

The form, function, and evolutionary significance of neural aromatization

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ABSTRACT

Songbirds have emerged as exceptional research subjects for helping us appreciate and understand estrogen synthesis and function in brain. In the context of recognizing the vertebrate-wide importance of brain aromatase expression, in this review we highlight where we believe studies of songbirds have provided clarification and conceptual insight. We follow by focusing on more recent studies of aromatase and neuroestrogen function in the hippocampus and the pallial auditory processing region NCM of songbirds. With perspectives drawn from this body of work, we speculate that the evolution of enhanced neural estrogen signaling, including in the mediation of social behaviors, may have given songbirds the resilience to radiate into one of the most successful vertebrate groups on the planet.

1. Introduction

Over the last several decades, songbirds (Passeriformes; Suborder Passeri or Oscines) have emerged as premier animal models for investigations into the neurobiology of vocal learning and communication, auditory processing, animal intelligence, socio-sexual behaviors, in addition to substantial work exploring the genetics underlying animal phenotypes. Perhaps not surprisingly, much of this integrated biology is coordinated by endocrine signaling, again the focus of substantial investigation. A notable endocrine specialization in songbirds involves the extensive expression of the estrogen-synthetic enzyme aromatase (Cyp19) in neural tissues. Stemming from work on songbirds, several significant advances to our understanding of the vertebrate-wide mechanisms and function of neuroestrogen synthesis have emerged over the past few years. In this review we highlight the role songbirds have played in the development of general concepts regarding the form and function of neuroestrogen synthesis. Following this overview, we then describe work in this fascinating avian Suborder where neuroestrogens have clearly defined roles in hippocampal-based learning and memory and pallial auditory-processing. Altogether, this work leads to new perspectives on the cellular and subcellular distribution of neuronal aromatase and the synthesis and secretion of estrogens at the synapse

(synaptocrinology), de novo neurosteroidogenesis, and the presence of aromatase in the conserved vertebrate social behavior network. This widespread importance of neuroestrogen signaling fuels speculation about the advances in neurotranscriptional regulation of the aromatase gene as an underlying feature of the extraordinary expansion of this, the largest of all avian Suborders, and the group of birds that attracts so much human attention.

1.1. Overview

The discovery in brain of aromatase activity, that is, the conversion of androgens into estrogens, by Naftolin and colleagues radically transformed our thinking about the interplay linking gonadal hormones and neural and behavioral processes (Naftolin et al., 1971, 1975). By showing that neuroestrogen synthesis was a highly conserved property of the vertebrate brain, work by Callard and colleagues (Callard et al., 1978) opened the door for a host of studies linking aromatase with basic neural physiology. Importantly, the identification of aromatase in brain provided clarification on observations that both androgens and estrogens were seen to stimulate certain neural functions and behaviors: androgens could exert their influence by first being converted into the bioactive estradiol locally in brain (Macluskus and Naftolin, 1981;

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McEwen, 1981).

1.1.1. Neuroanatomy and cell biology

Early work showed that aromatase activity in the mammalian brain was largely present in neurons (e.g. Canick et al., 1986). The enzyme was known to be associated with microsomal subfractions of tissue preparations indicating that aromatase protein was largely bound to endoplasmic reticulum. While studying aromatase in the quail hypothalamus that included isolation of purified microsomal and synaptosomal subfractions, aromatase activity was detected in both (Schlinger and Callard, 1989). The relative abundance of aromatase in the synaptosomal fractions in particular changed in response to photoperiod consistent with a change in gonadal hormone-dependent expression of hypothalamic aromatase and the activation of some masculine behaviors (e.g. Balthazart, 1990). These results led to the prediction that aromatase could be found in synaptic terminals where it might have a role in producing behaviorally active estrogens. Additional concrete evidence confirmed aromatase present at vertebrate synapses (Naftolin et al., 1996). In that case, immunocytochemistry using an antibody against human placental aromatase together with light and electron microscopy, convincingly showed aromatase immunoprotein in axons and axon terminals of rat, quail, monkey and human hypothalamic and limbic brain tissues.

Around this same time songbirds emerged as interesting model systems for exploring the anatomy and physiology of brain aromatase. Aromatase activity was found to be widely distributed in the zebra finch brain (Vockel et al., 1990a,b; Schlinger and Arnold, 1991) the organ that appeared to be the primary site of estrogen synthesis in males (Schlinger and Arnold, 1991, 1992a, 1993). Measures of aromatase showed that like other mammals and birds, aromatase was present in conserved hypothalamic and limbic tissues (Vockel et al., 1990a,b; Balthazart et al., 1996). However, aromatase was also found in diverse brain areas, notably in pallial regions now seen as homologous with mammalian cortical structures (Saldanha and Schlinger, 1997; Schlinger, 1997; Silverin et al., 2004; Soma et al., 1999, 2000a, 2003) and hippocampus (Saldanha et al., 1998, 1999; Rensel et al., 2015). Further, unlike mammals, but similar to teleost fishes (Forlano et al., 2001), the developing songbird brain appeared to maintain its distribution and robust expression through adulthood, a characteristic which belied the contiguity of neuroestrogen synthesis and action throughout the animals' lifespan (Roselli and Resko, 1993, 2001; Jacobs et al., 1999). More specifically, while the abundance of aromatase expression appears to decrease with age in the mammalian brain (Roselli and Resko, 2001), no such decline is apparent in songbirds and teleost fish. It is noted however, that teleosts seem to express aromatase with greater abundance and in a variety of cell types within the brain (Forlano et al., 2001). Importantly for us here, this expansion of aromatase expression within a number of these regions may have first appeared in sub-oscines (Tyranni) compared to other avian groups (Silverin et al., 2000, 2004; Soma et al., 2000b), becoming more pronounced in the Oscine suborder (Fig. 1).

Much previous work on avian neuroendocrine control of behavior had focused on captive-held species like doves and quail. What made songbirds suddenly so interesting? Songbirds had long attracted the attention of ethologists for their remarkable ability to learn their often complex and beautiful songs (Thorpe, 1961). The capacity to learn vocalizations and then use these songs in reproductive and aggressive contexts was found to arise from a conspicuous neural circuitry or song system that drove the function of the bird's syrinx and respiratory system to enable production of each species' acoustically distinct song (Nottebohm et al., 1976). With the discovery of this intricate neural song system songbirds became extraordinary model organisms in neurobiology (Marler and Slabbekoorn, 2004). Importantly, the discovery of a significant capacity for estradiol synthesis by the songbird brain arose at a time when there was intriguing evidence that estradiol was a crucial signaling molecule in the development and sexual differentiation of the

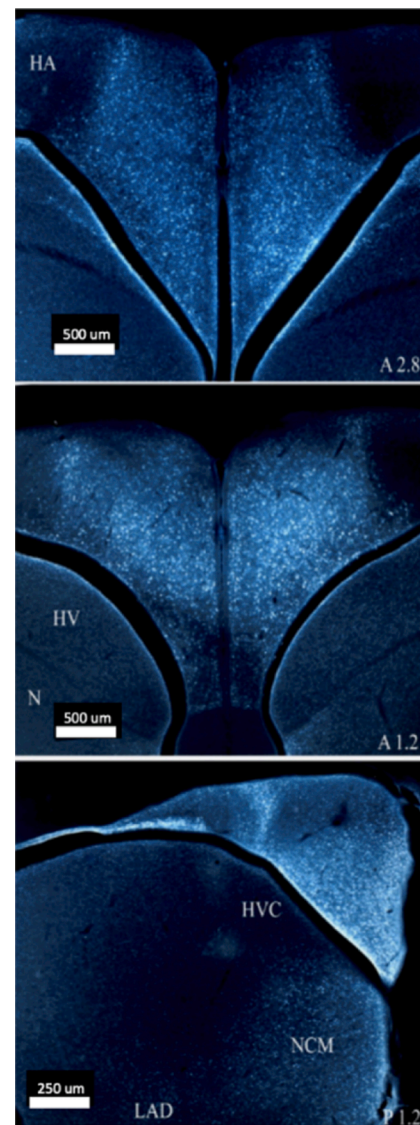


Fig. 1. Aromatase expression (punctate/granular light blue fluorescence) demarcates the hippocampus and caudomedial nidopallium (NCM) in a male black-capped chickadee (*Parus atricapillus*). Hyperstriatum accessorium (HA), hyperstriatum ventral (HV), nidopallium (N), lamina arcopallium dorsalis (LAD). Mag bars (lower left) and AP coordinates (Stokes and Nottebohm, 1974; lower right) are shown. (For interpretation of the references to colour in this figure legend, the reader is referred to the web version of this article.)

neural song system of these birds (e.g. Gurney and Konishi, 1980). These circuits were conspicuously sexually dimorphic in some species, such as zebra finches, where singing behavior was also performed in a sex-specific fashion. Studies from diverse perspectives placed estradiol in a unique position to drive the development of several key elements of this circuitry (Holloway and Clayton, 2001). Because little estradiol appeared to be secreted by the testes of some male songbirds, the abundant aromatase in brain near song control circuits strongly suggested a link between neuroestrogen synthesis and song learning and production (Schlinger et al., 2001; Schlinger and Arnold, 1992b). Although the role of estradiol in song development has received substantial interest and fueled an extensive research the origins of sex differences in the neural system controlling song is decidedly complicated as the role of chromosome complements have also emerged as major players in this phenomenon (Agate et al., 2003).

In addition to this possible role for neuroestrogen synthesis and the development and production of birdsong, male songbirds appeared to

express estrogen-dependent reproductive and aggressive behaviors, as seen in many other vertebrates. Importantly, in some species, aggressive behaviors were activated by estradiol outside of the breeding season when circulating gonadal steroids were low, giving brain steroidogenesis, and neuroestrogen synthesis in particular, a likely role in activation of songbird aggression (Soma et al., 2000a, 2000b). Additional neurosteroidogenic mechanisms appear to be involved as well (Pradhan et al., 2010 and see Section 1.1.3). Thus, highly conserved estrogen-dependent circuits underlying vertebrate social behavior were likely controlled by estradiol synthesized in the brain. Importantly, in several documented cases in songbirds, the activation of aggression occurred quite rapidly suggesting rapid membrane actions of estradiol mediating these effects (reviewed by Heimovics et al., 2018). There is now an abundance of data showing that estrogens can modulate neural circuits and behaviors within a very fast time-frame involving one of several possible membrane receptor mechanisms (see below). The question remains as to whether these rapid actions involve a) locally synthesized neuroestrogens possibly including aromatizable precursors, b) rapid secretion of peripheral estrogens or aromatizable androgens, or c) rapid secretion of steroidal substrates such as DHEA. These remain active areas of investigation (Heimovics et al., 2018; Balthazart et al., 2018).

The distribution and abundance of aromatase had been investigated using biochemical measures of activity, but the advent of in situ hybridization procedures using zebra finch specific antisense oligomers (Shen et al., 1995) as well as the production of high quality aromatase-specific antibodies for use in immunocytochemical studies (Balthazart et al., 1996) greatly increased our appreciation of the neuroanatomy of

songbird aromatase. When studied using an antibody specifically directed at zebra finch aromatase (Saldanha et al., 2000) and combined with light and electron microscopy, aromatase was detected in abundance along presumptive axons and axon terminals in the pre-optic area, hippocampus, song motor nucleus HVC (as a proper name), and the caudo-medial nidopallium (NCM), an auditory processing pallial region of the avian brain (Peterson et al., 2005). Strikingly, in some of these regions aromatase-positive soma were absent, suggesting that any estrogens formed were produced solely in processes or terminals. Indeed, Peterson et al. (2005) also detected sex-differences in the number of aromatase-positive synapses across all brain regions, perhaps contributing to some of estradiol-dependent sex-differences in sex-behavior, song production and spatial memory capabilities exhibited by these adult birds. More specifically, males had more presynaptic boutons that expressed aromatase relative to females as measured by immunoelectron microscopy (Peterson et al., 2005), and this sex difference was also apparent in studies the measured aromatase activity in synaptosomes, but not microsomes (Rohmann et al., 2007) (Fig. 2).

1.1.2. Regulation of brain estrogen synthesis

A key feature of vertebrate brain aromatase is that it is subject to regulation. Earlier, researchers were aware that aromatase in diencephalic regions was subject to seasonal and hormone-dependent changes in activity and expression. These changes were, in general, found to result from regulation by circulating gonadal steroids largely impacting aromatase gene expression (Balthazart et al., 1990; Lephart, 1996; Pasmanik et al., 1988; Roselli and Resko, 2001; Steimer and Hutchison,

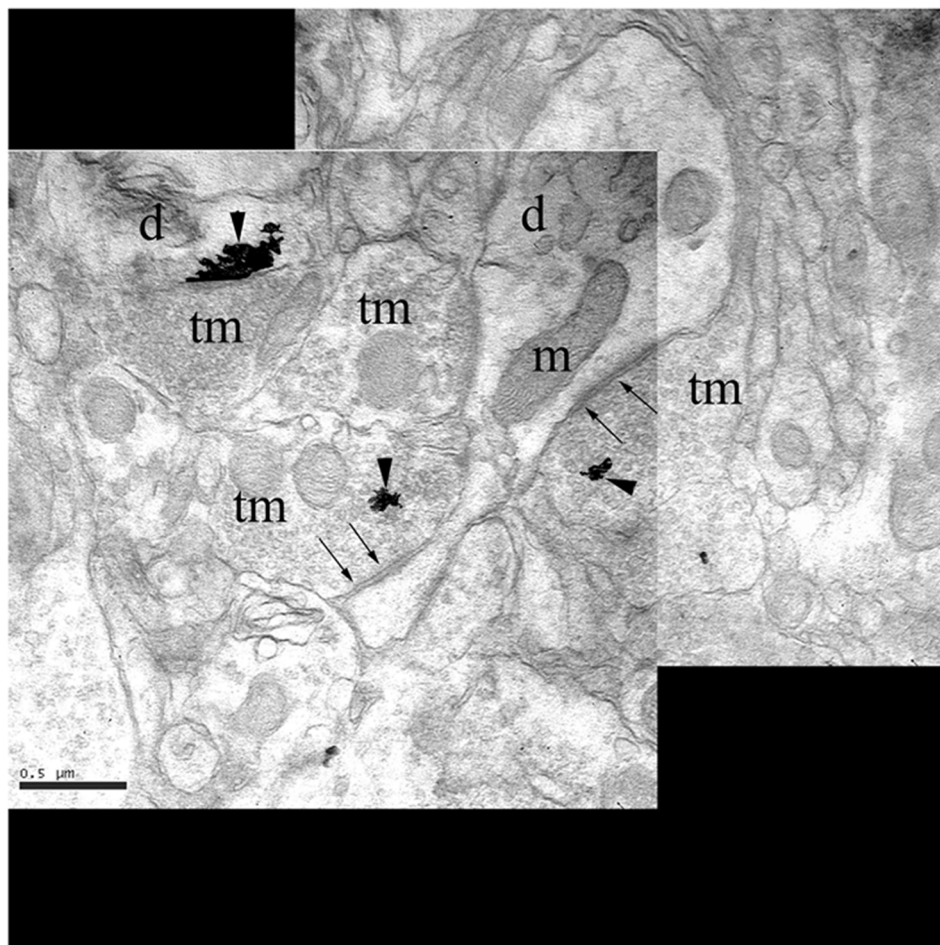


Fig. 2. Processed (sharpened and contrast enhanced) image depicting two aromatase-expressing presynaptic boutons and one aromatase-expression post-synaptic dendrite in the preoptic area of the zebra finch hypothalamus. Terminal (tm), dendrite (d), immunoprotein (arrowheads), mitochondrion (m), arrows show direction of information transfer (pre-to-post synapse).

1981). Seasonal and/or hormone-dependent changes in brain aromatase was also observed in songbirds, largely in the diencephalon (Vockel et al., 1990b) but also in diverse areas of the brain including in the telencephalon (Foidart et al., 1998; Soma et al., 1999; Fusani et al., 2001; Silverin et al., 2004), the hippocampus and in the auditory processing region NCM (Soma et al., 2003; although see Wacker et al., 2010). Key studies in quail diencephalon showed that in addition to long-term transcriptional regulation of aromatase protein, brain aromatase activity could also be regulated on a rapid time-scale. This rapid regulation was due to changing phosphorylation states of the aromatase protein, including ATP and robust calcium-sensitivity (Balthazart et al., 2003, 2005), in which phosphorylating conditions down-regulated aromatase activity and changes in estrogen-dependent neural functions.

Subsequent studies showed that rapid regulation of brain aromatase was also occurring in the songbird brain with some newer cellular and anatomical perspectives (Remage-Healey et al., 2010a, 2010b see below; Cornil et al., 2012; Comito et al., 2016). Biochemical studies showed that under elevated phosphorylating conditions, aromatase was down-regulated in preparations of the whole telencephalon (Cornil et al., 2012) of male zebra finches as well as specifically in the hippocampus, NCM and hypothalamus of both males and females (Comito et al., 2016). However, differences across these brain regions were detected suggesting that the local neurochemical environment may impact the degree to which Ca^{++} -dependent phosphorylation regulates aromatase activity. Additional evidence for neurochemical differences were detected when the concentration of ATP in the assays was modulated. Unlike what had been reported in quail hypothalamus, a low concentration of ATP produced a robust down-regulation of aromatase, whereas a high dose of ATP diminished this effect (Comito et al., 2016). Although these latter results were observed in vitro, these results provide potential evidence for a regional and temporal and even species specificity in the rapid modulation of aromatase activity that may bear on local neuroendocrine function.

An especially important consideration for interpretation of regional differences comes from studies showing that the compartmentalization of aromatase can influence how aromatase might be regulated. As discussed previously, the presence of aromatase in distal fibers and pre-synaptic boutons is well conserved across several vertebrate species. This sequestration could permit the transport of the aromatase protein from the soma to the synapse, resulting in distinct pools of aromatase which may be subject to differential regulation and/or response by steroid sensitive neural targets. Indeed, direct evidence supporting a differential regulation of synaptosomal and microsomal aromatase activity subsequently appeared in studies that separated the synaptosome from microsomes. Briefly, following lysis and successive spins, synaptosomal and microsomal pellets were prepared from the whole telencephalons of male zebra finches followed by exposure to a concentrated phosphorylating environment (high PO_4^-), or a phosphorylating environment with the calcium chelator EGTA or the kinase inhibitor bisindolylmaleimide (BIS). The data revealed that synaptosomal aromatase activity was potentially inhibited under high PO_4^- and this inhibition was prevented in the presence of EGTA and BIS. In microsomal pellets however, high PO_4^- had little to no effect on aromatase activity, suggesting that aromatase in synaptosomes may be more responsive to phosphorylation relative to that in microsomes (Cornil et al., 2012). These findings from studies of the songbird brain support the idea that microsomal and synaptosomal pools of aromatase may be differentially regulated providing opportunities for differences in concentrations of locally formed estradiol in the face of exposure to similar levels of androgenic substrate.

The preceding discussion pointing to changes in aromatase across various time-domains suggests that levels of estradiol also change across temporal and spatial domains (Schlinger, 2015). Yet, because estradiol is functional at quite low concentrations it requires sensitive procedures for its measurement. In an effort to detect changes in brain estrogens on a relatively rapid time-scale, microdialysis procedures were developed

and coupled with sensitive ELISAs that became available to measure estradiol. These procedures were successfully applied to the zebra finch pallium, and while the ELISAs were most sensitive to 17-beta-estradiol, the antibody did cross-react against other estrogens, hence we use the term 'neuroestrogens' here. Aromatase expression is quite abundant in the NCM, an auditory processing region of the zebra finch brain and this site was selected for investigation (Remage-Healey et al., 2008). Here estrogens could be detected in dialysate samples collected over 30 min. Importantly, those levels were shown to change significantly and within 30 mins when birds were exposed to zebra finch song. Those levels declined back to baseline within 30 min after the song stimulus was removed. There were no detectable changes in blood estradiol, or in estrogen levels outside of NCM all supporting the view that aromatase was up-and-down regulated on a relatively brief time-scale within NCM to rapidly change local neural levels of estrogens during periods of social and sensory stimulation. Patterns of elevation in response to song tutoring were also demonstrated in the NCM of juvenile male and female zebra finches (Chao et al., 2015) indicating that neuroestrogens may impact song learning or consolidation directly. Elevated estrogen levels in the songbird shown by microdialysis were substantiated also by sensitive radioimmunoassay procedures applied after steroidal extraction of microdissected regions of the zebra finch brain (Charlier et al., 2010).

The song-induced increase in NCM neuroestrogen levels was not unexpected. Separate studies showed that aromatase activity in the posterior telencephalon that contained NCM was indeed elevated when males were singing (Remage-Healey et al., 2009). Notably, the increase in aromatase activity was detected largely within synaptosomal, not microsomal, preparations of NCM, suggesting that estradiol levels specifically increase at NCM synapses in response to song playback.

As noted earlier, targeted phosphorylation of aromatase leads to changes in androgen-binding capacity and/or catalytic activity. Subsequent studies, also in the quail brain, revealed that the neurotransmitter glutamate could, via a Ca^{++} -dependent mechanism, rapidly down-regulate neural aromatase (Balthazart et al., 2006). In vivo retrodialysis of glutamate into the NCM of male zebra finches caused a rapid (within 30mins) suppression of local neuroestrogen levels (Remage-Healey et al., 2008), a result consistent with a rapid down-regulation of local aromatase activity. These independent biochemical and neuroanatomical studies collectively pointed to mechanisms whereby sensory-induced neural activation, via excitatory pathways, could result in in Ca^{++} -dependent phosphorylation or phosphatase activity that could, in turn, alter local estrogen levels in brain. Pre-synaptic boutons appeared to be one important effector site for this collection of processes. Studies of songbirds were decisive in the development of a synaptocrine hypothesis whereby the synthesis and action of aromatase at the synapse could be viewed as neuroscientists view any other neurotransmitter/neuromodulatory system (Saldanha et al., 2011; Remage-Healey et al., 2011a, 2010b; see also Balthazart and Ball, 2006).

1.1.3. Neurosteroidogenesis

Estrogen synthesis requires androgen as its substrate raising the question of the source of the substrate for synaptic aromatization. Prevailing dogma asserted that the gonads would likely be that source in most cases, though the adrenals could also contribute androgens systemically. Another compelling possibility is that the brain itself synthesized androgenic precursors for brain aromatization. Cholesterol transporters and steroidogenic enzymes required to synthesize testosterone had been identified in the brains of a variety of vertebrates (Baulieu 1997; Compagnone and Mellon 2000) with songbirds added to this list (Vanson et al., 1996; London et al., 2003, 2006; London and Schlinger, 2007; Soma et al., 2004). Deciphering the contribution of peripheral versus central steroidogenesis can be difficult. However, studies showing steroidal control of song and aggression outside of the breeding season, when gonadal steroids circulate at low levels, argues for a potential role for neurosteroids upstream of the aromatase reaction

(Soma et al., 2000a, 2000b; Pradhan et al., 2010). This view places the aromatase reaction in a terminal position of a more complete neurosteroidogenic pathway.

The brain might also receive substrates that do not require de novo steroidogenesis, but which might still require enzyme catalyzed conversion into aromatizable products. For example, the hormone dehydroepiandrosterone (DHEA) can be synthesized by the adrenals and, upon reaching the brain, can be metabolized by the enzyme 3 β -HSD (3 β -hydroxysteroid dehydrogenase/isomerase) and 17 β -HSD (17 β -hydroxysteroid dehydrogenase) into testosterone for aromatization into estradiol. Evidence in songbirds for both DHEA in blood and for brain 3 β -HSD and 17 β -HSD suggest this process is indeed possible (Tam and Schlinger, 2007; Cam and Schlinger, 1998; Soma et al., 2004) of behavioral significance (Pradhan et al., 2010) and generalizable to other vertebrates (Soma et al., 2015).

Detection of steroidogenic enzyme mRNA and/or protein expression provide clues to the possibility of steroid synthesis in brain. Perhaps more convincing measures involve direct detection of neural sex steroid levels greater than that in blood, and varying in concentration across discrete brain regions. Two approaches have been used to assess sex steroids in the songbird brain. One involves microdissection of brain regions followed by extraction of steroids, isolation using chromatography and quantification using radioimmunoassay or ELISA (Heimovics et al., 2016) or Mass Spectroscopy (Jalabert et al., 2021). In this latter study of song sparrows (*Melospiza melodia*), although estrogens were undetected in brain during the non-breeding season (when estrogens have been shown to activate aggressive behaviors e.g. Soma et al., 2000a, 2000b), progesterone was found to be elevated when gonadal circulating progesterone was low suggesting the neuroprogesterone synthesis as a likely possibility.

A second approach to assess neurosteroidogenesis involves in vivo microdialysis, as described previously to measure estrogens (also see similar approaches in rhesus macaques, rats, and quail (Kenealy et al., 2013, 2017; Sato and Woolley, 2016; de Bournonville et al., 2021), but which can also be used to assess aromatizable substrates as well. For example, when an aromatase inhibitor was retrodialyzed into the zebra finch NCM and steroids simultaneously measured in the dialysate, neuroestrogen levels decreased as expected (Remage-Healey et al., 2008) but, simultaneously, testosterone levels increased. This result would most likely be expected if a local pool of testosterone was the substrate for NCM aromatization. Presumably, if testosterone was delivered continuously to NCM via vascular flow, then the testosterone levels might have remained constant. One conclusion is that testosterone was synthesized in the NCM itself. If that was indeed the case, the source of the testosterone precursors remained unknown, but these results were consistent with the tantalizing idea that substantive neurosteroidogenesis was a piece of the brain aromatase story.

Since the initial microdialysis observations of rapid changes in NCM neuroestrogen levels (Remage-Healey et al., 2008), there have been reports of changes in substrate concentrations, predominantly the androgen testosterone. While the majority of work in this field has examined dynamic, phosphorylation-dependent changes in aromatase activity and production of estrogens in the brain, local neuroestrogen provision could depend on the relative changes in substrate availability, such as testosterone, and more 'passive' conversion into neuroestrogens. Indeed, in many systems the enzymes responsible for androgen synthesis are expressed and active in the brain (Tsutsui, 2011), including the songbird NCM (London et al., 2003, 2006; Soma et al., 2004; Tomaszewski and Dzibur, 2013), and androgenic synthesis can be rapidly regulated by external stimuli (Soma et al., 2004). In a direct test of these ideas, during brief periods of song playback, local levels of testosterone were measured in the NCM of adult female zebra finches, concomitantly with local levels of estrogens (de Bournonville et al., 2020). Replicating prior observations, de Bournonville et al showed elevation in local levels of NCM neuroestrogens during song playback. Consistent with the above predictions, the local levels of testosterone systematically dropped

during song playback simultaneously and both of these steroid dynamics recovered once the playback stimulus was turned off. In follow-up experiments, the same playback design was repeated alongside retrodialysis delivery into NCM of fadrozole, the aromatase inhibitor to suppress local estrogen production, and showed that local testosterone levels were elevated in response to playback over 700% from baseline. The next follow-up experiment repeated this same playback design but also included retrodialysis of fadrozole and trilostane, an inhibitor of 3 β -HSD that would, in turn, block androgen synthesis. In this case, when both neuroestrogen and neuroandrogen synthesis was blocked in the left NCM females showed no significant changes in local levels of androgens in response to song playback. This study of a songbird provides evidence that testosterone is made in the brain and changes in response to sensory stimuli. Relevant to these observations is that acoustic startle reflex in rats is sensitive to androgen synthesis inhibition (Frau et al., 2014), and sensory stimuli can cause synthesis of multiple classes of androgens in the brain (Soma et al., 2015) (Fig. 3).

In summary, an enormous body of previous work has demonstrated highly important and diverse roles for estrogen synthesis by the vertebrate brain, with studies of songbirds paving the way for many new insights. Work on songbirds has continued to advance and raised new perspectives in regard to the function of brain aromatase in songbirds. We summarize what we see as the most significant of these in the remainder of this review. As mentioned previously, these advances fall into the following classes (a) hippocampal-based learning and memory and (b) pallial auditory-processing. We conclude this review with some considerations for future research and speculation about the importance of aromatase in songbird radiation.

2. Neuroestrogens, the hippocampus, and learning and memory in songbirds

Songbirds are an extremely diverse group of birds with numerous species that exhibit exceptional spatial memory and problem-solving capabilities (Sherry et al., 1989; Krebs et al., 1989; Basil et al., 1996; Clayton and Dickinson, 1998). Since many of these behaviors are potentially modulated by hormones (Marler et al., 1988; Oberlander et al., 2004), songbirds have emerged as excellent animal models for studying the role of E2 in the regulation of spatial learning and memory.

A peculiarity of the passerine brain is that unlike every non-songbird studied, the hippocampal formation (HP) contains high levels of aromatase (Saldanha et al., 1998, 2012). Indeed, the aromatase transcript and enzyme activity are readily detectable in the finch HP (Shen et al., 1995; Saldanha et al., 1998, 1999, 2000). Ultrastructural examinations reveals an abundance of fibers including pre-synaptic boutons and post-synaptic dendrites that express the aromatase protein (Peterson et al., 2005). In some species such as the zebra finch, HP aromatase activity is comparable to that in the ovary, and 2–3 times higher than that in the male preoptic area (POA; Schlinger and Arnold, 1991; Saldanha et al., 1998). Since ovarian estrogens and aromatization in the POA are known modulators of organismal physiology and behavior, it is possible that aromatization in the HP may also modulate behavioral endpoints including HP-dependent memory function. Manipulation of circulating E2 levels supports a role of estrogens in spatial learning and memory in zebra finches and possibly also in the corvid scrub jay (*Aphelocoma californica*, Rensel et al., 2013, 2015). However, a more conclusive approach is to systematically manipulate HP aromatase independent of other tissues or other brain regions.

The abundance of aromatase in neuronal fibers of the HP, coupled with the superficial location of this brain area in birds, makes direct manipulation of the avian HP more tractable, thereby permitting a direct test of the role of targeted E2 provision on HP-dependent behavior. Bailey et al. (2013) tested the role of HP aromatase on the acquisition and performance of spatial memory in adult male zebra finches following either (a) targeted chemical ablation of the HP or (b) localized inhibition of HP aromatase by the placement of a silastic ATD (1,4,6-

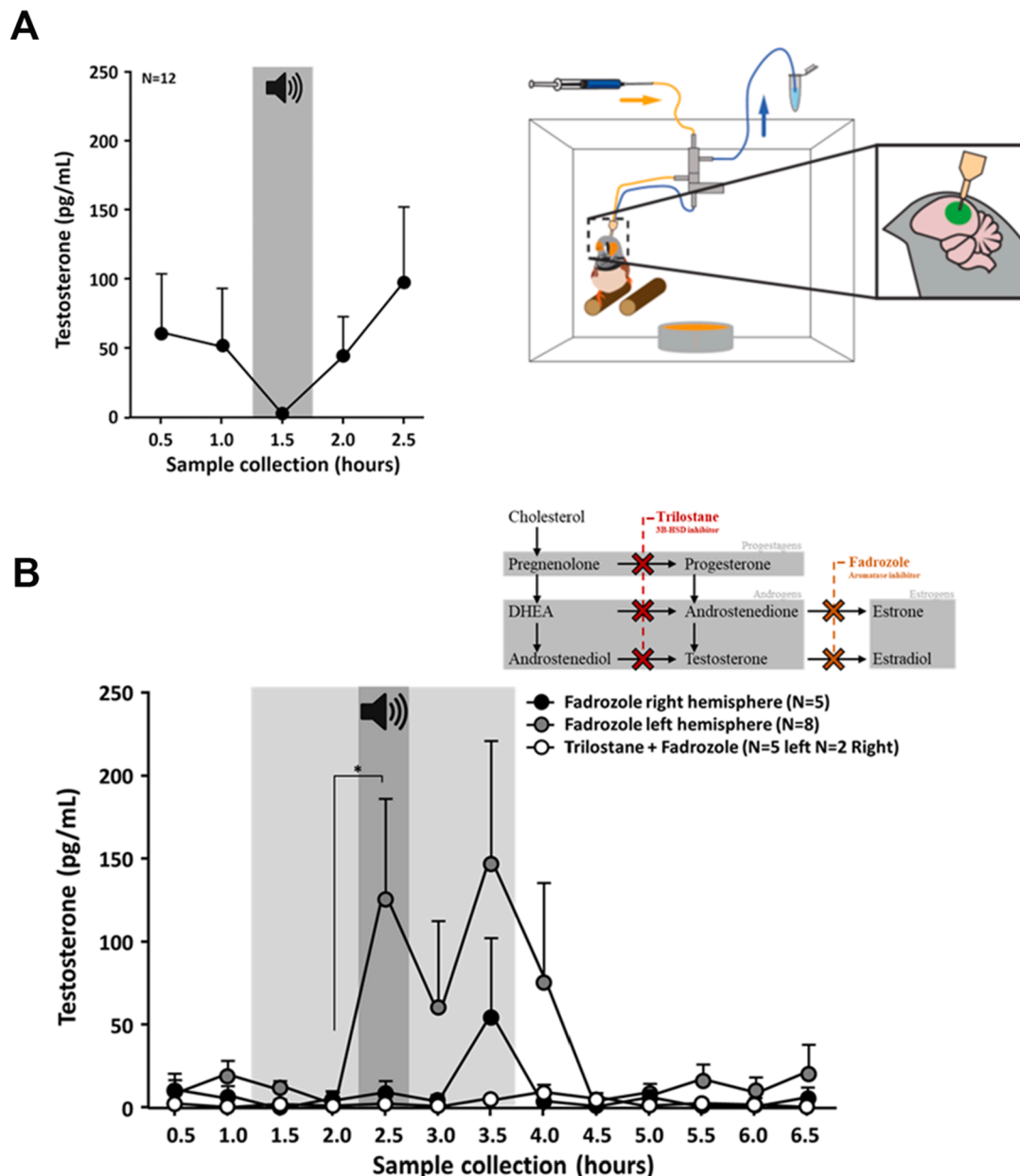


Fig. 3. Microdialysis detection of brain testosterone in zebra finch NCM shows rapid regulation. (A) Left, Testosterone levels in NCM of females. While neuroestrogen levels are elevated during playback (not illustrated, refer to [de Bournonville et al., 2020](#)), song playback (gray shading) is associated with mean decreases in levels of testosterone (mean $\pm$ SEM). Right, Schematic of microdialysis in an awake, behaving zebra finch inside a sound attenuation chamber permitting undisturbed sampling of dialysates and delivery of artificial cerebrospinal fluid (aCSF) and steroidogenic enzyme inhibitors. Schematic courtesy of Dr. Daniel M. Vahaba. Male shown for illustration purposes. (B). Top, experimental design for administration of steroidogenic enzyme inhibitors for aromatase (Fadrozole) and/or 3-beta-hydroxysteroid dehydrogenase (Trilostane) via retrodialysis. Bottom, during blockade of estradiol synthesis via aromatase testosterone levels in NCM are elevated in the left hemisphere (grey circles) during song playback (speaker, dark gray period) but not in the right hemisphere (black circles). By contrast, during blockade of local neuro-testosterone synthesis, no such elevation is observed. * $p < 0.05$. Adapted from [de Bournonville et al., 2020](#) Hormones and Behavior.

Androstatriene-3,17-dione)-filled pellet placed atop the HP. Control subjects received sham lesions or an empty silastic pellet. All subjects were then challenged to acquire a food-finding, spatial memory task. HP-lesions and ATD impaired the acquisition of spatial memories relative to their respective control groups. Further, in a subsequent probe trial, HP-lesioned and ATD birds took longer, and made more mistakes prior to finding the (previously) baited cup ([Bailey et al., 2013](#)). Upon completion of the behavioral experiment E2 levels in the HP, caudomedial nidopallium (NCM) and the POA were measured; the latter two areas representing brain nuclei where aromatase was not manipulated. HP E2 content was significantly lower in birds treated with ATD relative to controls. However, both NCM and POA E2 levels were statistically

indistinguishable in HP-ATD birds and controls, suggesting that the experimental manipulation was successful in inhibiting HP aromatase, but did not affect aromatase in other brain areas. Lastly, circulating E2 was also similar in HP-ATD and control animals ([Bailey et al., 2013](#)). The data suggest that not only does HP aromatase support memory function, but HP aromatization may be as important for HP-dependent spatial memory function as the very integrity of HP circuits.

In a subsequent study [Bailey et al. \(2017\)](#) replicated the impairment of HP-dependent spatial memory following ATD application, and went on to show that replacement with local E2, or simply the agonism of GPER1 (G-protein coupled estrogen receptor 1) despite the inhibition of HP-aromatase, restored memory function to levels comparable to

control animals. Finally, while ATD dramatically decreased the expression of post-synaptic density (PSD95), replacement with E2 or agonism of GPER1 resulted in restoration of expression levels comparable to control birds (Bailey et al., 2017). Thus, aromatase in the HP may function via the local provision of E2 and transduction via GPER1, to result in increases in synaptic strength and/or efficacy. The precise nature of this strengthening and the GPER1 dependent signaling pathways involved, remain to be elucidated.

There are striking similarities between the patterns of data described above and the effects of local E2 on spatial memory function in rodents. Experiments using HP cultures point to a role for locally derived E2 in the support of HP function. Kretz et al. (2004) demonstrated the necessity of E2 synthesis in HP slice cultures on multiple indices of synaptic plasticity including function. More specifically, HP cultures treated with letrozole showed lower levels of E2, but also expressed less spinophilin and synaptophysin, fewer synaptic profiles and impaired LTP relative to controls. Almost every effect of aromatase inhibition was rescued upon concomitant replenishment with exogenous E2. Since these studies were conducted on slice cultures, the cellular and ultrastructural source of E2 is unclear, although it remains possible that HP neurons themselves are the source of local E2 in the rodent (Prange-Kiel et al., 2006; Zhou et al., 2010).

Accordingly, in gonadectomized mice of both sexes, intra-HP administration of the aromatase inhibitor letrozole impairs spatial memory function, perhaps via an effect on memory consolidation (Tuscher et al., 2016; Koss and Frick, 2019). While the cellular and ultrastructural source of HP E2 remains to be determined in rodents, the data suggest that local aromatization within the HP supports memory function, an effect conserved across multiple vertebrate species and, importantly, independent of circulating E2 (Bailey et al., 2013; Bailey and Saldanha, 2015; Bailey et al., 2017; Koss and Frick, 2019; Taxier et al., 2020). It is noteworthy that a role for local aromatization in the severity of HP-dependent seizure activity has been reported in the rat (Sato and Woolley, 2016). This latter result adds physiological support for neuroestrogen synthesis in the rodent HP. In total, the implication of these studies across species is that neuroestrogen synthesis is an important regulator of human hippocampal function and cognition, in both healthy and dysregulated states.

More recently, the neuronal source of aromatase and its role in memory function was tested in a transgenic mouse with a conditional knockout of aromatase in the forebrain, but not the hindbrain. Lu et al. (2019) showed that knocking out the aromatase gene in the forebrain not only impaired spine density, local E2 content, and LTP in the HP, but also decreased performance on HP-dependent, but not HP-independent behavioral tasks. Mice lacking aromatase in excitatory neurons of the forebrain demonstrated a decrement in the induction of LTP relative to wild type conspecifics. More specifically, tetanic stimulation of Schaeffer collaterals resulted in the expected induction of LTP as measured in the postsynaptic neurons within CA1. However, mice lacking forebrain aromatase showed a dramatic decrease in the slope of excitatory postsynaptic potentials (epsp) relative to the slope of epsps in wild-types. Further, while performance on the Barnes maze, object placement and context dependent associative learning were all impaired in male mice lacking aromatase in the forebrain and ovariectomized female conditional knockouts, performance on tests of depressive- or anxiety-like behaviors appeared unaffected (Lu et al., 2019). While these recent studies await replication, they do suggest that modulation of HP-dependent memory function by neuronal and perhaps synaptic aromatization may be a conserved trait across many vertebrates.

Data first obtained from songbirds and confirmed in rodents, strongly argue in support of a role for targeted provision of estrogens perhaps in fibers and synapses, in the expression of behavior. As described below corroborative evidence for this idea, also in songbirds, has also been described in a completely different part of the telencephalon; the caudomedial nidopallium and its role in auditory physiology.

3. Neuroestrogens, the NCM and auditory processing in songbirds

The large body of work identifying rapid neuroestrogen synthesis in specific brain regions like the quail hypothalamus, songbird auditory pallium, songbird and rat hippocampus, and rhesus hypothalamus (see above) drove interest in whether there were resultant rapid actions on the neurophysiology of those regions and their downstream targets. This prediction was bolstered by considerable evidence for membrane estrogen receptors that mediated rapid effects in neurons in the hypothalamus and hippocampus (e.g. Kelly and Rønnekleiv, 2002; Woolley, 2007), and knowledge about such effects has grown substantially over the past 15 years (Rudolph et al., 2016; Ogawa et al., 2020; Tozzi et al., 2020; Evans, 2019; Sheppard et al., 2019). In parallel, studies that used peripheral aromatase blockers showed that estrogen synthesis was linked to auditory functions in songbirds, more generally (Alward et al., 2016; Yoder et al., 2012), and that the songbird NCM in particular contained neurons expressing the membrane G-protein estrogen receptor GPER1 (Acharya and Veney, 2012; Krentzel et al., 2018). Studies of songbirds have provided key evidence for a connection between local synthesis of estrogens and their direct actions on the neurophysiology of local circuits.

The view of neuroestrogens as genuine neuromodulators was expanded by neurophysiology studies in songbirds focused on the pallial NCM and its targets. The region contains dense aromatase expression as described above, and further study showed evidence for clusters of aromatase neurons with tightly-packed, soma-somatic contacts (Ikeda et al., 2017). This level of cellular interconnection and possible involvement of gap junctions, drove interest in the electrophysiology of NCM neurons and in particular aromatase-expressing neurons. Co-expression of aromatase and the calcium-buffering protein parvalbumin showed clearly that a portion of aromatase neurons in NCM form a population of fast-spiking interneurons (Ikeda et al., 2017), which aligned with evidence collected from post-mortem samples of human temporal cortex (Yague et al., 2006). The electrophysiology of aromatase neurons in the local and far-ranging sensory circuit in the songbird pallium became a key area of investigation.

In anesthetized *in vivo* electrophysiology recordings, estrogens had clear acute actions on the sensory coding of auditory neurons in NCM, in both adults and juveniles (Remage-Healey et al., 2010a, 2010b; Remage-Healey and Joshi, 2012; Vahaba et al., 2017; Krentzel et al., 2018). Estradiol in particular, rapidly increased auditory-evoked firing rates and the occurrence of burst firing in NCM neurons, whereas the aromatase inhibitor fadrozole did the opposite (Remage-Healey et al., 2010a,b, 2012; Remage-Healey and Joshi, 2012). The rapid actions of neuroestrogens in NCM had an influence on the larger interconnected network of song system neurons, as not only did estradiol enhance the representation of song stimuli in NCM, but this also led to alterations in stimulus selectivity in downstream sensorimotor regions HVC and a motor interface nucleus Nif (Remage-Healey and Joshi, 2012; Pawlisch and Remage-Healey, 2015). Therefore, the rapid effects of estrogens on auditory pallial function were distributed through the wider network, and likely involved interactions with a host of other neuromodulators. In support of this broadened perspective, further evidence showed that catecholamines and neuroestrogens each independently enhanced the auditory gain and coding in the NCM, and these events were likewise transmitted downstream to HVC (Ikeda et al., 2015; Lee et al., 2018). This set the stage for a more detailed basis for anatomical and physiological interactions between dopamine-receptor expressing NCM neurons and aromatase-expressing neurons in NCM (Macedo-Lima et al., 2021) (Fig. 4).

Along with the rapid regulation of estradiol levels and neurophysiology in NCM, the functional significance of neuroestrogen synthesis and action became of primary interest. Local estrogen synthesis blockade within the NCM disrupted some aspects of auditory learning in both adults (Macedo-Lima and Remage-Healey, 2020) and juveniles

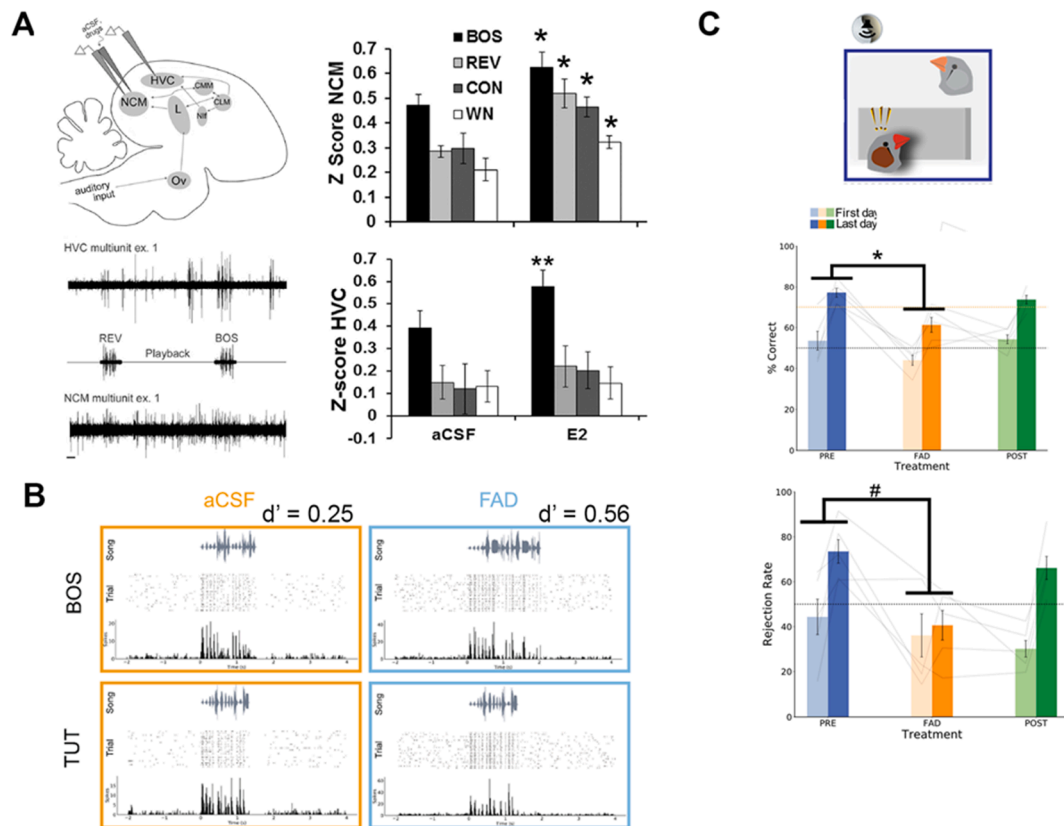


Fig. 4. Electrophysiology and behavioral studies of estrogen synthesis and action in NCM of male zebra finches. (A). Top left, schematic of dual-site extracellular recordings in NCM and HVC of male zebra finches while manipulating estradiol levels within NCM. Bottom left, examples of simultaneous extracellular voltage traces in HVC (top) and NCM (bottom) in response to acoustic playback of the bird's own song (BOS) and a reversed BOS (REV). Note the inherent neural selectivity in HVC for BOS, whereas NCM neurons respond similarly to both stimuli. At right, estradiol (E2) retrodialyzed into NCM rapidly enhances the responses to all four classes of sound stimuli (BOS, REV, CON = conspecific song, WN = white noise) in NCM, whereas the same treatment leads to enhanced responses to BOS only in HVC. Adapted from Remage-Healey and Joshi, 2012, Journal of Neuroscience. (B). Extracellular NCM recordings from birds treated with aCSF vs FAD during development after they reach adulthood. Plots show a song spectrogram (top), a raster plot of single-unit spiking activity in response to repeated presentations of stimuli (middle), and a *peri-stimulus time histogram* in 10 ms bins (bottom) for both the presentation of bird's own song (BOS) and the song of the adult tutor from which the bird learned song (TUT). The average neural selectivity for BOS stimuli across all animals in the study is shown as a mean d' value for aCSF vs. FAD treated animals (see Vahaba et al., 2020 for more details). Adapted from Vahaba et al., 2020 Scientific Reports. (C). A socially-reinforced operant experiment in which animals are separated temporarily across 'smart glass' barriers provides a way to assess auditory learning in a go/nogo paradigm (top). Intracranial injection of FAD (orange bars) impairs performance on the auditory learning task, as measured by % correct (middle) and rejection rate (bottom), whereas injection of vehicle saline (blue and green bars) has no effect on the learning paradigm. Adapted from Macedo-Lima and Remage-Healey, 2020 Hormones and Behavior. * $p < 0.05$; ** $p < 0.01$, # $p < 0.10$ for all panels. (For interpretation of the references to colour in this figure legend, the reader is referred to the web version of this article.)

(Vahaba et al., 2020), as well as immediate-early gene induction and fMRI responses to song in NCM (De Groof et al., 2017; Krentzel et al., 2019). These multiple lines of evidence demonstrated that brain-derived estrogens can have minute-by-minute actions on auditory coding and learning. Thus, NCM neuroestrogen synthesis, presumably including that occurring directly within synaptic terminals, was shown to exert rapid neurophysiological and behavioral responses, commensurate with a role as a neuromodulator in the pallidum.

Studies on the localized expression of aromatase in the HP and NCM described above have revealed many basic properties of neuroestrogen synthesis, action, regulation and function. One question requiring an answer, is how these seemingly distinct actions of aromatase on neurophysiology and behavior may be coordinated in real-time to orchestrate discrete, yet integrated organismal behavior.

4. The social behavior network: Temporal coordination via aromatase-dependent function.

What would spatially distinct and/or independently regulated pools of aromatase in the brain afford the organism? The answers may lie in a circuit of brain areas remarkably well conserved across multiple

vertebrate species. Newman (1999) elegantly described a connected set of forebrain and midbrain nuclei that seemed critically important for the expression of various social behaviors in mammals. All of these nuclei were individual contributors to many specific behaviors that necessitate social interaction such as sex behavior, aggression, parental behavior, pair bonding and affiliation. Moreover, some of these brain nuclei were also nodes in pathways responsible for social recognition, communication, and responses to social stress. However, the model went further to suggest that although these nuclei were individually important, it was the patterns and coordination of pathways among these nuclei that was necessary for the expression of the complete suite of social behaviors (Newman, 1999; reviewed in Goodson, 2005).

The nodes of the social network are the extended medial bed nucleus of the stria terminalis including the medial amygdala (BnST/mAMY), lateral septum (LS), medial preoptic area (POA), anterior hypothalamus (AH), ventromedial hypothalamus (VMN), and the portions of the midbrain including the periaqueductal gray and ventral tegmentum (PAG/VTA). Comparative studies using tract-tracing and shared neurochemical expression have documented two important properties of the social behavior network. Firstly, every one of these nuclei is reciprocally connected to every other nucleus; a characteristic that is observed in

individual species across the vertebrate kingdom from teleost fish to mammals (see O'Connell and Hofmann, 2011). Another shared property of these nodes is the consistent expression of androgen and/or estrogen receptors, suggesting that all these brain nuclei can be modulated not only by their reciprocal connectivity, but also by endocrine factors such as lipophilic sex-steroids (Goodson, 2005; O'Connell and Hofmann, 2011) (Fig. 5).

The highly localized expression of aromatase is at least one additional property that links the components of the social behavior network. Aromatase expression has been documented in two or more of the network's nodes in every vertebrate studied (Maruska et al., 2020; Coumilleau and Kah, 2014; Cohen and Wade, 2011; Saldanha et al., 2000; Roselli and Resko, 1993, 2001). Based upon our studies of aromatase expression and aromatase-dependent function in the zebra finch, we propose an aromatase (and neuroestradiol)-dependent system that coordinates the activity of multiple network nodes, and integrates social behavior and the social behavior network with sensorimotor function. We propose (1) that local aromatization of circulating androgens in discrete neural areas underlies temporally coordinated estrogen provision at multiple nodes of the social behavior network, and (2) that the expression of aromatase in specific ultracellular compartments is a plausible mechanism underlying the integration of sensory and pre-motor circuits necessary for patterns of species-specific social behaviors.

In the zebra finch, as in other vertebrates, every node within the social behavior network contains neurons that express aromatase where aromatase is expressed in both somata and their proximal fibers (Shen

et al., 1995; Saldanha et al., 2000; see Fig. 3). This is less true in two targets of the social behavior network, the HP and HVC, both of which contain an abundance of synaptic, but few if any somal aromatase expression (Peterson et al., 2005). Importantly, the HP and HVC are critical parts of neural circuits underlying spatial memory and singing, behaviors that can contribute to social behaviors like territoriality, foraging, social recognition, courtship and perhaps aggression (see Ubaka and Tsutsui, 2014).

Since the product of aromatization cannot be stored like proteins including neurotransmitters, E2 production and response at the synapse is likely to occur very rapidly. It is precisely this rapid characteristic that may allow for temporal synchrony across the social behavior network. By coordinating E2 production at this high temporal resolution, the hypothesis predicts that multiple nodes of the SBN could produce E2 almost simultaneously, thus coordinating the behaviors modulated by each node. This is likely since nodes of the social behavior network are interconnected, and because behaviors modulated by individual nodes are often expressed in synchrony. Aggression during appetitive sex-behaviors, or during parental behavior are two examples of the coincident, coordinated behaviors mediated by nodes within the social behavior network. We propose that songbirds are excellent models to test the following hypotheses. (1) coordination of the social behavior network by aromatization is necessary for the behavioral output that is influenced by social behaviors, and (2) dendritic and presynaptic aromatization underlies the integration of sensorimotor function with the social behavior network. Indeed, evidence already points to a clear relationship between the abundance of aromatase in the SBN and the intensity of social behavior output in songbirds (Rosvall et al., 2012).

In canaries (*Serinus canaria*), Alward et al. (2013) implanted testosterone (T) directly into the POA and documented increases in song rate, but not stereotypy relative to controls. Additionally, T implants in the POA also increased HVC volume, an effect often coincident with increased singing. In agreement, in the same species, an inhibition of the PAG results in an increased latency to sing (Haakenson et al., 2020). Both the POA and PAG, nodes of the social behavior network, are sites of E2 action from local aromatization, but also from aromatase expression in neurons that project directly from the POA to the PAG (Balthazart et al., 1992; Cornil et al., 2006; Carere et al., 2007). In further support, rapid actions of E2 have been described in the expression of canary morning song (Alward et al., 2018), and in the regulation of aggression in male song sparrows during the non-breeding season (Heimovics et al., 2018; see Introduction). The latter effect is believed to reflect local aromatization in the ventromedial telencephalon, a brain region that contains nucleus taeniae, another important node in the social behavior network (Soma et al., 2003). These observations are consistent with the idea that aromatization in components of the social behavior network can regulate the expression of social behaviors (Alward et al., 2013; Heimovics et al., 2018; Haakenson et al., 2020). More specifically, multiple nodes in the social behavior network may be coordinated by aromatization, and aromatase in the social behavior network can influence social behaviors mediated by sensory-motor integrative brain areas (like HVC) outside the network. To the best of our knowledge this remains to be directly tested. A comprehensive and systematic experiment that selectively inhibits aromatase in isolated components of the social behavior network and its target nuclei, and their role in behavior remains to be reported.

5. Summary and conclusions

In closing, the dramatic behavioral, anatomical, and physiological dimorphisms demonstrated by songbirds in general, and zebra finches in particular, has helped widen our understanding about fundamental aspects of neuroendocrinology. Beginning with stark behavioral and anatomical differences in the brain, to adult neurogenesis, and further to synaptocrine signaling; the research community has exploited the remarkable features of songbird species to find answers to questions

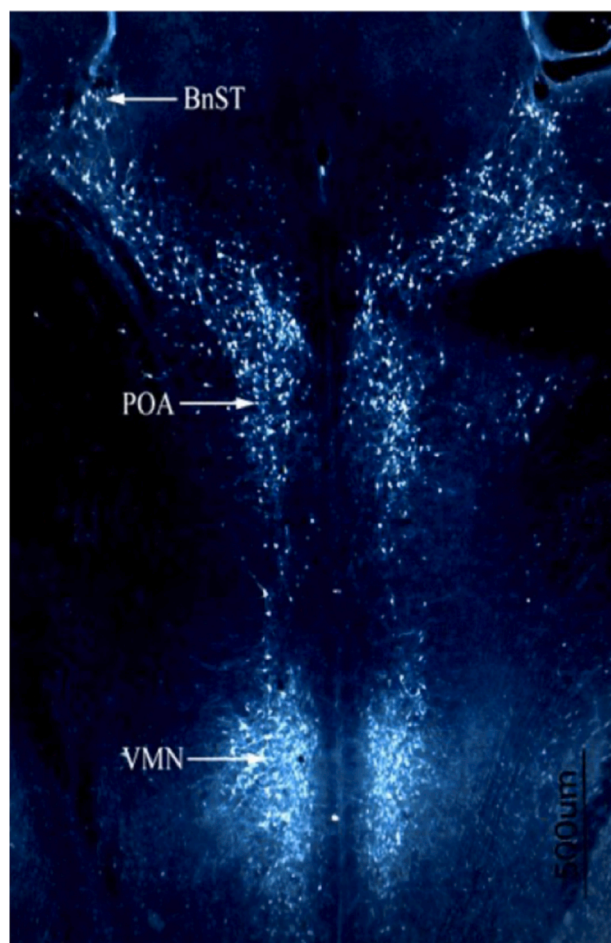


Fig. 5. Aromatase expression in the Bed Nucleus of the Stria Terminalis (BnST), Preoptic Area (POA), and Ventromedial Nucleus of the Hypothalamus (VMN) of an adult male black-capped chickadee (*Parus atricapillus*). All three brain areas are nodes within the Social Behavior Network.

about the interactions among hormones, brain, and behavior. What has emerged is a tantalizing association among neuroendocrine characteristics seemingly unique to songbirds, and their incredible adaptability, resilience, and radiation across multiple, diverse habitats and ecosystems. First, the interconnected network necessary for song learning and singing is especially pronounced in, and highly conserved within, oscine songbirds. Second, the demonstration of telencephalic neuroestrogen synthesis at levels comparable or even higher than the ovary, is scarcely matched by any other order of homeotherm. Thirdly, among homeotherms, the sustained and seasonal capability to produce, guide, and incorporate new neurons into functional circuits, is yet another trait seemingly unique to songbirds. Finally, the role of neuroestrogens in protecting the brain from impending toxicity and brain damage, has not been reported in a comparable timeframe or amplitude, in any other vertebrate (see Saldanha, 2020). Could the neuroplasticity, constitutive and inducible neuroestrogen synthesis, and their effects on social behaviors be linked to the songbird radiation?

Recent studies indicate Passerine birds appeared about 47 million years ago with the Sub-oscine-Oscine split occurring approximately 10 million years later (Oliveros et al., 2019). The oscine suborder has long been recognized to be distinct from Suboscines based on their unique syringeal musculature, as well as other morphological traits. Their capacity to learn vocalizations, a behavioral trait apparently not found in suboscines, was also hypothesized to be a phenotype that led to their highly successful radiation (Adkins-Regan, 2005). The idea that song learning and production was a crucial determinant of the success of this immense Suborder was further strengthened by the discovery of the complex neural song system controlling song and song learning, a system that is present, though poorly developed in Sub-oscines.

An additional feature that appears, to the best of our knowledge, to separate songbirds from other avian groups is their elevated expression of aromatase in several brain regions (Saldanha et al., 2000). As described in Section 1.1.1 of this review, both the auditory processing region NCM and the HP (as well as other regions) are seen to possess a greater capacity to make estradiol in passerine species as compared to non-passerine species with even greater expression in the oscine versus the suboscine Suborders. Thus, we believe it appropriate to consider that the expanded transcriptional regulation of aromatase in the songbird brain was a crucial determinant of the success and radiation of this clade of birds. Localized neuroestrogen synthesis may have led to generalized improved neural plasticity and auditory categorization among songbirds. Neuroestrogen production may have allowed for the evolution of increased spatial memory and intelligence. Some species, such as the Corvids, captured this latter trait and evolved exceptional problem solving and spatial memory capabilities. Other groups exploited their increased estrogen-dependent auditory capabilities allowing emergence of learned vocalization and production of complex songs. Some species took advantage of this enhanced ability to make neuroestrogens to develop expanded social relationships by actions on their social behavior network. Altogether, these capabilities made for a unique set of advantages possessed by the Oscine songbirds that allowed them to become the most speciose group of birds in the world.

Of course, while brain aromatase has been examined in a number of oscine species and several non-passerine species, few suboscines have been studied. Obviously the expression and regulatory elements controlling aromatase expression need to be examined in a larger group of birds. Nevertheless, much has been made of the neural song system and its importance to the oscine clade despite the fact that few suboscines have been studied (e.g. Soma et al., 2000b; Liu et al., 2013). Further research can only help in deconstructing this speculation into careful and informed hypotheses that will continue to fuel our curiosity and fascination with songbirds.

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Declaration of Competing Interest

The authors declare that they have no known competing financial interests or personal relationships that could have appeared to influence the work reported in this paper.

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