动物学报 52(Supplement): 238–241, 2006
Acta Zoologica Sinica

S14-1 Integrating steroid synthesis with steroid action: multiple mechanisms in birds

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Abstract Sex steroids of gonadal origin influence avian behavior, anatomy and physiology. There is evidence, however, that sex steroids can be synthesized in non-gonadal tissues and have actions unrelated directly to reproduction. We describe evidence in birds for sex steroid synthesis in the adrenals and brain. The expression and activity of steroidogenic enzymes outside of the gonads creates opportunities for birds to use sex steroids as potent signaling molecules on nonreproductive tissues without disadvantageous activation of reproductive systems.

Key words Steroid hormones, Brain, Aggression, Song, Reproduction

1 Introduction

The sex steroid hormones progesterone (Prog), testosterone (T) and estradiol (E_2) are powerful signaling molecules influencing a wide range of anatomical and physiological systems. These hormones play a significant role in reproduction; and the gonads are the dominant source of these steroids. Upon reaching the circulatory system, they have access to all tissues and then act on cells that express the appropriate steroid receptors and their coactivators. In some cases, sex steroids may act on tissues associated with nonreproductive functions. In these cases, the sex steroids can be synthesized outside of the gonads, principally in the adrenals and in the brain. We propose that the synthesis of sex steroids in these non-gonadal sites is a mechanism for taking advantage of the potency of these hormones, allowing them to be used in nonreproductive contexts without inappropriately activating reproductive tissues. Birds are useful animal models for exploring this hypothesis.

Do birds synthesize sex steroids in non-gonadal tissues, and if so, why? We will present evidence that the avian adrenals and brain can synthesize androgens. We describe two systems involving songbirds in which non-gonadal androgens may have significant actions on the brain to control behaviors that can be expressed independently of such behaviors as copulation which are traditionally associated with reproduction *per se*. In the first system, an inactive androgen may be secreted by the adrenals and then metabolized into active androgens and estrogens in the brain to activate aggressive behavior in winter. In the second system, active sex steroids may be synthesized locally in the developing brain to masculinize some neural circuits. In both of these systems, non-gonadal sex steroids are used as signaling molecules, and their actions are restricted to neural circuits and behaviors that can be expressed in nonreproductive contexts.

What evidence exists that sex steroids are synthesized outside of the gonads? Steroid synthesis by a tissue can be inferred if removal of that tissue (such as castration) reduces circulating hormone levels. It is possible also to infer synthesis in tissue if the concentration of hormone there exceeds the concentration of that hormone in blood or if the concentration of hormone in the venous output of that tissue exceeds the concentration of hormone in its arterial supply. Direct evidence of steroid synthesis, moreover, can be determined if the tissue itself has the activity or mRNA expression of the enzymes and transporters needed for steroid synthesis.

From such inferences, steroidogenesis is easy to document in vertebrate gonads during periods of reproduction, especially in birds. At this time they express steroidogenic enzymes in large amounts to produce the large quantities of hormone needed to elevate blood levels (e.g., Freking et al., 2000). In cases where steroid synthesis is coupled to local actions, evidence for synthesis is more difficult to document because the amount of steroid produced is small. Often, the best approach for identifying steroid synthesis under these conditions is to use sensitive molecular or biochemical techniques to identify expression or activity of steroid synthetic enzymes (Tsutsui and Schlinger, 2002). It is also important to examine the appropriate animal models at life-history stages when non-gonadal steroidogenic enzymes might be expressed.

Sex steroids are synthesized from cholesterol in the following way. Cholesterol is first transported into mitochondria where it is converted into pregnenolone by the side-chain cleavage enzyme (CYP11A1). Pregnenolone is

then converted into either Prog by the enzyme 3β-hydroxysteroid dehydrogenase (3β-HSD) or into the inactive androgen dehydroepiandrosterone (DHEA) by the 17α-hydroxylase enzyme (CYP17). Androstenedione (AE) is then produced from Prog by the actions of CYP17 or by the actions of 3β-HSD on DHEA. AE is converted to T by the enzyme 17β-HSD. And T is converted to E_2 by the enzyme aromatase. These reactions occur in mitochondria and the endoplasmic reticulum. Because they are lipophilic, steroidal products diffuse passively out of cells to affect neighboring cells or distant cells after entering the systemic circulation (Miller, 1988).

Typically, these enzymes are co-expressed in a single cell (as in the testicular Leydig cell) or in adjacent cells within a tissue (as in theca and granulosa of the ovarian follicle). In some cases, however, they can be expressed in different tissues. Thus there can be substantial physical separation of the reactions along the steroidogenic pathway within an organism. For example, estrogens have many important actions on the avian male brain, despite low E_2 concentrations in blood. The T in blood is produced in the testes by action of the synthetic enzymes side-chain cleavage, 3β-HSD and CYP17 (Freking et al., 2000). Circulating T is then converted into E_2 in the brain by the neural expression of aromatase (see above; Schlinger, 1997). Here we discuss evidence that the avian brain can express other enzymes of the steroidogenic pathway.

2 DHEA and aggression in non-breeding birds

Many birds abandon territories over winter in favor of flocks. In the non-breeding season, the gonads and secondary sex characters (e.g., cloacal protuberance, wattles) are generally regressed, and circulating T and E_2 are usually non-detectable. Some species, however, aggressively defend territories during the non-breeding season, even though plasma T is at basal levels (Soma and Wingfield, 1999). Winter territoriality may be dissociated from plasma T because of the costs of circulating T (e.g., Ketterson et al., 1992). In seasonally breeding birds, sex steroid hormones in general circulation may have particularly high costs during the non-breeding season. The non-breeding season may extend over most of the year and can be a difficult time for birds, especially small birds, because of cold temperatures and reduced food supply (Wingfield et al., 2001). Diurnal species also cannot forage during the long nights of winter. Overall, T treatment has been shown to reduce overwinter survival in songbirds (Dufty, 1989). Many costs of T during the non-breeding season may be energetic: T increases metabolic rate (e.g., Buchanan et al., 2001) and decreases fat stores, which are important for insulation and surviving snow and ice storms during winter. Other costs of T involve suppression of the immune system (Casto et al., 2001). T treatment may also stimulate reproductive behaviors that are inappropriate during the winter (Logan, 1992). During the spring breeding season, in contrast, high levels of circulating T are useful for coordinating multiple physiological systems in preparation for reproduction.

In song sparrows (*Melospiza melodia*), males are territorial during the breeding season (spring) when plasma T is high. Yet they are also highly aggressive during the non-breeding season (autumn and winter), when plasma T is non-detectable and the testes are completely regressed (Soma and Wingfield, 1999). Furthermore, castration has no effect on non-breeding aggression. Thus, aggression may be independent of sex steroids such as T in autumn and winter. This hypothesis was tested in three field experiments by treating wild song sparrows with the pharmacological inhibitors of aromatase and androgen receptor binding. In particular, blockade of estrogen synthesis by an aromatase inhibitor strongly decreased male aggression in the non-breeding season. The effects of the aromatase inhibitor, however, can be rescued by estrogen replacement (Soma et al., 1999, 2000a,b). These data indicate that sex steroids, particularly estrogens, are important for the expression of aggressive behavior in the non-breeding season, even though plasma levels of sex steroids are non-detectable and castration has no effect.

Subsequent studies have addressed the source of androgen substrate for brain aromatase in the non-breeding season, because plasma levels of aromatizable androgens (testosterone and androstenedione) are basal then. Although Dehydroepiandrosterone (DHEA) is a largely inert androgen precursor, it can be converted locally into active sex steroids within tissues that express the appropriate enzymes. Interestingly, plasma levels of DHEA are elevated in non-breeding song sparrows (Soma and Wingfield, 2001). Further, DHEA concentrations in the adrenals of winter birds are high, suggesting adrenal secretion of DHEA. In a separate experiment, treatment of wild non-breeding males with DHEA increased territorial song and the size of a brain region that controls song behavior (Soma et al., 2002b). DHEA treatment did not, however, stimulate the growth of secondary sex characters such as the cloacal protuberance. These data raise the hypothesis that DHEA is converted into active sex steroids in the brain but not in peripheral tissues. Recent results suggest that DHEA treatment does not inhibit immune responses in sparrows, in contrast to T treatment (N. Owen-Ashley and J. Wingfield, pers. comm.).

Current studies are examining regional and seasonal differences in song sparrow brain aromatase using biochemical and molecular techniques. *In situ* hybridization reveals that aromatase mRNA is highly expressed in the hypothalamus and telencephalon. Interestingly, biochemical measurement of aromatase activity in the avian amygdala (nucleus taeniae) shows that seasonal changes in aromatase activity correspond to seasonal changes in aggression, as in its reduction at molt (Soma et al., 2003). As DHEA has important behavioral and neural effects in songbirds, its metabolism in the song sparrow brain is also under examination. We have designed and validated a biochemical assay to measure its conversion to androstenedione and estrogen by

the sequential activities of 3β-HSD and aromatase (Vanson et al., 1996; Soma et al., 2002a). Excitingly, the song sparrow brain can convert DHEA to androgens and estrogens, with highest levels of 3β-HSD activity in the diencephalon and telencephalon. We are now comparing seasonal differences in DHEA metabolism by the brain, with the prediction that it will be highest in the non-breeding season.

Taken together, the data support the novel hypothesis that in non-breeding song sparrows, the adrenals secrete DHEA, which is then metabolized into sex steroids in the brain to maintain aggression. With DHEA instead of T in the circulation, non-breeding song sparrows may circumvent the costs of circulating T, while continuing to provide sex steroids to appropriate neural circuits. A similar mechanism may be operating in a wide variety of species.

3 Neural steroid synthesis in the developing avian brain

Evidence is accumulating that the avian brain may not only metabolize steroids but has the capacity as well to fully synthesize steroids from cholesterol. These "neurosteroids" may influence the development of neural circuits in songbirds. The zebra finch (*Taeniopygia guttata*) is sexually dimorphic in its behavior and underlying neural song circuit. Adult males sing and possess a series of brain nuclei that control song output, but females never sing and lack a functional song system. Studies show that these brain areas can be masculinized by steroids, especially by E_2. Specifically, treatment of females within the first 3 weeks post-hatch masculinizes the neural song system sufficiently to allow them to sing song as adults (Arnold and Schlinger, 1993). The source of these steroids was assumed to be the gonads. Most studies, however, fail to support the idea that the gonads are their natural source.

If males develop their functional song system under the influence of gonadally-derived steroids, then manipulations of gonads should impact the organization of the song system. Contrary to this hypothesis, castration of genetic male zebra finches nine days post-hatch does not block the male pattern of song circuit organization; and induction of functional testicular tissue in genetic females is not sufficient for the development of the mail song system (Wade et al., 1999; Arnold and Schlinger, 1993). In addition, it would be expected that circulating levels of steroids would be higher in males than females during the steroid-sensitive period if the gonads were secreting the necessary steroids. No such difference, however, has been measured consistently during the first three weeks of post-hatch development (Arnold and Schlinger, 1993). Further, male and female zebra finch gonads show equal capacity to synthesize androgens based on enzymatic activity and enzyme mRNA levels. The testes do not express the enzyme aromatase in significant amounts, but the ovary does, rendering the testes unable to supply greater concentrations of E_2 to the brain than ovaries (Freking et al., 2000). Therefore, an alternative source must exist for the masculinizing steroids.

Evidence that the brain is the steroid-synthesizing tissue has emerged for the zebra finch song system. Holloway and Clayton (2001) prepared slices of male and female brains from developing birds and cultured them. These slices contained major song areas that are sexually dimorphic in the nuclei and inter-nuclear connections. The normal masculine song nuclei connections were present in male slices but not in female. If a female slice was put in culture with a male slice, however, the male pattern of connections appeared in the female, suggesting that the male slice was secreting a diffusible factor that impacted the female. An assay of the culture media for male and female slices then found the level of estrogen to be higher in that from the male slice. Since the brain is isolated from the gonads, these data not only suggest a sexual difference in the ability of the brain to synthesize estrogens, but also that the brain itself has the capacity to synthesize steroids *de novo*.

Molecular and biochemical techniques have enabled the identification of steroidogenic enzymes in the brain of zebra finches and other avian species. Extensive evidence shows that aromatase is abundant in the zebra finch brain (Schlinger, 1997). Biochemistry has measured levels of 3β-HSD activity in cultures from developing zebra finch telencephalon (Vanson et al., 1996), as well as in the adult songbird telencephalon (see above). In the zebra finch, highly sensitive RT-PCR has shown the presence of mRNA for CYP11A1, 3β-HSD, CYP17, and aromatase in developing telencephalon as well (Schlinger et al., 2000). *In situ* hybridization, moreover, confirms the expression pattern of CYP17 mRNA in developing and adult zebra finch telencephalon and other brain areas (London and Schlinger, 2002).

In another avian species, the common quail (*Coturnix coturnix*), evidence for expression in the brain of all steroidogenic enzymes necessary for active steroid synthesis has been shown (Tsutsui and Schlinger, 2002). Taken together, these data suggest that the zebra finch brain will be found to expresses the complete suite of enzymes necessary to synthesize steroids independently of the gonads. Brain-derived steroids could then act to specifically organize the song system into a functional neural circuit. Song can be used in nonreproductive contexts, so its development is uncoupled from the organization of neural circuits underlying male and female copulatory behavior. In this way, the song circuit can develop in females of those species in which females sing without masculinizing other sex-related events.

Although we have presented evidence for adrenal and neural sex steroid synthesis, further work is required to confirm that the amount of androgen produced is sufficient to influence target sites in the avian brain as we suggest. We predict that additional studies of other bird species at appropriate life history stages will reveal the involvement of non-gonadal sex steroids in other behavioral and physiological systems.

Acknowledgements This project was supported by grants

NIH MH61994 and MH19384. K. K. Soma is a NIH postdoctoral fellow. B. Schlinger, who found himself unable to present this paper in person, extends heartfelt thanks to Dr. Leonida Fusani for doing so.

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