2007). Furthermore, E2 levels are suppressed in the auditory forebrain of juvenile songbirds during a song learning event (Chao et al., 2015), and this suppression during tutoring is followed by a subsequent post-training elevation in E2. These findings that E2 is suppressed during a training event and subsequently elevated

may explain similar observations that E2 is elevated post-training in other vertebrates (Chao et al., 2015; Tuscher et al., November 2013 (San Diego, CA, USA)). Together, these observations lead to the hypothesis that E2 levels are "rebounding" from neuroestrogen suppression during a learning event. Therefore it is important to clarify the functional role of reduced neural E2 production in the acquisition of sensory experience, in songbirds, rats and other model systems. In particular, key future research directions include understanding the acute control mechanisms for *in vivo* brain aromatase activity (such as calcium-dependent phosphorylation of the enzyme (Cornil et al., 2013)), as well as improving our temporal resolution for the fluctuations in neuroestradiol during and following discrete learning events.

While suppressing E2 could facilitate learning, elevated E2 may actually interfere with the encoding of recent experience. In corvids (Rensel et al., 2015) and finches (Rensel et al., 2013), exogenous E2 interferes with hippocampal-dependent spatial memory, which is consistent with recent findings in the prefrontal cortex in aged nonhuman primates (Lacreuse et al., 2014). Thus, it may be that the plasticity-enhancing effects of E2 may be deleterious to the faithful initial encoding of a novel sensory stimulus (Korol and Pisani, 2015). As such, it remains important to consider the balance between potential cognitively-enhancing, as well as –impairing roles for brain-derived E2 in the encoding and consolidation of recent experience. This is especially important when considering the timing of fluctuations in local E2 levels in higher cognitive circuits.

Conclusions and future directions

Thus far, we have presented work illustrating the largely conserved expression of aromatase in brain regions associated with learning and memory, proposed functional roles for E2 synthesis within these regions as it relates to memory consolidation, and suggested an alternative possibility that local suppression of E2 may be an important modulator for experience encoding. It is clear that more work is needed to further clarify the pluripotent mechanisms by which brain E2 signaling contributes to learning and memory.

The study of estrogen signaling in learning & memory has been largely focused on spatial navigation and object recognition memory in adult animals within the hippocampus. It will be interesting and necessary to expand the study of acute E2 production in cognition to include: 1) novel memory types (e.g. sensory: auditory and olfactory (Yoder et al., 2012; Dillon et al., 2013)); 2) ages across the lifespan (e.g. critical periods early in development, especially in relation to sensorimotor learning); 3) aromatase-enriched regions outside of the hippocampus (e.g. medial amygdala), and 4) areas of the brain in which neurophysiological signatures of experiential learning can be readily accessed. Broadening the range of research initiatives (i.e., across neural structures, age, memory-type, and species) is now necessary to build a generalized understanding of E2's role in cognition. Moreover, there is little information about fluctuating steroid levels in oft studied brain regions involved in cognition. For example, we now have the opportunity to determine *in vivo* changes in central E2 levels during and following training in regions such as the hippocampus.

Other burgeoning areas of steroid-mediated learning and memory include E2's apparent effect on epigenetic alterations. Epigenetic mechanisms, namely histone acetylation and DNA methylation, appear to mediate several aspects of learning and memory, and recent evidence suggests that E2's enhancement of memory consolidation relies on local chromatin modifications (Zhao et al., 2012). While there is no direct evidence for rapid neural aromatization regulating epigenetics, future studies should begin testing the effect of aromatase inhibitors on subsequent epigenetic changes and memory retrieval.

Another exciting prospect for future work is neuroestrogens' potential role in facilitating critical period plasticity for sensorimotor learning. HVC (proper name; functionally similar to Broca's area) is a requisite telencephalic sensorimotor nucleus for vocal learning, and integrates both auditory input and vocal output in songbirds. During development, rapid dendritic spine remodeling occurs within HVC immediately after initial tutoring experience, and the amount of spine remodeling post-tutoring is a strong predictor for vocal development and model imitation (Roberts et al., 2012). E2 is required for both the development of the sensorimotor circuit (including HVC) and for proper tutor song imitation. Therefore, acute fluctuations in brain-derived E2 may facilitate memory consolidation during development in estrogen-sensitive forebrain regions (such as NCM), which project to and modulate downstream auditory representations in HVC (Remage-Healey and Joshi, 2012). It is interesting to note that a role for E2 in vocal communication learning has been recently implicated in human infants, as well (Schaadt et al., 2015).

Research on the role of brain-derived estrogens in learning and memory has just begun. Expanding the research spotlight to include novel structures, behaviors, species, now presents an exciting jumping off point to explore the way that rapid changes in brain estrogen fluctuations regulate the encoding of recent experience.

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CHAPTER V

BLOCKING NEUROESTROGEN SYNTHESIS TRANSFORMS NEURAL REPRESENTATIONS OF LEARNED SONG, BUT NOT IMITATION ACCURACY IN DEVELOPING SONGBIRDS

Abstract

Birdsong, like human speech, is learned early in life by first memorizing an auditory model. Once memorized, birds compare their own burgeoning vocalizations to their auditory memory, and adjust their song to match the model. While much is known about this latter part of vocal learning, less is known about how initial auditory experiences are formed and consolidated. In adults and developing songbirds, there is strong evidence suggesting the caudomedial nidopallium (NCM), a higher order auditory forebrain area, is the site of auditory memory consolidation. However, the mechanisms that facilitate this consolidation are unknown. One likely mechanism is brain-derived 17β-estradiol (E2). E2 is important in the hippocampus for post-learning memory consolidation. Further, circulating E2 is elevated during the auditory memory phase, and in NCM immediately after song learning sessions, suggesting it functions to encode recent auditory experience. Therefore, we tested whether E2 production was necessary for auditory memory consolidation in development. Our results demonstrate that while systemic estrogen synthesis blockade regulates song production, inhibiting E2 synthesis locally within NCM does not prevent song learning. However, early life E2 manipulations in NCM transform neural

representations of birds' own song and its model song in both NCM and a downstream sensorimotor nucleus (HVC). Taken together, these findings suggest that E2 plays a complex role during development, and demonstrate that contrary to our initial predictions, unilateral post-training estrogen synthesis blockade in the auditory cortex does not negatively impact vocal learning.

Introduction

While many animals use sounds to communicate with one another (vocal communication), the ability to learn to vocally communicate is relatively rare (Petkov and Jarvis, 2012). In vocal learning animals, such as humans and songbirds, vocal learning occurs across two main phases: an auditory memorization ('sensory') phase, followed by a sensorimotor phase ('babbling', error correction/feedback) (Kuhl, 2010; Derégnaucourt, 2011). While much is known about sensorimotor learning, how of auditory memories form early in life is less clear.

One brain region likely involved in storing auditory memories is the caudomedial nidopallium (NCM) (Bolhuis and Moorman, 2015). NCM, comparable to mammalian secondary auditory cortex, is required for accurate song learning. Blocking ERK-signaling bilaterally in NCM during tutoring leads to poor song imitation (London and Clayton, 2008). Tutoring naïve juvenile songbirds leads to an increased proportion of tutor-song-selective neurons in NCM (Yanagihara and Yazaki-Sugiyama, 2016). Further, bilateral NCM lesions abolish innate preference for tutor song in adults (Gobes and Bolhuis, 2007; but see Canopoli et al., 2016; Canopoli et al., 2017). Thus, NCM contains a putative tutor 'engram'; however, the

mechanisms that enable auditory memory formation and consolidation remain unknown.

Consolidating recent experience in other contexts and systems require presynaptic signaling molecules ('neuromodulators'), such as brain-derived estrogens. 17β-estradiol (E2; a predominant estrogen) is a candidate neuromodulator required for auditory memory consolidation due to its faciliatory role in adult hippocampal-dependent cognition, across taxa (Vierk et al., 2012; Srivastava et al., 2013; Luine, 2014) (Woolley and McEwen, 1992; Packard and Teather, 1997b; Packard, 1998; Woolley, 2007; Frick, 2012; Bailey et al., 2013; Rensel et al., 2013; Bailey and Saldanha, 2015; Rensel et al., 2015; Barth et al., 2016; Tuscher et al., 2016b; Bailey et al., 2017; Blaustein, 2017; Bayer et al., 2018), but see (Korol and Pisani, 2015). Additionally, both circulating and brain-manufactured estrogens ('neuroestrogens') typically enhance hearing (Caras, 2013; Caras and Remage-Healey, 2016), and are associated with language and verbal memory (Fernandez et al., 2003; Zimmerman et al., 2011; Anthoni et al., 2012; Wermke et al., 2014; Schaadt et al., 2015). Together, current evidence suggests that neuroestrogen signaling may facilitate the consolidation of recent auditory experience.

Neuroestrogen rapidly enhances auditory physiology within NCM across the lifespan (Remage-Healey et al., 2010b; Remage-Healey and Joshi, 2012; Vahaba et al., 2017). Thus, it is possible that one functional role of E2 acting within the auditory forebrain is to facilitate song memory consolidation. NCM is uniquely enriched with estrogen synthase (aromatase) in vocal learning birds (Silverin et

al., 2000), suggesting its presence is distinctly important for song learning. Further, systemic inhibition of estrogen synthesis during training and testing results in impaired auditory recognition in adult zebra finches (Yoder et al., 2012). However, it's unclear how neuroestrogens may affect song learning during tutor memorization. Currently, there is limited evidence for the functional role of E2 during the vocal learning critical period. In songbirds, circulating E2 levels rise during the sensory phase, and at least in swamp sparrows, predict future tutor imitation success (Pröve, 1983; Weichel et al., 1986; Marler et al., 1987; Marler et al., 1988), as in humans with language (Wermke et al., 2014; Quast et al., 2016). Moreover, the expression of GPER1 (a membrane-bound estrogen receptor proposed to mediate the rapid effects of E2) peaks in the telencephalon of male songbirds during the sensory phase (Acharya and Veney, 2011). As with E2-dependent learning in rodents, E2 levels are rapidly elevated in NCM immediately after a song learning session (Chao et al., 2015).

The aim of the present study was to determine whether E2 synthesis is necessary for the consolidation of a recent auditory experience and the eventual vocal imitation of a tutor model. Based on prior findings, we postulated that elevated E2 levels in the auditory forebrain aid in memory consolidation following individual learning bouts. We tested whether the eventual degree of vocal similarity between the social model (tutor) and the pupil in adulthood would be impaired by inhibiting neuroestrogen synthesis in NCM during and immediately after bouts of vocal communication learning.

Methods & Materials

All methods and procedures were approved by the Institutional Animal Care and Use Committee (IACUC) at the University of Massachusetts Amherst.

Immunocytochemistry

Animals, perfusion, and sectioning

Male juvenile zebra finches (*n* = 6) were selected from mixed-sex breeding aviaries maintained on a 14:10 light:dark cycle. Male sensorimotor subjects (*n* = 3; 65, 71, and 71 dph) were identified by their sexually dimorphic plumage (orange cheek feathers; brown and black badge feathers). Sensory-aged male subjects without dimorphic plumage (*n* = 3; 20, 26, and 34 dph) were identified by PCR (see *Sex Determination* below). All subjects were obtained from our breeding colony and were exposed to adult song up until the day of the perfusion. Birds were euthanized via anesthetic overdose (isoflurane) and transcardially perfused with 20 - 30 mL of 0.1M phosphate buffer saline (PBS) followed by 35 mL of 4% paraformaldehyde (PFA). After perfusion, brains were extracted and placed into 4% PFA for 24 hours at 4° C. Brains were then transferred to a 30% sucrose-0.1 M PBS solution for 24 – 48 hours at 4° C. Once fixed, brains were submerged in an opaque tissue-embedding medium (O.C.T. compound; Tissue-Plus; Fisher Health-Care) and frozen at -80° C. Brains were thawed on wet ice on the day of sectioning and hemisected using a razor blade to allow us to carefully distinguish hemispheres. Brains were sectioned at 35 µm in the sagittal plane at -20° C using a cryostat (Leica CM3050 S). Each

hemisphere was separately collected into two series for lateral sections, and four series for medial sections. Medial sections were determined by the emergence of cerebellum. Sectioned tissue was placed in cryoprotectant medium in 12-well plates, which was wrapped with Parafilm and stored at -20° C until immunocytochemistry.

Antibodies

Antibodies and dilutions for aromatase and parvalbumin were identical to those used in Ikeda et al. (2017). Briefly, we used a polyclonal anti- aromatase primary antibody raised in rabbit (1:2,000; a generous gift from Dr. Colin Saldanha), and a monoclonal anti-parvalbumin primary antibody raised in mouse (1:10,000; Millipore MAB1572; RRID: AB_2174013). Secondary antibodies included goat anti-rabbit Alexa 488 (1:500; Thermo Fisher Scientific Inc.), and goat anti-mouse Alexa 647 (1:100; Thermo Fisher Scientific Inc.).

Procedure

Brain sections were first manually washed 3 x in 0.1 M PB, followed by 3 x 15-minute washes in 0.1 M PB on a plate shaker, followed by a 2-hour incubation at room temperature with 10% normal goat serum (Vector) in 0.3 % PBT. Tissue was then transferred to a 10% normal goat serum-0.3% PBT solution containing the primary antibodies and incubated at room temperature for 60 minutes. Afterwards, plates were tightly wrapped in parafilm and placed on an orbital shaker in a cold room at 4° C for 48 hours. On day 3, tissue was washed 3 x 15 minutes in 0.1 % PBT before being transferred to the secondary antibody-

containing solution made in 0.3% PBT for 60 minutes. At this point, tissue was kept in the dark to prevent any florescent bleaching. Tissue was washed again 3 x 10 minutes in 0.1% PBT, and finally transferred to 0.1 M PB, wrapped in parafilm, and stored at 4° C. Several days later, tissue was slide mounted, covered with ProLong Diamond Antifade Mountant with DAPI (Thermo Fisher Scientific Inc.), cover slipped, and placed in an opaque slide box and stored at 4° C.

Confocal imaging

Fluorescently-labelled tissue was imaged using a confocal microscope (Nikon A1 Resonant Confocal) with NIS-Elements imaging software. The laser strength and gain were determined independently for each antibody/fluorescent channel of interest. Once the levels were determined, the same setting was applied across all sections per fluorescent channel. NCM was located anatomically by the presence of cerebellum and the absence of aromatase-rich nucleus taenia (TnA; lateral boundary of NCM). An overview/reference image at 10x was obtained for each section followed by subregion (dNCM and vNCM) z-stacks obtained at 60x (1 µm z-steps for 15 µm).

Image analysis

An experimenter blind to subjects' ages and hemisphere quantified the total number immunostained cells for each fluorescent channel using ImageJ 1.52h (Schneider et al., 2012). We measured immunopositive-neurons two ways. Initially, we quantified aromatase and parvalbumin immunopositive-cells by

calculating their expression as a percentage of DAPI to normalize for relative cell density across sections and subjects (e.g. Aromatase+ cells % of DAPI = total # of aromatase+ cells / total # of DAPI+ cells). Additionally, we also quantified cell density relative to image volume to provide a more standardized report of its expression using the following equation:

$$Cell\ density\ (mm^3) = \frac{total\ \#\ of\ immunopositive{-}cells}{image\ volume}$$

Juvenile Song Learning

Animals

Juvenile male zebra finches (*Taeniopygia guttata*) were obtained from our breeding aviaries (*N* = 34; *n*= 6 for systemic experiments; *n* = 28 for microdialysis experiments). Nest boxes with an active clutch of young zebra finches (<10 dph) were observed to identify the putative mother. Once identified, the mother, offspring, and their nest box were placed in a cage within a sound-attenuation chamber (Eckel Acoustics), either as a single-family group, or, in a few rare instances, two adjacent cages of females with siblings were placed in the same chamber. Some breeding pairs were also isolated before laying a clutch (*n* = 3). In these instances, the adult male was left in the sound-attenuation chamber until the fledglings were ~13 dph. The remaining fledgling were removed from the breeding colony by 13 dph (range = 5 – 17 dph), which is well before the putative opening of the critical period for song learning (~20 – 25 dph). Birds were confirmed to be male via sex determination PCR at ~22 dph. By ~30 dph, most birds were isolated from their siblings and mom (range = 29 – 39 dph; *n* = 2 birds

that were >38 dph; most birds were 29 – 31 dph) and placed in a new cage and sound-attenuation chamber along with an unrelated adult companion female. An omnidirectional microphone (Countryman) was placed in the chamber and song was continuously recorded for the remainder of the experiment using Sound Analysis Pro (Tchernichovski et al., 2000).

For microdialysis subjects, a total of 20 birds were successfully treated with FAD or aCSF (*n* = 5 subjects per hemisphere per treatment). An additional eight subjects experienced non-health related technical issues during microdialysis (e.g. clogged microdialysis probe) that resulted in them being prematurely disconnected but retained as surgery control subjects ('cannula'-only subjects). One of these failed microdialysis subjects was deprived of any tutoring or adult male song until after 131 dph and served as an isolate control subject.

Timeline

Systemic

For systemically-treated subjects, birds were co-housed with an adult companion female throughout the entire experiment. Tutoring began at 40 dph (see *Tutoring regiment*) and was immediately followed by oral administration of the assigned treatment. Tutoring continued every other day for 20 days (i.e. 10 days of total tutoring), ending at 60 dph. Peripheral FAD treatment suppresses E2 synthesis for up to 48 hours (Wade et al., 1994). Thus, there was one 'washout' day without any treatments between each tutoring session. Birds were

returned to group housing at 131 dph, and after at least 6 weeks (~196 dph), were re-captured to record song and terminal electrophysiology recordings.

Microdialysis

Guide cannulae were unilaterally implanted in NCM several days after being initially isolated with a companion female. Several days following surgery, birds were connected to the microdialysis apparatus in a new sound-attenuation chamber without any companion birds. One day later, daily tutoring began for two to three days. After the last tutor session, birds were disconnected from the microdialysis setup and placed in a sound-attenuation chamber with an adult female companion bird in an adjacent cage. Companion females were switched every two weeks. Birds were returned to a group setting (all-male aviary in breeding room, or in a mixed-sex sound-attenuation chamber in same-sex cage) at 131 dph. After at least 6 weeks had elapsed, birds were returned to a sound-attenuation chamber for follow-up song recording and subsequent electrophysiology experiments. After electrophysiology recordings, birds were sacrificed, and brains were extracted for future sectioning and histological examination.

Sex determination

Zebra finches begin to develop sexually dimorphic plumage at ~30 - 40 dph. Therefore, we used established methods (Griffiths et al., 1998) as we have previously described (Chao et al., 2015; Vahaba et al., 2017) to determine juvenile birds' sex. Briefly, DNA for sex determination PCR was extracted from

whole blood obtained from the ulnar vein typically at ~22 dph (median age = 22 dph; range = 18 – 30 dph). Identified males were retained for the experiment, whereas females were returned to their original breeding aviary along with their mother once the youngest male fledgling reached ~30 dph.

Pharmacological inhibition of aromatase

For systemic experiments, birds were fed 30 µL of either saline (0.9% NaCl in $_{dd}$H20) or FAD (1 mg/mL in 0.9% NaCl) immediately following tutoring cessation. This dose is similar to previous studies that demonstrate significantly reduced aromatase activity and/or estradiol levels in zebra finches (Wade et al., 1994; Saldanha et al., 2000; Saldanha et al., 2004; Remage-Healey et al., 2010b; Rensel et al., 2013). Microdialysis subjects were retrodialyzed with artificial cerebrospinal fluid (aCSF) and 100 µM FAD in aCSF prepared as in previous experiments (Remage-Healey et al., 2008; Remage-Healey et al., 2010b; Remage-Healey et al., 2012; Chao et al., 2015).

In Vivo Microdialysis

Surgery

A unilateral CMA-7 microdialysis guide cannula with obdurator (CMA Microdialysis, CMA 7, ref. no. P000138) was implanted several days after isolation with a companion female (median age = 39 dph; range = 35 – 47 dph), as in previous studies (Remage-Healey et al., 2008; Ikeda et al., 2014; Chao et al., 2015). Birds were food deprived 30 minutes prior to surgery, and then received an intramuscular injection of Equithesin (30 – 40 µL, typically). Twenty

minutes later, birds were swaddled in a Kim wipe, and placed atop a heating pad and secured via ear bars at 45° to our custom surgical stereotaxic apparatus (Herb Adams Engineering). Head feathers were removed and a 20 µL subcutaneous injection of 2% lidocaine was administered underneath the scalp, which was subsequently resected to expose the outer layer of skull. The midsagittal sinus bifurcation (MSB) was then identified and used as a 0-point anatomical reference. A unilateral fenestra was then made over one hemisphere of NCM (coordinates: rostral = 1.20 mm, lateral = ± 0.90 mm), and the dura was carefully resected. A CMA-7 guide cannula with obdurator was then descended approximately 1.0 mm ventral into the proximate region of NCM (ventral range of NCM at this coordinate is 0.80 – 1.40 mm). The guide cannula was secured using cyanoacrylate and dental cement, and the exposed scalp and incision area sealed with cyanoacrylate. Birds recovered on a heating pad in a cage with *ad libitum* food and water until awake, after which they were transferred back to their sound-attenuation chamber in a separate cage from the companion female.

Acute neural injury induces glial aromatase production in birds, with aromatase responses peaking at 72 hours, and persisting up to six weeks after insult (Peterson et al., 2004; Wynne et al., 2008; Balthazart and Ball, 2013). To reduce the confound of injury-induced aromatase upregulation from the guide cannula surgery, birds were given at least three days to recover prior to starting microdialysis (median = 4 days; range = 3 – 5 days) to allow for injury-induced glial aromatase levels to subside (Saldanha et al., 2013).

Microdialysis

After the recovery period, birds were connected to the microdialysis apparatus in a new sound-attenuation chamber. The obdurator was replaced with a CMA-7 microdialysis probe (1 mm membrane length, CMA Microdialysis, ref. no. P000082), which was then connected to a dual-channel microdialysis swivel (375/D/22QM; Instech Labs) via fluorinated ethylene propylene (FEP) inlet and outlet tubing. Once the bird was connected, aCSF was dialyzed at a rate of 2 µL/min by a syringe pump located outside of the chamber (PHD 1000, Harvard Apparatus). After being hooked-up, all birds were observed to ensure they were healthy as evidenced by eating, drinking, and the ability to comfortably navigate the cage. Dialysate samples were collected every hour during the day (~09:00 - ~18:00 pm), yielding ~120 µL of dialysate per sample. Perfusate was dialyzed at a rate of 2 µL/min for the entire duration of the microdialysis experiment. Several hours after the final tutor session, FEP tubing was disconnected and birds were returned to a sound-attenuation chamber in a separate cage alongside an adult companion female. As described in similar studies (London and Clayton, 2008), guide cannulae eventually detach after experiments as the skull develops and expands, without any obvious deleterious health effects, typically 12 days after the last day of microdialysis (range = 6 – 38 days post-final microdialysis day; in one case, this did not occur until 154 days after microdialysis).

Tutoring regimen

All birds were naïve to song before the tutoring period. After tutoring, all birds were returned to an individual sound-attenuation chamber with an unrelated

adult female companion in an adjacent cage. Including a companion female is atypical for most experimental studies of song learning in the lab, and there is some evidence to suggest that adult females may impact song development in juvenile male zebra finches (Kojima and Doupe, 2011) and cowbirds (King et al., 2005). However, we opted to include a companion female as isolating subjects is less naturalistic for zebra finches (a highly gregarious songbird), and likely a great deal more stressful for developing subjects.

Passive audiovisual tutoring playback

In an initial pilot experiment, we were curious whether an automated passive playback tutoring design would enable accurate song learning/imitation in adulthood, as used in other song tutoring studies (Deshpande et al., 2014; Chao et al., 2015). Similar early isolation procedures as with the systemic and microdialysis subjects were used on a separate set of birds (n = 8). Otherwise unmanipulated subjects were isolated from their mother and siblings ~37 dph, and daily tutoring began at 42 dph until 47 dph (5 sessions total). Tutoring began at ~10:00 each day and lasted for one hour. During the tutoring session, a 60-minute tutoring video was played on a USB-powered LCD monitor (Lilliput 7-in) alongside song broadcasted via an adjacent speaker (Sony; model # SRS-TP1WHI). The video and song were obtained from an adult male zebra finch singing directed song to a female. At 48 dph, birds were reunited with an adult female companion and kept in isolation until 111 dph, after which time they were returned to a mixed-sex aviary. Song was recorded throughout the entirety of the experiment. Overall, birds tutored with passive audiovisual methods produced

poor copies of the tutor song (*n* = 6; mean ± SEM; similarity = 41.09% ± 0.07%; range = 23.37% - 64.98%), likely due to zebra finches requiring active/self-solicited learning (e.g. operant tutoring) and/or social instruction (reviewed in Derégnaucourt, 2011). Therefore, all remaining subjects were exposed to a hybrid live-tutoring with passive song playback of that tutor that yielded more reliable tutor song imitation.

Live tutoring with audio playback – systemic subjects

Audio visual tutoring methods did not yield successful tutor imitations. Therefore, we opted for a tutoring paradigm that included a live-male tutor alongside passive audio playback as in London and Clayton (2008). Unlike some songbird species that can learn song from passive audio playbacks (e.g. Thorpe, 1958; Marler and Peters, 1988), zebra finches require either operantly-evoked playbacks or social instruction (Tchernichovski et al., 2001; Derégnaucourt, 2011; Deregnaucourt et al., 2013). We developed a tutor playback that combined passive audio playback alongside a live adult male. While operant playback has been used successfully to tutor zebra finches, we wanted to target the post-tutoring period with higher temporal precision. Operant training is pupil initiated and can span a long time period, whereas a controlled, timed playback allowed us to target the period immediately after training (i.e. the putative auditory memory consolidation period). To that end, we first identified a non-breeding adult male from our colony that was vocally active, and sang in the presence of an observer. The tutor was placed in a sound-attenuation chamber with an adult female and female-directed song was recorded, from which a 60-minute tutoring

playback file was created. The same tutor playback and adult male was used for all systemically-treated subjects, as well as several of the microdialysis subjects (*n* = 22). After the original tutor perished, a new adult male was recruited, and a similar one-hour tutor playback file was created and presented to the remainder of subjects (*n* = 15).

The tutor playback file consisted of a 12-minute clip with 40 unique song bouts that was repeated five times. Each song bout contained 2 - 8 motifs, and included introductory notes. The 12-minute clip was assembled from 12 individual 1-minute blocks, where each block contained 30 seconds of song (4 – 5 song bouts per song period, each separated by 5 seconds of silence) followed by 30 seconds of silence. The final tutoring playback file was amplified to ~70dB (A-weighted) and bandpass filtered at 0.3 – 15 kHz (Adobe Audition), and played through a portable speaker (Sony, model# SRS-TP1WHI) placed inside the sound-attenuation chamber.

The tutor was placed in an individual cage and kept in a sound-attenuation chamber with other adult zebra finches at least 24 hours before the day of tutoring. On the day of tutoring, an experimenter placed the tutor cage beside the pupil's cage. After a 10-minute acclimation period without any song playback, the tutoring playback recording began. Immediately after the end of the tutor playback file, the tutor was removed from the pupil's chamber.

Bioacoustic analysis

Automated song analysis

Percent similarity, accuracy, and % sequence similarity was analyzed using SAP (Tchernichovski et al., 2000). Ten motifs of the tutor song were each compared to ten motifs of each pupil's song from 130 dph using default settings for asymmetric mean values, yielding 100 comparisons per subject. Similar methods were used for measuring Weiner entropy (WE) and entropy variance (EV) across development in systemic subjects. As only half of the systemically-treated subjects produced song pre-tutoring (n = 3; 1 FAD subject and 2 saline subjects), we averaged pre-tutoring WE and EV across all subjects to compare with relative to 49 dph, which was the first day all subjects produced song.

Manual song similarity analysis

In addition to automated song similarity methods, we also measured the number of tutor syllables copied by each subject and the quality of each copy. Coded and randomized motifs were qualitatively analyzed on a syllable-by-syllable basis as being either 'good', 'poor', or 'not available' relative to the tutor song by three experimenters blind to treatment conditions and subject identification. We confirmed that raters agreed across multiple dimensions by performing inter-rater reliability measurements using an unweighted Fleiss's Kappa. Raters were in excellent agreement in assessing syllable accuracy (K = 0.563, $p < 0.001$), assessing the syllables pupils were likely imitating (K = 0.657, $p < 0.001$), and on the total number of syllables copied from a tutor by a pupil (K

= 0.455, $p < 0.001$). Moreover, raters' intra-reliability was similarity high: raters agreed on 60.46% of syllable accuracy, 65.12% on pupil syllables that reflect the tutor syllable, and 58.14% on both the accuracy and imitated syllable in the pupil's song. Further, raters' similarity scores were well-matched to the SAP measurements: there was a significant positive correlation between all raters visual similarity scoring and SAP's % similarity measurement ($r(97) = 0.75$, $p < 0.001$; ***Fig. 12C***).

Singing rate

An experimenter blind to treatment conditions measured the daily number of song bouts and their length for the entire pre-tutoring period (3 – 5 days pre-tutoring), tutoring period (10 days; tutor-off days), and every 5 days after the last day of tutoring until 130 dph (14 days). An individual song bout was defined as being at least 1 s in total duration and considered unique if 500 ms of silence elapsed between singing periods. Song bouts were analyzed for one 3-hour period per analyzed day (14:00 – 17:00). These methods were adapted from previous studies measuring song rate (Meitzen et al., 2007; Aronov et al., 2008; Meitzen et al., 2009; Alward et al., 2013).

Adult song plasticity

In a subset of birds ($n = 23$), we compared birds' own song at 130dph (putative closure of the critical period for song learning) and song after being exposed to other adult male song ($\geq$6 weeks post-130 dph return). We used simple qualitative measurements to assess whether song had changed (either

'yes' or 'no' based on visual comparisons of several song files from each time point) instead of more thorough bioacoustic analyses as treatment did not appear to affect the likelihood of changing adult song (see *Results*), which was the main question of the experiments.

Behavior

Female two-choice song phonotaxis

Female songbirds use song to evaluate a potential mate (Zann, 1996; Tomaszycki and Adkins-Regan, 2005; Holveck and Riebel, 2007). Therefore, in addition to measuring song similarity, we also tested whether less subtle song features were affected by treatment by measuring song preference in adult female zebra finches. A 13" x 10" cage was placed in the center of a sound-attenuation chamber alongside speakers set on either side of it. Three ground-level perches were placed in the left- and right-most extreme side of the cage floor. A piece of cardboard cage matting was placed on the cage floor and divided into quarters with colored tape: left, left of middle, right of middle, and right. A non-breeding adult female zebra finch from our aviary (N = 12) was placed in the two-choice cage and isolated for ~24 hours before the playback experiment began to increase salience of the future song playback. On Day 2, a 30 min song file was presented starting at ~13:00. The song file consisted of a 2-minute clip repeated 15 times. The first minute of the 2-minute clip contained adult song solely from one FAD or saline bird, whereas the second minute of the 2-minute clip contained song from only one bird of the opposite treatment

condition. Each 1-minute clip included 5 s of song, followed by 5 s of silence, which was repeated 4 times (40 s total), and followed by 20 s of silence (60 s total) played on one side of the speakers. The 1-minute clip of the second bird was broadcasted on the opposite speaker in a similar manner. The same 2-minute clip was repeated 15 times (30 mins total). On Day 3, a different playback file was played at a similar time (~13:00) with new song stimuli played on opposite speakers compared to Day 2 to account for potential side-bias (e.g. if FAD song was broadcasted on the left speaker on Day 2, a new FAD song was broadcasted on the right speaker on Day 3). Females were returned to the aviary after the cessation of Day 3 playbacks. Birds were excluded from analysis if they spent the entire time in the middle/neutral zone (one bird was excluded from analysis from both days, and another bird was excluded from just one day of analysis). Total time spent near either the FAD or saline side was measured. Additionally, a FAD preference ratio was calculated similar to Remage-Healey et al. (2010b):

$$FAD\ Preference\ Ratio = \frac{\sum time\ spent\ near\ FAD\ speaker}{\sum time\ spent\ near\ FAD\ speaker + \sum time\ spent\ near\ saline\ speaker}$$

Microdialysis tutoring session behavior

Pupils who are more ‘attentive’ to the tutor during song learning sessions produce more similar copies of the tutor song in adulthood (Chen et al., 2016). As such, we explored whether treatment affected pupils’ behavior during tutoring sessions. Subjects were videotaped for 3 one-hour periods during each tutoring day, including: 1) the hour just prior to tutoring onset; 2) the tutoring period (~70

mins; 10-minute acclimation period + 60 min audio playback); and 3) the hour immediately after tutor offset. Three 10-minute clips per tutoring period for each subject were created for future behavioral scoring, including: 1) tutor acclimation period; 2) the beginning of tutor playback; and 3) 20 – 30 mins into the tutor playback period. Videos were scored for numerous behaviors by an experimenter blind to subjects' treatment conditions using JWatcher (Blumstein and Daniel, 2007). Behaviors quantified included: events (eating; drinking; perch hops; grooming/preening; jumps; flights; feather ruffling; head scratching), and states (resting/sleeping; tutor zone; outside of tutor zone; not in view).

Electrophysiology

Surgery

As others have reported (e.g. London and Clayton, 2008), guide cannulae implanted during development eventually dissociate from the skull, and the wound heals normally (see *Methods*), which allowed us to perform electrophysiology recordings from formerly dialyzed birds in adulthood. Surgical methods for the electrophysiology experiments were similar to previous procedures (Remage-Healey and Joshi, 2012; Ikeda et al., 2015; Vahaba et al., 2017; Krentzel et al., 2018), the main difference being a lack of an implanted microdialysis probe in the current experiment. At least six weeks following birds being returned to the aviary (median age on day of surgery = 227 dph, range = 158 – 526 dph), birds were recaptured, placed in a cage with a companion adult female, and song was recorded. On the day of the surgery, birds were initially

food deprived for 30 minutes. Afterwards, an intramuscular injection of Equithesin was administered, and 20 minutes later, birds were swaddled in a Kim wipe, and placed atop a heating pad and secured via ear bars to a custom surgical stereotaxic apparatus (50° head angle). The bird's beak was opened and placed in a beak holder. Once the bird was secured, head feathers were removed, and a 20 µL subcutaneous injection of 2% lidocaine was administered underneath the scalp, which was subsequently resected to expose the outer layer of skull. The MSB was then identified and used as our 0-point anatomical reference. A positioning needle was placed over the MSB, and adjusted to bilaterally mark NCM (rostral: -1.4 mm; lateral: ± 1.1 mm) and HVC (lateral: ± 2.40 mm). A piece of silver wire was inserted between the skull leaflets over the cerebellum to serve as a reference ground. A custom-fabricated metal head-post was then affixed above the beak and skull using dental cement and cyanoacrylate, followed by sealing the exposed scalp with cyanoacrylate. After surgery, birds were placed on heating pad within a recovery cage and provided with *ad libitum* food and water. Once birds awoke, they were returned to their sound-attenuation chamber in a separate cage from the companion female.

Anesthetized extracellular recordings

Extracellular, multiunit electrophysiological recordings were obtained from NCM and HVC in anesthetized subjects (n = 21 birds [aCSF = 8 birds; FAD = 8 birds; cannula = 5 birds]; single-units x treatment x region: aCSF = 20 HVC units; 49 NCM units; FAD = 31 HVC units; 48 NCM units; cannula = 14 HVC units; 18 NCM units) using Spike2 (version 7.04, Cambridge Electronic Design) at a

sampling rate of 16.67 kHZ, bandpass-filtered at 0.3 – 5 kHz. On the day of the experiment, birds were starved for 30 mins, followed by three intramuscular injections of 20% urethane on alternating sides of pectoral muscle (~100 µL total; ~33 µL per injection). Injections were administered every 45 minutes. Once anesthetized, birds were brought up to the recording room, wrapped in a Kim wipe, placed on a heating pad, and affixed to a custom head-post stereotaxic apparatus. The outer- and inner-leaflet of skull and dura was then exposed over the HVC and NCM of one hemisphere. A drop of silicone oil was placed over the exposed brain to prevent the tissue from drying out. Individual carbon-fiber electrodes (CarboStar-1; Kation) were advanced into the proximate region of NCM and HVC based on: 1) anatomical location (~0.80 – 1.40 mm ventral; and ~0.50 mm ventral, respectively); and 2) characteristic spontaneous- and stimulus-evoked firing rates. In anesthetized adult songbirds, HVC preferentially responds to playbacks of birds' own song (BOS) (Margoliash, 1983, 1986). As such, we played BOS along with other songs in our search stimuli set (see below) and used a combination of characteristic spontaneous activity and neural responses to BOS as an indication of placement within HVC. After a completed playback trial, electrodes were advanced 100 – 150 µm dorsal/ventral along the same track, and, if the region-specific characteristic firing persisted, a new recording was obtained. Once a track was past anatomical limits and/or ceased to display characteristic firing patterns, an electrolytic lesion presented at the most recent site for future anatomical confirmation. After one hemisphere was complete, the contralateral hemisphere was exposed and recorded. At the end of

the experiment, birds were rapidly decapitated, and their brains were extracted and placed in a 20% sucrose-formalin solution for future sectioning and histology. In addition to recording from successful subjects (i.e. aCSF and FAD treated subjects), we also recorded from subjects that whose microdialysis cannulae became non-functional during the tutoring experiment. We present these data as a visual comparison as surgery controls (noted as 'Cannula' subjects) but due to the variability for microdialysis failure in these subjects, we omitted them from our statistical model.

Auditory stimuli and playback

All stimuli were adjusted to ~70 dB (A-weighted) and bandpass filtered to 0.3 – 15 kHz (Adobe Audition). Two sets of stimuli were used during the recordings. A *search* set was composed of two unique conspecific songs (i.e. zebra finch; CON), birds' own song (BOS), reverse BOS (REV-BOS), and white noise (WN). The *experimental* set was composed of two novel CONs, BOS, REV-BOS, tutor's song (TUT), reverse TUT (REV-TUT), and WN. Search stimuli were presented manually by the experimenter to confirm putative NCM and HVC sites, whereas the experimental set were played automatically and randomized via a custom written script in Spike. For experimental playbacks, each stimulus was pseudorandomly played once per block, with a total of 20 blocks being presented for each playback period. Stimuli were separated by a 10 s inter-stimulus interval ± 0 - 2 s of random time.

Single-unit spike sorting

Individual single units were sorted using default parameters in Spike2 (v.7.04, Cambridge Electronic Design; as in Vahaba et al., 2017). Units were retained for analysis if they: 1) were distinctly clustered in a principal component analysis space (apart from noise and other units); 2) had an interspike interval of > 1 ms; and 3) were auditory responsive by visual inspection of the peristimulus time histogram and raster plot.

Data analysis

Single-unit electrophysiology recordings were analyzed using similar methods as in Vahaba et al. (2017). Briefly, spontaneous firing rates were defined as the total number of waveform events (spikes) occurring in a 2-second period prior to the onset of an auditory stimulus, whereas stimulus-evoked firing was defined as the number of spikes during a 2-second window starting at the onset of an auditory stimulus. The total number of spikes per stimulus were divided by the number of stimulus iterations to yield firing rates (Hz). Firing rates were also z-transformed to normalize data and account for variability across subjects and units using the following equation:

$$Z\text{-}score = \frac{\bar{S} - \bar{B}}{\sqrt{Var(S) + Var(B) - 2Covar(S,B)}}$$

Where *S* and *B* represents the number of stimulus-evoked and spontaneous spikes, respectively; $\bar{S}$ and $\bar{B}$ represent the mean number of stimulus and spontaneous spikes for a given stimulus.

We also analyzed stimulus selectivity using d prime (d'; Green and Swets, 1966), a psychophysics metric of discriminability used for assessing neural responses to a given stimulus relative to a different stimulus (e.g. Bauer et al., 2008; Remage-Healey and Joshi, 2012; Moseley et al., 2017), using the following equation:

$$d'_{A-B} = \frac{2(RS[STIM_A] - RS[STIM_B])}{\sqrt{\sigma^2[STIM_A] + \sigma^2[STIM_B]}}$$

Where *RS* is the response strength (mean stimulus-evoked firing rate subtracted from the mean spontaneous firing rate), $STIM_A$ represents the focal stimulus of interest, $STIM_B$ represents the relative stimulus to compare other stimuli to, and σ^2 is the RS variance for a given stimulus. WN was used as the comparison stimulus for NCM recordings, and CON1 for HVC recordings (see *Results*).

Adult Habituation Experiment

Subjects

A separate set of otherwise untreated adult male zebra finches (*n* = 22) were removed from our single-sex aviary (median age on day of electrophysiology recording = 274 dph; all males at least 120 dph) and placed in a cage within a sound-attenuation chambers alongside an adult companion female while song was recorded using Sound Analysis Pro (Tchernichovski et al., 2000). Birds were kept in the same cage until the day of the surgery which typically occurred after 3 days of isolation (mode = 3 days isolation pre-surgery; range = 0 – 6 days).

Surgery

The surgery methods used for this experiment were nearly identical to the one above. The main difference was that the skull was exposed solely over both hemispheres of NCM. After bilateral marking of NCM (coordinates = rostral: -1.20 mm; lateral = +/- 1.10 mm), the outer and inner leaflets of skull were carefully removed, leaving the dura intact as much as possible. Following silver wire implantation, a silicone dural sealant (Kwik-Sil, World Precision Instruments [WPI]) was placed over the exposed skull.

Auditory Training & Drug Administration

Awake birds were placed in a custom-fabricated restraint tube and brought into the recording room. After being secured to the head-post stereotaxic apparatus, 200 iterations of a single adult male zebra finch song (two motifs within one song bout, including intro notes) was presented (TRAIN) with a 12 s ISI. Training lasted 46 minutes in total. Immediately after the last TRAIN iteration, ~100 nL of either artificial cerebrospinal fluid (aCSF) or 100 µM FAD in aCSF were locally administered via pre-loaded glass micropipettes broken back to ~24 µm internal diameter, which were left in place for >2 minutes following injection to prevent dispersal. This volume has been successfully used in previous studies and appears to disperse across the extent of NCM (Tremere et al., 2009; Remage-Healey and Joshi, 2012). Pipettes were successively descended ventral 1.10 mm in NCM, followed by pressure-injections (Pneumatic PicoPump, PV830; World Precision Instruments). Following drug treatments, the exposure was

sealed with a lower tear-strength silicone adhesive (Kwik Cast), cured, and then the bird was returned to his cage.

Electrophysiology

Awake, restrained birds were brought back to the recording room for electrophysiology recordings 6 or 20 hours after training. Birds were non-anesthetized as habituation is not typically observed in anesthetized songbirds (Remage-Healey et al., 2010b), but see (Ono et al., 2016). Parylene-coated tungsten electrodes (0.5 or 2MΩ; A-M Systems) were descended bilaterally into the approximate drug injection region from *Training*. Recordings were amplified using an A-M system amplifier and obtained through a connected 1401 board and Spike2 (CED). A set of stimuli were first presented to the bird *search stimuli* to confirm the recording site displayed NCM characteristic-like auditory responses. After site confirmation, experimental stimuli were presented to the bird while neural activity was continuously recorded. Each recording site was electrolytically lesioned following playback. Recording sites/exposures were once again covered with silicone adhesive, and birds were either sacrificed via rapid decapitation immediately after recordings (n = 8) or 2-3 days later (n = 15) to allow for lesion sites to become more pronounced and readily observable in sectioned tissue (e.g. allow time for gliosis). Extracted brains were placed in 20% sucrose-formalin for attempted future sectioning and histological verification of recording and drug site via Nissl stain.

Auditory Stimuli & Playback

All auditory stimuli were presented at ~70 dB. Search stimuli consisted of a unique set of non-familiar conspecific song not used in the experimental stimuli set. Experimental playback stimuli presented during neural recordings included the trained conspecific song (TRAIN) and its reverse (REV-TRAIN), three novel conspecifics (CON1, CON2, CON3) and one reversed (REV-CON3), bird's own song (BOS) and its reverse (REV-BOS), and white noise (WN). To ensure birds were unfamiliar with the song presented, several stimuli were graciously adapted from an online zebra finch song repository (http://people.bu.edu/timothyg/song_website/). We also used two songs from birds in our own breeding colony as they had been removed long before the experiment began. Birds were presented with 25 consecutive iterations of each experimental stimulus with a 12 second ISI in blocks (e.g. 25 CON1 playbacks, then 25 CON2 playbacks, then 25 WN playbacks, etc.), as in previous experiments (e.g. Yoder et al., 2012).

Analysis

Analyses were inspired from previous studies with minor changes (e.g. Yoder et al., 2012). Briefly, multi-unit recordings were analyzed root mean squared (RMS) in Spike2 for the stimulus and baseline period. The stimulus period included the entire duration of playback stimulus + 100 ms after offset, whereas the baseline period was defined as a 500 ms period preceding stimulus onset. Mean baseline RMS was derived across the entire recording period, whereas mean stimulus RMS was calculated for each individual stimulus. Data

were filtered on a per trial (i.e. stimulus repetition) basis. First, any trial exceeding two-times the mean RMS for either baseline or stimulus RMS (separately) was excluded. After, any trial above/below 2.5 standard deviations was then excluded for both stimulus and baseline RMS. Finally, a grand mean baseline RMS (derived from the entire recording period; across stimuli) was subtracted from stimulus RMS values, yielding an adjusted RMS. Slope was derived from trials 1 – 25 using the *lm*() function via the *stats* package in R.

Statistical analysis

All statistical analyses were performed using R (R Core Team, 2018) via RStudio (RStudio Team, 2016) using several packages, including: *tidyverse*; *plyr*; *sciplot*; *irr*; *corrplot*; *data.table*; and *Hmisc*. Histology data (% DAPI; cell density) were analyzed using a two-way ANOVA (NCM subregion X phase). Singing rates were analyzed using a two-way ANOVA (treatment X time of day). Pearson's correlation was used to analyze changes in Weiner entropy relative to eventual song similarity at 130dph. One-way ANOVAs were employed to assess systemic treatments effect on song learning outcomes (separate analyses for per cent similarity, sequential similarity, and accuracy). Female phonotaxis data were analyzed using two-way ANOVAs (treatment X trial day). For microdialyzed subjects, automated and manual song similarity analyses were analyzed using a two-way ANOVA (treatment X hemisphere). Inter-rater reliability for manual song similarity scoring was analyzed using an unweighted Fleiss's kappa. The comparison between automated (SAP) and manual (visual) song similarity was

measured using Pearson's correlation. Tutoring behavior was analyzed using a mixed-effects ANOVA (tutoring day [within] X treatment [between]), and a correlation matrix adjusted for multiple comparisons (adjusted $\alpha = 0.00048$). For behavioral analyses, we restricted our data to the first 10 minutes of tutoring for only days 1 and 2 to be consistent as some subjects received three days of tutoring. A chi-squared was used to compare distributions of adult song plasticity across treatment. For electrophysiology measurements, a three-way ANOVA was employed (treatment X recording hemisphere X stimulus). Finally, for adult habituation neural recordings, adaption slopes were compared using two-way ANOVAs (treatment type [aCSF/nothing vs. FAD] X stimulus type [familiar vs. trained]). All *post hoc* comparisons were performed using Tukey's honestly significant difference (HSD) test. *P*-values < 0.05 were considered significant. Data from 'cannula' subjects were omitted from any statistical model and are plotted throughout the manuscript as a visual comparison (see *Results*).

Results

Cell density is region- and age-dependent in developing auditory forebrain while aromatase and parvalbumin expression are unchanging

We first sought to confirm the presence of aromatase in NCM across development. While previous studies have characterized aromatase expression developing songbird brains, both directly (protein: Saldanha et al., 2000) and indirectly (Vockel et al., 1988; Jacobs et al., 1999; Saldanha et al., 1999; Chao et al., 2015), information on aromatase protein expression specifically within NCM

between the sensory and sensorimotor phase of the song learning period has not been assessed, to our knowledge. In addition to aromatase, we were also curious as to whether transitions between learning phases were associated with differences in expression of the calcium buffering-protein parvalbumin. Parvalbumin is a a marker for a unique subpopulation of inhibitory interneurons (Tremblay et al., 2016), is co-localized with aromatase in NCM (Ikeda et al., 2017), and its presence often denotes changes in critical period plasticity within mammalian visual cortex (Hensch, 2005), as well as songbird song circuits (Balmer et al., 2009). We focused solely on males as they were the sex of interest for subsequent song and physiology experiments in this study. Although we collected both hemispheres of NCM for this experiment, we excluded hemisphere as a factor in our statistical model as we were underpowered. Qualitatively, we found similar expression of aromatase and parvalbumin across both hemispheres of sensory- and sensorimotor-aged subjects (see ***Tables 1 & 2***).

We divided our subjects into two age groups reflecting the two different developmental song learning phases: sensory- and sensorimotor-aged (20-34 and 65-71 dph, respectively). Overall, our density measures revealed comparable aromatase, parvalbumin, and aromatase-parvalbumin co-expression in both dorsal and ventral NCM across development (aromatase: $F_{(1, 31)} = 2.458$, $p = 0.127$; parvalbumin: $F_{(1, 31)} = 0.035$, $p = 0.854$; aromatase-parvalbumin: $F_{(1, 31)} = 0.003$, $p = 0.957$), age (aromatase: $F_{(1, 31)} = 2.218$, $p = 0.147$; parvalbumin: $F_{(1, 31)} = 0.277$, $p = 0.602$; aromatase-parvalbumin: $F_{(1, 31)} = 0.339$, $p = 0.565$), without

any significant interactions between age and hemisphere (aromatase: $F_{(1, 31)}$ = 0.048; parvalbumin: $F_{(1, 31)}$ = 0.751; aromatase-parvalbumin: $F_{(1, 31)}$ = 0.757; p > 0.3 for all tests; ***Table 1***).

Phase	*Subregion*	*Hemisphere*	*Subjects*	*Images*	*Aromatase (mm^3) ± SEM*	*Parvalbumin (mm^3) ± SEM*	*Aromatase-Parvalbumin (mm^3) ± SEM*	*DAPI (mm3) ± SEM*
Sensory	dNCM	L	2	3	153.45 ± 8.22	15.74 ± 3.55	14.26 ± 6.16	572.50 ± 58.36
		R	3	4	159.36 ± 14.34	13.65 ± 1.74	9.59 ± 3.04	553.32 ± 24.90
	vNCM	L	2	3	139.68 ± 5.48	14.26 ± 1.30	21.15 ± 7.09	467.25 ± 86.08
		R	3	5	146.08 ± 14.10	17.71 ± 2.09	10.33 ± 4.64	455.64 ± 44.55
Sensorimotor	dNCM	L	3	6	139.93 ± 13.44	17.46 ± 2.10	10.33 ± 2.98	467.74 ± 14.49
		R	2	4	149.03 ± 16.82	15.86 ± 1.52	15.86 ± 5.12	469.22 ± 50.22
	vNCM	L	3	6	119.76 ± 12.44	14.26 ± 2.31	10.08 ± 1.92	418.80 ± 28.69
		R	2	4	135.38 ± 21.69	18.44 ± 2.29	10.33 ± 4.39	393.96 ± 44.88

Table 3: Density measures for antibody staining in developing NCM.

Values represent mean cell density (mm^3) +/- the standard error of the mean.

Phase	*Subregion*	*Hemisphere*	*Subjects*	*Images*	*Aromatase*	*Parvalbumin*	*Aromatase-Parvalbumin*
Sensory	dNCM	L	2	3	27.08640214 ± 1.43	2.791449126 ± 0.7	2.503478983 ± 1.1
		R	3	4	29.22530938 ± 3.61	2.480539556 ± 0.3	1.705615841 ± 0.5
	vNCM	L	2	3	32.0912762 ± 6.19	3.180484319 ± 0.4	4.210794372 ± 0.9
		R	3	5	32.68685651 ± 2.68	4.0846586 ± 0.7	2.177982138 ± 1
Sensorimotor	dNCM	L	3	6	29.8562547 ± 2.48	3.699471363 ± 0.3	2.215532402 ± 0.6
		R	2	4	32.20574987 ± 2.96	3.530142779 ± 0.6	3.130796798 ± 0.8
	vNCM	L	3	6	28.28308721 ± 1.43	3.470999878 ± 0.6	2.458244997 ± 0.5
		R	2	4	35.01997611 ± 5.26	4.734797837 ± 0.4	2.400053955 ± 1

Table 4: Protein expression relative to cell density (% DAPI).

Values represent mean number of immunopositive-cells relative to the number of DAPI+ neurons (% DAPI) +/- the standard error of the mean.

Interestingly, we observed a significantly higher DAPI expression in dorsal NCM compared to ventral NCM ($F_{(1, 31)} = 8.128$, $p = 0.008$), as well as higher DAPI expression in sensory-aged animals compared to sensorimotor-aged subjects ($F_{(1, 31)} = 6.291$, $p = 0.018$; ***Fig. 9C,D***). No significant interactions emerged between region and age ($F_{(1, 31)} = 0.587$, $p = 0.449$). Similar results were found when we normalized counts for the markers of interest (aromatase and parvalbumin) to the relative amount of DAPI to account for subject and image variability (***Fig. 9A,B***; see ***Tables 3 & 4*** for all descriptive data for density and % of DAPI measurements). Overall, these findings confirm that aromatase and parvalbumin are present in the developing auditory forebrain, and that NCM appears to undergo cellular pruning as birds develop while maintaining subregion differences in cell density.

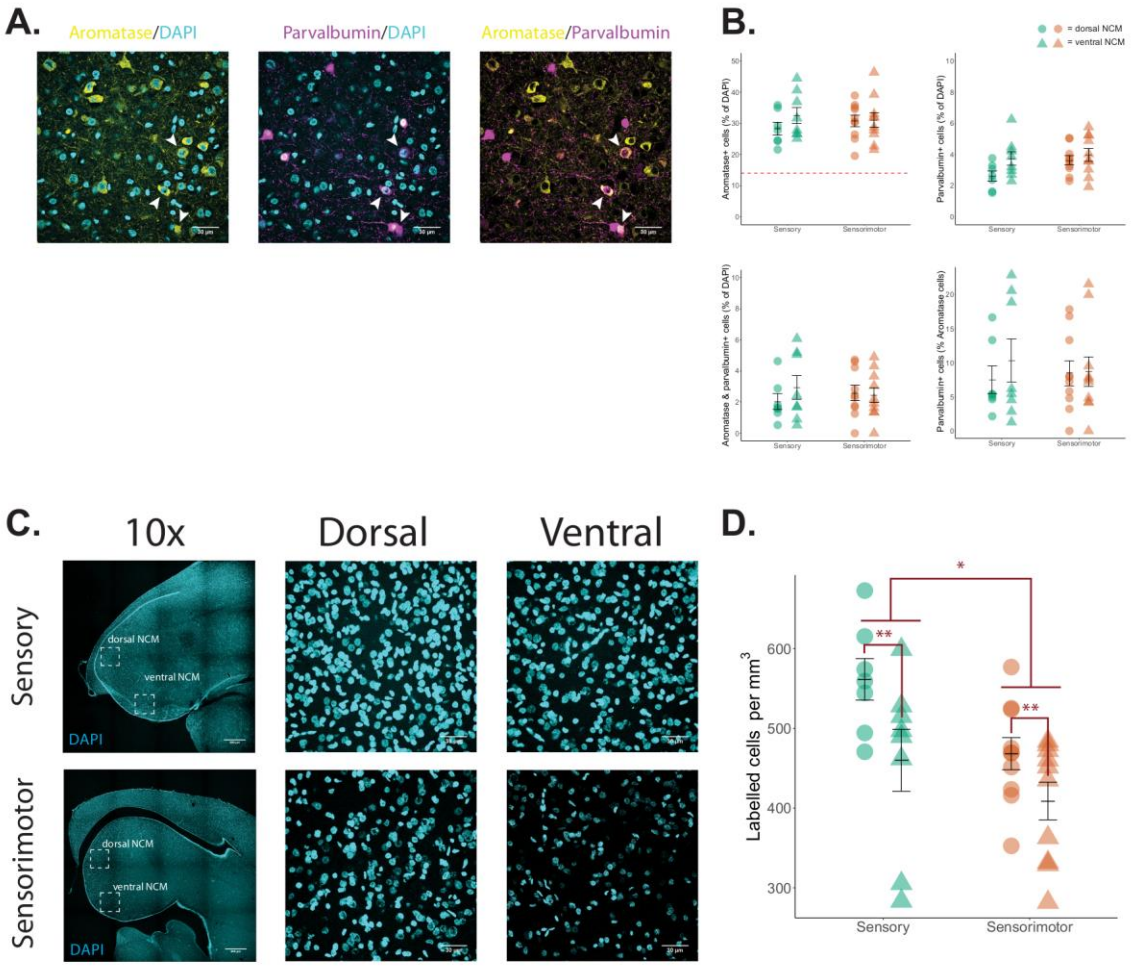

Figure 9: Changes in neuronal density and aromatase and parvalbumin expression in NCM across development.
A, Aromatase, parvalbumin, aromatase parvalbumin co-expression, respectively, from an exemplar sensory-aged male bird (26 dph; right hemisphere; ventral NCM). Pseudo-colored: yellow, aromatase; cyan, DAPI; magenta, parvalbumin. Each image from a single slice of a z-stack taken at 60x magnification. Scale bar = 30 µm. White arrowheads indicate aromatase and parvalbumin co-expression. B, Expression of aromatase, parvalbumin, and aromatase/parvalbumin co-expression, respectively, relative to the expression of DAPI (%), and parvalbumin co-expression relative to total aromatase expression (%). Overall, there are no significant differences in expression by age or NCM subregion. Circles = dorsal NCM; triangles = ventral NCM; green = sensory-aged birds; orange = sensorimotor-aged birds. C, DAPI expression across development; top row: sensory-aged bird (25 dph; right NCM); bottom row: sensorimotor-aged bird (71 dph; right NCM). 10x images taken from a 4 x 4 stitched image. Dorsal and ventral NCM images taken from a z-project max intensity 60x image. D, Cell density (DAPI expression) by region and age. Dorsal NCM shows higher cell density than ventral NCM. Similarly, sensory-aged birds have higher overall cell density across subregions compared to sensorimotor-aged subjects. * = $p < 0.05$; ** = $p < 0.001$.

Song learning is unaffected by global estrogen synthesis inhibition during development

Systemic administration: Birds in this experiment received an oral administration of either FAD or saline every other day for 20 days immediately following tutoring. Initially, we measured singing rates of systemically-treated animals before (<40 dph) and during the tutoring period (40 – 60 dph) as global inhibition of estrogen synthesis in adult songbirds reduces song production (Alward et al., 2016b). Pre-tutoring, birds sang at comparable rates independent of the time of day or future treatment group (treatment: $F_{(1, 13)} = 2.466$, $p = 0.140$; time of day: $F_{(1, 13)} = 1.797$, $p = 0.203$; treatment * time of day: $F_{(1, 13)} = 0.719$, $p = 0.412$; ***Fig. 10A,B***). However, during the tutoring period, FAD treatment significantly suppressed singing rates (FAD = 63.8 ± 13.6 bouts; saline = 116.0 ± 14.4 bouts; $F_{(1, 103)} = 6.623$, $p = 0.012$; Tukey's HSD: $p = 0.012$) independent of time of day ($F_{(1, 103)} = 0.\ 222$, $p = 0.\ 639$) or an interaction between time of day and treatment ($F_{(1, 103)} = 1.882$, $p = 0.173$; ***Fig. 10A,B***). Interestingly, while initial song production was reduced during development, eventual song similarity at 130 dph (one-way ANOVA (treatment); $F_{(1, 4)} = 0.064$), accuracy ($F_{(1, 4)} = 0.021$) , and sequential similarity ($F_{(1, 4)} = 0.095$) were statistically similar when both FAD and saline subjects reached adulthood ($p > 0.77$; ***Fig. 10D & Table 5***). Additionally, while FAD birds appeared to exhibit a lower tutor song similarity score early in development (49 dph), there was no effect of treatment ($F_{(1, 4)} = 0.427$, $p = 0.549$), nor an interaction of treatment with age ($F_{(4, 16)} = 0.569$, $p = 0.689$). There was, however, a significant increase in song similarity as birds

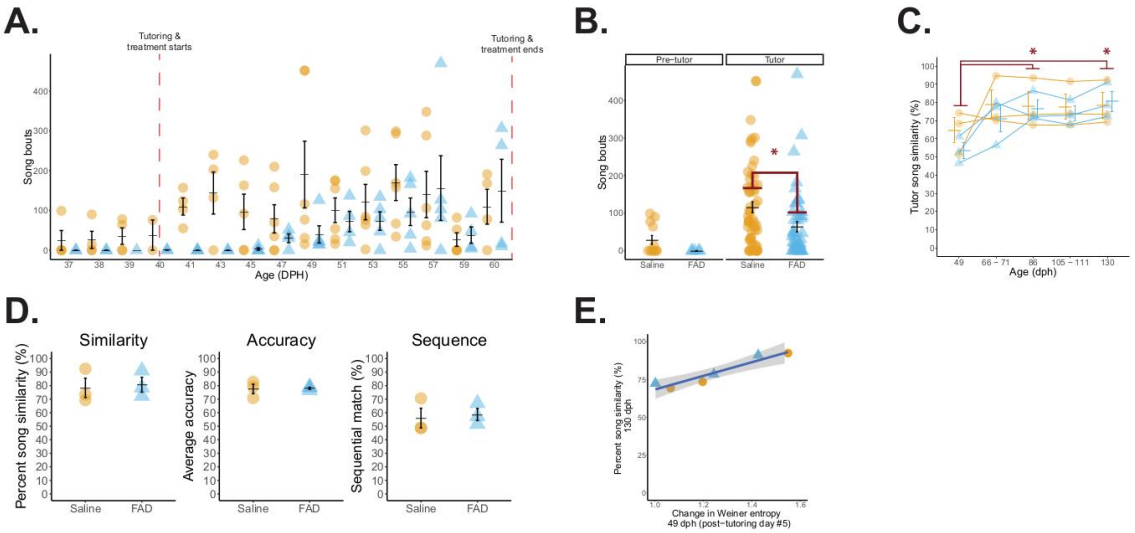

Figure 10: Systemic estrogen synthesis inhibition suppresses song production without impacting tutor song copying.
A, Daily number of song bouts before and across the tutoring/treatment period. **B**, Birds sing at similar rates before treatment/tutoring; however, systemic FAD treatment reduces song production (p = 0.012). Circles/orange = saline-treated birds (n = 3); triangles/blue = FAD birds (n = 3). **C**, Song similarity is lowest at 49 dph despite treatment (effect of age: p = 0.005; * is relative to 49 dph). **D**, At 130 dph, tutor song similarity, accuracy, and sequence similarity, respectively, are all similar across treatments. **E,** Change in Weiner entropy at 49 dph (post-tutoring day #5) predicts eventual percent song similarity to the tutor at 130 dph, independent of treatment (r^2 = 0.903*; p* = 0.004). * = $p < 0.05$.

reached adulthood (age: $F_{(4, 16)} = 5.528$, $p = 0.005$; *post-hocs*: $p < 0.05$ for 49 dph vs. 86 & 130 dph; all other age comparisons non-significant, $p > 0.06$; ***Fig. 10C***). Together, these data show that global estrogen synthesis is required for song production and does not impact eventual tutor song imitation.

Developmental changes (relative to pre-tutoring values) in Weiner entropy (WE) and entropy variance (EV) during tutoring predict adult tutor song fidelity (Deshpande et al., 2014). Independent of treatment, we tested this relationship for birds in the present experiment to assess whether they developed along a 'typical' song learning trajectory. In agreement with the previous report, we found a strong, significant positive correlation between change in WE at 49 dph and

Method	*Treatment*	*Hemisphere*	*Subjects*	*Similarity*	*Accuracy*	*Sequential match*
Systemic	Saline	-	3	78.38 ± 7.13	77.52 ± 3.52	56.01 ± 7.28
	FAD	-	3	80.66 ± 5.50	78.04 ± 0.79	58.64 ± 4.47
Microdialysis	aCSF	Left	5	60.71 ± 9.19	74.40 ± 2.07	60.57 ± 3.04
		Right	5	63.51 ± 7.53	71.69 ± 2.31	64.17 ± 4.47
	FAD	Left	5	52.79 ± 9.22	75.74 ± 1.07	68.20 ± 9.04
		Right	5	54.15 ± 9.13	74.43 ± 1.37	69.99 ± 8.91
	Cannula	Left	3	49.12 ± 15.34	69.32 ± 0.91	72.31 ± 14.86
		Right	4	64.50 ± 9.70	71.04 ± 1.80	51.50 ± 4.92
	Isolate	Left	1	24.56 ± -	68.40 ± -	85.01 ± -

Table 5: Automated song similarity measurements.
Values represent mean +/- the standard error of the mean for each song similarity metric.

percent song similarity in adulthood (130dph); $r(4) = -0.951$, $p = 0.004$, as well as a similar significant correlation when we considered entropy variance instead of WE ($r(4) = 0.863$, $p = 0.027$; ***Fig. 10E***). Therefore, while systemic FAD treatment did not impact song learning, developing song was predictive of eventual similarity, indicating that our daily treatment regimen did not impair a 'normal' song learning trajectory.

Female phonotaxis behavior: While song similarity data can provide information on how well a bird imitates a model song, there are likely subtle song features that are affected by early-life manipulations that may not be captured by automated analyses. As adult female zebra finches use courtship song to evaluate potential life-long mates (Zann, 1996), we asked whether a females' song preference was impacted by a males' drug treatment during development. We found a significant interaction between treatment and trial day ($F_{(1, 17)} = 7.30$,

p = 0.151). Follow-up analyses revealed that on the first day of phonotaxis, females spent more time near the speaker broadcasting a FAD-treated birds' song (p = 0.015), whereas on the second day there was nonsignificant tendency for preferring control birds' song (p = 0.059; ***Fig. 11A***). We also evaluated a 'FAD preference ratio' for day 1 vs. day 2. Visually, it appears that females initially prefer FAD song and then 'switch' preferences on day 2, but this was not statistically significant ($F_{(1, 8)}$ = 2.958, p = 0.124; ***Fig. 11B***). We also confirmed that there was no overall side bias (p = 0.0989) nor inherent preference for a specific male's song independent of treatment (p = 0.557). Thus, systemic estrogen synthesis blockade during development did not negatively impact song features important for eventual female mate-choice selection.

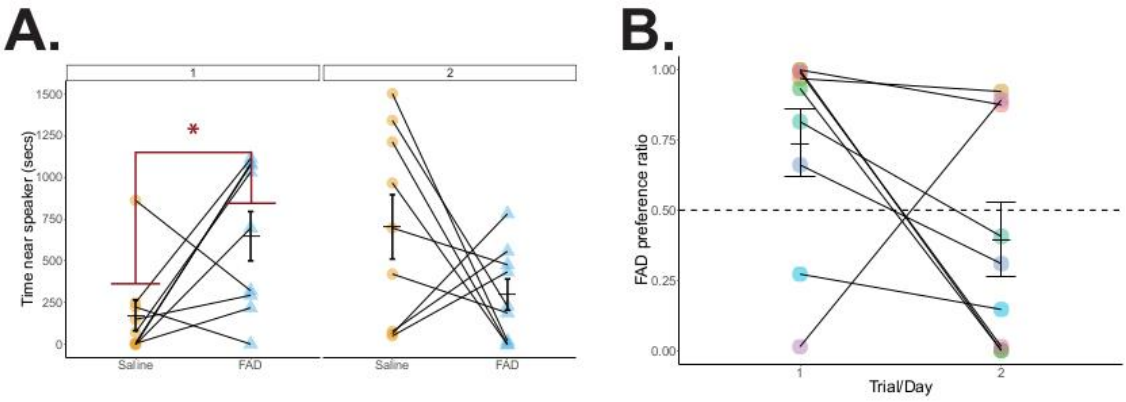

Figure 11: Female songbirds temporarily prefer E2-suppressed adult song. **A**, Adult female songbirds spend more time near a speaker broadcasting adult song from systemic E2 suppressed males on day 1, but not 2, in a two-day phonotaxis experiment (p = 0.015). **B**, Preference ratios for FAD song relative to control song is similar across days. * = p < 0.05.

Song learning is unaffected by inhibition of local estrogen synthesis in NCM during development

In vivo *microdialysis with social + playback tutoring*: Systemic treatments yielded no effect of aromatase blockade, but leaves open the possibility that

temporally-precise, site-directed manipulations within NCM may yield changes in auditory memorization. As with systemically-administered subjects, central unilateral FAD treatment did not modify eventual tutor imitation, nor did the cannulated hemisphere or interaction between treatment and hemisphere affect percent similarity ($F_{(1, 16)}$, treatment = 0.965; hemisphere = 0.056; treatment * hemisphere = 0.007; $p > 0.340$), accuracy ($F_{(1, 16)}$, treatment = 1.325; hemisphere = 1.277; treatment * hemisphere = 0.157; $p > 0.266$), or sequence similarity ($F_{(1, 16)}$, treatment = 0.950; hemisphere = 0.153; treatment * hemisphere = 0.017; $p > 0.343$; ***Fig. 12A & Table 5***). Therefore, contrary to our original prediction, unilateral central estrogen synthesis blockade in NCM did not impair tutor song memorization and eventual imitation.

Manual song similarity quantification: Whole motif similarity measurements via SAP is the conventional method to objectively analyze tutor similarity for zebra finches (Tchernichovski et al., 2000). Inspection of spectrograms suggested that SAP similarity measurements were not capturing the full extent of tutor song similarity (***Fig. 12B***: high % SAP song similarity for *Cannula* subject, but visually and acoustically dissimilar; opposite issue with *aCSF* subject). To address this, we employed visual song similarity measures in the spirit of early songbird bioacoustic research studies that relied solely on visual spectrographic assessment (Borror and Reese, 1953; Thorpe, 1954; Eales, 1985). In accordance with this match between SAP and when visual scoring methods, there were no significant effects for visually-scored song similarity (average percent copied) by cannulated hemisphere ($F_{(1, 16)} = 0.227$, $p = 0.640$), treatment

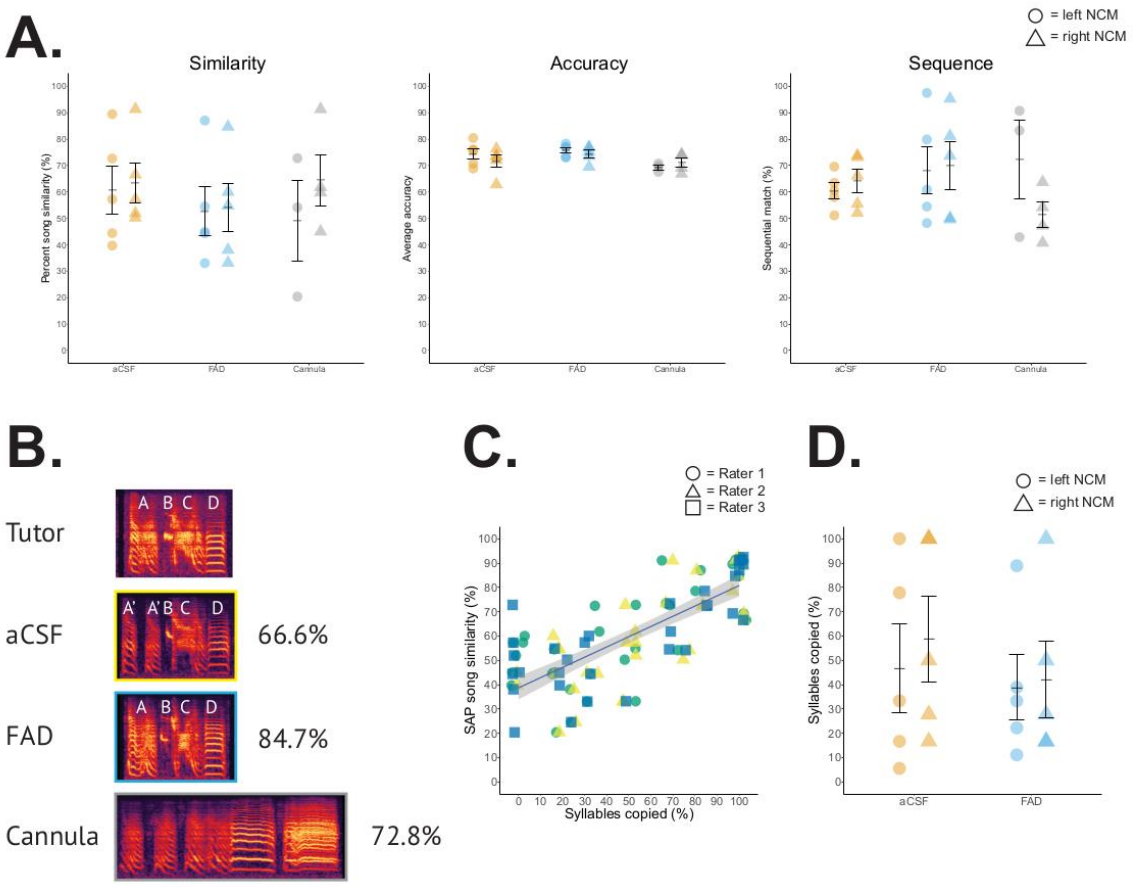

Figure 12: Song copying is unaffected by central estrogen production inhibition via *in vivo* microdialysis.
A, 130 dph song similarity, accuracy, and sequence similarity, respectively, are all comparable across aCSF- and FAD-treated birds. Cannula 'surgery controls' are graphed for visual comparison. Orange = aCSF; blue = FAD; grey = cannula; circle = left NCM; triangle = right NCM. **B**, Example song spectrograms and their average song similarity % relative to tutor. Letters denote syllables; A' = partial syllable derived from A. Note the seemingly high similarity of both the aCSF and FAD motif, yet divergent song similarity scores (aCSF bird = right NCM; FAD bird = right NCM; similarity score is averaged across 100 motif comparisons, see *Methods*). **C**, Manual song similarity measurements are strongly correlated with automated methods; color/shape denotes unique rater (r^2 = 0.563, p < 0.001); jitter added to reveal overlap. **D**, As with automated methods, manual song similarity scoring reveals comparable tutor song copying across treatments.

($F_{(1, 16)}$ = 0.561, p = 0.465), nor an interaction between either factor ($F_{(1, 16)}$ = 0.074, p = 0.789; ***Fig. 12D***). Therefore, irrespective of bioacoustic assessment, unilateral blockade of neuroestrogen production in the auditory forebrain during

and immediately after song learning did not impair auditory memorization of the tutor song.

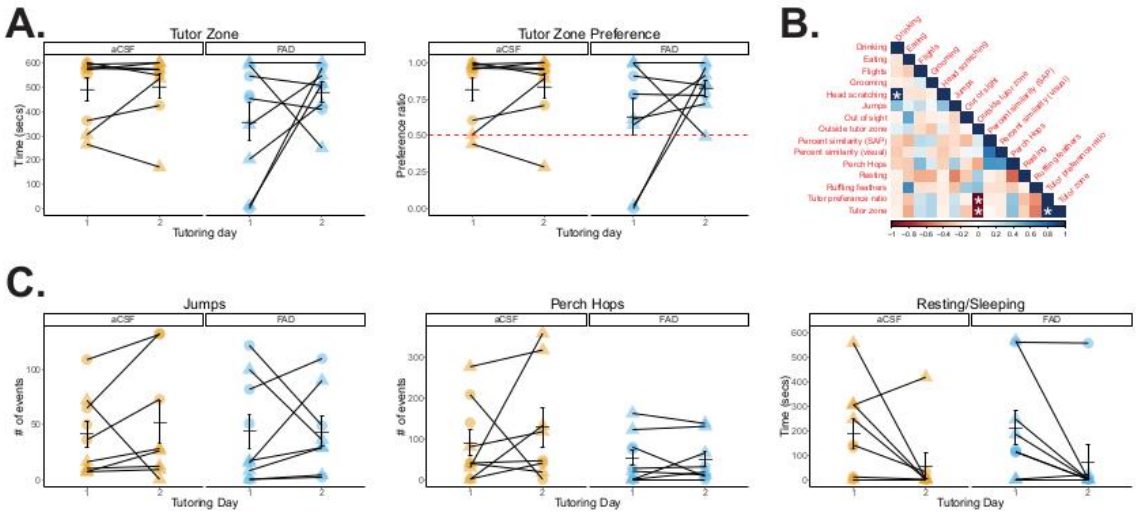

Figure 13: Juvenile male songbirds are similarly attentive to the tutor during microdialysis.
The time a bird spent near a live adult male tutor during *in vivo* microdialysis is similar across treatments, targeted hemispheres, and tutoring day. **A**, Behavior presented is from the first 10 minutes of song playback alongside live male presentation (see *Methods*). Orange = aCSF; blue = FAD; circle = left NCM; triangle = right NCM. Tutor preference ratios are similar across treatments. **B**, Correlogram of tutoring behavior and song similarity measurements reveal significant correlations (more time spent near the tutor negatively associated with time spent away from the tutor; tutor zone time positively correlated with tutor preference ration), and novel findings (positive correlation of head scratching and drinking); $p < 0.0005$ (adjusted α; Bonferroni correction). Behavior data presented is from the first 10 minutes of tutor playback across days 1 and 2 of tutoring. **C**, Motor activity is statistically similar across treatment and tutoring days.

Tutoring behavior: Attention plays a critical role for vocal learning early in development (e.g. Chen et al., 2016). Since estrogens can modulate attention in rodents (see references in Sommer et al., 2018) we explored whether FAD treatment impaired measures of attention during tutoring sessions in a subset of subjects (FAD *n* = 9; aCSF *n* = 9). Overall, we found no effect of treatment on the amount of time pupils spent near the tutor ('tutor zone'; a proxy for tutor attention)

on either tutoring day ($F_{(1, 14)}$; $p > 0.190$ for main effects and interaction; ***Fig. 13A***). The time spent near the tutor is one obvious behavior to explore with clear predictions about its impact on eventual song copying. However, as there are not many quantitative data to our knowledge on pupil behavior during tutoring, we also explored whether the other behaviors we scored might also be predictive of future tutor song similarity. We generated a correlogram that included all tutor session behaviors, as well as song similarity measurements (both visual and SAP derived). Overall, there were few significant correlations of interest pertaining to song similarity and behavior that emerged (***Fig. 13B***).

Another possibility is that FAD treatment may impair locomotion. We explored whether two common motor behaviors (jumping and perch hopping), as well as time spent resting/sleeping were affected by pharmacological exposure. Overall, neither treatment nor tutoring day affected jumping or perch hops ($F_{(1, 14)}$; $p > 0.158$ for main effects and interaction; ***Fig. 13C***); however, birds spent more time resting irrespective of treatment on the second day of tutoring, suggesting that the novelty of an adult male wanes after the first session ($F_{(1, 14)} = 7.938$, $p = 0.0137$; all other analyses $p > 0.808$; ***Fig. 13C***). These results suggest that as with song similarity, behavior during a social learning session is similarly unaffected by unilateral central neuroestrogen synthesis blockade.

Song changes after exposure to adult male conspecifics: We noticed highly aberrant song types in several formerly microdialyzed subjects independent of treatment at 131 dph (X^2 ($N = 23$) = 1.189, $p = 0.552$), which is well beyond the putative 'closing' of the critical period for song learning and song

should be highly stable (***Fig. 1***4). Aberrant songs were always highly variable (i.e. not crystallized/stereotyped) at 130 dph and resulted in high stereotypy after being exposed to other adult male birds, and typically involved dropping and/or adding new syllables (6/8 subjects added, dropped, or modified syllables). These results suggest that, in addition to age, experience gates the song learning critical period closure, which has been described in other studies on lab-reared tutored and isolate zebra finches (Eales, 1985; Morrison and Nottebohm, 1993; Jones et al., 1996; Deregnaucourt et al., 2013).

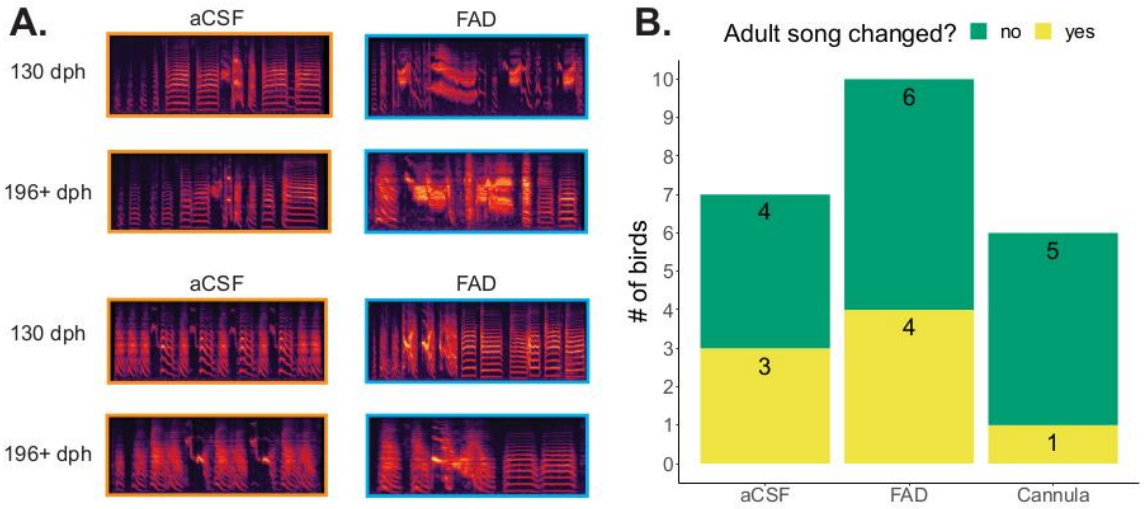

Figure 14: Song changes in formerly microdialyzed subjects after exposure to adult male song at 130 dph.
A, Spectrogram examples from two aCSF and two FAD microdialysis subjects. *Top row*: song at 130dph; *Bottom row*: song at 6 weeks+ 130dph after subjects were exposed to conspecific adult males. **B,** Histogram demonstrating that treatment had no bearing on whether microdialysis subjects altered their adult song after exposure to conspecific males.

Neuroestrogen suppression in development leads to enhanced neural representations of birds' own song and tutor song in HVC of adults

In a subset of formerly microdialyzed birds (21 out of 28 birds), we obtained neural recordings from two brain regions associated with song learning

and tutor memory representation: NCM and HVC. Relative to the implanted cannula site, recordings were obtained from both the contralateral and ipsilateral hemisphere to the site of microdialysis cannulation (i.e. contralateral = recording from non-dialyzed hemisphere; ipsilateral = recording from dialyzed hemisphere).

NCM: We first explored whether treatment impacted NCM firing properties. Spontaneous firing rates were unaffected by recording hemisphere, treatment, and there was no interaction between the two factors ($F_{(1, 93)}$ = 0.238, 0.003, and 0.779, respectively; $p > 0.60$; ***Fig. 15A***). Contrary to spontaneous firing, stimulus-evoked firing was significantly affected by a recording hemisphere x treatment interaction ($F_{(1, 651)} = 7.938$, $p = 0.005$) as well as there being a main effect for treatment ($F_{(1,6)} = 4.334$, $p = 0.038$) and stimulus ($F_{(6, 651)} = 7.670$, $p < 0.001$). Follow-up analyses revealed that the stimulus effect was driven mainly by an overall lower response to WN (WN < BOS, CON1, CON2, and REV-BOS), and a higher response evoked by CON1 (CON1 > REV-TUT; Tukey's HSD, $p < 0.02$ for all stimulus comparison; ***Fig. 15B***). To avoid pseudo-replication (Picciotto, 2018), and because of the main effect of stimulus, we opted to perform follow-up analyses on just CON1 data for NCM. Follow-up analyses did not yield any significant differences between recording hemispheres for stimulus-evoked firing in FAD-treated ($F_{(1, 46)} = 0.513$, $p = 0.478$) nor aCSF-treated subjects ($F_{(1,47)} = 0.734$, $p = 0.396$).

While raw firing rate data are informative, it is also useful to consider normalized auditory response rates (*z*-score) which accounts for recording site variability in spontaneous and stimulus-evoked activity (e.g. Vahaba et al., 2017).

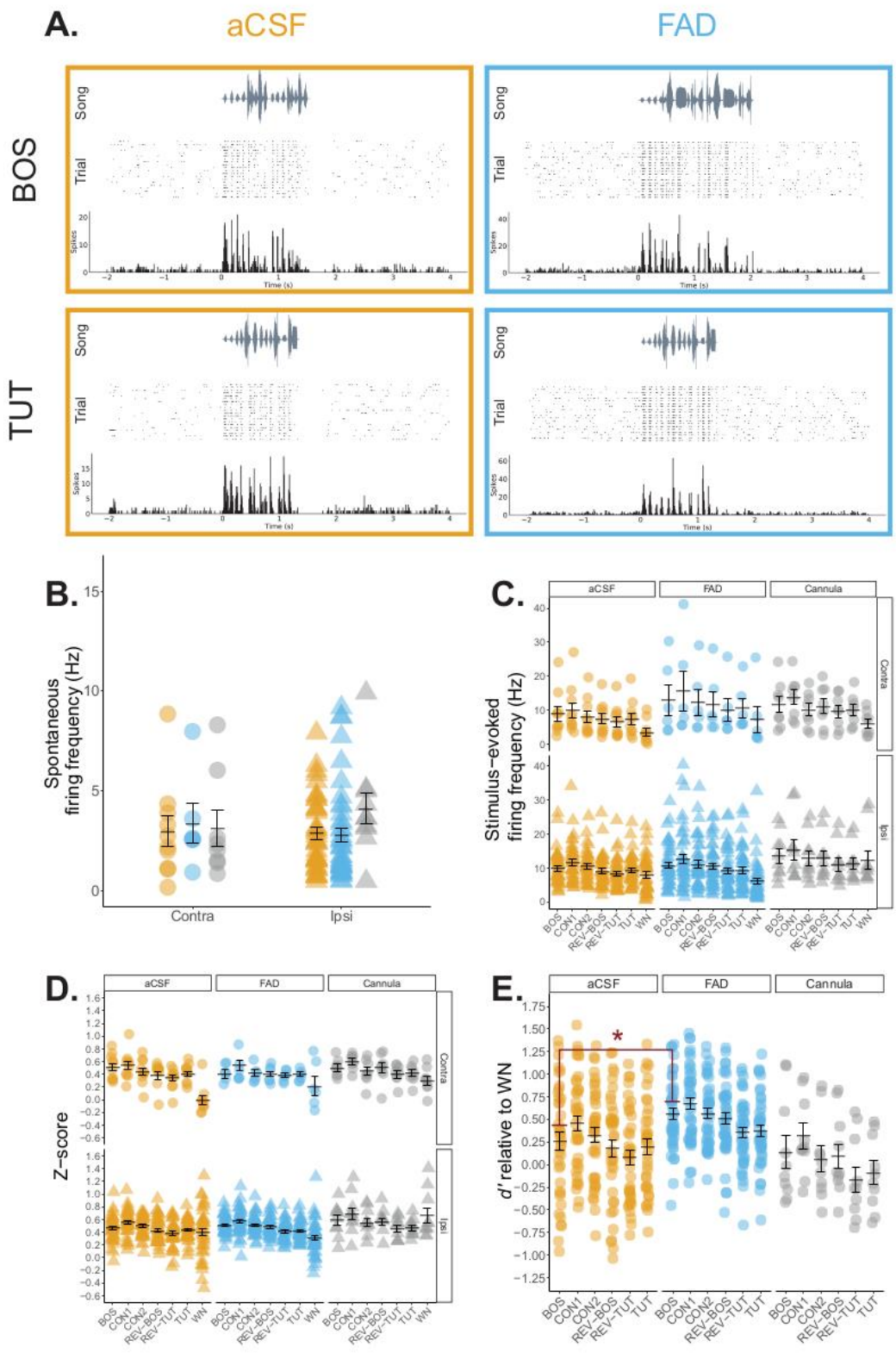

Figure 15: Single-unit recordings in NCM reveal modest differences in auditory responses in adulthood.

Representative NCM single-unit recordings from an aCSF and FAD in response to presentations of birds' own song (BOS) and tutor song. Each recording includes a song spectrogram (*Top*), and raster plot (*Middle*) with corresponding peri-stimulus time histogram in 10 ms bins (*Bottom*) across a 6 second period. The same unit is presented for each treatment across the two stimuli. **B,** Spontaneous firing rates were unaffected by developmental microdialysis treatment. Orange = aCSF; blue = FAD; grey = cannula; circle = contralateral hemisphere (relative to microdialysis site); triangle = ipsilateral hemisphere (relative to microdialysis site). **C**, Stimulus-evoked firing rates were significantly lower for WN and overall higher for CON1. A recording hemisphere x treatment interaction was significant; however, post hoc analyses limited to CON1 found no statistical differences for either treatment. **D**, Analysis of normalized auditory response (z-score) yielded a significant stimulus x recording hemisphere effect: contralateral NCM responded less to WN compared to all other stimuli, whereas forward conspecific stimuli elicited higher responses in the ipsilateral NCM, irrespective of treatment. **E,** Ipsilateral *d'* values relative to WN. BOS selectivity was higher in FAD songbirds in the ipsilateral hemisphere. BOS = birds' own song; CON1; CON2 = conspecific song; REV-BOS = reverse bird's own song; REV-TUT = reverse tutor song; TUT = tutor song. * = $p < 0.05$.

Analyses revealed a significant main effect of stimulus ($F_{(6, 651)} = 17.643$, $p < 0.001$) and recording hemisphere ($F_{(1, 651)} = 12.935$, $p < 0.001$), as well as a significant interaction between stimulus and recording hemisphere ($F_{(6, 651)} = 3.051$, $p = 0.006$; ***Fig. 15C***). In contralateral NCM, WN elicited a significantly lower z-score compared to all other stimuli ($p < 0.001$ for all stimulus comparisons). In contrast, z-scores were typically higher for non-reversed conspecific stimuli in the ipsilateral hemisphere regardless of treatment (CON1 > REV-BOS, REV-TUT, TUT, and WN; BOS > REV-TUT and WN; CON2 > REV-TUT and WN; $p < 0.05$ for all stimulus comparisons). Overall, the results in NCM suggest that irrespective of treatment, forward, conspecific stimuli (i.e. CON1, CON2, and BOS) reliably evoke the highest normalized auditory responses in the cannulated hemisphere.

Our initial impetus in recording from microdialyzed subjects was to test whether representations of learned stimuli (i.e. BOS and TUT) were different based on treatment early in development. To address this question, we calculated d prime (***d'***; see *Methods*) relative to WN to determine stimulus selectivity, as described in previous studies (Adret et al., 2012; Yanagihara and Yazaki-Sugiyama, 2016; Moseley et al., 2017). We limited our analyses to TUT and BOS as these were the learning-related auditory stimuli of interest that may have been impacted by treatment. Because of our earlier findings for auditory response profiles, we compared *d*' scores separately by recording hemisphere. Treatment did not impact overall TUT selectivity for either contralateral ($F_{(1, 14)}$ =2.222, p = 0.158) or ipsilateral ($F_{(1, 79)}$ = 2.861, p = 0.095) recording sites in NCM. However, FAD subjects demonstrated significantly stronger BOS selectivity in the ipsilateral cannulated ($F_{(1, 79)}$ = 6.371, p = 0.014; ***Fig. 15D***), but not contralateral hemisphere ($F_{(1,14)}$ = 3.93, p = 0.067; ***Fig. 16***). Taken together, unilateral E2 suppression in NCM during development enhances BOS representation in NCM relative to control birds.

HVC: The sensorimotor nucleus HVC contains a population of tutor-song-selective cells (Volman, 1993; Prather et al., 2008; Vallentin et al., 2016; Moseley et al., 2017) and receives E2-sensitive, indirect projections from NCM in part via the nucleus interfacialis of the nidopallium (Nif; Remage-Healey and Joshi, 2012; Pawlisch and Remage-Healey, 2015). To determine whether suppressing E2 synthesis in development affected downstream representations of either BOS or tutor song, we also recorded from HVC. Baseline firing rates were similar across

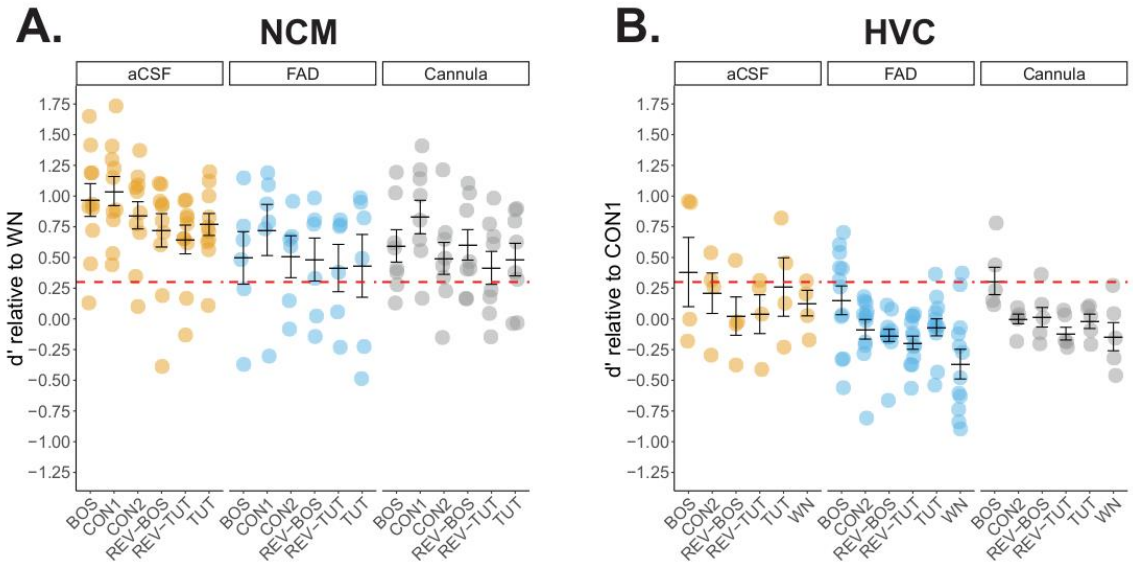

Figure 16: Contralateral d' selectivity in single NCM and HVC neurons. **A,** NCM; statistical analyses were performed for TUT and BOS, only (see *Results*). Other stimuli plotted for visual comparison. Irrespective of stimulus, responses were significantly higher in aCSF treated subjects in both ipsilateral and contralateral NCM. **B,** HVC; statistical analyses were performed for TUT and BOS, only (see *Results*). Other stimuli plotted for visual comparison. Both TUT and BOS selectivity were statistically similar in contralateral HVC.

treatments, recording hemisphere, and no interaction between the two factors were found ($F_{(1, 47)}$, $p > 0.132$; ***Fig. 17A***). For stimulus-evoked firing, there was a main effect of stimulus ($F_{(6, 329)} = 5.83$, $p < 0.001$) and recording hemisphere (ipsilateral > contralateral; $F_{(1, 329)} = 10.661$, $p = 0.001$; ***Fig. 17B***). All other effects and interactions were non-significant ($p > 0.131$). Follow-up analyses revealed that BOS elicited a significantly higher evoked firing response compared to all stimuli except TUT (BOS > CON1, CON2, REV-BOS, REV-TUT, and WN; $p < 0.05$); no other stimulus comparisons were significantly difference.

As with NCM, we also analyzed normalized auditory response in HVC. There was a significant effect of stimulus ($F_{(6, 329)} = 10.384$, $p < 0.001$), treatment ($F_{(1, 329)} = 11.297$, $p < 0.001$), as well as a significant interaction between

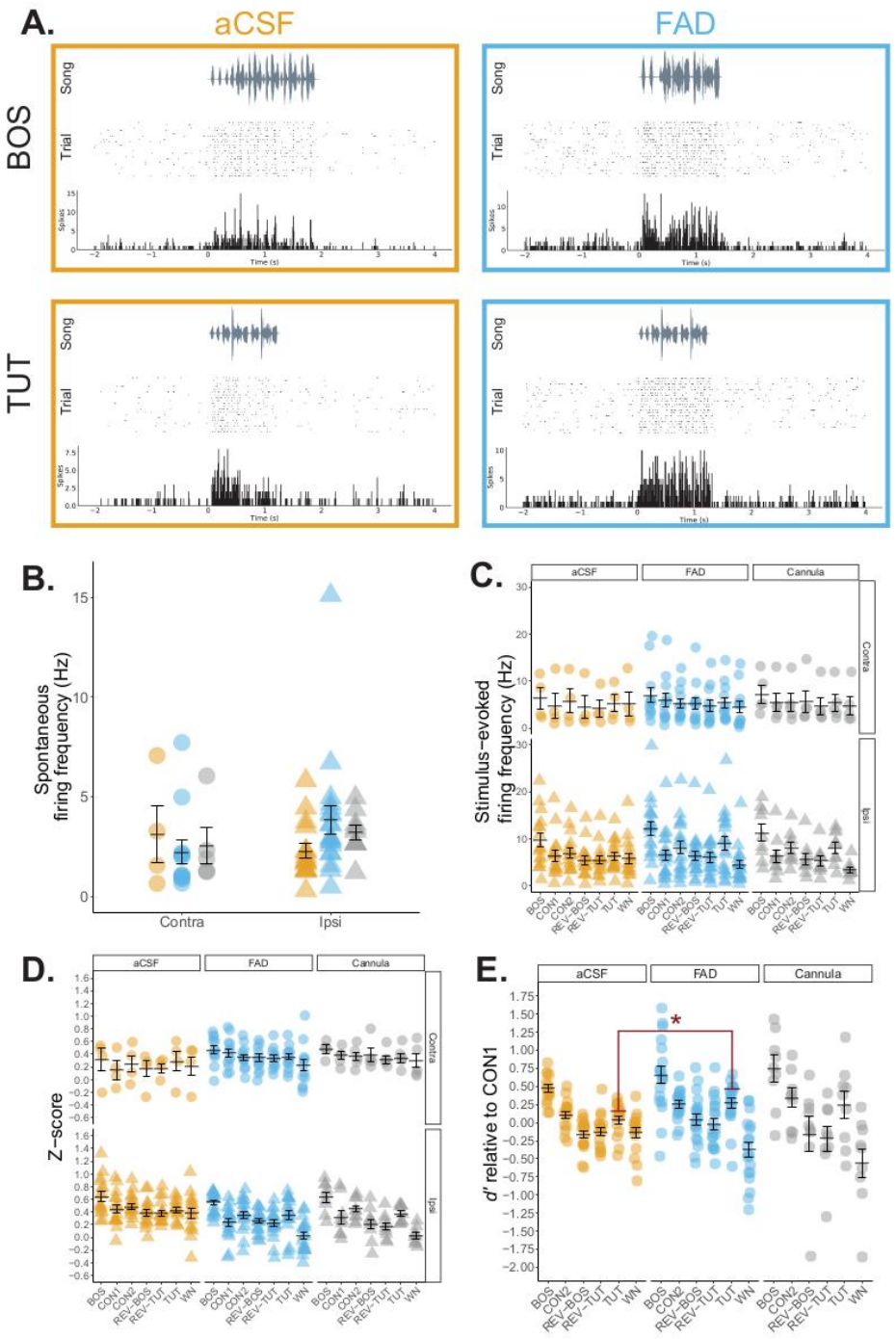

Figure 17: Tutor song selectivity is elevated in single HVC neurons of formerly estrogen-suppressed adult songbirds.

A, Representative HVC single-unit recordings from an aCSF and FAD in response to presentations of birds' own song (BOS) and tutor song. Each recording includes a song spectrogram (*Top*), and raster plot (*Middle*) with corresponding peri-stimulus time histogram in 10 ms bins (*Bottom*) across a 6 second period. The same unit is presented for each treatment across the two stimuli. **B,** Spontaneous firing rates were similar across treatments. Orange = aCSF; blue = FAD; grey = cannula; circle = contralateral hemisphere (relative to microdialysis site); triangle = ipsilateral hemisphere (relative to microdialysis site). **C**, Stimulus-evoked firing rates were significantly higher for BOS compared to all other stimuli except for TUT. Further, ipsilateral HVC displayed higher overall stimulus-evoked firing rates compared to contralateral HVC, independent of treatment. **D**, Analysis of normalized auditory response (z-score) yielded similar results as with firing rate; namely, a significantly higher response to BOS over all other stimuli independent of treatment, as well as a significantly suppressed response to WN compared to CON2 and TUT. **E,** Contralateral d' values relative to CON1. TUT selectivity is significantly higher in FAD subjects solely in the contralateral hemisphere. BOS = bird's own song; CON1; CON2 = conspecific song; REV-BOS = reverse bird's own song; REV-TUT = reverse tutor song; TUT = tutor song. * = $p < 0.05$.

recording hemisphere and treatment ($F_{(1, 329)} = 25.745$, $p < 0.001$; ***Fig. 17C***). All other main effects and interactions were non-significant ($p > 0.176$). BOS elicited a significantly higher response than did all other stimuli (BOS > CON1, CON2, REV-BOS, REV-TUT, TUT, and WN; $p < 0.016$). Conversely, HVC was less responsive to WN compared to select forward conspecific stimuli (WN < CON2 and TUT; $p < 0.009$). Based on the enhanced response to BOS for both z-score and stimulus-evoked firing, we opted to focus our follow-up tests on BOS. No significant differences were found for treatment for either the contralateral ($F_{(1, 14)} = 1.097$, $p = 0.313$) or the ipsilateral ($F_{(1, 33)} = 1.223$, $p = 0.277$) hemisphere.

For selectivity analyses, we focused solely on BOS and TUT relative to CON1 and tested whether TUT and BOS were differently represented between treatments. As there was a significant effect of stimulus and recording

hemisphere, we analyzed the effect of treatment on TUT and BOS selectivity separately by hemisphere. BOS selectivity was statistically similar across treatments across both the ipsilateral ($F_{(1, 33)}$ = 1.691, p = 0.202; ***Fig. 17D***), and contralateral hemisphere ($F_{(1, 14)}$ = 0.804, p = 0.385; ***Fig. 16B***). In contrast, HVC units were more selective for TUT in the ipsilateral hemisphere of FAD subjects ($F_{(1, 33)}$ = 5.82, p = 0.022; ***Fig. 17D***), but not contralateral hemisphere ($F_{(1, 14)}$ = 3.45, p = 0.084; ***Fig. 16B***). Taken together, unilateral E2 synthesis inhibition appears to enhance the neural selectivity for tutor song independent of the animal's ability to imitate the tutor's song.

Adult songbirds are unaffected by post-training inhibition of estrogen synthesis in NCM

As with juvenile songbirds, E2 is also acutely synthesized in the NCM of adult songbirds (Remage-Healey et al., 2008; Remage-Healey et al., 2012). Therefore, we also tested whether neuroestrogen production is involved in consolidating recent auditory experience in adult male zebra finches using a well-established auditory adaptation paradigm (see ***Methods***). Overall, adaptation rates (slope) were significantly shallower (lower) for familiar vs. novel stimuli (familiar = -0.28 ±0.4, novel = -0.49 ± 0.06; $F_{(1, 122)}$ = 4.150, p = 0.044), independent of treatment ($F_{(2, 122)}$ = 1.182, p = 0.310) or an interaction between treatment and stimulus type ($F_{(2, 122)}$ = 0.349, p = 0.706; ***Fig. 18***). Thus, unilateral estrogen synthesis in NCM immediately post-training did not adversely impact memory consolidation across development and in adulthood.

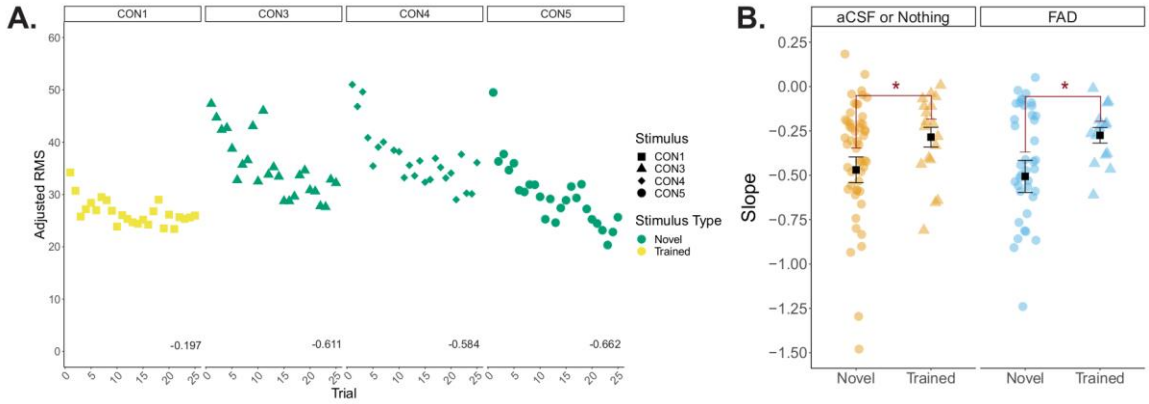

Figure 18: Neural adaptation to learned song is reduced in adult NCM independent of post-training E2 synthesis inhibition.
A, An exemplar multiunit response in the NCM of an untreated hemisphere. Adjusted RMS declines at a faster rate (steeper slope) for novel song (CON3, CON4, and CON5) compared to a shallower slope (slower adaptation) for the recently exposed song (CON1). Slopes for each stimulus is shown at the bottom of each panel. **B,** Average slope per stimulus in aCSF or non-treated hemispheres compared to FAD-treated hemispheres in NCM; slope derived from multi-unit RMS. Orange = aCSF or no treatment; blue = FAD; circles = novel stimuli (three unique CON per bird); triangles = trained stimulus (a single unique CON). The y-axis has been compressed for clarity and five slope data points were omitted (-3.37, -2.72, -2.56, -1.58, 0.51). * = significant main effect of stimulus type (novel vs. trained); $p < 0.05$. CON = conspecific song.

Discussion

Our findings suggest that while aromatase is present in developing auditory cortex, global and unilateral neuroestrogen production is not required for tutor song memorization. However, neuroestrogen blockade leads to suppressed singing rates during development and enhanced neural representations of tutor song in a downstream sensorimotor nucleus when measured in adulthood. Taken together, this study is the first, to our knowledge, that tests the involvement of estrogen synthesis in consolidating an ethologically-relevant sensory memory within the developing auditory forebrain. Therefore, this study

extends our knowledge of the role, region, and age in which estrogen is involved in learning.

Developmental and regional shifts in neuronal cell density in NCM independent of changes in aromatase expression or inhibitory interneurons

We observed a decline in NCM cell density in sensorimotor-aged birds compared to sensory-aged subjects. Only one prior study, to our knowledge, has assessed the cell density of NCM across development and found no regional nor age differences in sensory-aged (20 and 30 dph) and adult male zebra finches (Stripling et al., 2001). It is unclear why our results diverge from those of Stripling et al. (2001). However, the findings suggest a form of experience-dependent network pruning that could explain heightened auditory responses in NCM in sensory- vs. sensorimotor-aged male songbirds (Vahaba et al., 2017). Alternatively, the volume of NCM many expand with age, leading to decreased density. To our knowledge, the volume of NCM across development has not been well characterized, and these ideas remain to be tested.

The density of cells in dorsal NCM was higher compared to ventral NCM in contrast to previous observations (Stripling et al., 2001). This effect was independent of age, suggesting an anatomical distinction in developing NCM that may persist in adulthood (M. Macedo-Lima & L. Remage-Healey, unpublished observations). Numerous studies have described dorsal/ventral differences in response to auditory stimuli in NCM, but there does not appear to be a consensus regional effect. For example, immediate-early gene (IEG) auditory

responses (i.e. *ZENK* induction in NCM in response to auditory playbacks) yield varying results depending on species: no differences between NCM subregions are reported in in adult male European starlings (Gentner et al., 2004) or adult male budgerigars (Eda-Fujiwara et al., 2012), whereas higher dNCM ZENK compared to vNCM has been reported in both adult female white-crowned sparrows (Sanford et al., 2010) and both sexes of adult black-capped chickadees (Phillmore et al., 2003; but see Avey et al., 2014). In contrast, extracellular recordings in the NCM of adult starlings find stronger experience-dependent changes in firing rates in ventral vs dorsal NCM (Thompson and Gentner, 2010), which were suggested to be attributed to a noted enhanced thalamic input from Field L to ventral NCM (Vates et al. 1996). Therefore, while subregion NCM divisions are anatomically distinct, the functional significance of this density difference across development is unclear, but are suggestive of regional differences in auditory responsiveness.

In addition to quantifying NCM neuronal density, we observed similar expression of aromatase and parvalbumin protein across the critical period for song learning. While aromatase expression has been assessed previously across development and in adults, we found that subregions within NCM of sensory- and sensorimotor-aged males possess a similar capacity for estrogen synthesis. As aromatase is similarly expressed in NCM across development, changes in precursor androgens may explain previously observed age-dependent differences in baseline E2 in NCM across the critical period (Chao, et al., 2014), specifically in parallel with the maturation of the testes. Further, our

findings with parvalbumin are in-line with recent findings that find that PV cell density is largely unchanged in across development in the NCM of in male and female zebra finches, as well as other auditory forebrain nuclei (Cornez et al., 2018). Therefore, PV-dependent inhibitory tone and estrogen production remain relatively unchanged across development, suggesting important roles throughout the juvenile period in males.

Brain estrogen synthesis and song production in developing songbirds

Our experiments with systemic FAD treatment suggest that E2 facilitates song production in juvenile songbirds. It is well established that singing is regulated by classic (genomic) steroid hormone action, such as E2, in adult songbirds. In adult male zebra finches, long-term aromatase inhibition leads to suppressed courtship displays, including song production (Walters and Harding, 1988). More recent studies have found that E2 production also appears to acutely facilitate song production in adult zebra finches (Alward et al., 2016b). Our data expand on this understanding that acute suppression of E2 production constrains singing to now include developing male songbirds. The neural locus of this effect of E2-withdrawal on song production is unknown, but likely to include social behavior network nuclei such as the aromatase-rich nucleus taenia (Saldanha et al., 2000; Ikebuchi et al., 2009). Androgens, namely testosterone, have classically been thought to be the critical hormone for the onset of motor production in developing songbirds. For example, plastic song emerges alongside the rise of testosterone in juvenile swamp sparrows (Marler et al., 1987). However, it has also been noted that circulating estrogens coincides with

the onset of subsong (Marler et al., 1987). Thus, our data suggest that E2, and the conversion of precursor androgens to E2 within specific brain areas, may play a more significant role in song production in development than previously thought.

Circulating and brain-derived estrogens are not required for tutor song memorization

Overall, systemic aromatase inhibition yielded minimal effects on eventual tutor song similarity. While tutor song similarity was slightly lower at 49 dph in FAD subjects, FAD-treated birds quickly ‘catch-up’ to comparable tutor song similarity levels as control birds, and produce songs of equal valence for adult female conspecifics. These results are novel given the relatively limited number of studies that have directly tested the role of hormones in song learning in male songbirds. Androgens crystallize plastic song (Korsia and Bottjer, 1991; Whaling et al., 1995; Bottjer and Johnson, 1997) and neural circuit development (Livingston and Mooney, 2001). In contrast, circulating estrogen levels are thought to promote plasticity due to their coincident rise in age-limited song learning in birds during the auditory memorization (“sensory”) phase of development (Pröve, 1983; Weichel et al., 1986; Marler et al., 1987; Marler et al., 1988; but see Adkins-Regan et al., 1990). While our sample size is limited, the data suggest that circulating estrogen synthesis is not required for tutor song memorization during development.

One important caveat for the systemic FAD experiment here is that our pharmacological treatment may have missed a putative ‘critical’ post-training

consolidation period (within ~30 mins immediately following tutoring). E2 is important for auditory processing in adult and juvenile songbirds; thus, we did not want to interfere with online auditory processing of the tutor song during a tutoring session/playback. Instead, we intentionally administered FAD immediately after the offset of tutoring to specifically target the post-training memory consolidation period as in studies on hippocampal E2 and memorization (Frick, 2015). Comparable systemic aromatase inhibition treatments in birds led to marked reductions in E2 and aromatase activity (Wade and Arnold, 1994; Remage-Healey et al., 2010b; Rensel et al., 2013; Alward et al., 2016b), and systemic injections lead to suppressed aromatase activity in NCM within 30 mins (Alward et al., 2016b; but see Krentzel et al., in submission). Thus, if systemic FAD actively suppresses E2 synthesis >30 minutes after administration, and the putative post-training auditory memory consolidation period is <30 minutes, it is important to consider that the pharmacokinetics of oral FAD may not sufficiently target the period of immediate post-training auditory memory consolidation.

In agreement with our systemic results, targeted unilateral suppression of E2 in NCM failed to prevent birds from eventually successfully imitating their tutor's song. Tutoring leads to an initial drop in acute E2 levels within NCM, followed by a rapid increase immediately after a tutoring session in juvenile songbirds (Chao et al., 2015). In our paradigm, FAD was presented at the onset of tutoring and for a one-hour period immediately following the tutor session, without any detectable differences in eventual song similarity. Therefore,

unilateral E2 synthesis in NCM does not appear to be required for auditory memory consolidation.

Additionally, juvenile songbirds are seemingly unaffected behaviorally by unilateral estrogen manipulations in the auditory forebrain. Birds spent comparable amounts of time near by the live tutor and were similarly active during tutoring sessions. These results add to a small but growing understanding of tutor and pupil behavior during song learning. To our knowledge, these results are one of two published studies that explicitly quantify pupil behavior during tutoring (lab-reared, or otherwise) (Chen et al., 2016). Juveniles are thought to preferentially learn from, and as an extension, imitate, more aggressive males who are mated or feed them early in development (Zann, 1996). While it is largely unknown how pupil behavior during tutoring affects song learning, one key behavior appears to be pupil 'attention' during tutoring (Chen et al., 2016). As unilateral E2 synthesis does not impact attention (in our study, time spent near the tutor), it follows that song learning/imitation are similarly unaffected.

Acute neuroestrogen suppression during development exerts enduring effects on neural representation of autogenous and tutor song into adulthood

Suppressing E2 strongly enhanced adult neural representations the tutor's song in HVC. HVC is a sensorimotor nucleus that dually represents both autogenous and tutor song in developing (Volman, 1993; Nick and Konishi, 2005a, b) and adult (Prather et al., 2010; Moseley et al., 2017) songbirds, and is necessary for song learning (Roberts et al., 2012). One possibility is that if E2 reduces singing in microdialyzed birds as in our systemic experiments, there may

be a ‘catch-up’ period that leads to enhanced salience, coding, or replay (Dave and Margoliash, 2000) of the social model’s song (tutor) once E2 synthesis inhibition is ‘released’ in NCM. Thus, our findings in-line with those of swamp sparrows, demonstrating that tutor and BOS selectivity is independent of vocal imitation accuracy in adulthood.

Interestingly, FAD treatments enhanced upstream BOS selectivity in NCM compared to control birds. Auditory forebrain neurons (including NCM) are typically selective for conspecific vocalizations over synthetic noises (e.g. tones) (Stripling et al., 1997; Stripling et al., 2001), and have been noted for having a subpopulation of BOS-selective cells (Janata and Margoliash, 1999; Grace et al., 2003; Amin et al., 2004; Yanagihara and Yazaki-Sugiyama, 2016). In particular, NCM contains experience-dependent tutor song and dual tutor song/BOS selective neurons during development (Yanagihara and Yazaki-Sugiyama, 2016). Auditory responses in NCM are rapidly modulated by estrogens in adult (Remage-Healey et al., 2010b; Remage-Healey and Joshi, 2012) and developing zebra finches (Vahaba et al., 2017). Therefore, in agreement with our findings in HVC with tutor song, acute manipulations of E2 in NCM during development appear to be important for changing representations of birds’ own song as well.

No study is without its limitations and ours is no exception. It is worth noting that our treatments were presented unilaterally, and there is thus a strong likelihood that contralateral NCM can compensate for depressed E2 production in our study, leading to robust tutor song memory and proper song imitation in adulthood. While NCM appears to have lateralized function both natively

(Moorman and Nicol, 2014), and with regards to E2 (Remage-Healey et al., 2010b; De Groof et al., 2017), there is scant evidence for lateralized expression of aromatase (Saldanha et al., 2000; Ikeda et al., 2017). Relatedly, there is the additional possibility that either acute (microdialysis) or chronic (systemic) administrations may lead to homeostatic increases in aromatase production and/or activity (e.g. Saldanha et al., 2000), or upregulation of E2 from other sources (e.g. gonadal; adrenal). For example, estrogen-suppressed adult zebra finches have increased aromatase protein levels in the hippocampus, but not NCM (Saldanha et al., 2000). As the hippocampus is not involved in vocal learning (Bailey et al., 2009), this is unlikely to explain our findings. Lastly, it is possible that cannulation-induced injuries across control and FAD treated subjects obscured any potential differences in song learning outcomes. That is, since guide cannulae dissociated on their own, brain injury from the cannula may lead to similarly poor song learning outcomes as with FAD treatment. However, this is unlikely to be true as both microdialysis and systemically-treated birds yielded comparable song similarity rates in adulthood.

Song learning is gated by experience

Our study also replicates the finding that experience with social partners, in addition to age, can regulate the closure of the critical period. Importantly, the lack of song crystallization by 130 dph was independent of treatment, further emphasizing that unilateral estrogen synthesis in NCM does not participate in modulating critical period plasticity in contrast to androgens which prematurely crystallize song and related neural circuits (reviewed above). Others have also

noted abnormal song in adulthood in lab-tutored songbirds (Eales, 1985, 1987; Morrison and Nottebohm, 1993; Slater et al., 1993; Jones et al., 1996; Zann, 1996; Deregnaucourt et al., 2013), and found similar changes such as dropped syllables, reduced syllable lengths, and increased stereotypy once abnormal singing birds were exposed to other adult males. While further bioacoustic analysis is required to unpack the current findings, our work highlights the important limitation of controlled lab tutoring paradigms, namely that it is both quality and quantity of experience that dictate the closure of critical period song plasticity.

Recent auditory experience consolidation is insensitive to estrogen synthesis blockade in adult NCM

Our results in adult animals build on a well-established paradigm in which recent auditory experience is encoded in adult and developing NCM (Chew et al., 1995; Stripling et al., 1997; Smulders and Jarvis, 2013; Miller-Sims and Bottjer, 2014; Ono et al., 2016). We find that auditory recognition in adult songbirds is unimpaired by unilateral inhibition of E2 synthesis post-training. Repeated exposures of a single conspecific song leads to neural ‘recognition’ up 48 hours later (Chew et al., 1995), which is impaired when global estrogen production is dampened (Yoder et al., 2012). Our findings suggest that while E2 is important for spatial memory consolidation in the hippocampus of both birds and rodents (Frick, 2015; Bailey et al., 2017), as well as chemosensory memories in the olfactory bulb (Dillon et al., 2013), this role does not extend to auditory cortex. In rodents, E2 is rapidly upregulated in dorsal hippocampus immediately following

an object recognition training session (Tuscher et al., 2016b). In contrast, repeated song exposure in male and female zebra finches leads to immediate increases in estrogen levels which tapers off following song playback or social exposure cessation (Remage-Healey et al., 2008; Remage-Healey et al., 2012). Therefore, a lack E2 production following acoustic communication exposure in adults may explain the lack of a role for E2 in NCM for consolidating the auditory experience.

Conclusion

Here, we demonstrate that estrogens exert a complex role in the auditory cortex of developing male songbirds. Our findings show the capacity to synthesize neuroestrogens remains high throughout development alongside substantial age- and subregion-dependent changes in NCM cell density. Systemic estrogen synthesis blockade led initially to suppressed singing behavior in juveniles following tutoring. Further, the data indicate that while song memorization is unimpaired by acute inhibition of E2 production following training in developing and adult songbirds, early life E2 manipulations in auditory forebrain lead to altered neural selectivity of autogenous and tutor song in NCM and downstream HVC in adulthood, respectively. Taken together, this study expands our understanding of the role of brain-derived estrogens in learning and memory. Historically, studies on rapid E2 signaling and learning have been largely focused on adults and hippocampal-dependent learning. Therefore, in addition to continuing to study the role of brain-derived estrogen signaling across a diverse range of animals (Remage-Healey et al., 2017), it remains important to

test its function across different ages (Gresack et al., 2007a, b) and brain regions.

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CHAPTER VI

DISCUSSION

In this dissertation, I examined the role of peripheral and brain-derived E2 in complex acoustic signal processing and memorization, as well as changing neural architecture and aromatase expression, across the lifespan in male zebra finches. Findings from the experiments provide a new understanding for the role of aromatase activity and neuroestrogen signaling during the critical period for vocal learning. Specifically, I first demonstrated that auditory coding is enhanced in sensory-aged birds, and that acute E2 signaling exerts a lateralized, and age-dependent effect on communication processing. Then, I showed that the capacity to synthesize E2 in NCM is comparable across development, as is the abundance of parvalbumin interneurons; however, neuronal density decreases with age and is highest in dorsal vs. ventral NCM. Further, I found evidence that aromatase inhibition reduces song production, but does not impact tutor song memorization. In contrast, I obtained neurophysiology results establishing that early life, central E2 synthesis blockade enhances long-term neural representations of autogenous and tutor song in adulthood. Finally, experiments in adult songbirds confirmed that, as with juveniles, post-training unilateral inhibition of E2 synthesis in NCM is not required for consolidating recent auditory experiences.

From my initial experiments, I found that NCM undergoes a developmental ‘switch-point’ initiated once birds begin producing their own developing song. In contrast to sensorimotor-aged birds, auditory encoding and classification is

elevated in sensory-aged birds, suggesting that NCM is highly attuned to salient social communication signals during the auditory memorization phase of song learning (Vahaba et al., 2017). It would be interesting to know whether this 'switch-point' finding extends to female zebra finches, who also encode and memorize their fathers' song during development, which they use to evaluate a potential mate's song (Miller, 1979), and may be localized to the auditory forebrain as well (Terpstra et al., 2006). I also found that rapid E2 signaling in NCM yields a lateralized and age-dependent effect on sensory coding. Auditory responses are dampened by E2 in sensory-aged birds across hemispheres, whereas sensorimotor-aged birds show either an enhanced or inhibitory auditory response to E2 depending on hemisphere. Adults show a rapid, large-scale increase in auditory response with local E2 administration (Remage-Healey et al., 2010b; Remage-Healey and Joshi, 2012), which may be functionally significant for audition and song recognition (Remage-Healey et al., 2010b). Further, the enhancing effects of E2 in the auditory lobule of songbirds appears to left-lateralized (Remage-Healey et al., 2010b; De Groof et al., 2017). Lateralized E2 effects in development may be important for song discrimination and/or auditory memory acquisition, as others have described (Moorman and Nicol, 2014).

While changes in juvenile auditory responses were more modest in contrast, future work should attempt to test the role of rapid E2 signaling in more ethologically-relevant (e.g. awake, freely-behaving animals) to better understand its role in a more naturalistic context. GABAergic tone is enhanced in

anesthetized birds, which along with state (i.e. sleep or awake), gates auditory selectivity and response profiles in adult and developing songbirds (Schmidt and Konishi, 1998; Cardin and Schmidt, 2003; Vallentin et al., 2016; Yanagihara and Yazaki-Sugiyama, 2016). Therefore, we still have a limited understanding of how natural fluctuations of E2 in songbird auditory cortex influence communication encoding.

In subsequent experiments, I described how central and circulating E2 regulates behavior, singing, and song learning, and found that, as with adults, E2 synthesis protracts song production (Alward et al., 2016b), independent of its effect on song learning. Moreover, I also found that while a bird's ability to imitate a model song is unimpaired by acute estrogen suppression in development, neural selectivity is affected and persists into adulthood. Specifically, aromatase inhibition led to enhanced BOS representation in NCM and tutor song in HVC. NCM is a broadly-selective auditory forebrain region that responds more to natural songs (e.g. song) over synthetic noises (e.g. tones) and more to conspecific over heterospecific song (Mello and Clayton, 1994; Stripling et al., 1997), which is modulated by E2 (Maney et al., 2006). One proposed role for NCM in adults is individual recognition (Chew et al., 1996; Gentner, 2004), which may also be modulated by E2: estrogen synthesis inhibition in NCM abolishes males' innate preference for their own song (Remage-Healey et al., 2010b). More recently, Yanagihara and Yazaki-Sugiyama (2016) described both BOS- and TUT-selective neurons in developing NCM. Therefore, NCM appears to have E2-sensitive representation of birds' own song. Blocking E2 production in

development may increase the distribution of neuronal selectivity to compensate for delayed and/or suppressed song production. I found that systemic FAD treatment decreased singing rates in developing animals. As such, if these findings extend to microdialyzed subjects, there may be a ‘catch-up’ period where previously inhibited E2 in NCM leads to decreased song production, resulting in either more rehearsal in development or increased song replay in HVC (Dave and Margoliash, 2000), which has putative reciprocal connections with NCM (Lynch et al., 2013). Increased hearing of BOS and/or replay may then translate to increased BOS representation in NCM in adulthood to compensate for a delayed song learning trajectory as observed in systemically-treated FAD birds.

In contrast to NCM, previous studies on HVC have established strong BOS-selectivity in anesthetized birds (Margoliash, 1983; Margoliash and Konishi, 1985; Margoliash, 1986), which is enhanced by E2. Administration of E2 in NCM enhances downstream BOS-selectivity in the HVC of adult songbirds (Remage-Healey and Joshi, 2012), suggesting that aromatase inhibition in the NCM of developing songbirds might trans-synaptically transform TUT selectivity in HVC . Tutor selectivity is observed in HVC during development (Volman, 1993; Nick and Konishi, 2005a, b) and in adulthood (Prather et al., 2010; Moseley et al., 2017). Therefore, my finding that formerly neuroestrogen-suppressed birds show an enhanced TUT response in HVC suggests reduced E2 levels in NCM translates to an elevated representation of song or songs learned during development. Thus, downregulation of E2 signaling in NCM during learning in

development may improve learning accuracy, in contrast with a permissive learning role in adults.

Alongside my finding that auditory memorization and recognition is similarly unimpaired in adult NCM, results in juveniles converge on the idea despite E2's role in auditory processing, unilateral estrogen synthesis in NCM is insufficient to modify auditory recognition and consolidation across the lifespan. These findings extend our understanding of estrogens and cognition to include a critical period-dependent, and ethologically-relevant learning task (song learning) in sensory cortex (NCM). Much of what is known about estrogens and cognition come from studies on adult hippocampus in rodents (Packard and Teather, 1997a; Zhao et al., 2010; Boulware et al., 2013; Tuscher et al., submitted) and birds (Bailey et al., 2013; Rensel et al., 2013; Bailey and Saldanha, 2015; Rensel et al., 2015; Bailey et al., 2017), and have generally shown an enhancing role for estrogens in cognition. It is interesting to note, however, that when older rodents are administered E2 following a training task, there does not appear to be a similar improved memory as with younger rodents (Gresack et al., 2007a, b). Therefore, in addition to testing a novel, aromatase-rich and E2-sensitive cortical brain region, we also expand our understanding of E2 and cognition to include ontogenetic learning in songbirds. One caveat is that all aromatase inhibition in this set of studies was presented unilaterally. Therefore, it remains to be tested whether bilateral estrogen synthesis blockade is sufficient to drive changes in auditory memorization in order to rule out a potential compensatory role of contralateral NCM.

Finally, the last set of experiments detailed changes in cell density in NCM, as well as quantified the expression of estrogen-synthesizing neurons and a sub-class of GABAergic cortical interneurons (PV+). While the capacity for E2 production and PV+ expression remain comparable across development, neuronal density peaks in sensory-aged birds, as well as across ages in dorsal NCM. While my findings are in contrast to previous reports on cell density in NCM across development, which suggests NCM is adult-like by 20-dph (Stripling et al., 2001), they do provide a potential explanation for elevated auditory responses in sensory-aged birds as I reported in Vahaba et al. (2017). In contrast to the well-described synaptic pruning that occurs during brain development, these results suggest a 'cellular' pruning in NCM with age. Alternatively, as the volume of NCM has not been formerly assessed, it may be that as the auditory lobule expands, cell density decreases, however this idea remains to be tested.

The initial impetus to carry out experiments on the role of E2 in developing auditory cortex originated from an exciting set of findings from our lab. Chao et al. (2015) found that when juvenile male zebra finches were presented with tutor song playbacks, E2 levels declined during the tutoring session, and were subsequently elevated above baseline levels immediately after the song learning session. While the finding that neuroestrogen synthesis increases following learning (as in adult rodent hippocampus Tuscher et al., 2016b) sparked the various experiments carried out in the dissertation, the suppression of E2 in NCM during song playback may provide a better context for the finding that E2 suppresses auditory response in sensory-aged birds. This result, in combination

with the finding that tutor memorization is impervious to E2 synthesis blockade, contrasts with work in adult songbirds and rodents (Remage-Healey et al., 2010b; Remage-Healey et al., 2012; Frick, 2015), and highlights the fact that estrogenic action and synthesis is not a uniformly positive signal and varies greatly depending on age, neural structure, and learning task (Korol and Pisani, 2015). In particular, the results suggest that brain-derived or administered E2 interferes within the auditory cortex in development, despite the high abundance of aromatase, and it may be functionally significant to down-regulate E2 in NCM to permit proper auditory encoding and consolidation. This idea complements the finding that acute and systemic aromatase inhibition in development leads to enhanced neural representation of autogenous and tutor song in auditory song nuclei (HVC and NCM). However, these ideas are highly speculative and warrant further investigation.

In summary, this dissertation has thoroughly tested the pleiotropic role of E2 production and signaling across the lifespan of adult male zebra finches. This work is important both in it of itself as a basic set of scientific experiments, as well as having potential translational value. Gene mutations for aromatase are linked to language impairments in humans (Anthoni et al., 2012). Further, E2 levels in 5-month old human infants are an early, positive predictor of eventual language ability (Wermke et al., 2014; Quast et al., 2016), as with song learning in sparrows (Marler et al., 1987). As various cognitive disorders are linked to impaired social communication, such as in autism spectrum disorder, studying how neuroestrogens regulate socially learned vocal communication early in

development may provide key insights into similar mechanisms in other vocal learning animals, such as humans.

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