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Frontiers in Neuroendocrinology 27 (2006) 247–274
Frontiers in Neuroendocrinology
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Review

Brain aromatase: New lessons from non-mammalian model systems

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Available online 10 July 2006

Abstract

This review highlights recent studies of the anatomical and functional implications of brain aromatase (estrogen synthase) expression in two vertebrate lineages, teleost fishes and songbirds, that show remarkably high levels of adult brain aromatase activity, protein and gene expression compared to other vertebrate groups. Teleosts and birds have proven to be important neuroethological models for investigating how local estrogen synthesis leads to changes in neural phenotypes that translate into behavior. Region-specific patterns of aromatase expression, and thus estrogen synthesis, include the vocal and auditory circuits that figure prominently into the life history adaptations of vocalizing teleosts and songbirds. Thus, by targeting, for example, vocal motor circuits without inappropriate steroid exposure to other steroid-dependent circuits, such as those involved in either copulatory or spawning behaviors, the neuroendocrine system can achieve temporal and spatial specificity in its modulation of neural circuits that lead to the performance of any one behavior.

Keywords: Brain aromatase; Estrogen; Neurosteroid; Glia; Songbird; Teleost; Vocal communication; Hearing; Sex differences; Neuroprotection; Learning and memory

1. Introduction

Aromatase (estrogen synthase), the enzyme that catalyzes the transformation of androgens into estrogens, is a member of the P450 superfamily of cytochrome enzymes, and a product of the CYP19 gene (Fig. 1). Unlike other members of P450 enzymes, aromatase is the only enzyme in vertebrates that is capable of creating an aromatic ring that characterizes estrogenic compounds [239]. It is well established that the conversion of androgens to estrogens within the central nervous system (CNS) is an essential means by which testosterone regulates many physiological and behavioral processes such as sexual differentiation of the brain, activation of male sexual behavior, and steroid-hormone feedback on the secretion of gonadotropic hormones [17,156]. Formulation of the "aromatization hypothesis" by MacLusky and Naftolin [160], that androgen effects on the differentiation of male-specific brain circuits and functions in vertebrates are actually mediated by brain-derived estrogens, arose from studies which demonstrated that estrogens mimic androgenic effects on sexual behavior, gonadotropin secretion, and brain sexual differentiation reviews: [58,156]. Since that time, brain aromatase has been shown to regulate neural plasticity by stimulating growth and migration of cells, protecting against neurodegeneration (e.g., Alzheimer's disease) and brain injury, regulating the reproductive endocrine axis and activating adult sexual behavior, and influencing learning and memory, stress, and mood (review: [157]).

Since the discovery of estrogen synthesis in the brain of fetal mammals (see [177]), numerous studies throughout all major vertebrate groups have shown the brain's ability to metabolize androgens to estrogens. Two vertebrate lineages in particular, teleost fishes and the oscine songbirds, show remarkably high levels of adult brain aromatase activity, protein and gene expression compared to other vertebrate

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doi:10.1016/j.yfrne.2006.05.002

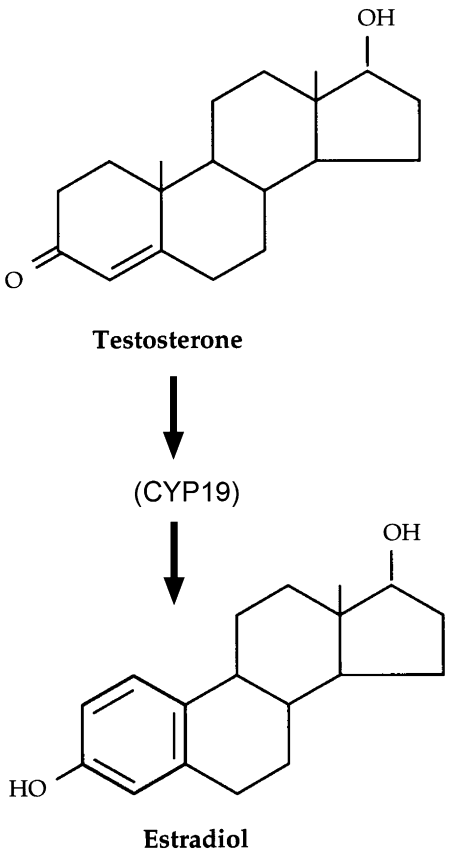


Fig. 1. Schematic depiction of aromatization by cytochrome P450 aromatase (encoded by the CYP19 gene) which catalyzes the conversion of testosterone to the aromatic compound, estradiol.

groups (see [53,208] and below). This paper will mainly review the anatomical and functional implications of brain aromatase expression in each of these non-mammalian groups that have provided important model systems for investigating the functional outcomes of estrogen synthesis, via both genomic and non-genomic pathways, in the nervous system. Studies of birds have played a prominent role in developing the concept that estrogens can organize and activate masculine neural circuits, like those involved in vocalization. Much of the discussion of teleosts focuses on one group, the toadfishes that include midshipman fish that depend upon vocal communication for successful reproduction (review: [33]). Studies of these teleosts, together with those of songbirds, have established neuroendocrine principles for the operation of well-defined neural circuitries leading to measurable, naturally selected behaviors, namely vocal communication signals.

1.1. Brain aromatase: An evolutionary perspective

Estrogen is an ancient signaling molecule and its importance is reflected in its conserved role in reproductive function throughout all vertebrates (reviews: [57,239]). Many examples of "vertebrate sex steroids," including estrogens, are now known to also be important in invertebrate reproduction [74,75]. Recent evidence suggests that estrogen receptors are the most ancient of all steroid receptors with their emergence dating prior to the deuterostome/protostome divergence [259]. Aquatic invertebrates are affected by estrogens and were presumed to concentrate estrogens from the environment, but new evidence shows aquatic invertebrates may make their own estrogens (see [153,203]) Aromatase activity has been detected in gonadal tissue, but not the head region, of the protochordate *Branchiostoma* (once known as *Amphioxus*, [59]) that shares many traits with vertebrates (see [196]). A Scleractinian coral exhibits aromatase activity along with high estrogen levels just prior to spawning, suggesting that corals possess all the enzymatic machinery necessary for steroid synthesis [266]. Thus, the role of endogenous steroid hormones in signaling reproductive events extends outside vertebrate lineages, suggesting that gonadal aromatase may have originated prior to vertebrates, more than 500 million years ago, earlier than previously thought [60].

Although aromatase activity is found in gonadal tissue of all vertebrates (as well as some invertebrates, see above), aromatase expression in brain is thought to have arisen in jawless fishes as evidenced by lampreys [55,58]. Therefore, expression of aromatase in both gonad and brain appear to be highly conserved vertebrate characters. Most vertebrates have a single gene, CYP19, which codes for cytochrome P450aromatase and, as demonstrated in mammals, is expressed in a number of extragonadal tissues (e.g., brain, fat, bone and placenta) where different promoters of the gene dictate tissue specificity (review: [240]). One exception in mammals is porcine aromatase in which distinct isoforms translated from different genes are expressed in different tissues [68,112].

Many recent investigations in teleost fishes have demonstrated two distinct forms of aromatase, CYP19a and CYP19b (or CYP19A1 and CYP19A2), which are preferentially expressed in ovary and brain, respectively, with each isoform sharing a higher identity to the same form in other species than to the other form within a species (review: [189]). The first evidence for two different aromatase genes was demonstrated in goldfish (*Carassius auratus*) and zebrafish (*Danio rerio*) [60] and later confirmed and found in these and many other diverse species of teleosts [42,69,98,113,141,145,149,151,152,255,257,264,268,284]. There is also evidence for CYP19 expression outside the brain and ovary in some teleosts (see [269]). A whole genome duplication event (tetraploidization) is thought to have occurred within the actinopterygian lineage, perhaps just prior to the teleost radiation [135]. Some of the duplicated genes, such as aromatase, are thought to have been preserved by subfunctionalization, whereby each gene may experience a loss or reduction of expression for different subfunctions (subset of a gene's function, either regulatory or coding) by degenerative mutations, so that both genes are required to fulfill the role of the ancestral gene [86] (see [195]). After preservation of both gene duplicates, the segregation of both ancestral gene subfunctions occurs by subfunction partitioning [195]. By fostering incompatibility among populations, lineage specific subfunction

partitioning of gene duplicates in general could have been responsible for the explosive radiation of teleosts, although the relative amount of subfunction partitioning that happened before or after the teleost radiation is unknown [195]. Investigating divergent groups of teleosts will help address this question, since there is evidence for single copies of aromatase in ancestral teleosts and non-teleosts (see below). Two different isozymes also offer functional diversity in addition to the regulation of timing and localization of aromatase expression. Indeed, Zhao et al. [285] have demonstrated that the goldfish brain aromatase isozyme has significantly higher activity than the ovarian form when expressed at the same levels *in vitro*. Similarly, different isozymes in Euorpean sea bass (*Dicentrarchus labrax*) also show different catalytic properties [107]. Eels, an ancestral group of teleosts (Elopomorpha), appear to have a single CYP19 gene [132,267], suggesting the aromatase gene duplication occurred after the divergence of the elopomorphs. Interestingly, sequence analysis of European eel CYP19 shows higher identity to brain (CYP19b) form in some fish, but higher similarities to gonadal (CYP19a) form in other fish [267].

To date, the aromatase gene has been investigated in only one elasmobranch species, the Atlantic stingray (*Dasyatis sabina*) and, like mammals, only one gene was identified [131]. Aromatase activity levels in nervous tissue of cartilaginous fishes are, again, like mammals, relatively low compared to teleosts [58]. When directly compared to a more recently derived teleost, the black porgy (*Acanthopargus schlegeli*), Japanese eel also showed relatively low brain aromatase activity, comparable to mammalian levels [136]. If ancestral fishes with a single aromatase gene also possess distinct promoters for tissue specificity, then mammals would reflect the ancestral phenotype. At least in *D. sabina*, this seems to be the case where, like mammals, aromatase transcripts expressed in extra-gonadal tissue (brain, pituitary and kidney) are initiated from an untranslated first exon [131]. Further insights into the evolution and function of brain aromatase would be elucidated by investigating brain aromatase activity and gene expression in non-teleosts such as lungfish, bichirs (*Polypterus*), chondrosteans (sturgeon, gars and bowfin) and other cartilaginous fish (e.g., sharks, skates, rays and chimaerids) (see [179]). An investigation of *Polypterus* hox genes revealed no evidence for genome duplication [67], and one would predict this ancient lineage of fish to have not only a single copy of CYP19, but also brain aromatase activity comparable to elasmobranchs. Since brain aromatase expression fluctuates, often quite dramatically, with season and reproductive state (see below), careful considerations must be taken when comparing aromatase activity and transcript levels across species.

1.2. Sexual dimorphism in brain aromatase, circuitry and behavior

In all vertebrates, steroid hormones participate in the development/organization of brain areas important in the production of sex-specific reproductive behaviors (review: [72]). In fact, many of testosterone's effects on the differentiation of male-specific brain circuits and functions are mediated by its conversion to estrogen in the brain by aromatase [17]. Perinatal exposure to estrogens in mammals and birds is also known to permanently affect neuronal/glial morphology and brain function in males (reviews: [10,142]), and sex differences in the brain also arise from aromatization during a critical developmental window (review: [176]). A number of recent studies in both teleost and avian species have now begun to provide a more complete picture of species variation in brain aromatase expression.

1.3. Teleosts

Since the work of Callard and colleagues [54], multiple studies across a broad range of species have substantiated that teleosts have extraordinarily high brain aromatase activity levels, especially in the forebrain which exhibits levels100–1000 times greater than that found in other vertebrates (reviews: [53,58,189]); aromatase mRNA levels are also very high (e.g., see [87,99]). These discoveries have generated much speculation as to the function and adaptive significance of this conserved character across teleosts. One area of intense interest has been the investigation of possible sex differences in aromatase protein and mRNA expression. Female goldfish, longhorn sculpin (*Myoxcephalus octodecimspinous*) and stickleback (*Gasterosteus aculeatus*) have higher activity in the telencephalon and preoptic area (POA), respectively, than males, while male and female European sea bass show the opposite relationship [42,43,56,108,186]. In medaka (*Oryzias latipes*), females have higher activity levels in homogenates of brain slices that contain periventricular hypothalamic areas, while males have higher levels in slices which contain the posterior preoptic area and suprachiasmatic nucleus [169]. In zebrafish, although quite variable, most males have higher aromatase mRNA in the telencephalon and hypothalamus compared to females [111], and brain aromatase may play a role in sex differentiation [264]. Similarly, male pejerrey (*Odontesthes bonariensis*) have higher mRNA expression in forebrain and midbrain homogenates [255]. Thus, although sex differences in brain aromatase are documented in teleosts and other vertebrates (see [202]), few examples have identified the functional significance of differences in the adult brain (see next section on birds).

One strategy to better understand sex differences in the brain is to study a model system that manifests an extreme example of a dimorphism linked to a behavior that differs either between or within the sexes. Midshipman fish, *Porichthys notatus*, provide one such model, in part, because they have two male phenotypes/morphs that differ in a large suite of behavioral, somatic, neural and endocrine traits (review: [27]). The presence of two distinct male morphs offers a unique opportunity to identify the biological principles that underlie natural variation in brain structure and function within the same sex (also see [200]). By focusing these efforts on auditory and vocal mechanisms shared by all vertebrates

(see below and later section on Audition), studies of midshipman fish have identified neuroendocrine principles that are also likely common to all vertebrate groups (review: [33]). Thus, an important component of these studies has been to establish male morph and male–female divergence in vocal-auditory traits and to show how steroidal and peptidergic hormones can influence the development, maintenance and seasonal expression of those traits. The two male morphs, types I and II, adopt fixed alternative reproductive strategies and are distinguishable by a variety of characteristics which correspond to morph-specific vocal and spawning traits [27]. As adults, type I males build and guard nests and actively "hum" to acoustically court females for spawning by simultaneously contracting large sonic muscles attached to the walls of the swim bladder ([50]; Fig. 2A and B). Type II males do not build nests or court females but instead mature at an earlier age and invest in gonad weight rather than body size to "sneak" or "satellite" spawn in competition with type I males (see [27]). Only type I males care for the young. The ratio of sonic muscle to body weight is 6 to 10-fold greater in type I males compared to either type II males or females. In contrast, the gonad to body weight ratio is on average close to nine times larger in type II males that have testes that are, on average, close to 9% of their body weight [23,50]. Many somatic, endocrinological and neurobiological characters that are divergent between male morphs are similar between type II males and females and parallel their convergence in vocalization and reproductive tactics [27].

Spanning the hindbrain–spinal cord junction lies the extensive sonic motor nucleus (SMN) that innervates the sonic swim bladder muscles. Developmental studies show that the SMN and sonic muscles share developmental origins with the vocal neurons and muscles of tetrapods; hence, the use of the term "vocal" when describing this motor system in teleosts [29,34]. Single neuron recording studies further support comparisons of vocal motor systems between teleosts and tetrapods (for example [147,277]). The SMN is inter- and intrasexually dimorphic, i.e., individual motor neurons comprising the nucleus and the nuclear volume itself is larger in type I males compared to type II males and females [23,24,27,28], and is androgen sensitive [26,35,48]. Other nearby premotor neurons comprising a vocal pattern generating circuit are also dimorphic ([28]; see Fig. 2C and D). In this regard, midshipman fish provide an ideal model to examine the influence of brain aromatase on the development and maintenance of dimorphic brain structures that directly control divergent reproductive tactics. Schlinger et al. [225] first demonstrated that the forebrain of all three reproductive morphs had the highest levels of aromatase activity, but were no different from one another. The hindbrain–spinal homogenates, however, which contain the dimorphic sonic motor nucleus, had significantly higher levels of activity in females and type II males (which were similar to each other) than type I males. These dimorphisms were later corroborated by measuring aromatase mRNA within the SMN by *in situ* hybridization technique, although females showed higher levels compared to males only in the pre-nesting period, while type II males expressed higher mRNA levels than both type I's and females during the nesting (spawning) period (see below) [89]. Since aromatase converts testosterone to estrogen, and estrogen does not have an effect on the morphology of the sonic motor nucleus [26,48], it was hypothesized that upregulation of aromatase in the "vocal" hindbrain–spinal region may prevent circulating testosterone, which is prevalent in both females and type II males, from masculinizing the central sonic motor system [87,225] and thus provide a mechanism for leading to either the development or maintenance of two male morphs. This was hypothesized, in part, because treating juvenile males with testosterone masculinized their SMN to be type I-like, while treating type II males in the same fashion had no effect except to upregulate aromatase mRNA in and around the SMN [26,35] (also see [88]). Thus, an increase in androgen metabolism, which parallels an increase in available androgens, may be an effective buffer to regulate steroid access to specific brain nuclei [35]. Manipulating brain aromatase levels directly during ontogeny to see effects on morph phenotype remains to be tested experimentally in this species.

Adult sex change in several teleosts is under social control [276] and rapid changes (within minutes) in behavior can occur even in the absence of gonads [101]. In the protandrous (male to female changing) black porgy (*A. schlegeli*) inhibition of brain aromatase prevents natural sex change to female [154]. In the protogynous (female to male changing) bluebanded goby (*Lythrypnus dalli*) females have significantly higher brain aromatase activity levels than males, but within minutes to hours of sex change, a significant increase in aggressiveness is coincident with a dramatic drop in brain aromatase activity (40%) [41]. Thus, brain estrogen levels likely shift prior to changes in gonadal structure and circulating steroids that may then maintain the sexual phenotype.

While certain regions of the promoter of CYP19b (CYP19A2) such as the estrogen response element (ERE) are consistently found in all teleosts (see below), other binding sites in the 5′-flanking region are variable, but may be predictive about species-specific mechanisms of brain and/or sexual differentiation. For instance, Steroidogenic Factor 1 (SF-1) that is involved in the regulation of P450 steroidogenic genes, and SRY/SOX 9 that are involved in male sexual differentiation in mammals, are found in both the CYP19a and b promoter in medaka, but only in CYP19a of zebrafish, goldfish and tilapia [65,151]. Interestingly, SF-1 distribution overlaps with aromatase in hypothalamic, but not telencephalic, brain areas in zebra finch [5]. In a comparison of different sex changing fish, a protandrous species (Asian sea bass or barramundi, *Lates calcarifer*) had progesterone responsive element (PRE) and SOX 9 binding sites, a protogynous species (humpback grouper, *Cromileptes altivelis*) had both PRE and androgen responsive element (ARE) sites, and a bi-directional changing species

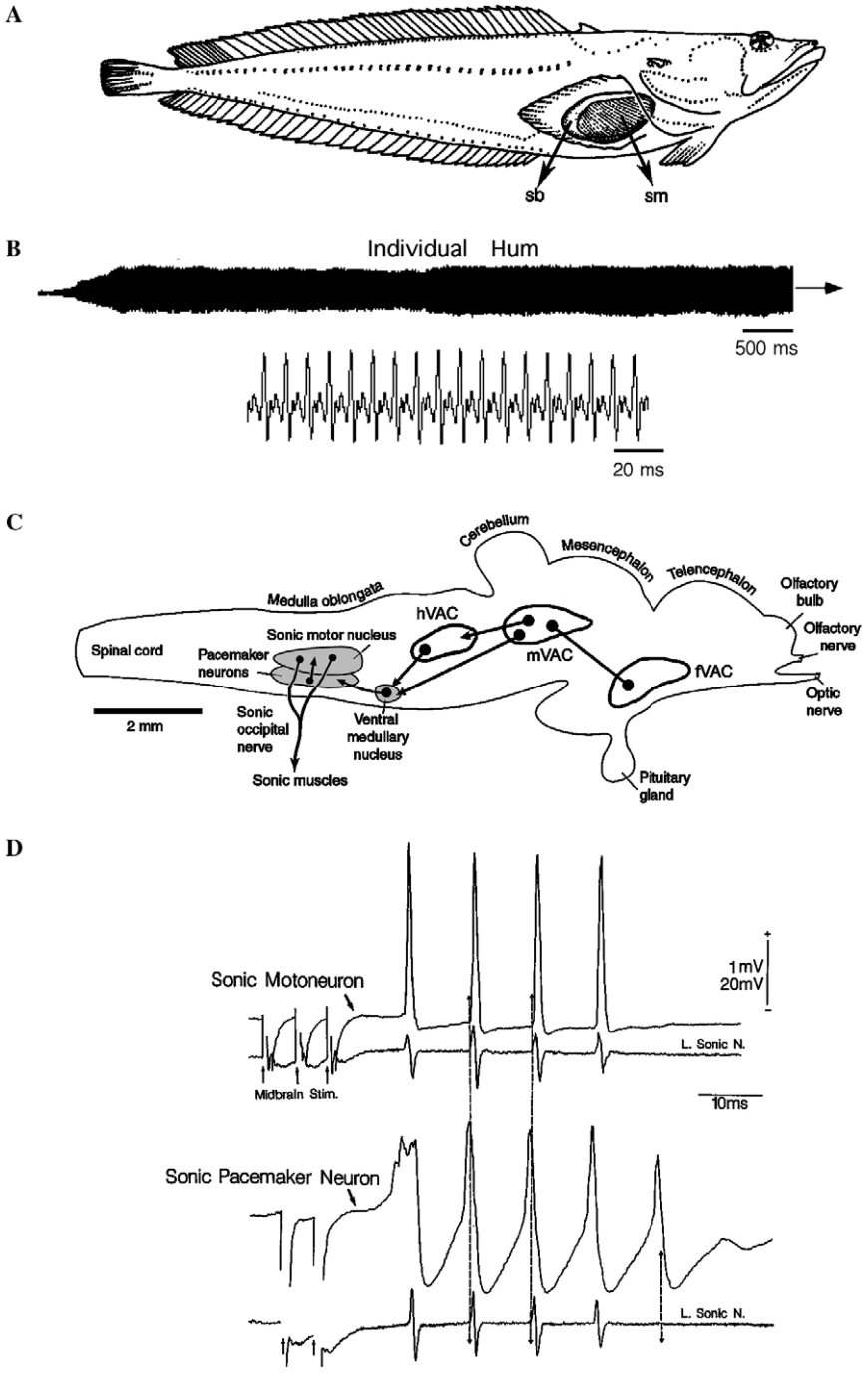


Fig. 2. Vocal motor network in midshipman fish. (A) Line drawing of a type I male. Sonic muscles (sm) are attached to the lateral walls of the swimbladder (sb). (B) Midshipman produce a long duration advertisement call known as a "hum" (shown here on two time scales; adapted from [30]). (C) Longitudinal view showing the relative position of vocal-acoustic integration centers (VAC) at forebrain (f), midbrain (m) and hindbrain (h) levels (see [109]). Central auditory nuclei provide input to each VAC (also see [31]). A hindbrain–spinal vocal pattern generator includes pacemaker neurons that provide bilateral, afferent input to the adjacent sonic motor nucleus that innervates the sonic muscles via occipital nerve roots that are comparable to the hypoglossal nerve [24,25,109]. A ventral medullary nucleus links the pacemaker-sonic motor circuit across the midline [25]. Lines connecting two dots represent reciprocal pathways. (D) Intracellular records from sonic motor (top) and pacemaker (bottom) neurons from a type I male (from [24]). Electrical stimulation of a mVAC site (see C) evokes a rhythmic sonic motor volley that is recorded extracellularly from an occipital nerve root and referred to as a "fictive vocalization" because its inter-pulse interval and total duration predict, respectively, the fundamental frequency and duration of natural calls. Each trace is the average of four records; top trace is DC-coupled, low-gain intracellular record, while bottom one is extracellular recording from the left (L) occipital nerve (N) root. Small arrows at the beginning of each of the lower traces indicate the onset of each electric stimulus pulse delivered to the midbrain. Each sonic nerve potential is aligned (hatched vertical lines) with the pacemaker and motor neurons' action potentials. Pacemaker neurons fire just prior to motor neurons that are nearly coincident with each pulse of the motor volley. Firing rates are independent of either stimulus number or frequency. Time scale and direction of polarity for all records are indicated in top trace. Amplitude bar represents 20 and 1 mV for intracellular and nerve records, respectively.

(broad-barred goby, *Gobiodon histrio*) had SOX 5, but not PRE, ARE or SOX 9 binding sites in CYP19b [98]. In addition, binding sites for transcription factors involved in neurogenesis and CNS development were also identified on CYP19b of medaka which may function to coordinate sexual differentiation and neural development [151].

1.4. Songbirds

Among avian species, a great deal of research has focused on songbirds, the Oscine suborder of the Order Passeriformes. Songbirds produce complex vocalizations by regulating respiration and thus airflow across a membranous syrinx that stretches across each of their two trachea [256]. A series of brain centers serve to control the output of the tracheosyringeal division of the hypoglossal motor nucleus that innervates syringeal muscles [279]. Most studies of the behavioral endocrinology of songbirds have focused on zebra finches and on canaries because, unlike most other altricial songbirds, these species breed well in captivity. Consequently, they are plentiful, individuals are available at all developmental ages for study and many aspects of their behavior, neuroanatomy and neurophysiology are well characterized.

The neural song system has been the focus of intense investigation into the mechanisms underlying song learning and song production (e.g., [197,47]). In some species, such as the zebra finch, this song system is well-developed in males that sing, but poorly developed in females that do not sing [181]. Therefore, the zebra finch has been investigated to determine the mechanisms underlying brain sexual differentiation. Some species, such as the canary, breed and thus sing seasonally, and the neural circuits underlying song fluctuate morphologically across the seasons [263]. These species have been studied to determine the mechanisms underlying this profound neuroplasticity. Because sex steroids are thought to participate in the activation of song, the development of neural sex differences and in aspects of neuroplasticity [223], neural steroid metabolism may be particularly important in this group of birds.

The importance of songbirds in behavioral neuroendocrine research has been well-documented recently [110,226,281]. Early work established that aromatizable androgens or estrogens activated song behavior in adults, influenced the capacity to learn song during critical developmental periods, and induced the early masculine growth of neural circuits in females of some species (review: [228]). Surprisingly, measures of sex steroids in songbird blood often detected relatively high levels of estrogens in males that, in some cases, were not reduced by castration (review: [222]). When sex steroids are found in blood, we assume they are produced by the gonads, or on some occasions, by the adrenals. Given such high levels of estrogen available to the brain from peripheral sources, there was a risk of overlooking a possible a role for brain aromatization in songbird neurobiology. Additional studies showed, however, that aromatase was expressed widely and in large amounts in the zebra finch brain [216,272], whereas testicular and adrenal aromatase was minimal [216]. These results prompted work on other songbirds which, like the zebra finch, expressed aromatase in several telencephalic regions at higher levels than are seen in non-passeriform species [219,93,201,205,206,237,238,245,250] (Fig. 3). Moreover, there is evidence that at least some of the estrogen detected in the blood of males is synthesized in the brain [205,217,220]. These studies collectively shifted attention back to the brain as the principle site of synthesis of neuroactive estrogens in songbirds.

There is strong evidence that steroids synthesized in the developing male zebra finch brain are responsible for inducing the masculine growth of the neural song control system. If young females (up to three weeks post hatching) are treated with 17β-estradiol they will develop a song system sufficiently well masculinized to allow for singing when the females reach adulthood [224]. These results lead to the conclusion that the male brain is naturally exposed to more sex steroids during the posthatching period to induce masculine song system growth. The evidence that this sex steroid is synthesized in the brain comes from three sources.

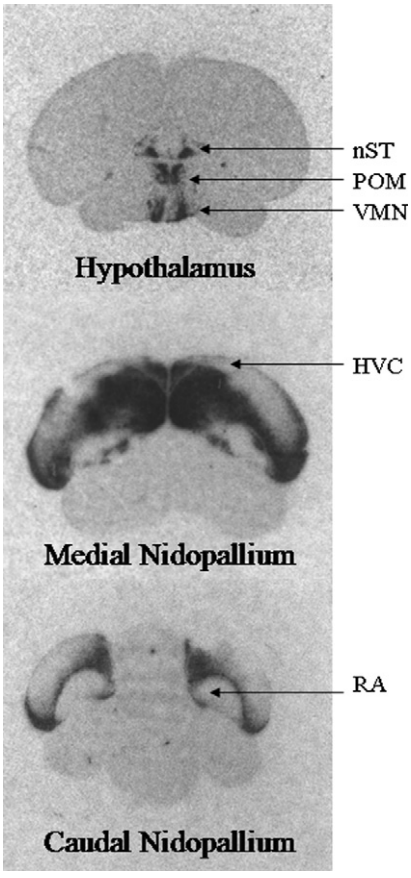


Fig. 3. Autoradiographic film images of an *in situ* hybridization depicting the distribution of aromatase mRNA (black) in medial (hypothalamus), mediocaudal (medial nidopallium) and caudal (caudal nidopallium) regions of the telencephalon of an adult male zebra finch. *Abbreviations*: NST, nucleus striae terminalis; POM, nucleus preopticus medialis and VMN, ventramedial nucleus of the hypothalamus. Song regions with no hybridization are HVC and RA (robust nucleus of the arcopallium).

First, with the exception of one experiment [129], measures of circulating sex steroids and peripheral steroidogenesis during the critical period of song system development in zebra finches do not support the view that males make more estrogens peripherally than do females [4,62,91,218]. Second, the finding that genetic females can be induced to develop with testes, but retain a feminine song system [106,273,274] indicates that sex steroids involved in song system masculinization are not naturally synthesized in the testes. Finally, estrogens can be measured in media taken from long-term cultured slices of the developing zebra finch brain and these estrogens appear to contribute to a feature of masculine song system development that occurs *in vitro* [125]. This latter study provides convincing evidence for estrogen synthesis *de novo* by the songbird brain with a specific and identifiable developmental function for this neuroestrogen [227].

In addition to evidence that neuroestrogens promote growth of the masculine song system in male zebra finches, there is also evidence that the masculine neural circuitry develops by constitutive sex-specific gene expression in brain [11]. Evidence exists for both steroid-dependent and steroid-independent effects on song system development [6,106,273,274]. The results can be reconciled by assuming that the fully masculine neural phenotype arises because estrogens act on the male brain with its background expression of genes on the Z chromosome. It is also possible that some of the genes expressed to a greater degree in the male brain are the steroidogenic factors that lead to the greater production of masculinizing neuroestrogens (e.g., [125]). Studies are ongoing in several laboratories to identify the full spectrum of genes and hormones that produce the functional neural song system.

2. Neuroanatomical basis for high aromatase activity

2.1. Teleosts

Only recently was the neuroanatomical basis for high enzyme levels in teleosts elucidated using species-specific mRNA probes and antibodies [87]. Using an antibody designed to recognize conserved teleost aromatases, distribution of the enzyme was first demonstrated in midshipman fish and was found consistently throughout ventricular areas in the brain and rostral spinal cord of adults (Figs. 4 and 5). Aromatase-immunoreactive (-ir) cell bodies line the entire periphery of the telencephalon, which is ventricular surface in teleosts because the lateral walls of the developing telencephalon turn outward, that is undergo a process of eversion as opposed to evagination in tetrapods [180](Fig. 5H–J). Aromatase-ir is also found around the olfactory bulb periphery and throughout preoptic areas which lie along the medial ventricle, and along the third ventricle and periventricular zones of the hypothalamus (Fig. 5G–K). In the midbrain, aromatase-ir cells line the periaqueductal gray with processes that extend to cover much of the tegmentum, while label is absent in both the

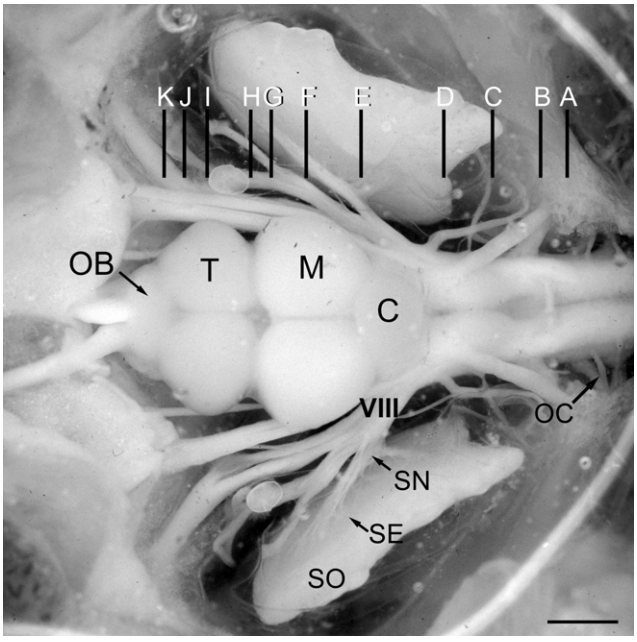
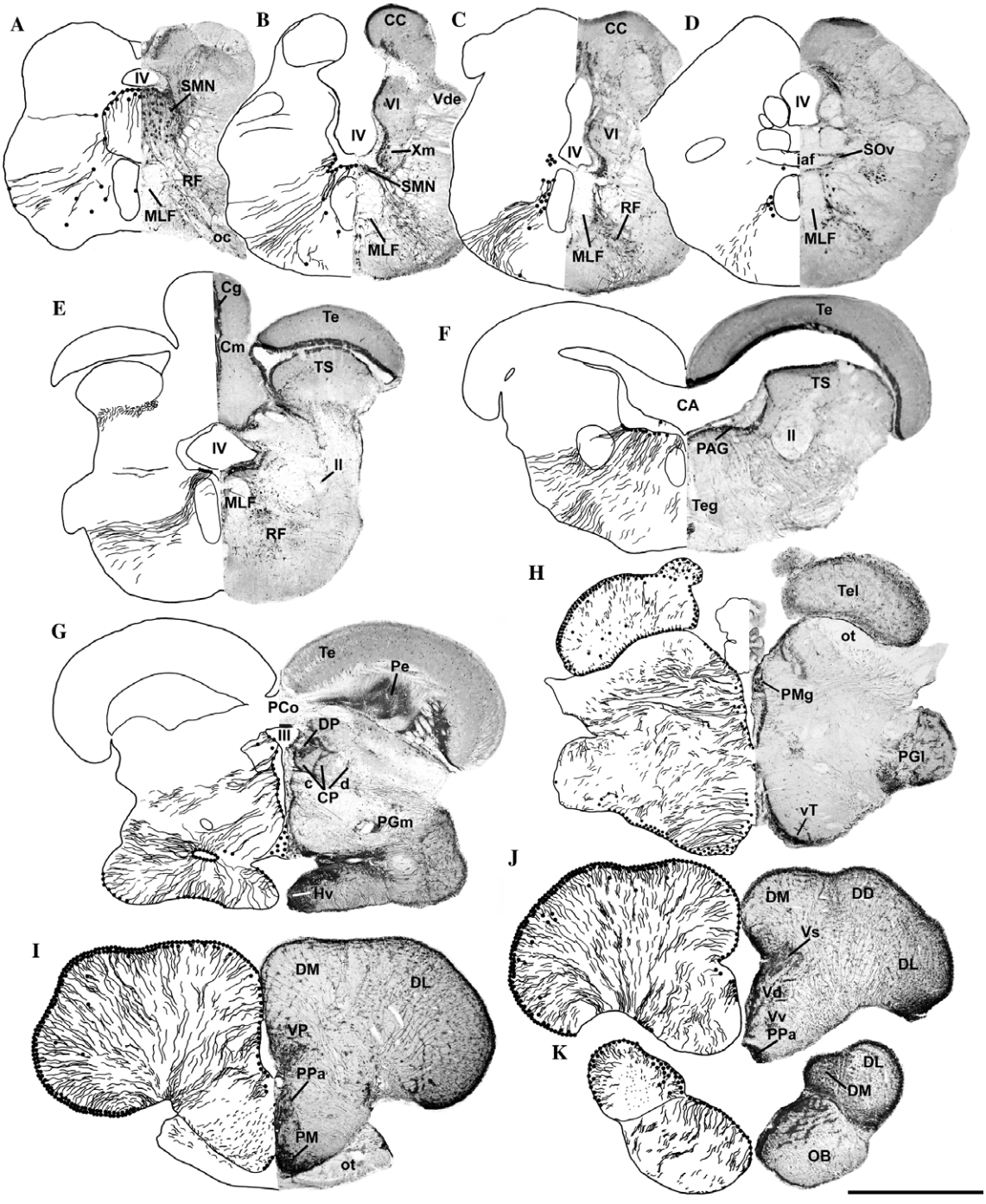


Fig. 4. Dorsal exposure of midshipman brain and inner ear indicating levels of transverse sections in Fig. 5, posterior to anterior (A–K), which demonstrate anatomical locations of aromatase-ir. *Abbreviations*: C, cerebellum; M, midbrain; OB, olfactory bulb; OC, occipital nerve; SE, saccular epithelium of the inner ear; SN, saccular branch of VIII; SO, saccular otolith; T, telencephalon and VIII, eighth nerve. Scale bar = 1.5 mm. Modified from [90].

tectum and torus semicircularis (homologs to the superior and inferior colliculus, respectively) (Fig. 5E and F). In the hindbrain, aromatase-ir is found along the fourth ventricle with processes coursing ventrolaterally through the reticular formation. Aromatase-ir cells also line the dorsal periphery of the SMN with their processes enshrouding the motor neurons (Fig. 5A–D) Importantly, this study corroborated relative numbers of aromatase-ir cells (and mRNA) with activity levels from different brain regions for this species (i.e., highest numbers of aromatase-ir cells in the telencephalon compared to midbrain and hindbrain) [87]. Specificity of aromatase expression in and around the SMN probably accounts for most of the aromatase activity found in the hindbrain and rostral spinal cord [225] (also see [185]). Brain aromatase in teleosts may be expressed exclusively in glial cells: the cellular morphology and pattern of aromatase-ir resembles radial glia seen in other vertebrates, including teleosts [7,138] and does not colocalize with neural-specific anti-Hu labeled cells or axons labeled by anti-acetylated tubulin, but co-localizes with anti-GFAP and vimentin, most notably in the telencephalon [87] (Fig. 6A–E). Prior to this study, other examples of aromatase expression in glia were restricted to post brain injury conditions in rodents and birds [95,96,192] (see below for further discussion of birds). Therefore, this was the first example of brain aromatase expression in radial glial cells in an adult vertebrate under normal conditions. Remarkably, very recent findings have also revealed constitutive aromatase expression in glial cells in human temporal cortex, basal forebrain and hypothalamus [133,283]. Thus, as is

the case in teleosts, aromatase expression in glial cells in humans likely mediates important functions outside of neuroprotection.

The pattern of aromatase distribution in radial glial cells throughout the brain, as first shown in midshipman fish was subsequently demonstrated, using the same antisera in trout (*Onchorynchus mykiss*) [172] in which localization to glia with another glial marker, protein S-100 was confirmed [189]. A similar pattern of brain aromatase distribution was recently shown in pejerrey fish also using this antibody [255], and in zebrafish, although a new antibody had to be generated to recognize brain aromatase due to problems with interspecific cross-reactivity [173]. Notable species differences are the presence of aromatase-ir in the tectum and torus semicircularis of trout, zebrafish and pejerry, but not in midshipman. Although cellular distribution is not known, aromatase activity was found at relatively high levels in both these areas in the African catfish (*Clarius gariepinus*), but absent in the tectum of stickleback [43,260]. Aromatase expression in glial cells appears to be a teleost character since its demonstration is now consistent in several distantly related teleosts. Whether this character is novel to teleosts or found in all actinopterygians requires further inquiry. With the exception of pejerry fish, all of the



above studies have corroborated immunocytochemistry (ICC) results with *in situ* hybridization (also see [111]). Remarkably, the mRNA pattern not only reflects location of aromatase-ir cell bodies but follows glial processes as well (Fig. 7), although this appears more pronounced in midshipman and zebrafish than trout [87,189]. There are two apparent exceptions so far to a glial-only distribution of aromatase in teleosts. The first is aromatase-positive neurons within the peripheral ganglion cell population that innervates the hair cells in the sacculus division of the inner ear of midshipman fish, the main auditory end organ in this species [90] (Fig. 6I). As discussed later, this trait relates to the steroid-sensitivity of the auditory encoding properties of the sacculus. The second case is in a weakly electric gymnotid fish (*Apteronotus leptorhynchus*) where mRNA is localized over relay cells that form part of the hindbrain circuitry that controls the rate of production of an electric organ discharge; abundant mRNA expression was also found in forebrain ventricular regions consistent with the glial pattern of expression in the above cited studies (H. Liu, M. Marchaterre, A.H. Bass and H. Zakon, submitted for publication).

2.2. Avian models

Early research examining hormonal effects on behavior of captive doves and Japanese quail established essential concepts of hormone action on the avian brain in the control of sexual behavior. In doves and quail, gonadal testosterone was found to be converted into 17β-estradiol largely in the hypothalamus/preoptic area where estrogen receptors were also expressed and where estrogens stimulated the performance of some components of courtship and aggressive behaviors [1–3,13,21,66,128,130,211,212,231]. Aromatase was also found to undergo regulation in the hypothalamus/preoptic area. Gonadal testosterone in breeding males increased aromatase activity at just the time when estrogen activation of reproductive behavior was required [215,230,252]. Although these kinds of studies in doves and quail laid the foundation for our appreciation of the pivotal role of brain aromatase in the control of behavior, studies of songbirds suggested that new concepts were needed.

Initial studies on brain aromatase in the zebra finch showed that, unlike doves and quail, activity could be measured in punches from virtually all areas of the songbird diencephalon and telencephalon [272]. A zebra finch aromatase cDNA was then cloned and aromatase probes generated for use in *in situ* hybridization analyses of its neuroanatomical distribution [233,234]. These studies identified aromatase expression throughout the telencephalon, especially in the hippocampus and the caudal nidopallium (Fig. 3). This general widespread neuroanatomical distribution has been confirmed in zebra finches and in other songbirds by additional *in situ* hybridization studies, as well as biochemical and immunocytochemical experiments [16,85,93,201,205–207,237,245,250]. Collectively, this work shows that songbirds differ from other avian groups by having evolved a quantitatively greater and anatomically more diverse capacity to synthesize estrogens in brain. Presumably, neural aromatase co-evolved functionally with the song control circuitry that is also unique in this avian group.

Surprisingly, neuroanatomical studies in zebra finches showed that there were few, if any, aromatase-expressing neurons in the song control system [208,234] despite evidence for activity in these nuclei [272]. Moreover, unlike the hypothalamus/preoptic area of all avian species studied, aromatase is generally not regionally co-localized with estrogen receptors throughout the telencephalon [134,174]. Much of the local action of estrogen in the songbird brain may depend upon the presence of aromatase at synapses. Steroids have multiple-documented functions in the brain, acting on intracellular receptors,

Fig. 5. Distribution of aromatase-ir in the brain of *P. notatus*. Left half of each section is a camera lucida drawing which shows aromatase-ir cell and fiber distribution; right half is a photomicrograph of ICC processed tissue with Nissl counterstain. (A and B) Aromatase-ir cells are concentrated dorsally and dorsolaterally within the sonic motor nucleus (SMN) and around the ventral half of the fourth ventricle (IV). Fewer aromatase-ir cells are found centrally within the SMN. Fibers course prominently through the SMN and spread ventrolaterally, concentrated along the reticular formation (RF). (C and D) Aromatase-ir cells are also found scattered ventrally at the level of the SMN, and just lateral to the medial longitudinal fasciculus (MLF) in areas of the rostral hindbrain. (E and F) Large numbers of aromatase-ir cells line the periventricular areas of the fourth ventricle (IV) and cerebral aqueduct (CA) in the caudal (E) and rostral (F) midbrain respectively. Fibers spread ventrolaterally and cover most of the tegmentum (Teg), while label is absent in the midbrain tectum (Te) and torus semicircularis (TS). (G) The third ventricle (III) in the diencephalon is completely lined with numerous aromatase-ir cells which project ventrolaterally. The periventricular areas of the hypothalamus (e.g., Hv, ventrolateral nucleus of the hypothalamus) also contain aromatase-ir cells that project radially. (H) Lateral projections originate from aromatase-ir cells along the medial ventricle in areas that include the gigantocellular division of the magnocellular preoptic nucleus (PMg) and ventral tuberal hypothalamus (vT), a vocal-acoustic integration center. (I and J) Aromatase-ir cells (2–4 layers thick) line the entire periphery of the telencephalic hemispheres (also see H, Tel), as well as the medial ventricular surface; fiber projections course ventromedially. Aromatase-ir cells are prominent in forebrain areas, e.g., the preoptic area (PPa, anterior parvocellular; PM, magnocellular). (K) Label extends well into the olfactory bulbs (OB), where numerous ir-cells line the dorsomedial edge and fiber projections course ventrolaterally. *Other abbreviations*: Ap, area postrema; CC, cerebellar crest; Cg, granule cell layer of the corpus of the cerebellum; Cm, molecular layer of the cerebellum; CP c/d, compact/diffuse division of the central posterior nucleus; DD, dorsal division of the dorsal telencephalon; DL, dorsolateral telencephalon; DM, dorsomedial telencephalon; DP, dorsal posterior nucleus of the thalamus; Hv, ventral periventricular hypothalamus; iaf, internal arcuate fibers; ll, lateral lemniscus; OC, occipital nerve; ot, optic tract; PAG, periaqueductal gray; PCo, posterior commissure; Pe, periventricular cell layer of the torus semicircularis; PGl, lateral division of nucleus preglomerulosus; PGm, medial division of nucleus preglomerulosus PL, paralemniscal midbrain tegmentum; SOv, ventral division of secondary octaval nucleus; Vd, dorsal nucleus of area ventralis; Vde, descending tract of the trigeminal nerve; Vl, vagal lobe; Vlh, ventrolateral hypothalamus; VP, posterior nucleus of area ventralis of the telencephalon; VS, supracommissural nucleus of the ventral telencephalon; Vv, ventral nucleus of area ventralis and Xm, vagal motor nucleus. Scale bar = 1 mm. Modified from [87].

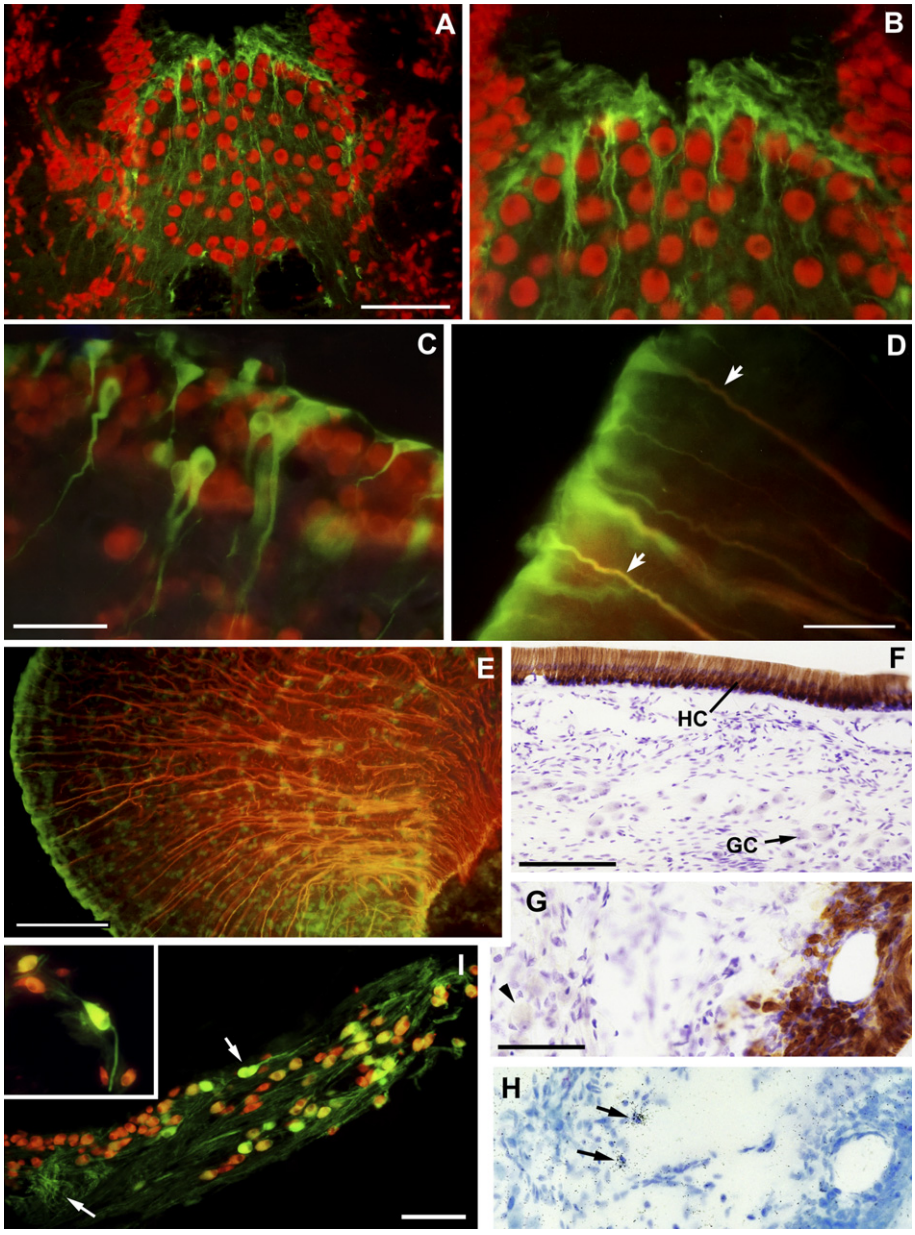


Fig. 6. Cellular identification of aromatase in the brain and inner ear of midshipman. (A) Photomicrograph of the sonic motor nucleus fluorescently double-labeled with teleost specific anti-aromatase (green) and neuronal specific anti-Hu (red). Scale bar = 240 μm. (B) Higher magnification of (A). Aromatase-ir cells are concentrated at the dorsal periphery and fibers course around motor neurons throughout the nucleus. Scale bar = 160 μm. (C) Dorsal telencephalon double-labeled with teleost anti-aromatase (green) and anti-Hu (red). Scale bar = 80 μm. (D and E) Co-localization of aromatase in radial glial cells in midshipman telencephalon. High (D) and low (E) magnification of cells which line the periphery of the telencephalic lobes double-labeled with anti-aromatase (green cell bodies) and anti-GFAP (yellow-orange fiber projections). Scale bar = 50 μm in (D) 200 μm in (E). (F) Sagittal section through the saccular epithelium of the inner ear shows the hair cell layer (HC), revealed by a hair-cell specific antibody, relative to ganglion cells (GC) that are positioned within the saccular branch of the eighth nerve that innervates the HC. (brown reaction product, Nissl counterstain). (G and H) Adjacent sections which show ERα mRNA by *in situ* hybridization label (H; arrows) in relation to the hair cell layer (brown) in G in a female midshipman. Arrowhead in G indicates relative position of a ganglion cell. Scale bar = 50 μm. (I) Double-label immunofluorescence using aromatase (green) and neuronal (soma) specific Hu (red) antibodies reveals aromatase in ganglion cell somata (bright yellow) and their processes (green) in the eighth nerve in a female midshipman. (I inset) high magnification of aromatase expression in bipolar ganglion cells (yellow) and processes (green) (center and upper left). Scale bar = 100; 50 μm for inset. Photomicrographs modified from [87,90].

membranes and at pre- and post-synaptic targets (e.g., [163,37]). Therefore, understanding the subcellular localization of a steroid metabolic enzyme like aromatase in a neuron may be especially important. For example, a somal localization of aromatase could yield estrogens for actions on either estrogen receptors or reduce the concentration of androgens, lessening their actions on androgen receptors, and ultimately influencing transcription in the adjacent nucleus. By contrast, aromatase might be trafficked to distant terminals where significant alterations in androgen or estrogen concentrations might influence pre- or post synaptic function.

Studies of aromatase in the avian brain have given us a unique window onto the subcellular localization of brain aromatase. Neuronal aromatase can be found in somata, processes and in synaptic terminals, including terminals located in brain areas devoid of somal aromatase. The first evidence that aromatase might be present in terminals came from subfractionation studies of the quail hypothalamus [213]. Although aromatase was known to be membrane-bound and associated predominantly with the endoplasmic reticulum (microsomes), in these experiments with brain tissue aromatase activity was found enriched in purified synaptosomal preparations [213]. This observation was later confirmed by electron micrographic studies showing aromatase immunoreactivity in terminals in the hypothalamus/preoptic area and the bed nucleus of the stria terminalis (BNST) of several vertebrate species [178]. In these areas, aromatase-positive synaptic terminals were seen to synapse upon aromatase-positive or aromatase-negative dendrites and somata. Unfortunately, the ultrastructure of aromatase-expression was not studied in other brain areas, most importantly areas that lacked aromatase-positive somata. It is difficult to ascribe a specific function for aromatase in any one subcellular compartment of neurons in the hypothalamus/BNST when the enzyme is present in many parts of the cell. Subsequent studies showed that aromatase is present in fibers of the quail spinal cord, a location where estrogens may rapidly modulate pain responses [79,80]. These studies indeed suggest that estrogens synthesized at the synapse may quickly regulate neurotransmission in nociceptive pathways.

Studies of the songbird brain have also provided evidence for aromatase in projection neurons. As mentioned previously, aromatase ICC stains cell bodies as well as fibers and putative terminals [14,15,178,208] (Fig. 8). Aromatase positive fibers can be abundant, with protein both in dendrites and in axons. In many cases, however, axons are seen to project in directions where the post-synaptic target is not obvious. Although it is possible that these fibers might return back to identified cell bodies in the same nucleus (as reported by [178]), the significant lengths of some axons (some exceeding several 100 micrometers), makes it more likely that they synapse on more distant, unidentified neurons.

The long-distance trafficking of aromatase may be particularly important in songbirds. Androgens and estrogens impact several song system nuclei (for review see [228]). Curiously, in some cases, these two steroids work antagonistically such as during periods of song learning when estrogens maintain the plasticity of circuits underlying learning whereas androgens crystallize song elements [46,278]. Because aromatase increases estrogen while simultaneously reducing androgens it occupies a potentially significant position in steroid-dependent song learning. Surprisingly, many song system nuclei have few if any cells that stain for aromatase using ICC or that hybridize to a songbird specific aromatase probe using *in situ* hybridization [208,233,234]. Consequently, a direct role for local aromatase in song learning seemed doubtful.

Paradoxically, however, aromatase could be measured in many brain regions [272] that show no evidence for aromatase somal expression [208]. The identification of aromatase in terminals and synapses now seems to resolve this question [194]. First, brain regions such as the nucleus HVC, a recognized site of androgen and estrogen action, has few if any aromatase-positive somata but many synaptic terminals containing aromatase [194]. Some of these terminals arise from axons reaching HVC from elsewhere in the brain [208]. Consequently, aromatase-expressing neurons may influence the steroidal environment of individual synapses or post-synaptic neurons in HVC thereby achieving a fine-scale control over steroid-mediated synaptic events. Such a locally restricted control of neural circuits is impossible to achieve by regulation of endocrine (gonadal) secretion alone.

Not only is aromatase present in synapses, but males also have more aromatase-positive synaptic terminals than do females in several brain regions [194]. Although there is considerable evidence for sex differences in estrogen action on the songbird brain, there has been little evidence for sex differences in peripheral or central estrogen synthesis [62,218,275]. It may be that aromatase-positive neurons are more complex than are similar neurons in females, leading to a greater number of aromatase-containing fibers [208] or synapses [194]. These differences could create a greater concentration of estrogen in males only at discrete behaviorally important neural sites.

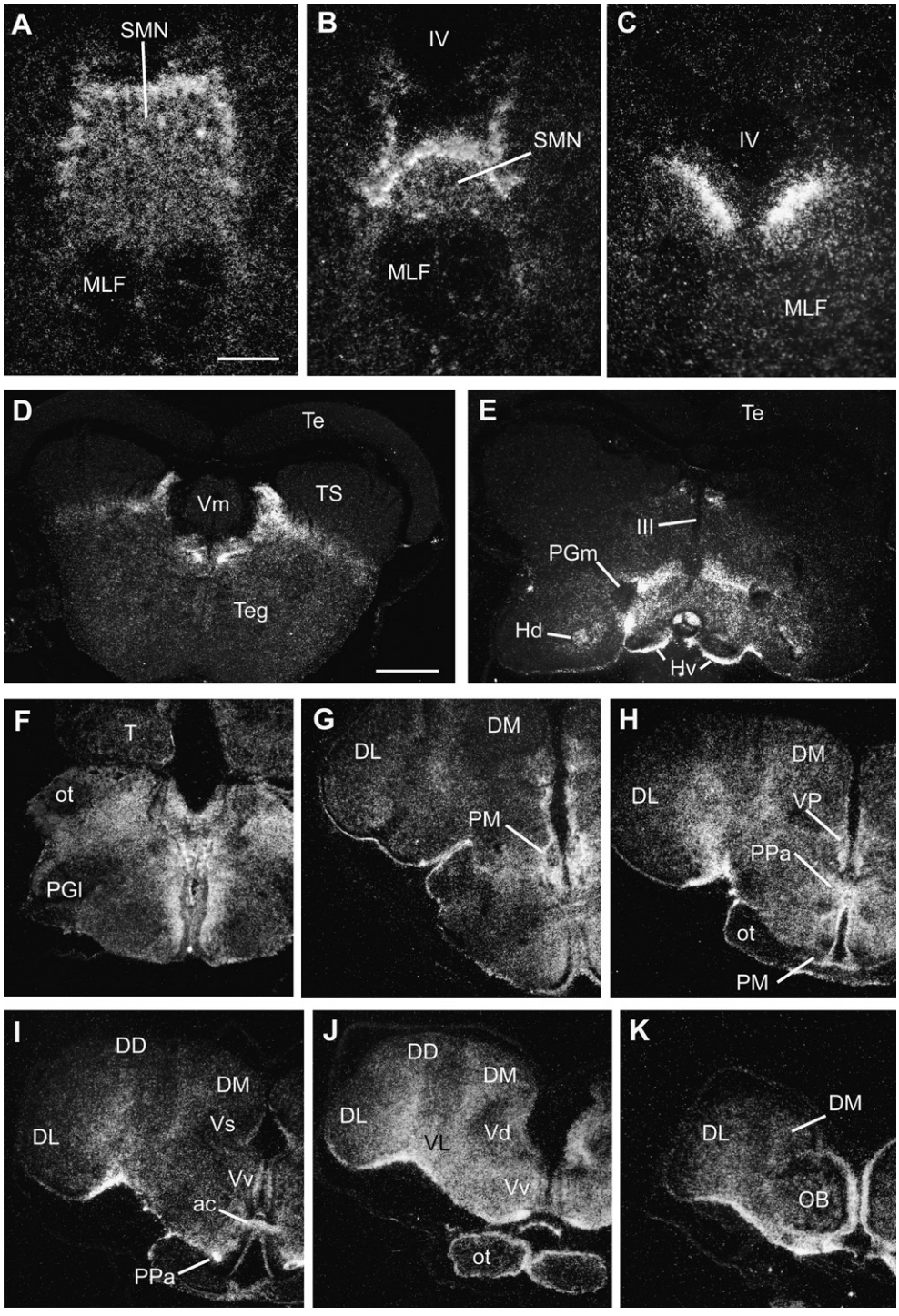
3. Seasonal variation and steroid regulation of brain aromatase

Many behaviors change seasonally in wild populations of teleosts and songbirds. Since seasonal fluctuations of sex steroids influence the expression of these behaviors, then the brain regions controlling each behavior will likely show seasonal and steroid-dependent shifts in aromatase expression.

3.1. Teleosts

As expected, like in other vertebrates, brain aromatase activity [43,56,108,162,187] and mRNA [89,99,140,141] expression levels fluctuate seasonally and with reproductive state in teleosts, implying an important role in reproductive physiology. Few studies [43] (see below), however, have made comparisons of brain aromatase activity or mRNA across seasons or between sexes in specific brain nuclei. Aromatase mRNA expression was quantified by *in situ* hybridization in the SMN and POA in type I male and female midshipman in relation to seasonal changes in circulating steroids and reproductive behavior [89]. Females showed highest aromatase expression in both areas during gonadal recrudescence (egg growth that occurs during the pre-nesting period), just prior to the migration into nesting

areas in the intertidal zone (see [27]), when both testosterone and 17β-estradiol are elevated [243], while type I males showed highest overall expression in both areas at the start of the nesting period when vocal courtship and territories are obtained and androgens are elevated [49,148,243]. Interestingly, highest aromatase expression in both the POA and SMN was found during the same time period, but only the SMN in both sexes showed dramatic, significant changes across seasons, which may reflect its function in a seasonally active behavior. Although aromatase in the midbrain periaqueductal gray, also part of the descending vocal motor pathway [109], was not quantified, it showed similar fluctuations to the SMN, while the anterior POA appeared to be less variable across the sampled time periods [89]. In teleosts in general, it appears that brain transcript levels mirror changes in circulating steroids [89,99], while peaks in circulating testosterone and 17β-estradiol precede peaks in activity [108,187]. Therefore, in some cases, sex differences in brain aromatase may be due to temporal sex differences in either circulating steroid levels or reproductive state



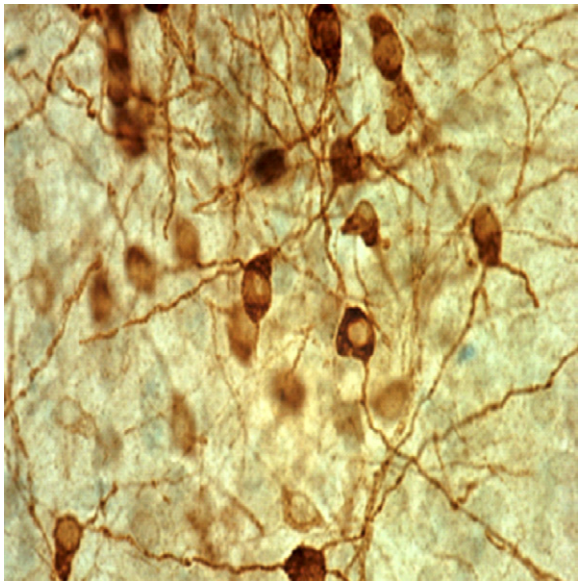


Fig. 8. Aromatase-immunoreactivity in neurons in the medial preoptic nucleus (POA) of an adult male zebra finch (for details see [208]).

[89,187,255]. Interestingly, both aromatase and estrogen receptor alpha (ERα) mRNA expression were found in the pineal of females in the spring pre-nesting period, about a month before inshore migration and spawning occurs, but not during reproductive or non-reproductive periods [90]. Since 17β-estradiol is documented to modulate melatonin release of pinealcytes *in vitro* [40], ERα may function to coordinate seasonal changes in photoperiod, in part, by "priming" the neuroendocrine system for reproduction in midshipman and other seasonally breeding vertebrates.

Both androgens and estrogens are known to upregulate brain aromatase mRNA and activity in teleosts and other vertebrates including birds [44,61,99,162] (see [17] for other vertebrates). The hypothesis that circulating steroids are responsible for seasonal changes in brain aromatase expression was tested in midshipman females by ovariectomy and replacement with testosterone, 17β-estradiol or control (no steroid) implants. Females with either testosterone or 17β-estradiol implants exhibited mRNA levels in the SMN and POA like those in recrudescing females (when natural circulating steroids peak), while females with control implants had expression levels in the SMN that resembled the levels of non-reproductive females (when circulating steroids are basal). Non-reproductive females, however, despite having very low or undetectable levels of circulating steroids, still maintained elevated aromatase mRNA in the POA in comparison to control implanted females [88]. This suggests that even minute levels of either steroids or estrogen precursors synthesized in the ovaries throughout the year may help to maintain elevated levels of aromatase expression in the POA.

The initial upregulation of brain aromatase mRNA expression may occur during gonadal recrudescence when circulating steroids start to rise and subsequent upregulation of the enzyme (i.e. local estrogen production) may exert a positive feedback on expression of the enzyme [61]. This feedback loop may explain, in part, why female midshipman have high brain aromatase activity during the nesting period when circulating steroids have sharply declined [89]. Numerous studies have demonstrated that 17β-estradiol upregulates brain aromatase activity and transcripts in teleosts [99,115,146,173,188] and an ERE (and in some instances 1/2 ERE) was consistently found in the promoter region of CYP19b across all species investigated zebrafish: [61,139,261]; goldfish: [258]; medaka, *O. latipes*: [151]; catfish, *Ictalurus punctatus*: [141]; Nile tilapia, *Oreochromis niloticus*: [65]; three species of sequential hermaphrodites, *L. calcarifer*, *C. altivelis*, *G. histrio*: [98]. Also, 17β-estradiol induction of brain aromatase transcripts can be blocked by an estrogen antagonist in zebrafish embryos, indicating a necessary role of estrogen receptor (ER) [146,173]. Paradoxically, in the few studies which have employed standard immunocytochemical and *in situ* hybridization methodologies in the same species, the distribution of aromatase and ER(s) do not appear to be colocalized, but coregionalized. In the forebrain, aromatase is exclusively expressed in radial glial cells whose processes cover large regions, while ERα appears to be localized to neurons in discrete nuclei largely in the POA and periventricular hypothalamic nuclei [90,146,172,173]. This discrepancy in distribution is exemplified in the midshipman where ERα is concentrated in specific regions of the dorsal

Fig. 7. Darkfield visualization of *in situ* hybridization throughout the brain of *P. notatus* using probes from partial cDNA cloning of midshipman brain aromatase. Pattern of signal in all brain regions is consistent with that found by ICC (see Figs. 5 and 6). (A) Aromatase mRNA signal clearly defines the sonic motor nucleus (SMN) boundary near level in Fig. 5A. Notice heaviest signal is around the dorsal and lateral periphery. (B) Hybridization signal in the rostral SMN near Fig. 5B. (C) Aromatase mRNA just below the fourth ventricle (IV) in the rostral medulla near Fig. 5E. (D) Hybridization signal in the caudal midbrain in between levels (E and F) in Fig. 5. As seen in ICC, there is a clear boundary of aromatase expression ventral to the tectum (Te) and torus semicircularis (TS). (E) Aromatase mRNA expression in the diencephalon near level G in Fig. 5. Signal is most apparent in areas of the hypothalamus (Hd, dorsal periventricular; Hv, ventral periventricular hypothalamus) and along the third ventricle. (F) Aromatase mRNA expression in the diencephalon–telencephalon transition area (near Fig. 5H) shows high signal along the midline ventricle through thalamic and hypothalamic regions, and dorsolateral just beneath the optic tract (ot). (G) The caudal telencephalon (between Fig. 5H–I) shows strong hybridization in the magnocellular preoptic nucleus (PM) and along the telencephalic periphery. (H) Strong hybridization in the anterior parvocellular division of the preoptic area (PPa), posterior nucleus of area ventralis of the telencephalon (VP), and ventrolateral area (near Fig. 5I). (I) Section just caudal to Fig. 5J at the level of the anterior commissure (ac) which shows very strong punctate signal within the PPa. (J) Anterior telencephalon (rostral to Fig. 5J) has high aromatase mRNA signal especially within ventrolateral (VL) and ventral nucleus of area ventralis (Vv), as well as within the optic tract (ot). (K) Aromatase mRNA expression is especially strong in the medial and ventral olfactory bulb (OB, near Fig. 5K). *Abbreviations*: Vm, molecular layer of the valvula; also see Fig. 5A–K. Scale bars = 200 μm (A–C); 400 μm (D–K). Modified from [87].

pallium (homologs to mammalian limbic areas), whereas aromatase mRNA and protein are diffusely expressed in glial processes throughout these areas [87,90]. Other areas in the midshipman brain where aromatase is abundant, but ERα expression is low or undetectable, as in the vocal-acoustic regions of the midbrain tegmentum and sonic motor nucleus, imply local estrogen acting through non-genomic pathways or through other ER receptors (see below, [90]). However, ERα transcripts were detectable by RT-PCR in glial cultures from adult trout brain, which indicate ERα may be present in radial glial cells, but at very low levels [172]. Through *in vitro* experimentation, Menuet et al. [173] clearly demonstrated in zebrafish that the CYP19b gene can be induced with low levels of ERα, both ERE and 1/2 ERE are required and importantly, that a glial cell context is necessary for maximum induction by 17β-estradiol. The authors also suggest glial-specific factors may be required for 17β-estradiol upregulation of aromatase in glial cells.

3.2. Songbirds

Many behaviors also change seasonally in wild songbirds. Copulatory behaviors may be expressed largely or solely during the breeding season when the gonads are well developed. On the other hand, some bird species sing year round or they establish territories both during the breeding and the non-breeding seasons. Assuming that sex steroids impact the expression of all these behaviors then we must assume that the brain regions controlling each behavior are exposed to unique steroidal environments. One way that this might be accomplished in males is if they were to regulate testosterone-metabolizing enzymes differently in different brain regions. The regulation of aromatase in the brains of seasonal breeding songbirds is indeed complex. For example, in Song sparrows of the Pacific Northwest (*Melospiza melodia morphna*), aromatase activity is elevated in the diencephalon when the birds are breeding [250], similar to what has been shown for non-songbird species [215,230]. Additionally, like the diencephalon, aromatase activity in the medial nidopallium of song sparrows is elevated when males are breeding suggesting that these regions experience their highest exposure to estrogen during the breeding season in contrast to little exposure at other times of the year. Contrary to this pattern, aromatase activity is elevated in both the spring and in the autumn in the ventromedial telencephalon. This region contains nucleus taenia (nT), a brain region that likely participates in the activation of aggressive behavior. Interestingly, aromatase activity in the nT of song sparrows is downregulated during molt, the only time of year when these birds show minimal aggressive behavior. Finally, aromatase activity was unchanged across the year in the hippocampus suggesting that estrogens act on hippocampal circuits year round. Presumably, each brain region requires estrogen at different times of the year. It is the region-specific regulation of aromatase that produces the requisite estrogens. The mechanisms underlying the region-specific regulation of aromatase is unknown but could involve steroid-dependent or steroid-independent changes in aromatase gene expression or direct regulation of protein activity itself (see below). The regulation of aromatase is not unique to regions of the song sparrow brain. Other songbirds show their own distinctive regional and seasonal patterns of expression or activity [201,245,271]. Additional studies of estrogen action in the brains of these birds are needed to determine how and why aromatase is differentially regulated across the songbird brain.

There is also evidence that steroids synthesized in brain may activate some behaviors of adult songbirds during non-breeding seasons. Males of many temperate-breeding bird species display territorial aggressiveness when environmental conditions are appropriate for breeding and when their reproductive systems are well developed [280]. Estrogens likely contribute to this aggressive behavior as gonadal androgens are aromatized to estrogen in brain [214,247,118,238]. Whereas most species abandon territories during the non-breeding season, others remain territorial year round. It has long been believed that steroids played no role in controlling non-breeding aggression because the testes are regressed and circulating T is basal. For some species, however, this view is changing with evidence that, despite low T-levels in blood outside of the breeding season, inhibition of sex steroid synthesis or action reduces territorial aggression [119,246,247].

Three possibilities seem most likely to explain these paradoxical observations. One possibility is that a steroid other than T or one of its metabolites activates non-breeding aggression. A likely candidate is the inactive androgen precursor dehydroepiandrosterone (DHEA) that has been found to circulate in the blood of male songbirds and is present in significant amounts in the non-breeding season [119,248,251]. Moreover, treatments of non-breeding male song sparrows with DHEA can activate aggression and induce growth of neural circuits controlling song [249]. These data suggest that DHEA, possibly derived from the adrenals of non-breeding birds, activates singing and aggression during the non-breeding season. Both aromatase and 3β-HSD (3β-hydroxysteroid dehydrogenase/Delta5–Delta4 isomerase) are present in the brains of non-breeding songbirds, making it likely that they act coordinately to create estrogens that activate these behaviors [246,251]. Conceptually, we can now view aromatase as working alone on circulating T levels or coordinately with other enzymes like 3β-HSD using additional substrates such as DHEA to activate behavior. In that there is also evidence for steroid-acute regulatory protein (StAR) and the side-chain cleavage enzyme CYP11A1 in the songbird brain (London and Schlinger, unpublished), we cannot rule out the possibility that the steroid-dependent activation of non-breeding aggression results from the complete pathway of sex steroidogenesis within the brains of non-breeding songbirds. Additional studies are needed to evaluate this hypothesis.

4. Functional considerations

The functional roles of locally produced estrogen in the brain remain the central challenge now that the tools to identify enzyme expression at the cellular level are available. As a central metabolite of androgens produced locally or peripherally, estrogens may: (1) bind to one of several nuclear estrogen receptors (ERα, ERβ and in teleosts, ERγ; see [120,171]) to modulate transcription of numerous genes, (2) bind to membrane receptors or activate signal transduction pathways for rapid modulation of cellular physiology, (3) bind to neurotransmitter receptor subunits, (4) interact with growth factors through receptor interactions (see [94]) or (5) perhaps be transported back into the circulation to act on peripheral tissues. Multiple estrogen receptors and non-genomic actions of locally produced estrogens allows for diverse signaling pathways complementary to high brain aromatase in teleosts [90].

Although initial experimental evidence for neurosteroidogenesis was gathered about 25 years ago (see [38]) and there have since been hundreds of papers published about neurosteroidogenic enzymes [36,170] and neurosteroids actions (e.g., [114,232,265]), we still do not have unequivocal evidence that neurosteroidogenesis plays a role in the natural lives of most vertebrate organisms. However, research on both vocalizing teleosts and songbirds has gathered evidence that points to a potentially important role for neurosteroidogenesis in the normal development or adult expression of steroid-dependent traits.

4.1. Audition

Gonadal steroids have been proposed to induce changes in auditory sensitivity among female humans during the menstrual cycle [164]. Studies of human and rodent females with Turner's syndrome that show a loss of ovarian estrogen production also exhibit a progressive loss of high-frequency hearing [126], while postmenopausal women show a positive relationship between increasing 17β-estradiol levels and hearing sensitivity [144]. Multiunit recording and evoked potential studies in amphibians and birds support the hypothesis that among seasonally breeding vertebrates, auditory mechanisms show seasonal shifts to accompany the changes in the spectral and/or temporal properties of vocalizations in order to maximize the detection, identification and localization of conspecifics [102,123,159,190].

Type I male midshipman fish produce advertisement hums that are long duration (min ≥ 1 h) with a fundamental frequency (F_0) near 90–100 Hz (@15–16 °C) with several prominent harmonics (Fig. 2B). Behavioral studies, including underwater playbacks, show that the hum functions as an advertisement call and that only reproductively active females with mature eggs show positive phonotactic responses to hums [50,165,168]. This observation led to the hypothesis that changing reproductive state shapes auditory mechanisms that encode male vocalizations.

Like other vertebrates, the inner ear of teleosts includes three non-otolithic organs, the semicircular canals, and three otolithic organs known as the sacculus, lagena and utriculus [33,83]. In midshipman, as in many teleosts, the sacculus is the main auditory end organ [71] and is innervated by the saccular branch of the eighth nerve (SN, Fig. 4). Neurophysiological studies of saccular nerve afferents in midshipman show that the frequency sensitivity of the sacculus matches the frequency content of their vocalizations. Since behavioral studies show that the efficacy of midshipman hums to evoke a positive phonotactic response can be mimicked by pure tones [165,167,168], tonal stimuli used in neurophysiological experiments are behaviorally relevant. Afferent responses are measured for both spike rate and the vector strength of synchronization, VS, a quantitative measure of the degree of phase-locking of spikes to the time-varying fine structure of an acoustic stimulus. Phase-locking measures (i.e., VS) provide the most accurate neurophysiological code of frequency in midshipman (and other teleosts) and can explain the frequency discrimination behaviors of teleost fish [82,166]. Phase-locking is also the most accurate measure of peripheral frequency encoding among terrestrial vertebrates for tone stimuli below 1 kHz (see [150] for overview). The importance of phase-locking mechanisms among all vertebrates is further emphasized in studies of humans showing that the loss of temporal coding mechanisms via phase-locking may contribute to deficits associated with sensorineural hearing loss [52].

For wild populations of non-reproductive fish, VS values gradually decline over the range of 60–360 Hz (Fig. 9A), while for animals in reproductive condition, VS remains relatively high up to 340 Hz and then gradually decline at 400 Hz (Fig. 9B). The enhanced phase locking up to almost 400 Hz reflects a dramatic improvement in the degree of temporal encoding by the sacculus to the second (~200 Hz) and third (~300 Hz) harmonics of the male's hum that often contain either as much or more energy as the fundamental frequency (~100 Hz, Fig. 9C). An increased ability of the sacculus to detect the hum's upper harmonics should also lead to a more robust encoding of fundamental frequency, as was shown in studies of saccular afferents [167]. This enhanced encoding mechanism should improve a female's ability to detect calling males in their nest, in part, because higher harmonics propagate farther in shallow water environments like those where midshipman nest because of the inverse relationship between water depth and the cutoff frequency of sound transmission [84] (also see [32]). It is further likely that the shift in frequency encoding capacity of the sacculus will also contribute to the female's ability to detect other vocal communication signals that are used in agonistic encounters and also have a broad frequency content (see [30]). As yet, it is not known if males will also exhibit steroid-dependent seasonal shifts in hearing, but this would also be adaptive for males as they too would benefit from enhanced detection of conspecifics in the shallow intertidal zone.

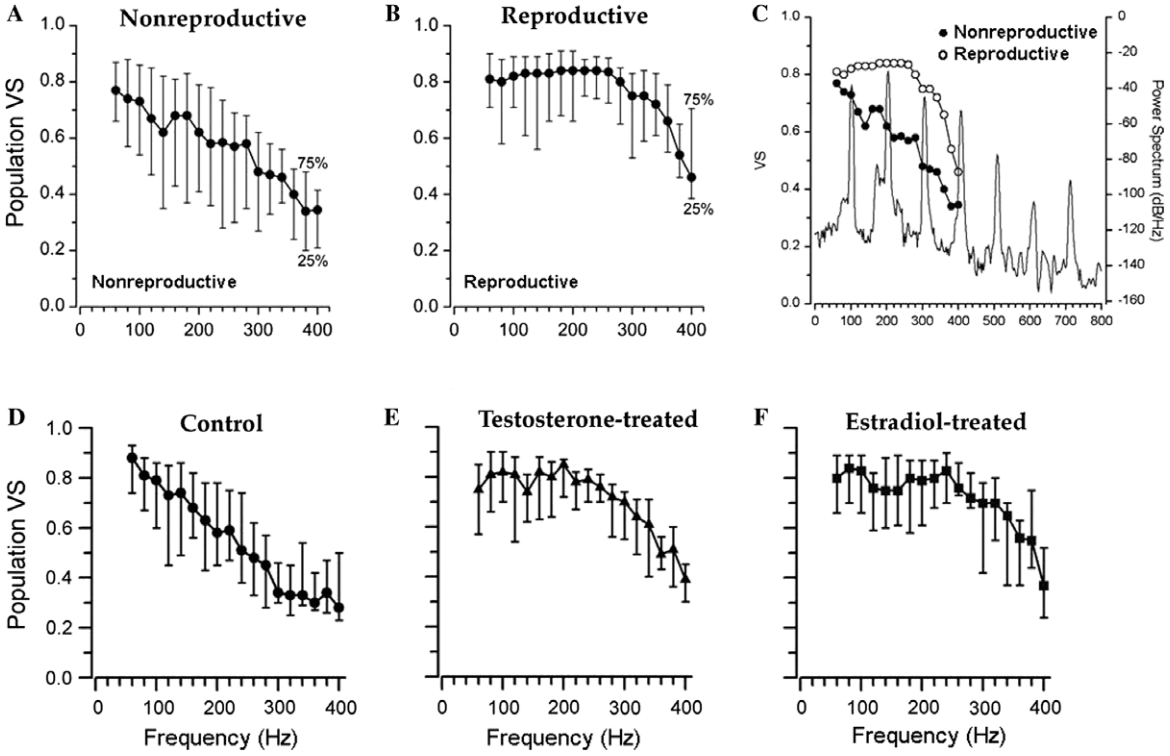


Fig. 9. Seasonal and steroid-dependent plasticity of vocal-auditory coupling in midshipman fish. Shown here are plots based on single neuron recordings from eighth nerve afferents that innervate the auditory-encoding sacculus division of the inner ear of adult females (see text for more background information). Each plot portrays the spike train responses of the entire population of saccular afferents from each study group to 500 ms duration, isopressure tone stimuli from 60 to 400 Hz presented at an intensity like that near a humming male at his nest (130 dB re 1 μPa; see [241,242] for individual neuron responses). Responses are quantified for vector strength of synchronization (VS) that describes the degree of phase-locking of spikes in response to the tone stimulus; a value of 1 would indicate perfect phase-locking. Only significant VS values are used to generate iso-intensity curves (see [241] for details of the analysis). (A and B) Iso-intensity curves for the entire population of saccular neurons recorded from non-reproductive (A, $n = 24$ animals, 88 neurons) and reproductive (B, $n = 18$ animals, 84 neurons) females show median (filled circles), 25th percentile (lower bar) and 75th percentile (upper bar) VS values. (C) The match between the power spectrum of a hum and the degree of frequency encoding of saccular afferent neurons recorded from both groups of females. The left *y*-axis indicates the VS values for the iso-intensity curves, while the right *y*-axis indicates the relative intensity (dB) values for the power spectrum. Frequency is plotted along the *x*-axis for both sets of measures. (D–F) Iso-intensity profiles compiled as above for an entire population of saccular afferents, but in this case recorded from control ($n = 11$ animals, 54 neurons), testosterone ($n = 9$ animals, 53 neurons) and 17β-estradiol ($n = 16$ animals, 54 neurons) implanted non-reproductive females. Note the nearly identical plots between the values for the steroid-treated non-reproductive females and the wild-caught reproductive females shown (B). Thus, steroid-treated, non-reproductive females, like wild caught females in reproductive condition that have naturally elevated levels of testosterone and 17β-estradiol, show robust encoding of the fundamental frequency (~100 Hz) and the upper frequency components [see (C)] of the male hum (A–C after [241]; D–F after [242]).

The discovery of seasonal changes in frequency encoding suggested that such shifts might be dependent, in part, on seasonal changes in circulating steroid hormone levels. Midshipman fish show increases in circulating levels of both testosterone and 17β-estradiol just prior to the onset of the nesting period, with levels dropping to basal values at the end of the breeding season [243]. A subsequent series of neurophysiological studies showed that ovariectomized, non-reproductive females given steroid implants that induce levels of either testosterone or 17β-estradiol like those observed in reproductive females, have iso-intensity curves that look nearly identical to those of reproductive females that have naturally elevated levels of testosterone and 17β-estradiol [242]. Thus, compared to non-reproductive controls (Fig. 9D), steroid-treated non-reproductive females (Fig. 9E and F) have VS values that are 50–100% greater over the 200–300 Hz frequency range of the male's hum. As with reproductive and non-reproductive females [241], there were no differences in auditory thresholds between control and steroid-treated females [242]. The similarity in the frequency encoding properties of saccular afferents in testosterone and 17β-estradiol-treated fish was consistent with the concurrent elevation of both steroids during the breeding season [243]. The increases in phase-locking precision were gradual over the 3–5 week treatments. By contrast, the iso-intensity profiles from non-reproductive females with testosterone implants for 9–14 days did not differ from controls. Together, these results suggest the steroid induced shifts in auditory encoding are dependent on transcriptional events, like those observed for the affects of steroids on morphological traits in midshipman fish [35,48,88] and vertebrates in general (e.g., see [263]).

In sum, the neurophysiological studies of peripheral afferent encoding in midshipman fish show that: (1) the yearly onset of reproductive behavior and increases in

circulating levels of 17β-estradiol and testosterone in females [243] are paralleled by enhanced phase-locking of saccular afferents to the upper harmonics of the male's mate call and (2) 3–5 week implants of either testosterone or 17β-estradiol induce the frequency encoding profile of reproductive females in ovariectomized, non-reproductive females. Since testosterone and 17β-estradiol -treated, non-reproductive females show essentially identical phase-locking profiles, the steroid-induced events could mainly be due to 17β-estradiol. This would also be consistent with differences in circulating levels of these two steroids; plasma levels of 17β-estradiol are almost two fold higher than those of testosterone in reproductive females [243].

Neurophysiological studies in terrestrial vertebrates that have been completed since the time of the midshipman study provide support for steroid and/or seasonal-dependent changes in neuronal activity. An *in vitro* study of the telencephalic nucleus robustus archistriatalis (RA) of adult white crowned sparrows (*Zonotrichias leucophrys gambelli*) that is a part of the descending vocal motor pathway reports seasonal changes in levels of spontaneous activity [184]. Treatments of males with either testosterone or a combination of the non-aromatizable androgen 5α-dihydrotestosterone (DHT) plus 17β-estradiol, together with a light:dark cycle that mimics breeding-like conditions, induces increases in spontaneous firing rates that are significantly different from those observed in animals in a non-reproductive condition. Local production of estrogen via telencephalic aromatase activity (see earlier discussion) is the likely source of telencephalic estrogen that contributes to similar mechanisms under more natural conditions. Other studies have also recently shown photoperiod-dependent shifts in the responses of single neurons to the bird's own song in a telencephalic nucleus, HVC [73] and this mechanism is also a likely candidate for steroid-dependent plasticity.

A second study of adult male leopard frogs (*Rana pipiens*) shows seasonal changes in the frequency tuning properties of single auditory neurons in the midbrain's torus semicircularis [102]. Although there were no observable differences in levels of spontaneous activity, there were significant seasonal increases and decreases in, respectively, the number of tonic and phasic units. Response latencies decreased, while there were increases in phase locking to amplitude-modulated signals among reproductive males. Seasonal shifts in all of these parameters could contribute to improved recognition of conspecific calls (see [102]). The possible role of steroids in this mechanism remains unexplored.

4.2. Vocal motor patterning

Several studies have identified the long-term influences of steroids on the development and maintenance of vocal motor systems in avian species [12,263] and midshipman fish [35,48,88,89]. Most of these actions are likely mediated by nuclear steroid receptor activation of transcription that typically requires a minimum of 30 min for detection, although transcriptional responses have been detected in 7.5 min [81]. Recent studies demonstrate the short-term actions of steroids, including estrogen, on the neurophysiology of the vocal motor system of midshipman fish. As with the auditory system of teleosts, we first need to provide some background on the vocal motor system of teleosts to fully appreciate this finding.

Midshipman fish, and other batrachoidid fishes that are generally referred to as toadfishes, vocalize using an extensive hindbrain–spinal vocal pattern generator that is readily activated by midbrain and forebrain nuclei (Fig. 2C). Sound is produced by the simultaneous contraction of a pair of muscles attached to the walls of the swim bladder (Fig. 2A). Each sonic muscle is innervated by the ipsilateral sonic motor nucleus (SMN) that extends from the caudal hindbrain into the rostral spinal cord. The SMN's sole input is from pacemaker neurons (PN); a single PN innervates both SMNs [24]. Pacemaker neurons receive input from a hindbrain ventral medullary nucleus (VM) that receives input, in turn, from other hindbrain and midbrain vocal sites; midbrain vocal nuclei receive input from the forebrain [25,109]. The firing of each pacemaker and motor neuron is correlated 1:1 with each summed potential of the rhythmic motor volley recorded intracranially from an occipital nerve root (Fig. 2D). The firing frequency of the motor volley determines the sonic muscle contraction rate and, in turn, the timing of each sound pulse [24] (also see [71]). The motor volley is referred to as a "fictive vocalization" because its duration and repetition rate predict, respectively, the duration and the fundamental frequency of signals such as the advertisement hum. Electrical

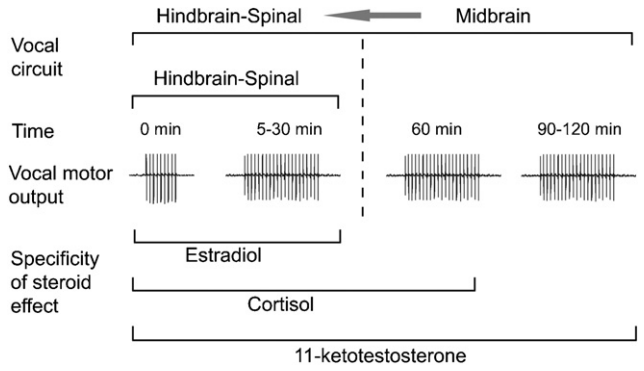


Fig. 10. Steroid-dependent modulation of fictive vocalizations (from [198]). Intramuscular injections of 17β-estradiol, cortisol, and the teleost specific androgen 11-ketotestosterone (11KT) all induce similar increases in the duration of the fictive vocalization as represented by neurophysiological recordings of the occipital nerve motor volley in the center of the figure (see Fig. 2D). However, each steroid has a specific effect on the longevity of the duration increase; 17β-estradiol has the briefest and 11KT the longest lasting effect. The region containing the hindbrain–spinal pattern generator circuit can be surgically isolated in vivo by making complete transections just rostral to hVAC and just caudal to the sonic motor nucleus (see Fig. 2). Studies of the isolated vocal pattern generating circuitry show that it is both necessary and sufficient for the rapid steroid effects lasting up to 30 min, while descending input from the midbrain is necessary for sustained effects beyond 30 min (see [198]).

stimulation of midbrain and forebrain sites (Fig. 2D) evokes fictive vocalizations in immobilized fish [24,109]. The VM–PN–SMN network is referred to as the hindbrain–spinal vocal pattern generator because a rhythmic sonic output is still observed with VM stimulation following *in vivo* surgical isolation of this region (Fig. 10) [198]. This neurophysiological model provides a simple model system for studies of steroid-dependent plasticity in vocal phenotypes.

Studies of type I male midshipman now show that estrogen, androgens and corticosteroids modulate the duration of fictive vocalizations within 5 min. Estrogen effects last for up to 30 min, while those of cortisol and the teleost-specific androgen 11-ketotestosterone last, respectively, for up to 60 and 120 min. Surgical isolation of the hindbrain–spinal region containing the vocal pattern generator show that this region alone can account for the immediate effects observed within 5–30 min, including the effects of estrogen (Fig. 10). Other more recent studies also show the effects of estrogen on the vocal pattern generator of type II males and females (L. Remage-Healey and A. Bass, unpublished observations). Rapid steroid effects on neuronal activity are well known in other systems [92,137,143]. The midshipman study was apparently the first demonstration of rapid steroid effects on the neural substrates of vertebrate vocalization. As such, these findings provide an experimental paradigm to study the physiological basis for rapid steroid effects on vocal neurons and link them to shifts in the temporal parameters of vocal behaviors (e.g., see [199] for field studies of vocal behavior in sonic fish).

5. Local estrogen action

Local estrogen production in the peripheral and central nervous systems, via aromatase conversion of testosterone to estrogen, may play a significant role in the effects of steroids on auditory and vocal mechanisms in both teleosts and birds.

5.1. Teleosts

Two pieces of anatomical evidence lend support to an estrogen-dependent mechanism that acts at the level of the inner ear. The peripheral ganglion cells that give rise to saccular afferents lie medial to the hair cell layer and in the adjacent portion of the saccular nerve as it extends towards the hindbrain (for central pathways, see [31]). A recent study shows aromatase-positive ganglion cells within the saccular branch of the eighth nerve adjacent to the saccular epithelium (Fig. 6I; [90]). Other experiments also show that midshipman-specific ERα mRNA is present in the saccular nerve branches adjacent to the hair cell layer of the sacculus (Fig. 6H; [90]). This is the first time that ERα mRNA has been identified in the inner ear of any vertebrate. There is no evidence for ERα mRNA expression in either ganglion or hair cells, although the signal is localized over small cells that resemble non-neural Schwann and satellite cells (A. Bass and P. Forlano, unpublished observations). Expression is also more apparent in pre-spawning females undergoing gonadal recrudescence during the seasonal peak of 17β-estradiol, just prior to the spawning season [243]. In sum, these recent anatomical findings support the hypothesis that estrogen production in the inner ear, by aromatase-positive ganglion cells provides a local pool of estrogen for both the induction and maintenance of enhanced temporal encoding in the sacculus of females while they are in reproductive condition. Further support for estrogen effects on hearing come from studies showing ERα immunoreactivity (not mRNA) in the cochlea of humans and rodents [253,254]. The neurophysiological studies in midshipman fish now provide a functional context for the presence of ERα in the inner ear (e.g., [253,254]).

As the neuroanatomical studies of the ear suggest, the effects of estrogen on vocal motor patterning may also depend on a local estrogen source. Recall that aromatase-positive glia enshroud the somata within the SMN (Fig. 6A and B). This character is found in all three reproductive morphs, although aromatase expression is greatest in type II males and females [89]. All three morphs show detectable levels of testosterone during the pre-nesting and nesting seasons [243], so that gonadally derived testosterone could provide a readily available substrate for estrogen modulation of vocal patterning.

5.2. Avian models

In addition to potentially highly localized actions of estrogens, the local formation of estrogen may be also regulated rapidly at rates substantially faster than could be achieved by regulation of gonadal steroid secretion. Studies in the Japanese quail suggest that estrogen concentrations might change relatively rapidly at the synapse by the rapid regulation of the aromatase enzyme itself. First, it appears that aromatase in the quail hypothalamus can undergo calcium-dependent phosphorylation that can decrease aromatase activity within minutes to hours [18,19]. Calcium may also inhibit aromatase activity within minutes via an interaction of the calcium–calmodulin complex with the aromatase molecule [20]. Treatments of hypothalamic explants with potassium or with glutamate receptor agonsists AMPA and kainate all inhibited aromatase over a similar time frame [18]. Such results suggest that neuronal aromatase is phosphorylated upon excitation, thereby locally reducing that neuron's capacity to synthesize estrogen. Just how such a decrease in aromatase is related to changes in synaptic estrogen concentrations or to changes in pre- or post-synaptic function is not clear. Nevertheless, the presence of aromatase at synapses that can undergo rapid regulation provides a degree of spatial and temporal steroidal targeting that is otherwise impossible to achieve in the face of a constant supply of aromatizable androgen from the gonads. Perhaps steroidogenesis occurs fully in or around specific synapses [124] so that some steroid-dependent

neural circuits are able to function independently from gonadal or adrenal steroids.

It is important to recognize that aromatase may not be the only sex-steroid metabolic enzyme to be compartmentalized in synaptic terminals. In birds, 5β-reductase is also released from synaptic terminals when they are lysed by a hyperosmotic shock [213]. This enzyme can inactivate testosterone [127] but it can also use progesterone as a substrate and participate in the synthesis of 5β-allopregnanolone, a compound that strongly potentiates $GABA_A$-induced post-synaptic hyperpolarization [64]. Synaptic 5β-reductase could play a powerful role in regulating either androgen- or estrogen-dependent post-synaptic function, or in GABAergic neurotransmission. Whatever its function, this cytoplasmic enzyme is expressed widely throughout the avian brain, making it likely that it acts together with aromatase to influence the local sex steroid environment.

5.3. Learning and memory in songbirds

Songbirds have attracted the attention of biologists and psychologists not only because of their capacity to learn and then sing complex songs but also because of their remarkable spatial memory capabilities [70]. It is well known that many bird species store and later retrieve food items, sometimes creating thousands of such caches scattered over large areas of the environment [39,100,121,175,236]. The neural mechanisms underlying these feats of memory are poorly understood, though, not unexpectedly, most research points to the hippocampus as an essential structure in the formation of the bird's spatial memories [117,235].

There is considerable research suggesting that estrogens are important for the optimal functioning of neural circuits underlying certain forms of memory (e.g., [78]). Consequently, the discovery that the songbird hippocampus was an important site of aromatase expression and activity [206] led to subsequent studies on a role for estrogens in hippocampal-based spatial memory functions. For example, female zebra finches treated with 17β-estradiol perform better on a spatial memory task than do untreated females or females treated with testosterone or the non-aromatizable androgen 5α-DHT [183]. The 17β-estradiol-treated females also had larger neurons and a lower cell density in the rostral end of their hippocampi suggesting that these were the sites where estrogens improved memory performance. Moreover, adult zebra finches treated with the aromatase-inhibitor fadrozole have overall smaller hippocampi than do untreated birds [229]. This finding suggests that endogenous estrogen, likely derived from local hippocampal aromatase activity, is essential for optimal hippocampal function in songbirds. Another point of interest is that birds that cache food do so mostly during the autumn and winter when neurogenesis is also elevated in some songbirds ([22], see also section below). It is at just these times that an elevation in hippocampal aromatase has been observed in another songbird species [245], providing additional support for a relationship between aromatase and hippocampal function.

It may well be that the effects of estrogen on hippocampal-based memory functions involve pre-or post-synaptic NMDA receptors. It appears that all neurons in the zebra finch hippocampi that express aromatase also express NR1, the requisite subunit of the functional NMDA receptor [209]. In addition, treatment of zebra finches with 17β-estradiol increases NR1-expression, the number of synapses on NR1-expressing neurons, and overall hippocampal synaptic density [209]. Consequently, mechanisms for synaptic strengthening are enhanced under the influence of estrogen. Additional research examining the relationship between estrogen and excitatory neurotransmission in the hippocampi of food-storing songbirds is warranted.

6. A role for glial aromatase in development

6.1. Teleosts

Neuroestrogens in teleosts have been proposed to contribute to their continual neurogenesis throughout life [87,99,189]. Although compelling, support for this comes more from studies in other vertebrates than in fishes. Brain aromatase activity peaks during periods of high neural proliferation in mammals, and locally produced estrogens influence neurotrophins (reviews: [157,262]). Furthermore, new brain cells are born in ventricular zones throughout the teleost brain [155,286,288] where high levels of aromatase is also expressed [87,172,173]. Recent evidence in mammals revealed radial glial themselves are neuronal and glial precursors [9,77], and experimental evidence suggests radial glia may be a source of new neurons in adult teleosts and also function to guide migrating neurons to their target area [287]. Whether new neurons migrate along aromatase-ir radial glia to reach their targets is unknown. Interestingly, detailed analysis of the promoter regions of CYP19b in medaka has revealed putative transcription factor binding sites known to be important for primary neurogenesis, and general CNS development [151]. Indeed, general characteristics of teleosts appear to be continual neurogenesis and remarkable plasticity in brain and behavior. The mechanisms through which abundant aromatase expression in glial cells contribute to these characters remains largely to be defined.

6.2. Songbirds

A fascinating feature of songbird neurobiology is that new neurons continue to be born throughout the life of the bird, with a portion of these neurons migrating into and becoming incorporated within functional neural circuits [182]. Adult songbirds retain a proliferative zone along the ventricles [103] with precursor cells that can differentiate into neurons or glia, including radial glia [105,8]. In the same fashion that is seen during development, these radial

glia form the scaffold upon which new neurons migrate [104].

Estrogens are suspected to play a role in this neuronal proliferation in the songbird brain. Although estrogens may not stimulate neurogenesis itself [51], they likely influence the proper survival, guidance and/or differentiation of newly born neurons. For example, more newly born neurons reach the song nucleus HVC when adult female canaries are exposed to estrogen [122]. These neurons seem not to express ER, but they do migrate through a layer of estrogen-receptor expressing cells [122]. It is likely that estrogens stimulate neurotrophin synthesis and secretion by these ER-expressing cells [244,76]. Presumably, these estrogen-dependent neurotrophins stimulate neuronal recruitment in the adult songbird brain.

It is unknown if brain aromatase plays a role in this estrogen-dependent neuronal recruitment, though there is good reason to believe that such a role exists. For example, in some seasonally breeding songbirds, selected song control nuclei like HVC enlarge at the onset of breeding and regress when breeding ends [263]. The increase in volume of nucleus HVC is largely the result of an increase in neuron number [263] and in males, these anatomical changes correlate with changes in the gonadal secretion of testosterone. Furthermore, testosterone or 17β-estradiol, can induce growth of song nuclei in non-breeding or castrated birds (for review see [228]). Given the elevated levels of aromatase in the songbird telencephalon it is likely that the estrogens that stimulate song system growth are synthesized in brain from peripheral testosterone. Unequivocal evidence for this idea has, however, not been obtained.

7. A role for glial aromatase in neuroprotection

Aromatase is expressed in neurons in the brain of adult birds and mammals [63,178,213] (Fig. 8). Surprisingly, aromatase was also found to be expressed in astrocytes in primary cell cultures of the zebra finch brain [221]. A similar observation was made in primary cultures of the mammalian brain [289]. These observations for glial aromatase proved perplexing until it was found that, after brain injury, aromatase is expressed in glia near the trauma site [96]. For example, within 24 h after a mechanical injury to the zebra finch telencephalon, aromatase mRNA and protein are upregulated in astrocytes near the injury site, an expression that persists for at least 7 days [192]. Aromatase is upregulated in other glia as well. For example, songbirds retain populations of radial glia along their ventricular borders [8]. If the zebra finch hippocampus is injured near the lateral ventricle where populations of radial glia are retained, then aromatase is also upregulated in radial glia [193] (Fig. 11). In songbirds, cells proliferate along this ventricular zone naturally and after neural injury. Following injury, some newly derived cells (labeled within 24 h with BrdU), appear to migrate along radial glia fibers that stain positively for aromatase [193]. Estrogens synthesized in these radial fibers may influence the differentiation or migration of newly born cells.

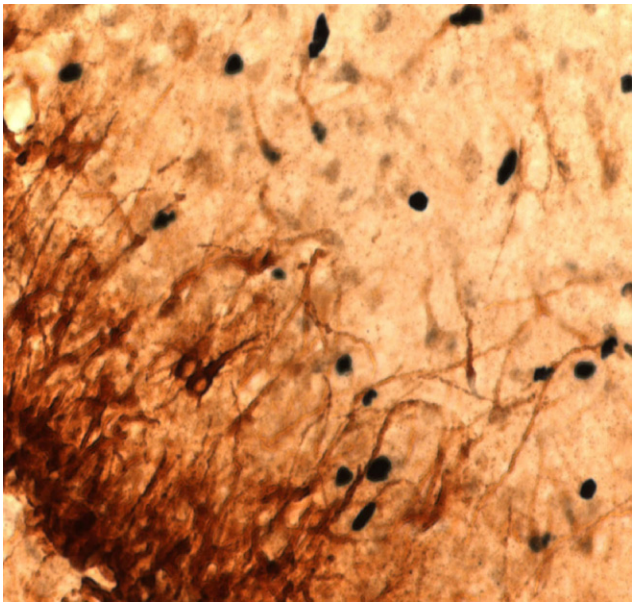


Fig. 11. Aromatase (brown) and BrdU (bromodeoxyuridine; black) immunoreactivity 48 h after neural injury to the zebra finch brain, 24 h after injection of BrdU. Note aromatase staining of radial glia along the lateral ventricle and their stained processes in association with mitotically active (BrdU-labeled) cells (for details see [193]). (For interpretation of the references to color in this figure legend, the reader is referred to the web version of this paper.)

The aromatase present in glia around sites of neural injury may function to lessen long-term damage. Wynne and Saldanha [282] made injections into both telencephalic hemispheres of adult male zebra finches that served to both create damage and deliver either saline (as a control to one hemisphere) or the aromatase inhibitor fadrozole (to the contralateral hemisphere). The size of each lesion was then measured after 72 h. The damage measured in the hemisphere that received fadrozole was significantly greater than that measured in the hemisphere that received saline, suggesting that the upregulation of aromatase in astrocytes around the injury site functions to limit the extent of damage.

It is likely that estrogens synthesized by glial aromatization limit a process of sustained cell death provoked by neural trauma. The density of aromatase-expressing astroctyes is correlated spatially with the density of injury-induced, mitotically active cells (Peterson, Lee and Schlinger, unpublished). Wynne and Saldanha [282] showed that injections of fadrozole combined with 17β-estradiol dramatically lessened the number of apoptotic cells found around the injection site as compared to an injection containing fadrozole alone [210]. Thus, injury-induced glial aromatase functions to limit secondary damage in the songbird brain. Experiments on the mammalian brain indicate that the upregulation of aromatase in glia is a conserved property of the vertebrate brain. Separate studies show that estrogens can be neuroprotective in mammals as well [97]. These kinds of experiments have important implications for clinical uses of sex steroids in combating longterm damage

associated with neural injury. Studies of brain aromatase in songbirds has led the way in building the concepts underlying this exciting therapeutic strategy.

8. Concluding comments

We now appreciate that neural aromatase synthesizes estrogens that play a much more extensive role in vertebrate neurobiology. Studies of birds and more recently of teleost fishes have given a unique insight into the role of aromatase in a diversity of mechanisms. Here, we have focused on some recent developments in our understanding of estrogen action in the nervous system of these two groups of non-mammalian vertebrates that have provided a number of model systems for neuroethological and behavioral neuroendocrinological research. This focus, developed in part, because of the discovery that, compared to other vertebrates, teleosts and songbirds show especially high levels of brain aromatase activity, protein and gene expression. As reviewed here, numerous methodologies have been used to investigate the functional significance of this trait. Many of these studies have also concentrated on the auditory/vocal communication circuitry because it has readily provided behavioral and physiological explanations for the existence of this exaggerated trait, i.e., high levels of aromatase expression, in teleosts and birds.

When defined as a neurosteroid, estrogen can either be synthesized in the nervous system or derived from circulating androgen precursors synthesized in non-neural sources including the gonads, adrenal glands and even the kidney (review: [37]; also see [45] for androgen sources in teleosts). One of the exciting avenues of research in the future rests upon the increasing evidence that the full spectrum of steroidogenic enzymes and transporters required to convert cholesterol into active sex steroids are present in the brain. Evidence for CYP17 (P450 17α-hydroxylase,17,20-lyase) mRNA expression, the enzyme that acts on pregnenolone to produce androgens, is found in the brain of fathead minnow [116] and this local androgen production may function to maintain high brain aromatase levels in teleosts. There also exists immunocytochemical evidence for 3β-HSD (3β-hydroxysteroid dehydrogenase/Delta5–Delta4 isomerase) which also plays a role in androgen synthesis in the brain of zebrafish as well as lungfish [161,204] and therefore it may not be surprising if androgens and androgen precursors are made *de novo* in the brain of teleosts.

Among songbirds, 3β-HSD activity was first measured in primary cell cultures of developing zebra finch brain [270]. Under these conditions pregnenolone was converted to progesterone and the relatively inactive androgen DHEA was converted to androstenedione, which was then further acted on by aromatase leading to the formation of estrogens. CYP17 is also expressed in the developing and adult zebra finch brain [158]. During early development (posthatch days 1 and 5), CYP17 is expressed along the lateral ventricles where neurogenesis is most active and where androgen receptors are also expressed [191]. Although no sex differences in CYP17 expression were detected, differences in the substrate available for CYP17 or downstream metabolic enzymes such as aromatase might create the different hormonal signals in males and females that lead to the development of sex-specific circuitry. Aromatase may represent the terminal reaction of a full steroidogenic enzyme cascade and collectively, these enzymes may synthesize the estrogens that participate in the sexual differentiation and maturation of the neural circuits controlling specific behaviors such as song. How steroidogenic enzymes are spatially and temporally related has yet to be determined, but remains an exciting avenue of investigation.

Gonadal sources of steroids may function to organize a subset of neural circuits, but the regulation of what steroids actually reach a particular target is best regulated locally. Circulating steroids can vary seasonally in many vertebrates and producing steroids locally in specific brain areas releases the dependence on a peripheral source tied to gonadal recrudescence. This is exemplified in sex-changing fish where the steroid environment in the brain can change long before gonadal sex change is completed [41]. As demonstrated in mammals and birds, circulating steroid levels are not indicative of estrogen action on specific targets including brain function (see [240], above). In some cases, the brain may even function as a "steroid thermostat," regulating the overall hormonal milieu of the animal. Certainly, the extensive distribution of aromatase in radial glial cells localized along the ventricular surfaces of the teleost brain would facilitate exchange of steroids between the brain, cerebral spinal fluid and circulation. In addition, the morphology and extensive distribution of radial glia processes greatly increases the surface area to which local estrogen production may reach its targets through paracrine action. Furthermore, evidence from studies in mammals suggests a complementary interaction between neurons and glia in the production of neurosteroids [94]. The mounting evidence for regional-specific patterns of aromatase expression and thus estrogen synthesis suggests that the conserved neural role for neurosteroidogenesis is in the development and maintenance of sex-specific, regional circuitry that would, for example, target steroids to vocal circuits in either birds or teleosts without inappropriate steroid exposure to other steroid-dependent circuits such as those involved in either avian copulatory or teleost spawning behaviors. Additional studies identifying the consequences of such local synthesis on the electroresponsive properties of neurons (e.g., [184,242]) on both rapid and slow time scales remains essential to understanding the adaptive significance of such mechanisms for the translation of brain function into behavior.

Acknowledgments

This research was supported by predoctoral training Grant NIMH 5T32MH015793 (P.M.F.), NIH DC00092 (A.H.B.), NSF IBN9987341 and 0516748 (A.H.B.) and

MH061994 (B.A.S.). We also thank C. Saldanha and S. Peterson for assistance with songbird photomicrographs.

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