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Neurosteroidogenesis: Insights from Studies of Songbirds

Barney A. Schlinger*

Departments of Integrative Biology and Physiology and Ecology and Evolutionary Biology, Laboratory of Neuroendocrinology, Brain Research Institute, UCLA, Los Angeles, CA. 90095

Luke Remage-Healey

Neuroscience and Behavior Program, University of Massachusetts, Amherst, MA 01003

Abstract

The long-held dogma that the brain is a target of steroids produced by peripheral organs has delayed widespread acceptance of the functional importance of neurosteroidogenesis. Comparative studies have been vital for establishing the key actions of gonadal and adrenal hormones on brain and behavior. No doubt, studies across diverse phyla will continue to be crucial for revealing the true significance of neurosteroidogenesis to proper function of the vertebrate brain. Here we review work out of our lab and others highlighting advances to our understanding of brain steroid synthesis and action using songbirds as animal models. These studies show that steroidogenic transporters and enzymes are present in the songbird brain and their expression and/or activities are subject to developmental, seasonal or short-term regulation. Our work in a songbird points to synaptic synthesis of neuroactive steroids and fast, peri-synaptic membrane actions. Combined with evidence for rapid steroidal control of behavior, these studies firmly establish a neuromodulatory role for avian neurosteroids. We hope this work will join with that of other species to embolden acceptance of neurosteroidal signaling as a core property of vertebrate neurobiology.

Keywords

Songbirds; Sex-steroids; Estrogens; Aromatase

Widespread acceptance for gonadal steroid control of brain and behavior involved decades of research on diverse animal species held in captivity or observed in nature. Whereas peripheral hormone effects can be profound they do not explain all sex steroid effects on the brain. The past few decades have witnessed the birth of a new conceptual framework that involves the actions of steroidal hormones synthesized in the brain itself or neurosteroids (1). Identifying how the brain balances its reliance on gonadal and neurosteroids remains a focus of research of many laboratories. To achieve advances in our appreciation of neurosteroidogenesis, studies have focused on traditional laboratory animal models (e.g. 2-4) as well as a variety of unique vertebrate species with novel neural and/or behavioral endpoints (e.g. 5-14).

Songbirds (Oscine Passeriform species) have played a unique role in the development of our understanding of hormonal control of brain and behavior (15, 16). This vertebrate group attracts great attention because they are a common and conspicuous feature of our world, like us they are largely diurnal, and they communicate using specialized visual and auditory signals. For those of us living in temperate latitudes, we are aware of their noticeable

*Correspondence: schlinge@lifesci.ucla.edu.

seasonal changes in behavior, the characteristic that led to their early role in demonstrating gonadal hormone control of reproductive behavior. (17) The elaborate learned songs produced seasonally by many songbirds stimulated neurobiologists to investigate the hormonal and neural basis for song learning and performance. These studies revealed a novel neural circuitry that is hormone-dependent, sexually dimorphic (in some species) and that exhibits an astonishing degree of neuroplasticity (18). Early studies identified estrogen as a principal agent stimulating masculine growth of the zebra finch song system, a species where adult males, but not females, sing, (19). Although we now know that hormones may only play a secondary role in sexually dimorphic song system development (20), this early work stimulated a flurry of research into basic endocrine physiology of the zebra finch (the most commonly studied captive songbird) and other songbird species (both captive and wild species). This work produced an inconsistent picture (21) that, together with intensified research into the natural behavior of many songbird species, led many investigators to question whether gonadal hormones controlled *all* steroid-dependent songbird behavior and corresponding features of the songbird brain.

Our interest in this area grew as we began to explore the tissue source for estrogens that were routinely measured in male songbird blood. To our surprise, outside of the brain, we could find little evidence for activity of the estrogen-synthetic enzyme aromatase in any male tissue (22). Vockel et al (23) had shown that they could detect aromatase activity in a variety of zebra finch brain regions above-and-beyond the typical hypothalamic/ limbic regions where aromatase was found in many other species. Together, these results indicated that the brain might be a source of estrogen detected in male blood, a hypothesis confirmed by *in vivo* measures of androgen to estrogen conversion in two songbird species (24-26). Not only did these observations shift our focus onto the brain as an important site of estrogen synthesis, but it also meant that most, if not all, actions of estrogens on the male songbird brain were the result of constitutive neuroestrogen synthesis.

Additional studies into aromatase mRNA and protein expression confirmed the widespread distribution of the enzyme (27, 28), but also raised questions. For example, what was the source of the androgen substrate for brain aromatization? Why did songbirds, as compared to other bird and many mammalian species, make so much estrogen in brain? Pursuit of these questions led to some unexpected properties of brain-hormone interactions in these birds.

Androgen Substrate for Brain Aromatization

Brain aromatization was largely thought to rely on gonadal androgens as the substrate for estrogen synthesis. In male songbirds, however, blood estrogen levels did not always correspond with expected or observed circulating testosterone levels suggesting the brain used an alternative substrate or tissue source of testosterone (29-31). We obtained evidence lending support for both of these possibilities. First, we confirmed activity of the enzyme 3 β -hydroxysteroid dehydrogenase/isomerase (3 β -HSD) in the zebra finch brain with the capacity to convert pregnenolone into progesterone as well as to convert the active androgen precursor dehydroepiandrosterone (DHEA) into androstenedione that could be further converted into estrogens (32-34). DHEA can be produced by the avian adrenals, but the small amount of adrenal androgen produced in zebra finch were lower than expected for a significant endocrine function (35). This raised the possibility that androgens might be synthesized directly in brain. To assess this possibility, we cloned in zebra finches the core cholesterol transport proteins and steroidogenic enzymes (steroidogenic acute regulatory protein, StAR; side-chain cleavage enzyme, SCC; 3 β -HSD; and 17 α -hydroxylase, CYP17) and then evaluated their expression in the zebra finch brain (36-39).

As was being observed in quail brain, (40- 42), we detected evidence for zebra finch brain neurosteroidogenesis by observing neural mRNA expression of the core members of the steroidogenic pathway. Expression, as determined by Northern blot, quantitative PCR and in situ hybridization analyses showed relatively widespread expression, though certain areas stood out (43). Areas of the adult brain of special relevance for the remainder of this paper include the caudomedial nidopallium (NCM), nucleus taeniae of the amygdala and hypothalamus. We continue to explore how these mRNAs are translated into functional protein in brain. Together, these data give us confidence that the complete pathway for the synthesis of estrogens from cholesterol is a feature of the zebra finch brain (44), a conclusion supported by studies of estrogen-synthesis in zebra finch brain slices cultured in vitro in the absence of significant steroidal substrates (45).

Why neurosteroidogenesis in songbirds?

In light of the widespread view that gonadal steroids activate vertebrate reproductive behaviors, two related features of adult songbird behavioral biology stood out as possible targets of neurosteroidogenic signaling. First, although territorial aggression is usually seen as a steroid-dependent behavior of adult reproductive males, many male songbirds are territorial year-round and, in some cases, both males and females are territorial including during the non-breeding season when circulating sex-steroids are basal (17, 46). Second, although singing behavior is usually seen as a hormone-dependent behavior of males defending breeding territories and soliciting females for mating, some birds sing outside of the breeding season and in some species both males and females sing (47). Furthermore, development of song circuits and phases of steroid-dependent song learning occur when there is little evidence of or expectation for elevated levels of gonadal sex-steroids (48). Some of this discord between gonadal function and steroid-dependent properties of songbird brain and behavior might be explained by neurosteroidogenesis. There are lines of evidence to support this conclusion.

Territorial Aggression

The best evidence for neurosteroidal activation of territorial behavior comes from studies of one race of North American oscine songbird, the song sparrow (*Melospiza melodia*). Sedentary populations of this species have been the focus of a lengthy study on the hormonal control of territorial aggression (see 49, 50). Relevant to our focus here are studies showing that this territorial behavior expressed in the non-breeding season remains activated by sex steroids (predominantly by estradiol) but circulating gonadal steroids at these times of year are basal (51, 52). Although circulating testosterone levels are low in non-breeding male song sparrows, the androgen precursor dehydroepiandrosterone (DHEA) is present in blood (53). Given evidence from zebra finch studies that 3 β -HSD and aromatase in brain can combine to produce estrogens from DHEA (described above) we considered the possibility that these two enzymes might function together in the non-breeding song sparrow brain to produce estrogens that activate territorial aggression.

Several research results support this conclusion. First, as was detected in zebra finches, song sparrows have considerable expression of aromatase throughout the brain (54). Second, aromatase in some brain regions, but not others, is subject to seasonal regulation. Interestingly, aromatase is elevated in the non-breeding season in brain in keeping with a role for neuroestrogen activation of aggressive behavior during the non-breeding season, notably in the region containing nucleus taeniae. Third, 3 β -HSD is expressed and active also in the song sparrow brain (55, 56), including in two regions likely involved in control of aggressive behavior, the diencephalon, containing the hypothalamus and the ventromedial telencephalon, containing nucleus taeniae. Importantly, 3 β -HSD, like aromatase, is subject to seasonal regulation, as it is elevated during the non-breeding season compared to the

breeding season in all measured brain regions. Such a result positions 3β -HSD to convert DHEA into a substrate for neural aromatization when circulating testosterone is low (56). Finally, there is emerging evidence from the Soma lab at the University of British Columbia for short-term (rapid) upregulation of 3β -HSD during aggressive interactions (56). These data show that the local neurosteroidal environment can rapidly fluctuate as concentrations of steroidal neuromodulators keep pace with changing behavioral contexts, a developing new concept in neuroendocrine signaling to be discussed further below.

These studies highlight a direct functional role for neural 3β -HSD. Additional studies are needed to determine if circulating DHEA is the sole substrate for brain 3β -HSD or whether neurally produced DHEA participates as well. As discussed previously, we have detected expression of CYP17 in the songbird brain (36, 37) as has been detected in the brains of other vertebrates (57) leaving open the possibility that the complete neurosteroidogenic pathway is activated on non-breeding song sparrows to activate aggression outside the breeding season. More research is needed also to evaluate whether pregnenolone, from either central or peripheral sources, is a substrate for brain 3β -HSD in the formation of neuroprogestins. Finally, and independent of brain steroid metabolism, we cannot dismiss the possibility that DHEA exerts direct effects on songbird neurobiology (58). Additional studies of song sparrows, other songbirds, and other non-avian animal models will undoubtedly expand our appreciation of brain 3β -HSD in neural development and in the activation of behavior.

Song

A key component of song learning involves higher-order auditory processing of song stimuli to enable proper recognition of song elements. Interestingly, one area of the songbird brain especially rich with aromatase, as well as other steroidogenic enzymes, is the NCM, a region that receives input from the auditory system and which projects to pre-motor nuclei for song production. Aware of this rich steroidal environment, we developed the ability to successfully use in vivo microdialysis to measure estrogens and androgens in the NCM of adult male zebra finches on a short time-scale (30mins) previously unattainable by other methods (59). This crucial advance set the stage for evaluating the flux of these steroids as awake, behaving birds were subject to a changing social environment and to changing auditory stimuli. This work showed that within NCM, but not adjacent brain regions (with either low aromatase or that received no auditory afferent projections), marked changes in estradiol occurred when males were exposed to females or to conspecific zebra finch song (59). Moreover, local levels of estradiol were reduced by exposure to an aromatase inhibitor (Fadrozole) or to glutamate, the latter result replicating what had been observed in vitro in quail hypothalamus (60). Lastly, in some cases levels of testosterone changed inversely with that of estradiol, indicating that rapid regulation of local aromatase was key to the observed changes in estradiol.

These results raised the question of what estrogens might be doing in NCM. Aware that male zebra finches express preferences for learned vs. novel songs (61), a behavior requiring auditory discrimination, we tested male song preference in birds retrodialyzed with Fadrozole locally into either the left or right NCM. During a 30min test session, inhibition of estrogen production in the right NCM abolished male preference for his own song (62). This result revealed a behavior that appeared to require the presence of estradiol and showed that the estrogen effect was occurring on a rapid and temporary time-scale.

The cellular basis for this rapid estrogen effect appears to depend on a neuromodulatory role of estradiol on burst firing of NCM neurons. Our electrophysiological studies showed that conspecific song-induced multiunit activity of NCM neurons was enhanced in the presence of estradiol (62). Exogenous estrogens promoted a switch from isolated spiking to a burst

firing pattern that occurred within minutes of application. In the presence of Fadrozole, song-evoked firing frequency and burst-firing were diminished showing that endogenous estrogen production was responsible for modulation in intact males. At about the same time, almost identical electrophysiological findings for a role of estrogens in the zebra finch NCM emerged from the lab of Rafael Pinaud (63) providing timely confirmation for results of both labs. More recently, Pinaud's lab has provided an even more detailed electrophysiological quantification of estrogen's actions in the songbird NCM to guide song discrimination (64). Likewise, new experiments in our lab show that the rapid estrogen actions in the zebra finch NCM are dependent on a membrane-specific receptor, further substantiating the rapid modulatory role of neuroestrogens (L. Ramage-Healey and B. Schlinger, unpublished observations). These studies combined with the above-mentioned behavioral observations provide a comprehensive view for why estrogen production is elevated in NCM during social interactions.

Synaptic Steroidogenesis

Neuromodulation involves synaptic release of neurochemicals that regulate the amplitude or frequency of post-synaptic membrane potentials. We have obtained particularly good evidence for synaptic estrogen synthesis in the songbird brain suggesting that this form of neurosteroidogenesis is a true form of neuromodulation {Balthazart, 2006 #3641} Saldanha et al., submitted). A crucial advance was that immuno-electron microscopy using a zebra finch specific aromatase antibody shows staining for aromatase in pre-synaptic boutons in several regions of the zebra finch brain (65), as had been shown previously in quail and other species (66, 67). Whereas some brain regions possess aromatase-positive somas and terminals, including the HPOA and NCM, making the subcellular source of estrogen hard to determine, some areas with aromatase-positive terminals have few if any aromatase-positive somas. These latter regions, including the hippocampus and nucleus HVC, may obtain virtually all of their estrogen from synaptic synthesis.

Even in regions with aromatase in multiple subcellular locations, synaptic aromatase may be most important. For example, there is much evidence for sexually-dimorphic neural provision of estrogen to the zebra finch brain. The only reliable sex-differences in brain aromatase are those found in synaptosomal preparations (68) or visualized by immuno-electron microscopy (65). Furthermore, when subcellular fractions of brain are prepared from singing and non-singing males, more aromatase is found in synapses of singers than non-singers whereas microsomal aromatase (presumably that obtained largely from somas) are not different (69). Compartmentalization of aromatase within presynaptic boutons appears crucial for providing sex- and song-specific estrogenic signals in the songbird brain.

The aromatase in synapses is likely that which is subject to rapid regulation. In quail hypothalamic explants, aromatase undergoes Ca^{++} -dependent phosphorylation that reduces aromatase activity within minutes (70-72). Treatments of these explants with K^{+} or with glutamate receptor agonists NMDA, AMPA and kainate all produce a similar rapid inhibition of aromatase (71). Although the subcellular localization of this aromatase is unknown, it might be aromatase at the synapse that is phosphorylated upon excitation, locally reducing the neuron's capacity to synthesize estrogen. Our microdialysis studies of zebra finch telencephalic estradiol and testosterone concentrations provide considerable support for the view that glutamatergic downregulation of aromatase occurs in vivo in the zebra finch NCM (59). Moreover, we have obtained evidence recently indicating not only that retrodialysis with K^{+} reduces NCM estrogens levels in vivo, resembling what is seen in quail hypothalamus in vitro, but the effect is blocked by omega-conotoxin, a synapse specific Ca^{++} -channel blocker (Ramage-Healey et al., submitted). These results lead us to conclude that the estrogens we measure in brain using in vivo microdialysis are synthesized

largely in synapses where flow of Ca^{++} through voltage-gated Ca^{++} channels serves as a key regulatory signal for estrogen production.

Conclusions

Results from these studies of songbirds shed new light on functional perspectives of neurosteroidogenesis. Expression in brain of genes involved in steroidogenesis may preadapt circuits to take advantage of these neuromodulatory signals when peripheral steroid signals are unavailable, when steroidal neuromodulation is required at a speed greater than can be achieved by peripheral synthesis and release, or when widespread exposure to steroids is undesirable. Synaptic neurosteroidogenesis may be a widespread feature of vertebrate neurobiology and a source of novel forms of neuromodulation (e.g. (73). Indeed, evidence is accumulating from several vertebrate model systems to support the concept that estradiol should, in many cases, be considered a classic neuromodulator (74-76). We are no longer restricted to viewing the whole brain as subject to steroid fluctuations based on seasonal and sexual cycles. By contrast, we can see that steroid concentrations can fluctuate in brain over long- or short time scales with cellular and subcellular precision, including at individual pre- and post-synaptic targets. Songbirds are perched to contribute new insights into this changing perspective on brain steroid chemistry.

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