RAPID SOCIAL REGULATION OF 3β-HSD ACTIVITY IN THE SONGBIRD BRAIN

by

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ABSTRACT

Rapid increases in plasma androgens are generally associated with short-term aggressive challenges in many breeding vertebrates. However, some animals such as song sparrows (<u>Melospiza</u> <u>melodia</u>) are aggressive year-round, even during the non-breeding season, when gonads are regressed and systemic testosterone (T) levels are non-detectable. In contrast, levels of the prohormone dehydroepiandrosterone (DHEA) are elevated year-round in the plasma and brain. The local conversion of brain DHEA to potent androgens may be critical in regulating non-breeding aggression. 3β-hydroxysteroid dehydrogenase/Δ4-Δ5isomerase (3β-HSD) catalyzes DHEA conversion to androstenedione (AE) and the cofactor NAD^+ assists in this transformation. In this thesis, I asked whether brain 3β-HSD activity is regulated by social encounters in seasonally breeding male songbirds. In Experiment 1, I looked at the long-term seasonal regulation of brain 3β-HSD activity. 3β-HSD activity was highest in the non-breeding season compared to the breeding season and molt. In Experiment 2, I hypothesized that brain 3β-HSD activity is rapidly regulated by short-term social encounters during the non-breeding season. A 30 min social challenge increased aggressive behavior. Without exogenous NAD^+, there was ~355% increase in 3β-HSD activity in the caudal telencephalon and ~615% increase in the medial central telencephalon compared to controls ($p<0.05$). With exogenous NAD^+, there was no effect of social challenge on 3β-HSD activity. These data suggest that endogenous cofactors play a critical role in the neuroendocrine response to social challenges. The increase in brain DHEA conversion to AE during social challenges may be a mechanism to rapidly increase local androgens in the non-breeding season, when there are many costs of systemic T.

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CO-AUTHORSHIP STATEMENT

I, D.S. Pradhan, conducted a major portion of the data analyses for Experiment 1. For Experiment 2, I assisted with the field work, and conducted all the laboratory work and data analyses myself. The following co-authors are listed on the manuscript: Amy E. M. Newman, Douglas W. Wacker, B.A. Schlinger, J.C. Wingfield, and Kiran K. Soma.

Experiment 1 was designed and conducted primarily by K. K. Soma, with the assistance of D.W. Wacker, and under the guidance of B.A. Schlinger and J.C. Wingfield.

For Experiment 2, A.E.M. Newman primarily conducted the field work by collecting behavioral data and performing all the dissections.

REGULATION OF STEROIDOGENIC ENZYMES

Introduction

It is well established that a change in the physical or social component of an animal's environment can influence its endocrine state to regulate behavior. Steroids regulate behavior in both the long-term and short-term via genomic and non-genomic mechanisms, respectively (1-3). In a dynamic environment, a stimulus may vary over time, and steroids are modulated to accommodate such changes. Changes in steroid levels are the result of changes in steroid synthesis and metabolism (4). Changes in steroidogenic enzymes may be crucial rate-limiting steps that govern behavioral transitions in changing environments (5, 6).

Enzymes involved in the biosynthesis of active steroid hormones are found in several organs such as the gonads, adrenal glands, liver, heart, placenta, and brain (7, 8). Substantial advances have been made in understanding the expression of genes coding for steroidogenic enzymes, the structure of these enzymes, and the reactions catalyzed by them (8) (see Fig. 1.1 for steroid synthesis pathway). Steroid-converting enzymes are often studied at a highly reductionist level, focusing on parameters such as substrate affinity and specificity, maximum velocity, optimal temperature and pH, cofactor requirements, subcellular localization, and regulation by end products (9, 10). This information can, in turn, allow integrative studies of how enzyme activities change as a function of age, endocrine condition, seasonal cues, stress, and social interactions (11, 12).

Gonadal Androgens and Behavior

Testosterone (T) is secreted by the testis, and, to a lesser extent, ovaries in many vertebrates and is important for the development of secondary sexual characteristics and activation of behavior. In male Japanese quail (Coturnix japonica), castration completely eliminates copulatory behaviors, which can be restored by T treatment (13, 14). Moreover, systemic T levels in male quail rapidly increase when presented with a receptive female or threatened by a conspecific male intruder (15, 16). This increase in systemic T is mediated by the hypothalamic-pituitary-gonadal (HPG) axis (17). Hypothalamic gonadotropin-releasing hormone stimulates the anterior pituitary to secrete luteinizing hormone (LH), which stimulates the gonads to secrete T. In the Leydig cells of the testes, steroidogenic acute regulatory (StAR) protein transports cholesterol to the inner mitochondrial membrane to initite steroid synthesis (18). However, the time frame in which T-synthesizing enzymes in the gonads are upregulated in the gonads remains unclear.

The traditional model of T action on behavior is that T secreted by the gonads is transported via the blood to distant target organs such as the brain, where T is metabolized to other steroids, such as estradiol (E_2) to regulate behavior. The aromatization model was formulated with two sets of discoveries. First, E_2 treatment can restore copulatory behavior in castrated quail (19). Second, aromatase inhibitors block the behavioral effects of T (20). Specifically, aromatase in the anterior hypothalamic-preoptic area (POA) is critical for reproductive behaviors (21).

Additionally, T also exerts its effects via conversion to other biologically active steroids. For example, 5α-reduction of T leads to the formation of 5α-dihydrotestosterone (5α-DHT or androstane-17β-ol-3-one) and 5α-androstane-3β,17β-diol (5α-3β-diol) (22, 23). In contrast, the 5β-reduced metabolites of T are considered biologically inactive. In long-term castrated doves (Streptopelia risoria) there is a negative correlation between 5β-

reductase activity and aggression. Administering E_2 in castrated doves can restore aggressive behavior (24). The reduction of T by 5β-reductase is considered an "inactivation shunt" (25). The balance between the concentrations of active and inactive metabolites of T regulates behavior (21).

In summary, conversion of gonadal T to E_2 by brain aromatase is important for the regulation of behavior. However, gonadal T secreted into the systemic circulation can reach many target organs. In the periphery, T has other functions, such as spermatogenesis and development of accessory reproductive organs and secondary sexual characteristics, which may not be necessary (e.g., outside the breeding season). Thus systemic increases in T are potentially costly (26, 27).

Neural Androgens and Behavior

Local production of androgens within target tissues may be an effective mechanism to reduce the costs of systemic T (26, 27). Androgens can be locally synthesized in the brain in at least two ways. First, the circulating prohormone, dehydroepiandrosterone (DHEA), can be converted within the brain to the active and aromatizable androgen, androstenedione (AE). Second, androgens can be synthesized de novo from cholesterol in the brain (Fig. 1.1). All of the sex steroidogenic enzymes and StAR are expressed and are active in the brain of vertebrates, including birds (7, 28). In song sparrows (Melospiza melodia), conversion of DHEA to active sex steroids within the brain may regulate non-breeding aggression (27, 29). It is not clear whether the effects of DHEA on behavior are attributable to peripheral (e.g. adrenal) or neural sources of DHEA. To understand how neural androgens regulate behavior, it is important to study the factors regulating steroidogenic enzymes.

Regulation of Steroidogenic Enzymes in the Brain

Many studies have documented effects of steroids on behavior. However, note that the reverse is also true: an organism's behavior can also influence its steroid levels, via changes in the activities of steroidogenic enzymes (17). For example, the degree of receptiveness of a female to a male dove's displays can rapidly stimulate her reproductive endocrine system, which has been termed behavioral 'self-feedback' (30).

Additionally, in seasonal breeders, the reproductive system of the female is also influenced by environmental cues, such as slow changes in photoperiod. Thus a combination of short-term and long-term regulation of steroidogenic enzymes is vital for the necessary feedback to affect behavior (Fig. 1.2). For the remainder of this review, I will focus on aspects of steroidogenic enzyme regulation.

Long-term Regulation of Steroidogenic Enzymes in the Brain

Enzymes can be regulated in the long-term by a combination of factors of the external environment and endogenous rhythms. Predictable changes in the environment, such as onset of seasons, which last for months, can stimulate transcription and translation of genes for steroidogenic enzymes (17). This may be coupled with an upregulation in synthesis of other machinery required for steroid action, such as steroid receptors. Steroid hormones released as a product of enzyme activity are secreted into the systemic circulation by endocrine glands. These steroids travel to distant target cells and bind to intracellular receptors to affect gene transcription, generating a feedback system. Genes continue to modulate steroidogenic enzyme expression over the course of predictable environmental changes. This genomic regulation of enzyme expression and activity, which may take hours or days to occur, is termed 'long-term', and prepares the organism to cope with predictable changes in the future.

Long-term effects of physical environment and life history stages on enzymes

Predictable seasonal changes in the physical environment, such as food availability, day length, temperature, and predator density have profound influences on the behavior and hormonal mileu of animals (31, 32). Many vertebrates go through marked morphological and physiological cycles, including growth of muscle tissue, body fat, and gonads. Seasonal growth of neural song control nuclei in songbirds is also regulated by changes in hormone levels across seasons (33). Field studies allow us to study the effects of several integrated parameters, while laboratory studies can enable us to isolate these factors.

Field studies allow the study of seasonal changes in physiology and behavior in a natural environment. For example, in the temperate zone, song sparrows go through seasonal changes in plasma T levels. Plasma T peaks during the breeding season, and remains basal during the molt and non-breeding seasons (34). Despite having low plasma T, song sparrows aggressively defend their territories during the non-breeding season. Along with seasonal changes in plasma T are seasonal changes in the activities of steroid-converting enzymes in the brain. Interestingly, brain aromatase activity shows region- and season-specific changes in these birds (35). In male European starlings (Sturnus vulgaris), four T metabolizing enzymes (aromatase, 17β-hydroxysteroid dehydrogenase, 5α-reductase and 5β-reductase) also show seasonal changes in activity in the diencephalon and telencephalon (36).

Temporal patterns of enzymes also vary as a function of life history stage and mating system during the breeding cycle, but few studies have looked at this phenomenon. In an Arctic breeding bird, the Lapland longspur (Calcarius lapponicus), activities of T metabolizing enzymes in the brain change within the short breeding season, which was correlated with changes in behavioral priorities (37). In this species, the peak in systemic T level is very brief, about 1-4 days during the territorial display phase and is low even during the time that males mate-guard females. Socially monogamous males who provide parental

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care have different T levels during the breeding season compared to polygamous males who do not show parental care (38). We can hypothesize that activity of brain steroidogenic enzymes in these animals also show specific patterns.

Animals respond to changes in photoperiod to adapt to seasons. Day length and temperature can be easily adjusted in the laboratory to mimic photoperiod cues and study effects on enzyme activity and behavior. For example, photo-inhibition (change from 16 hour long days to 12 hour days) affects aromatase activity, but not 5α-reductase or 17β-HSD in specific brain regions in Siberian hamsters (39). Japanese quails exposed to long days, but not short days display sexual behavior (40). In long days, 5α- and 5β-reductase activity are also affected in the pituitary (40). In another experiment, in quail, exposure to long days increased brain aromatase activity, but not brain 5α- or 5β-reductase, in the mitochondrial and microsomal fractions (41).

Long-term effects of social environment on steroidogenic enzymes

Presence of a reproductively mature female can affect hormone levels and behavior of males. Plasma DHT levels increased in male mallards exposed to females over 10 days, compared to isolated males (42). Repeated social stimulation over the course of days has been correlated with plasma hormone levels in other bird species (42-44).

Few studies have looked at the long-term effects of social stimulation on activities of T metabolizing enzymes in the brain. In one study, male Japanese quails were exposed to a different social situation each day: a male was presented with a sexually receptive female for 5 min, or a stuffed female (for 1 min), or two males were allowed to interact aggressively for 1 min (15). Sexual behavior and frequency of crows were recorded, T levels in plasma were measured before and after social stimulation, and brain and cloacal tissue were collected at the end of the experiment. The first group of males was stimulated with each social

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condition once before being sacrificed after 6 days. The second group was stimulated with each social condition twice after being sacrificed after 9 days. There was a strong positive correlation between social behavior and active products of in vitro metabolism of T in the anterior hypothalamus, and negatively correlation between behavior and 5β-reduced (inactive) metabolites (15).

Exposure to social interaction via stimulation of visual or tactile stimuli with females can also affect brain aromatase. Hutchison and Steimer performed a study (45), were they divided sexually mature male doves into 3 groups. In Group A, visually isolated males could hear, but not see other doves; in Group B, males were paired with a sexually receptive female; and Group C consisted of males who could see and hear Group B pairs. In Experiment 1, animals were socially stimulated for 2 and in Experiment 2 animals were socially stimulated for 4 days. Male doves given an opportunity to interact with a female had higher aromatase activity in the POA compared to isolated males or males who were only stimulated visually by a female. Interestingly, the increase in aromatase activity was specific to brain regions implicated in reproductive behavior. Moreover, aromatase activity increased irrespective of the duration of social stimulation. Other T metabolizing enzymes were not affected by sexual interaction (45).

Long-term effects of steroids on steroidogenic enzymes

Several studies have investigated the regulation of brain steroroidogenic enzymes by endocrine factors such as steroids. Studies involving treatment of gonadectomized animals with steroids will provide insight into the function of neurosteroids. For example, in female rats, neurally produced progesterone has been implicated in follicular maturation. In the female rat, estradiol benzoate increases brain 3β-HSD mRNA expression 24 and 44 hr after treatment and 3β-HSD activity after 44 hours (46). Note that 3β-HSD converts

pregnenolone to progesterone (Fig. 1.1). In another study, in vivo treatment of castrated zebra finches with T for 10 days increases aromatase activity (47) and mRNA in the brain (48). In an in vitro study of zebra finch brain cultures, long term (72 hour) E_2 treatment decreased aromatase activity and expression, but had no effect on 5α- and 5β-reductase (49).

Short-term Regulation of Steroidogenic Enzymes in the Brain

Steroidogenic enzymes can be regulated in the short-term by sudden, unpredictable changes in the environment, which may require an organism to rapidly modify its behavior. Rapid changes in steroidogenic enzyme activity lead to rapid changes in steroid synthesis, resulting in behavioral change. Short-term Changes in enzyme activity are likely to involve non-genomic mechanisms since the behavioral switch occurs very quickly, within 15 to 30 min (50). However, some experiments have looked at shorter times, but there are constraints of the ability to reliably measure steroidogenic enzyme activity in a very short time-scale.

Short-term effects of steroids on steroidogenic enzymes

The regulation of steroid biosynthesis in the gonads and adrenals has been studied in great detail (4, 8). The discovery that steroid biosynthesis can be rapidly regulated in the brain, however, is fairly recent. Neurally produced steroids could limit their own synthesis via local negative feedback loops (10) and this might be important for creating short steroid pulses at particular synapses. In zebra finch brain, within only 10 min, E_2 inhibits 3β-HSD activity in a dose-dependent manner. Moreover, there is a sex difference in this E_2 effect, with a greater effect in females than in males (51). Similar to studies on subcellular localization of brain aromatase (41), we are currently investigating the subcellular localization of 3β-HSD in the zebra finch brain (L. Lau, D. Pradhan, and K. Soma

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unpublished). Treatment of specific subcellular compartments with E_2 will help elucidate the regulation of brain 3β-HSD.

Short-term effects of stress on steroidogenic enzymes

Predators can be a short-term stressor for animals in their natural environment. Stress activates the hypothalamic-pituitary-adrenal axis (HPA), stimulating the adrenals to release glucocorticoids. DHEA, released by the adrenal glands, is also regulated by stress in humans and song sparrows (52, 53). Since DHEA has anti-glucocorticoid effects in the nervous system (54), neural metabolism of DHEA active sex steroids may be beneficial (55). Interestingly, brain 3β-HSD activity decreases with 10 min restraint stress in zebra finches (56). In contrast, in quail, brain aromatase activity increases with 15 min restraint (57). The functional significance of these neurochemical changes in different species remains unclear. Current studies are examining how brain 3β-HSD and aromatase are affected by 30 min restraint stress across different seasons (D. Pradhan, A. Newman, L. Lau, and K. Soma). This is will be an excellent system to study both long-term and short-term effects of stress in wild animals. Thus, scientists working with animals on behavior-endocrine relationships must pay attention to rapid effects of handling stress on steroidogenic enzymes.

Short-term effects of social interactions on enzymes

In male-male social encounters, there have been reports of rapid increases in circulating androgens (presumably from the gonads) in many vertebrate species such as songbirds (58, 59), fish (60, 61), amphibians (62) and mammals (63). The mechanisms by which gonadal T synthesis is rapidly upregulated are not clear, but it could involve StAR or phosphorylation of P450c17 (64, 65).

There have been few studies documenting the rapid effects of social interaction on brain steroidogenic enzyme activity. In one study, intact male Japanese quail were either handled (control), or allowed to see a female or allowed to physically interact with a female for 1, 5, or 15 min. In the POA, aromatase activity significantly decreased at 5 min after interaction, but not at 1 or 15 min. Moreover, dopaminergic activity decreased after 1 min and reached baseline levels after 5 min (66). This suggests that the rapid change in dopamine precedes changes in aromatase. Moreover, there is evidence that dopamine regulates aromatase activity in the quail brain (67).

In another study, in sex changing fish, there was no significant change in brain or gonadal aromatase activity of females within 10, 20, or 30 min after male removal, even though aggressive behavior rapidly increased after male removal (M. Grober, submitted). However, there was a significant decrease in brain aromatase one day after male removal (68). These results indicate that the time-scale for rapid changes depend upon the type of stimulus and the particular behavior being measured.

Mechanisms for short-term regulation of brain steroidogenic enzymes

Over the past decade, considerable progress has been made in understanding the mechanisms underlying the rapid regulation of brain aromatase. The aromatase protein has multiple phosphorylation sites, and there is evidence that Ca^{2+} dependent phosphorylation can rapidly downregulate aromatase activity in brain homogenates (69). Catecholamines such as dopamine are rapidly released and can also rapidly affect aromatase activity (70). These mechanisms could be applied to understand the rapid regulation of other steroidogenic enzymes. For example, similar to aromatase, preliminary studies show that 3β-HSD protein consists of multiple phosphorylation sites (T. Charlier and K. Soma, unpublished). In the brain, 3β-HSD protein is necessary to synthesize active sex steroids from DHEA. In

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summary, sudden changes in the environment could cause rapid release of neurotransmitters (e.g., dopamine, GABA) in the brain, which could act via kinases to increase Ca^{2+} mediated phosphorylation of steroidoidogenic enzymes. Increased activity of these enzymes would change levels of steroids to influence behavior.

Objectives

The goal of this thesis is to examine the potential for social regulation of brain DHEA metabolism through changes in 3β-HSD activity during the non-breeding season in wild male song sparrows. Through preliminary studies (Experiment 1), we found that 3β-HSD activity is modulated by seasonally. Based on these results, I performed Experiment 2, where I investigated whether a short-term, 30 min social challenge can alter 3β-HSD activity. Objectives of Experiment 2 are to determine:

1. Regional differences in brain 3β-HSD activity during the non-breeding season.

2. Effects of short-term social challenges on brain 3β-HSD activity.

Findings from this study can serve as a framework for future experiments to look at the behavior-endocrine dynamics over the course of an aggressive challenge. It can also be applied to better understand the neuroendocrine control of behavior in species that undergo seasonal changes in gonadal hormones, or have low levels of circulating hormones throughout the year, such as tropical species (71).

Figure 1.1 Simplified diagram of sex steroid synthesis.
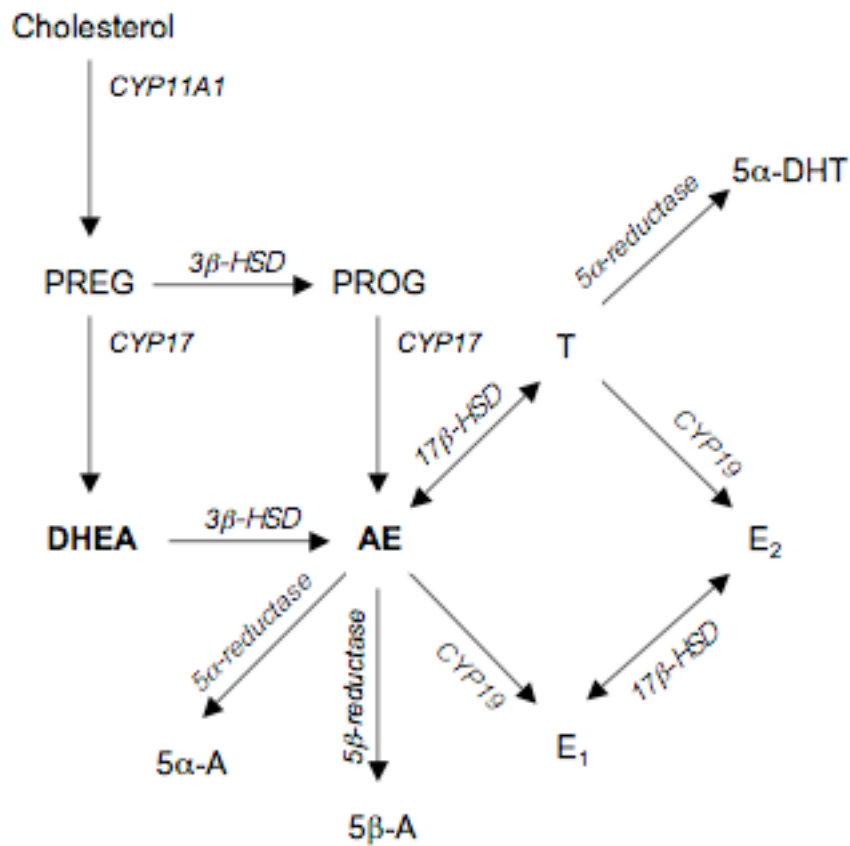
Steroids: PREG, pregnenolone; PROG, progesterone; AE, androstenedione. Enzymes: CYP11A1, cytochrome P450 side chain cleavage; CYP17, cytochrome P450 17α-hydroxylase/C17,20 lyase, 3β-HSD, 3β-hydroxysteroid dehydrogenase. The enzyme 3β-HSD metabolizes DHEA into AE. AE can then be converted to other steroids: 5α-A, 5α-androstanedione; 5β-A, 5β-androstanedione; E_1, estrone; E_2, estradiol; T, testosterone, DHT, dihydrotestosterone.

Figure 1.2   Bidirectional relationship between physiology and behavior.

Physical and Social Environment

Short-term change
in steroidogenic enzymes

Long-term change
In steroidogenic enzymes

Rapid change
in steroids

Slow change
in steroids

Non-genomic
mechanisms

Genomic
mechanisms
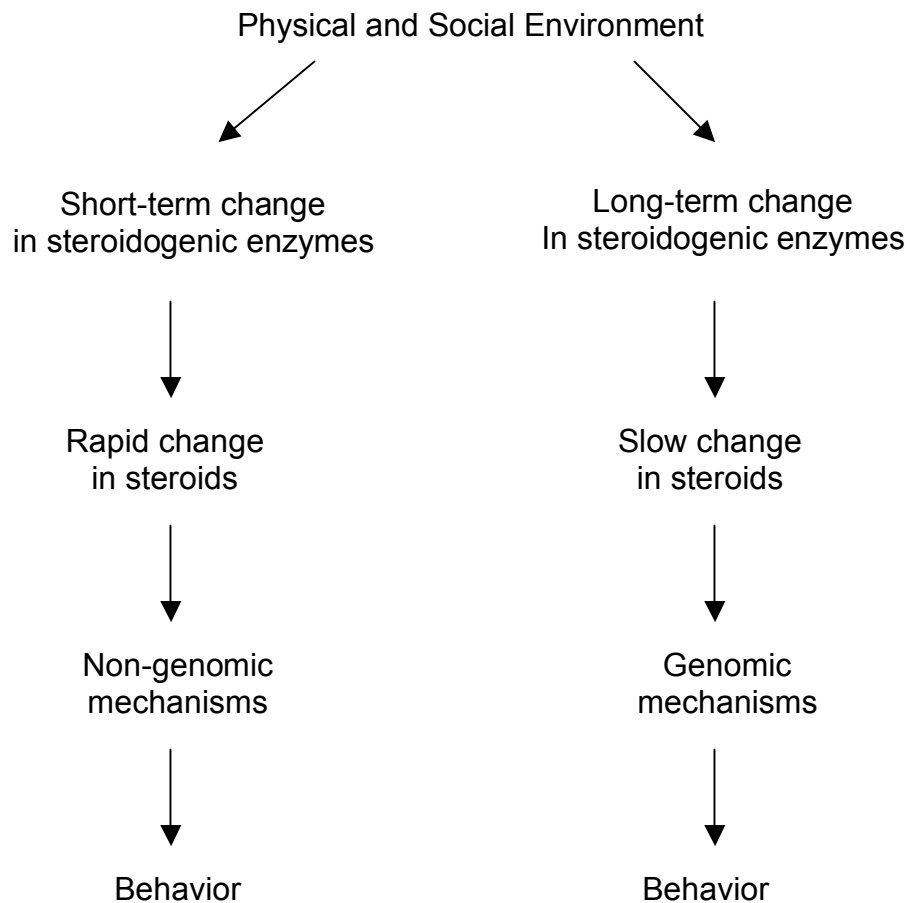
Behavior

Behavior

The physical and social environment can regulate brain steroidogenic enzymes in the long- and short-term, which results in changes in local levels of steroids via genomic or non-genomic mechanisms. The consequent change in behavior can also regulate steroidogenic enzymes. This can occur within an individual, and its behavior can regulate the behavioral and physiological response in another individual.

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SEASONAL AND SOCIAL REGULATION OF 3β-HSD ACTIVITY IN THE SONGBIRD BRAIN[1]

Introduction

Pioneering studies investigating inter-male aggression in song sparrows, <u>Melospiza</u> <u>melodia</u>, have provided support for the 'challenge hypothesis', which predicts an increase in plasma testosterone (T) levels during periods of social instability (1). The challenge hypothesis applies to many vertebrates, including humans (2). In song sparrows, plasma T level is high during the breeding season, compared to other times of the year, and transiently increases further during social challenges (1). However, the link between aggression and androgens is less clear during the non-breeding season. Song sparrows and several other species maintain high levels of territorial aggression outside the breeding season despite low levels of circulating T (3-7). One hypothesis is that local androgens within the brain regulate non-breeding aggression (8). This may be a mechanism of local, rather than systemic, steroid signaling, in order to circumvent the 'costs' of high systemic T during the non-breeding season (8-10).

Song sparrows are an excellent species to investigate the mechanisms that regulate aggressive behavior during the non-breeding season. They maintain year-round territories in the Pacific Northwest and respond similarly vigorously to simulated territorial intrusions (STI) during the breeding and non-breeding seasons (4). Intriguingly, during the non-breeding season, their gonads are completely regressed, they have low systemic T levels, and castration does not affect aggression (4). Importantly, aggressive challenges do not increase systemic androgens in the molt and non-breeding season.

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Systemic levels of the prohormone dehydroepiandrosterone (DHEA) are high in non-breeding song sparrows (11). DHEA implants during the non-breeding season increase territorial singing and the size of a song control nucleus (HVC) by almost 50% (12). DHEA has no known intracellular receptor (13, 14) and is considered an inactive neurosteroid that must be converted to active sex steroids such as T and estradiol (E_2) (15). Reducing estrogen synthesis in wintering song sparrows significantly reduces aggressive behavior and HVC size (16). These data suggest that DHEA is metabolized to active sex steroids such as E_2 locally in the brain to regulate non-breeding aggression.

Steroidogenic enzymes in the brain may be a link between the environment and behavior. The songbird brain expresses all the enzymes necessary for the downstream metabolism of DHEA to active sex steroids (17, 18). 3β-hydroxysteroid dehydrogenase/Δ4-Δ5 isomerase (3β-HSD) catalyzes the conversion of DHEA to androstenedione (AE), an active and aromatizable androgen (19), and uses NAD^+ as a cofactor. Thus conversion of brain DHEA to AE via brain 3β-HSD may be critical for regulating territorial behavior during the non-breeding season.

In Experiment 1, we studied the long-term seasonal regulation of brain 3β-HSD activity by measuring the conversion of DHEA to AE during the breeding, molt, and non-breeding seasons. In Experiment 2, we examined the short-term social regulation of brain 3β-HSD activity by social encounters during the non-breeding season.

Methods

Experiment 1: Seasonal regulation of brain 3β-HSD activity

Field Protocol. Free-living wild male song sparrows (n=18 total) were captured near Seattle, WA, with 3.58 ± 0.41 min conspecific song playback during the breeding (May 4-5), molt (Aug 30-31), and non-breeding (November 14-16) seasons of 2002. Experimental protocols complied with University of Washington Animal Care Committee.

Within 3.08 ± 0.37 min of capture, the subject was rapidly sacrificed, and trunk blood was collected. While in the field, blood was kept on wet ice, and the brain was quickly dissected according to (20) and stored on dry ice. Upon returning to the laboratory, blood was centrifuged and plasma samples were stored at -20°C, and brain tissues were stored at -80°C until assayed.

Hormone assays. Briefly, DHEA and T were extracted from plasma using dichloromethane and separated using diatomaceous earth/glycol columns and measured via radioimmunoassay as described in (11). All the samples were run in a single assay, and the intra-assay variability was 3.25% for DHEA and 7.1% for T. The antibodies used were: DHEA (Endocrine Sciences, Calabasas, CA D7-421) and T (Wien Laboratories, Saccasunna, NJ, T3003).

3β-HSD activity assay with exogenous NAD^{+}. We measured the in vitro conversion of [³H]DHEA to [³H]AE and [³H]5β-androstanedione (5β-A). The assay procedure was followed from Soma et al (21), with few modifications. First, a timecourse was performed to determine a suitable incubation time. We used whole brain homogenates pooled from 2-3 animals and compared 3β-HSD activity in the breeding and non-breeding seasons. Incubation times ranged from 60 to 300 min. For samples from both seasons, AE was the major product formed, and AE formation increased linearly from 120 to 240 min (data not

presented) and we chose 180 min as the appropriate incubation duration. Next, we measured

3β-HSD activity in different brain regions and processed a maximum of three regions at a

time. Each region was homogenized in 200 μL sucrose phosphate (SPO_4) buffer, and 180 μL

was incubated at 41°C with 200 nM [1,2,6,7-^{3}H]DHEA (specific activity = 74 Ci/mmol), 1.5

mg AE cold-trap, 20 μL NAD^+ were added. Tritiated AE was recrystalized to constant

specific activity, which confirmed identity of the metabolites. Trilostane, a 3β-HSD

inhibitor, decreased [^{3}H]AE formation by 98.5%, while fadrozole, an aromatase inhibitor,

specifically and completely abolished [^{3}H]estrone (E_1) + [^{3}H]E_2 formation.

Experiment 2: Social regulation of brain 3β-HSD activity

Field Protocol. Free-living song sparrows (n=18 total) were studied during the non-breeding

season, November 23 – December 8, 2005 near Vancouver, BC (49° 12'N, 123° 01'W).

Experimental protocols complied with University of British Columbia Animal Care and the

Canadian Council for Animal Care permits.

Individual territories of resident song sparrows were mapped at least 2 days prior to

the study. Prior to each trial, a mist net was installed, furled and camouflaged near the

ground. The resident was then exposed to a simulated territorial intrusion (STI) for 30 min.

The STI consisted of a live caged conspecific decoy and conspecific song playback placed in

the approximate center of the territory. Control animals were exposed to similar conditions;

however, the cage was empty and no playback was used. Territorial singing and defense

displays made by the resident bird were recorded for 30 min. Immediately after, the mist net

was unfurled, and the resident was captured using conspecific playback. STI stimulated

animals were captured within 1.64 ± 0.53 min and controls were captured within 1.36 ± 0.6

min (t=0.332, p=0.744). Blood samples were then taken from the brachial and jugular veins

(results to be presented separately). Control subjects were sacrificed 4.19 ± 0.24 min after capture and STI subjects were sacrificed 4.13 ± 0.27 min after capture (t=0.184, p=0.857).

In the field, the brain was rapidly dissected into left and right regions and immediately frozen on dry ice. All the dissection procedures were same as (20). The following tissues were collected 1) rostral, medial central, lateral central, caudal, dorsal (contains HVC), and ventromedial telencephalon (contains taenial amygdala). 2) caudomedial neostriatum (NCM) (\ song perception); 3) hippocampus (learning and memory); 4) rostral and caudal diencephalon (motivated behaviors); 5) cerebellum. Cerebellum and hippocampus served as control regions. Tissue was stored at -80°C until assayed.

Experiment 2A: 3β-HSD activity assay with exogenous NAD$^+$. We determined 3β-HSD activity by measuring the in vitro conversion of [^{3}H]DHEA to [^{3}H]AE, and added NAD$^+$ exogenously as a cofactor. This facilitated the production and accumulation of [^{3}H]AE. In these experiments, we were also able to measure low quantities of [^{3}H]5β-A. All the samples were processed as in (21, 22). Briefly, first, using pooled samples, we performed a time course where we compared 3β-HSD activity in brain homogenates and supernatants (homogenates centrifuged at 1000 x g at 4°C for 30 min) of non-breeding animals, using incubation times from 10 to 180 min (data not shown). Activity in supernatants was 6x the homogenates and was non-linear by 10 min. Hence we chose supernatants for the subsequent assays and examined earlier time-points in the next assay. We measured 3β-HSD activity from 2.5 to 60 min; [^{3}H]AE increased linearly from 2.5 to 15 min (Fig. 2.1A) and hence we chose 5 min as the appropriate duration for reactions with NAD+.

Brain tissue from either the right or left side (randomized) of each region was assayed for 3β-HSD activity. Brain tissue was homogenized on ice, (2 second bursts/pulses, 3 to 6 times) in 300 μL ice-cold sugar-phosphate buffer, and centrifuged at 1000 g at 4°C for 30 min. 180 μL of the supernatant was incubated with 200 nM [1,2,6,7-^{3}H]DHEA (specific activity 74.5 Ci/mmol) and 20 μL NAD$^+$ (final concentration, 1 mM) at 41°C, with shaking (speed 90 RPM). After 5 min, the reaction was terminated by snap-freezing in methanol/dry ice. Samples were then wrapped in parafilm and stored at -20°C. To determine procedural losses, tubes containing a known amount of repurified [1,2,6,7-^{3}H]AE were processed in parallel. Control tubes contained everything but tissue. Steroids were extracted by adding 3 mL diethyl ether to each sample and each sample was vortexed for 30 sec and centrifuged at 2100 RPM, at 4°C for 5 min. After snap-freezing in methanol:dry ice bath, the supernatant was decanted into a separate tube. This procedure was repeated two times, after which the samples were dried down under nitrogen. The dried residues obtained after ether extraction were resuspended in dichloromethane:methanol (1:1), and radioinert DHEA, AE, and 5β-A were added as markers. Samples were spotted onto silica gel plates, and run in chloroform:ethyl acetate (4:1) for 18 min (2 times). Steroids were visualized under UV light after spraying with primulin. The appropriate bands were scraped from the plates, tritiated steroids were eluted from the silica with 900 μL methanol:water (8:1), and 200 μL aliquots were counted in a scintillation counter (BeckmanCoulter LS 6500). The dpm were corrected for background values and recovery (average = 80%), and all data are presented as fmole per mg protein per min. Lastly, to confirm the identity of products formed, the eluates from TLC were injected through HPLC and the retention time for [^{3}H]AE was noted (Fig. 2.2).

Experiment 2B: 3β-HSD activity assay without exogenous NAD⁺. In some telencephalic

regions, we did not add exogenous NAD^+. In these preparations, the forward reaction of

[³H]DHEA conversion to [³H]AE by 3β-HSD was driven by endogenous NAD^+. This assay

allowed us to measure the production of [³H]AE, and also allowed the conversion of the

formed [³H]AE to [³H]5α-A, [³H]5β-A, [³H]E$_1$, and [³H]E$_2$. Thus we were also able to

assess activities of 5α-reductase, 5β-reductase, and aromatase. In a timecourse, [³H]AE

increased linearly from 45 to 120 min of incubation and we chose 90 min as the appropriate

duration (Fig. 2.1B).

Tissues from the rostral and caudal telencephalon were homogenized in 300 μL

buffer, and medial central telencephalon was homogenized in 500 μL buffer and centrifuged.

The medial central telencephalon was split between the two assays, with and without NAD^+.

200 μL of the supernatant from each region was then incubated with 200 nM [³H]DHEA for

90 min with shaking, after which the reaction was terminated.

Following the extraction using diethyl ether (2 times), androgens and estrogens were

separated first using phenolic partitioning as described in (23). One microliter of CCl$_4$ and 1

μL NaOH (1N) were added to the dried extracts. Samples were then centrifuged at 2100

RPM, 4°C for 5 min, with no brake. Following this, 700 μL of the estrogen containing

NaOH top layer was collected and transferred to a new tube. An equal volume of CCl$_4$ was

added to the new tube, and centrifuged as above, before 400 μL of NaOH layer was collected

and transferred to a new tube. An equal volume of dH$_2$O and 2 mL ethyl acetate was added

to the NaOH. Samples were vortexed for 20 sec and the NaOH was transferred to a new tube

with a Pasteur pipette. This procedure was repeated twice more to ensure the complete

transfer of estrogens from NaOH to ethyl acetate. The ethyl acetate tubes corresponding to

the sample sample were combined and dried down. One milliliter dH$_2$O was added to the

tubes containing the remaining androgens and centrifuged as above. One and a half milliliter of the CCl_4 containing the androgen layer was extracted. All of these procedures were conducted at 4°C and the samples were then dried down with nitrogen at 40°C. Both estrogens and androgens were then resuspended in dichloromethane:methanol (1:1) and radioinert steroids of interest. Resulting samples were spotted onto TLC plates for separation. Estrogens were run twice for 23 min in 3:1 ether:hexane tanks, and plates were then kept under iodine fumes for visualizing $[^3H]E_1$ and $[^3H]E_2$, which were then scraped off the plates. Androgens were run twice for 18 min in 4:1 chroloroform:ethyl acetate tanks. $[^3H]AE$ was the major androgen metabolite, $[^3H]5\beta$-A was the minor metabolite, and $[^3H]5\alpha$-A was non-detectable. Tritiated silica was then resuspended with 800 μL methanol and 100 μL dH_2O, vortexed for 30 sec and centrifuged at 2800 RPM, 4°C for 10 min. Four hundred microlitres of the supernatant was counted in 5 mL scintillation fluid for 2 min per sample. Recoveries for estrogens were 14 % for estrogens and 85 % for androgens.

Statistics. All data are expressed as mean ± SEM. Data were transformed where appropriate, and analyzed using SPSS 11.0 or Prism 4.0 for Mac OS X. For Experiment 1, we first performed the one-way analysis of variance (ANOVA) to compare seasonal changes in plasma hormones and body condition, followed by Fisher's protected least significant difference (LSD) test as a post hoc. For each brain region, differences among the three seasons were analyzed using one-way ANOVA. When the ANOVA was significant, we performed a Fisher's LSD test to determine differences between the seasons. For Experiment 2, to investigate the differences in 3β-HSD activity between control and STI stimulated samples, each brain region was analyzed using separate t tests. For samples without exogenously added NAD^+, we performed correlation analysis between aggressive behavior

and 3β-HSD activity. Errors for percentage increase in 3β-HSD activity were calculated

according to (24). Values of $p < 0.05$ (two-tailed) were considered significant.

Results

<u>Experiment 1: Seasonal regulation of brain 3β-HSD activity</u>

In this experiment, we investigated the seasonal regulation of brain 3β-HSD activity in a

seasonally breeding songbird. Male song sparrows were captured during the breeding, molt,

and non-breeding seasons (n=18 total). First, we examined the plasma levels of DHEA and

T in each season (Table 2.1). Overall, there were significant effects of season on plasma

DHEA and T (DHEA: $F_{2, 18}$=3.205, p=0.048; T: $F_{2, 18}$=24.063, p<0.0001). Plasma DHEA

levels were highest in the breeding season, reduced during molt, and increased again during

the non-breeding season. Plasma T levels were highest in the breeding season and basal

during the molt and non-breeding season. Testis volume and the androgen-dependent cloacal

protuberance were regressed outside of the breeding season (Table 2.1).

Next, we measured 3β-HSD activity in brains collected in different seasons by

quantifying the conversion of [^{3}H]DHEA to [^{3}H]AE and [^{3}H]5β-A in brain homogenates

(Fig. 2.3). 3β-HSD activity changed seasonally in the central medial telencephalon ($F_{2, 14}$=6.098, p=0.012), being lowest in the breeding season compared to molt (Fisher LSD,

p=0.008) and non-breeding season (p=0.012). 3β-HSD activity also significantly differed

among the three seasons in the caudal telencephalon ($F_{2, 15}$=3.841 p=0.045), and was higher

in the non-breeding season compared to molt (Fisher LSD, p=0.033) and breeding season

(Fisher LSD, p=0.027).   Seasonal changes in 3β-HSD activity were also observed in the

ventromedial telencephalon ($F_{2, 15}$=3.857, p=0.045) and caudal diencephalon ($F_{2, 15}$= 4.266,

p=0.034), and was highest in the non-breeding season for both (p<0.05). In the rostral

diencephalon, no significant change in 3β-HSD activity was observed ($F_{2, 14}$=1.037, p=0.380). Taken together, these data suggest that brain 3β-HSD activity is preferentially upregulated during the non-breeding season.

Experiment 2: Social Regulation of brain 3β-HSD activity

The results from Experiment 1 raised the hypothesis that high DHEA metabolism during the non-breeding season may be a mechanism to locally elevate androgen levels in the brain to mediate aggression. We further examined this hypothesis by testing the prediction that aggressive interactions would increase 3β-HSD activity. During the non-breeding season of 2005, we exposed free-living male song sparrows to a simulated territorial intrusion (STI) for 30 min. Stimulated animals responded strongly, by singing, wing-waving, and flying close to the decoy (25). The response latency of STI stimulated birds was 1.23 ± 0.47 min and the song latency was 2.78 ± 2.37 min, indicating the rapid effect of STI on territorial behavior.

Experiment 2A: 3β-HSD activity assay with exogenous NAD$^+$

Similar to Pradhan et al (22), we measured 3β-HSD activity in supernatants prepared from brain homogenates, because 3β-HSD activity in supernatants was 6x higher than in homogenates (see methods). In Experiment 2A, we added 1 mM NAD$^+$ exogenously, which maximized [^{3}H]AE formation and inhibited [^{3}H]AE conversion by aromatase (21). Under these in vitro assay conditions, there was no significant effect of STI on 3β-HSD activity in any brain region, except for the cerebellum, in which STI decreased 3β-HSD activity (Table 2.2). Note that 3β-HSD activity is ~8x higher in the rostral diencephalon than caudal diencephalon. Similar to previous studies, 5β-A was a minor product formed (21, 22). To assess 5β-reductase activity, we expressed [^{3}H]5β-A as a percentage of total 3β-HSD

metabolites ($[^3H]AE + [^3H]5\beta$-A) (Table 2.1). STI significantly increased 5β-reductase activity in the dorsal telencephalon (p=0.036) and significantly decreased 5β-reductase activity in the rostral diencephalon (p=0.022).

Experiment 2B: 3β-HSD activity assay without exogenous NAD$^+$

Next, we focused on telencephalic regions and did not add NAD$^+$ exogenously. These assay conditions permitted us to measure the activities of other steroidogenic enzymes that convert AE, such as aromatase, 5α- and 5β-reductase (21). In the central medial telencephalon, STI significantly increased 3β-HSD activity 615 ± 118 % (t_{15}=2.998, p=0.009; Fig. 2.4). Similarly, in the caudal telencephalon, STI significantly increased 3β-HSD activity 355 ± 18 % (t_{13}=3.721, p=0.003; Fig. 2.2). Note that both these regions have androgen and estrogen receptors (26) and also contain parts of the limbic system (central medial telencephalon contains the septum). There are also neuronal tracks that connect to the diencephalon from these regions. In contrast, there was no effect of STI on 3β-HSD activity in the rostral telencephalon, which has not been implicated in the control of aggression (control = 0.97 ± 0.72 fmole/mg protein/min; STI = 0.88 ± 0.34 fmole/mg protein/min; t_{16}=0.119, p=0.907).

Further, 3β-HSD activity was positively correlated with the time the subject spent within 1 m of the decoy (central medial telencephalon: Pearson correlation, r=0.56, p=0.0204; caudal telencephalon, Pearson correlation, r=0.73, p= 0.0021). More time spent in close proximity to the intruder reflects a more aggressive response (27).

Lastly, we expressed each downstream product of $^3[H]$DHEA as a percentage of total metabolites, to calculate the indices of 5β-reductase, 5α-reductase, and aromatase activities (21, 22). Percentage of $^3[H]$AE was higher in STI stimulated subjects in the central medial

telencephalon (p=0.038) and the caudal telencephalon (p=0.043) (Table 2.3). We expressed ^{3}[H]E$_1$ + ^{3}[H]E$_2$ as a percentage of total ^{3}[H]DHEA products to evaluate aromatase activity. Surprisingly, aromatase activity significantly decreased in the central medial telencephalon (p=0.018) and caudal telencephalon (p=0.008). Similarly, we expressed ^{3}[H]5β-A as a percentage of total ^{3}[H]DHEA metabolites to evaluate 5β-reductase activity. There was no effect of STI on 5β-reductase activity on any region and no 5α-A was produced in any region, indicating very low 5α-reductase activity. Taken together, these results indicate that during the STI, there was a net increase in active androgens.

Discussion

Taken together, the results from this study suggest a mechanism for regulation of aggression during the non-breeding in free-living song sparrows, when plasma T levels are low. First, wintering song sparrows have higher systemic DHEA compared to T. Second, elevated brain 3β-HSD activity during the non-breeding season suggests increased conversion of DHEA to active sex steroids. Third, 3β-HSD activity is rapidly (within 30 min) regulated by social challenges during the non-breeding season, consistent with non-genomic control mechanisms (28), and this was specific to behaviorally relevant forebrain regions. The conversion of DHEA to active androgens in local regions of the brain appears critical during the non-breeding season, indicating a shift from systemic to local signaling during the winter (8). Lastly, we also show that caution should be exercised in interpreting data from studies in which saturating concentrations of cofactor are exogenously added to maximize product formation.

Seasonal regulation of brain androgen synthesis

Compared to the breeding season and molt, 3β-HSD activity was highest during the non-breeding season in several behaviorally relevant brain regions (Fig 2.3). 3β-HSD activity is a critical first step in converting the prohormone DHEA to active androgens and estrogens (29). In song sparrows, aromatase activity also changes seasonally (30). A combination of elevated 3β-HSD and aromatase activity can increase E_2 levels locally, since E_2 regulates aggression during the non-breeding season (16).

There are seasonal changes in steroidogenic enzyme activities in other species as well. For example, in Lapland longspurs, brain aromatase activity changes across the breeding season (32). Interestingly, these changes are most pronounced in the caudal telencephalon, suggesting that this region is particularly sensitive to seasonal and behavioral regulation (32) (see Fig. 2.4). In male European starlings, four T converting enzymes (aromatase, 17β-hydroxysteroid dehydrogenase, 5α-reductase and 5β-reductase) also show seasonal changes in activity in the diencephalon and telencephalon (33). Additionally, studies in goldfish have revealed that there is a dramatic increase in aromatase activity in the anterior hypothalamus/preoptic area during gonadal maturation and the spawning season (34).

Other hormones, such as the pineal hormone melatonin, that undergo seasonal changes may also affect non-breeding aggression. For example, chronic melatonin treatment (mimicking short-days) increases aggression in Siberian hamsters (31). Our preliminary evidence suggests that long-term (10 days) melatonin treatment to song sparrows increases 3β-HSD activity, perhaps via gene transcription (K. Soma, unpublished).

Social regulation of brain androgen synthesis

Rapid, non-genomic mechanisms of steroid action in the brain have been implicated in several recent studies. Here, we stimulated wild songbirds to a STI for 30 min and measured 3β-HSD activity in brain regions that contain parts of the social behavior network and regulate motivated behaviors such as aggression and reproduction (35, 36). In STI stimulated subjects, 3β-HSD activity rapidly increased ~615% in the medial central telencephalon and ~355% in the caudal telencephalon. These rapid changes in 3β-HSD activity are much greater than previously reported rapid changes in aromatase activity (37-39). Rapid increases in aromatase activity lead to increases in E_2; an increase in 3β-HSD activity early in the steroidogenic pathway may be a mechanism to rapidly and transiently increase E_2 at important synapses (38), which would then regulate behavior. Relevant to our present findings, studies have shown that E_2 rapidly increases aggression in mice housed in short days, but not long days (40).

However, contrary to our expectation that aromatase activity will increase as a result of aggressive encounters, surprisingly, our results show that the opposite is true (Table 2.3). The data indicate that 30 min of STI causes a decrease in estrogens and a build-up of AE, which may signal via androgen receptors to increase aggression. One explanation is that, social interactions are dynamic, and so is the steroid environment within the brain as a result of steroidogenic enzyme activity. Over the 30 min of stimulation, the intensity of aggressive behavior can vary over time, depending upon the perception of threat. Once the intruder leaves the territory, and the threat subsides, it might be useful to rapidly terminate active steroids and upregulate inactivating enzymes.

Rapid release and re-uptake of steroids at specific synapses could be mechanisms to transiently increase local steroid levels (28). This hypothesis could be tested by measuring 3β-HSD and aromatase activities in shorter time intervals, and also post-STI. All these

studies could also be compared with parallel studies in the breeding season. Previous studies have found that after the removal of the decoy, wintering song sparrows are less persistent during the post-STI period compared to the breeding season. When treated with T, non-breeding song sparrows maintain aggression post-STI (4). Thus, we predict that in the spring, post-STI aggressive behavior will be coupled with increased 3β-HSD activity, while in the winter, decreased aggressive behavior post-STI will be coupled with decreased 3β-HSD activity.

Possible mechanisms for short-term regulation of brain 3β-HSD

There are several possible mechanisms by which 3β-HSD activity may be modulated rapidly. In this study, we found that 3β-HSD was regulated only when no exogenous NAD^+ was added. This suggests that 3β-HSD may be regulated via bioavailability of endogenous NAD^+, and this hypothesis must be tested in future studies. In most in vitro enzyme activity assays, cofactors are exogenously added to maximize the particular reaction. In vivo, cells maintain ~ 1 mM NAD^+ concentration compared to ~1 nM steroid concentrations (41). Cellular redox states are maintained by NAD^+/NADH concentration gradients, which in turn determine the direction of the steroid conversion (41). Thus cofactors may play a critical role in the rapid regulation of 3β-HSD activity. However, regulation of intermediate steps of metabolism and regeneration of cofactors are poorly understood and in need of further investigation (42).

In this experiment, centrifugation of homogenized brain tissue at 1000 g generated supernatants enriched with mitochondria, synaptosomes, and microsomes (43). Since mitochondria recycle NAD^+, and contain NAD^+, we may have measured 3β-HSD activity specifically in these organelles. Previous studies have reported that 3β-HSD activity is low

or non-detectable in microsomal fractions without exogenous NAD^+ (44, 45) (L. Lau, D. Pradhan, K. Soma, unpublished results). Addition of exogenous NAD^+ however, maximized mitochondrial and microsomal 3β-HSD activity, causing a ceiling effect, which may have diluted the effects of regulation of mitochondrial 3β-HSD activity. Since synaptosomes also contain high levels of aromatase (46, 47), this assay provides evidence for the potential for rapid regulation of androgen synthesis at a very local level.

3β-HSD may also be regulated by mechanisms similar to aromatase. Like aromatase, 3β-HSD amino acid sequence contains several possible phosphorylation sites (T. Charlier and K. Soma, unpublished), and studies from quail indicate that rapid changes in aromatase occur via Ca^{2+} mediated phosphorylation by protein kinases (38). In addition, aromatase can be rapidly (within 5 min) modulated by specific glutamate receptor agonists (kainate and AMPA) (48). Thus, the social environment could rapidly modulate endogenous neurotransmitters such as dopamine, GABA, and various neuropeptides, increasing Ca^{2+} mediated phosphorylation of 3β-HSD via kinases.

<u>Conclusions</u>

Together, our findings suggest that 3β-HSD activity is high during the non-breeding season in male song sparrows, and is increased even further by short-term conspecific social challenges. Local androgen levels in the brain may be under an opposite regulatory pattern compared to systemic androgen levels (Fig. 2.5). We propose that the increased baseline activity of brain 3β-HSD might be a mechanism to prime the steroidogenic response, so that local levels of androgens can be rapidly increased during periods of social instability.

Owing to the numerous 'costs' of high levels of systemic T (49), there is considerable evidence that alternate mechanisms are necessary to regulate aggressive behavior in several vertebrates. Regulation may involve rapid physiological changes localized within the brain

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itself, and include multiple molecular and biochemical machinery such as cofactors, receptors, substrates, and enzymes expression and activity. Moreover, both long- and short-term endocrine control mechanisms are important, depending on behavioral ecology, life history strategies, and seasonal cues. These factors would collectively ensure the necessary hormonal feedback for maintaining or reducing aggressive behavior locally, without involving peripheral androgen levels.

Table 2.1   Seasonal changes in plasma steroids in free-living adult male song sparrows.

	Breeding	Molt	Non-breeding	F	p
Plasma T (ng/ml)	4.82 ± 1.64^a	0.28 ± 0.06^b	0.20 ± 0.03^b	24.063	<0.0001
Plasma DHEA (ng/ml)	0.48 ± 0.06^a	0.31 ± 0.02^b	0.42 ± 0.04^{ab}	3.205	0.048
Testis volume (mm^3)	2913.31 ± 350.48^a	73.28 ± 58.58^b	6.91 ± 0.63^c	65.403	<0.0001
Cloacal protuberance (mm)	7.48 ± 0.29^a	5.07 ± 0.37^b	3.66 ± 0.22^c	40.643	<0.0001

Note: Within rows, values with different superscripts differ significantly from each other. n=6 per group. Testosterone (T), plasma dehydroepiandrosterone (DHEA), testes volume, and cloacal protuberance length.

Table 2.2   Effect of simulated territorial intrusions on brain 3β-HSD activity in free-living non-breeding song sparrows.

|	Total metabolites fmole AE + 5β-A / mg protein / min			5β-A / Total %		
	Control	STI	p	Control	STI	p
Rostral telencephalon	156.66 ± 30.63 (9)	127.35 ± 18.52 (9)	0.235	1.06 ± 0.69	1.78 ± 1.38	0.649
Central medial telencephalon	107.61 ± 27.86 (9)	119.84 ± 23.52 (9)	0.757	2.53 ± 0.98	3.38 ± 1.58	0.656
Central lateral telencephalon	20.14 ± 2.48 (9)	31.49 ± 8.00 (9)	0.101	7.96 ± 4.18	6.29 ± 1.56	0.713
Caudal telencephalon	44.53 ± 7.02 (7)	65.66 ± 7.23 (7)	0.519	0.33 ± 0.31	1.30 ± 0.36	0.067
Dorsal telencephalon	19.71 ± 5.96 (9)	21.6 ± 5.43 (8)	0.232	**20.48 ± 8.65**	**53.14 ± 11.49**	**0.036***
Ventromedial Telencephalon	42.04 ± 11.55 (9)	37.37 ± 21.91 (9)	0.188	32.00 ± 11.30	10.27 ± 6.8	0.158
NCM	70.80 ± 11.35 (7)	60.03 ± 12.88 (9)	0.606	2.31 ± 2.00	5.19 ± 2.3	0.377
Hippocampus	22.58 ± 5.81 (8)	11.22 ± 4.94 (9)	0.082	54.18 ± 15.51	37.18 ± 12.25	0.398
Rostral diencephalon	1175.19 ± 537.20 (9)	600.75 ± 2226.25 (9)	0.339	**1.30 ± 0.51**	**0.18 ± 0.07**	**0.022***
Caudal diencephalon	126.52 ± 27.01 (8)	184.42 ± 75.57 (8)	0.483	3.34 ± 1.78	1.46 ± 0.82	0.351
Cerebellum	**164.92 ± 45.42** (9)	**70.65 ± 16.66** (9)	**0.002****	0.67 ± 0.37	11.74 ± 11.04	0.331

Note: [3H]DHEA was added as a substrate and NAD$^+$ was exogenously added as a cofactor. The sum of the metabolites is an index of 3β-HSD activity and 5β-A % is an index of 5β-reductase activity. Numbers in parenthesis denote sample sizes. Values in **boldface** indicate a significant difference between groups. *p<0.05, **p<0.01.

Table 2.3   Effect of simulated territorial intrusions on [^{3}H]DHEA conversion to [^{3}H]AE and [^{3}H]AE metabolites in the brain in the absence of exogenously added NAD$^+$.

|	Control	STI	p
Rostral telencephalon			
AE/Total (%)	16.6 ± 10.98	62.84 ± 15.88	0.077
5β-A/Total (%)	12.05 ± 10.93	6.45 ± 3.48	0.889
E$_1$+E$_2$/Total (%)	60.23 ± 15.93	30.71 ± 15.22	0.189
Central medial telencephalon			
AE/Total (%)	48.59 ± 15.56	89.23 ± 8.55	**0.038***
5β-A/Total (%)	14.16 ± 8.45	0.68 ± 0.68	0.108
E$_1$+E$_2$/Total (%)	37.24 ± 13.12	10.08 ± 8.62	**0.018***
Caudal telencephalon			
AE/Total (%)	92.76 ± 1.45	96.11 ± 0.56	**0.043***
5β-A/Total (%)	1.35 ± 0.76	2.32 ± 0.54	0.130
E$_1$+E$_2$/Total (%)	5.90 ± 1.82	1.37 ± 0.40	**0.008****

Note: Formed [^{3}H]AE was converted to 5β-A, E$_1$ and E$_2$. No 5α-A was produced. p values are from *t* tests. **Boldface** indidcates p ≤ 0.05.

Figure 2.1 Timecourse of 3β-HSD activity with [³H]DHEA as a substrate in non-breeding adult male song sparrow brain.

A.

B.
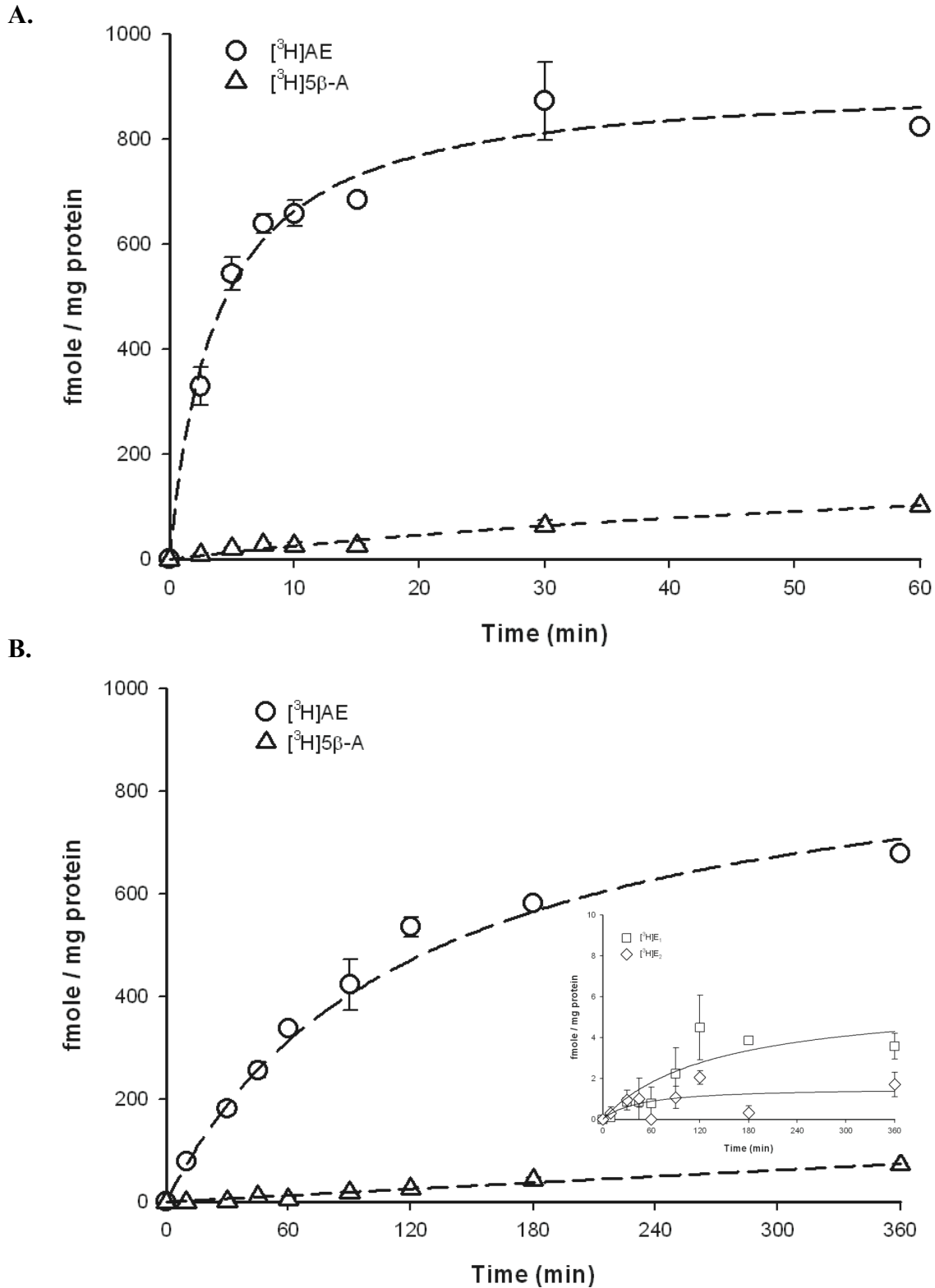
(A) Androgens measured with the addition of 1 mM NAD$^+$, from 2.5 to 60 min, n = 3 replicates per timepoint. (B) Androgens measured without addition of NAD$^+$, from 10 to 360 min. n = 2 replicates per timepoint. (Inset) Estrogens measured without addition of NAD$^+$.

**Figure 2.2   Representative HPLC chromatograph illustrating the peak and retention times of [³H]AE.**
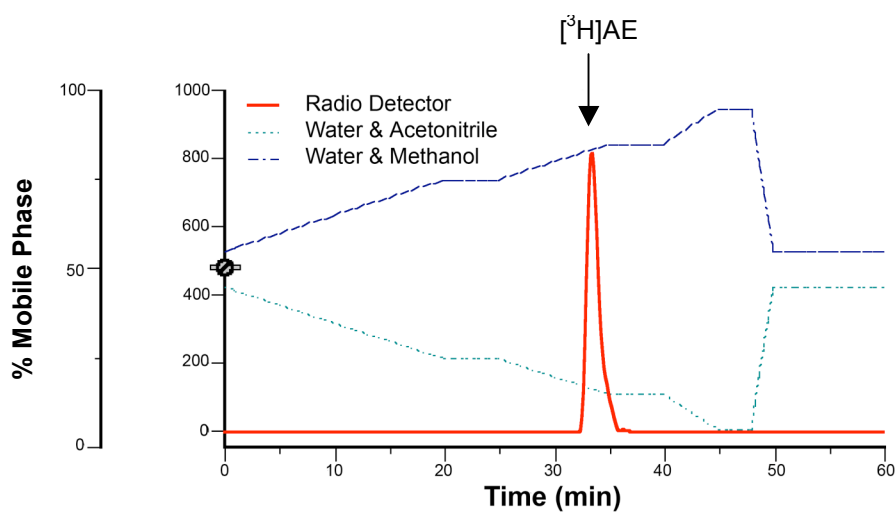
Non-breeding male song sparrow brain supernatants were incubated with 200 nM [³H]DHEA for 5 min. Steroids were separated using TLC and dried eluates were resuspended with methanol and injected through HPLC.

**Figure 2.3 Seasonal profiles of baseline 3β-HSD activity in wild male song sparrows.**
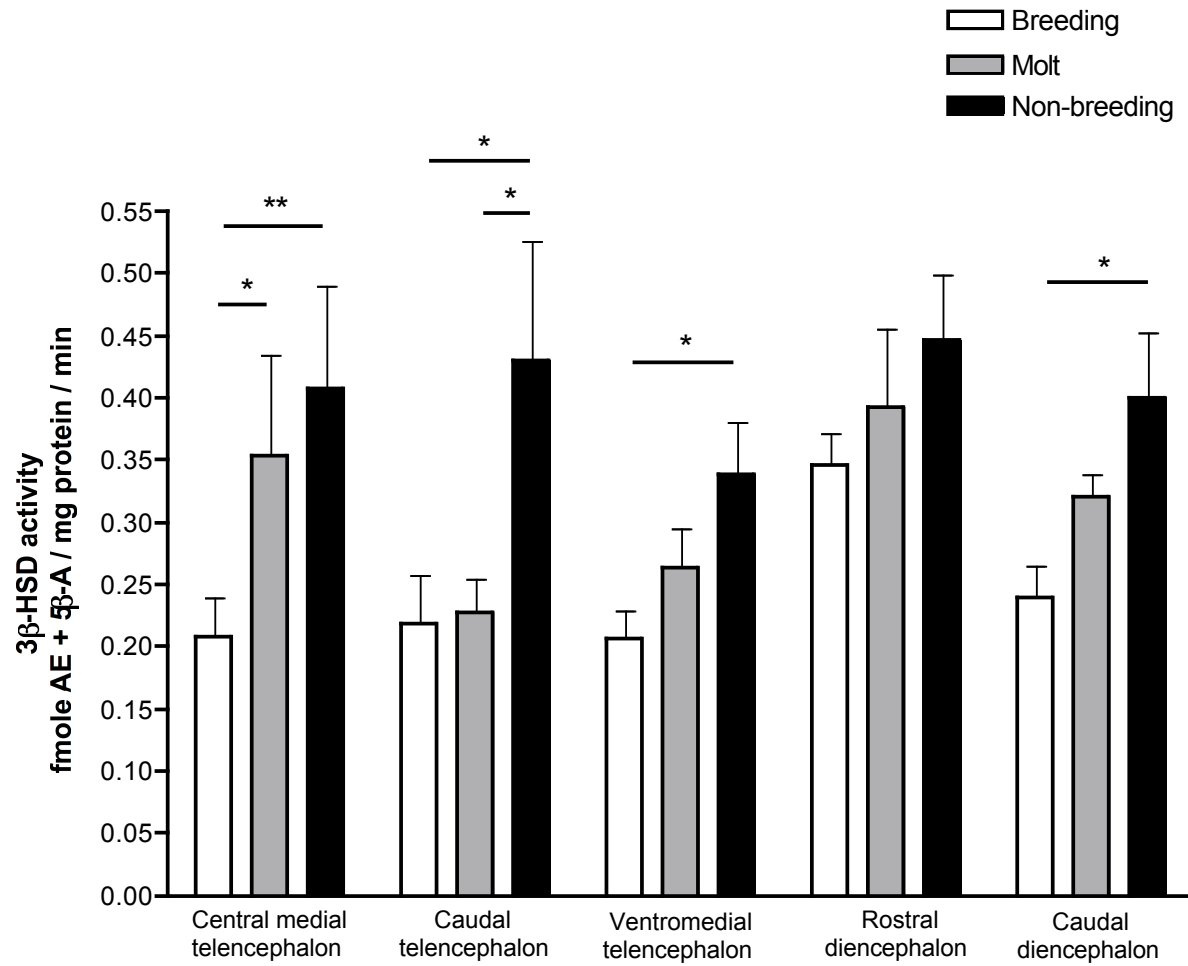
[³H]DHEA was converted to [³H]AE and [³H]5β-A. 3β-HSD activity was significantly higher in several regions (n=6). *p<0.05, **p<0.01.

Figure 2.4 Effect of simulated territorial intrusions on brain 3β-HSD activity in free-living non-breeding song sparrows.
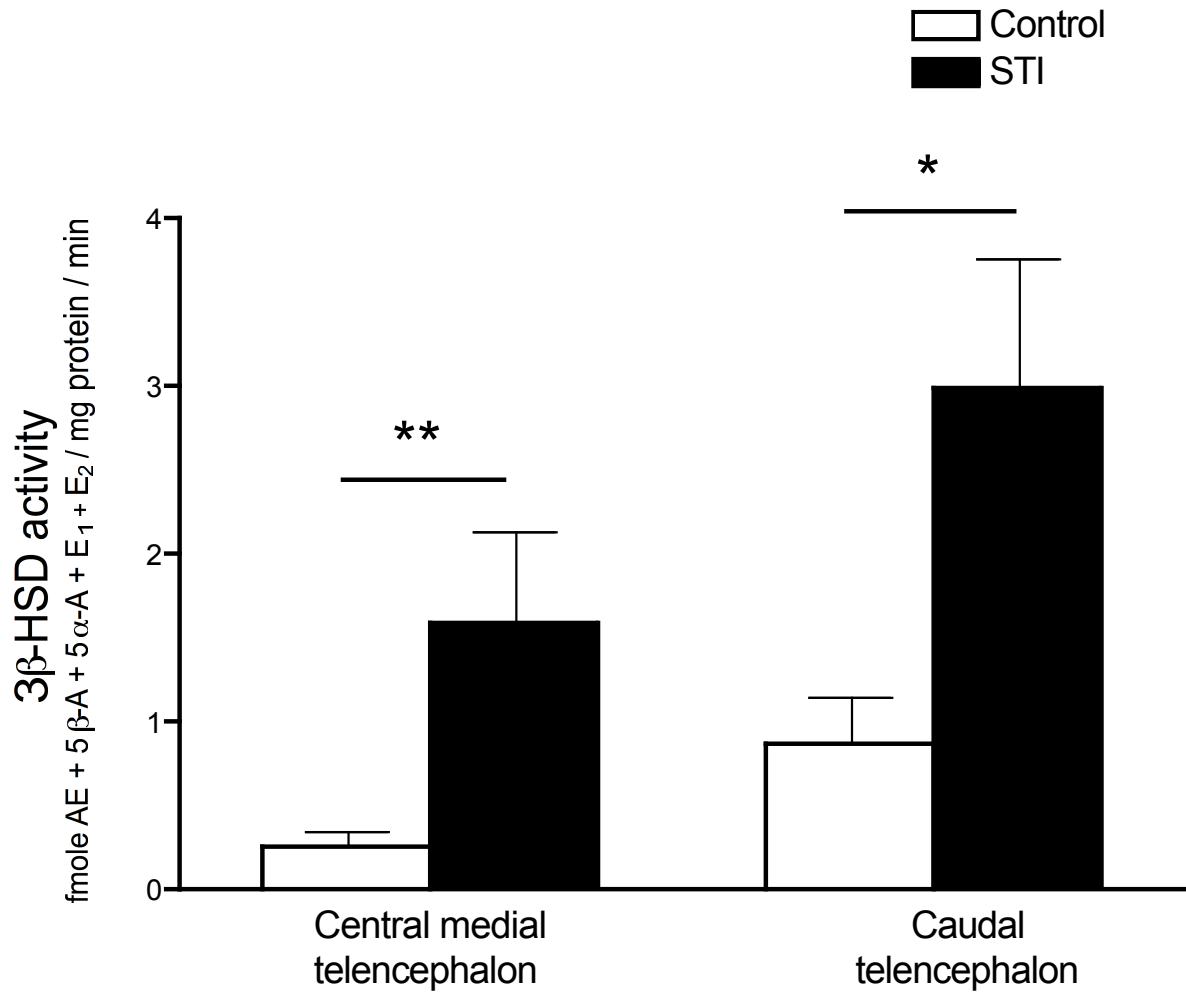
[^{3}H]DHEA was added as a substrate and no NAD$^+$ was added exogenously. 3β-HSD activity rapidly increased in the central medial telencephalon (n=8 control, n=8 STI) and caudal telencephalon (n=7 control, n=9 STI). *p<0.05, ** p<0.01

Figure 2.5   Hypothetical levels of systemic and local androgens in seasonally breeding male songbirds.
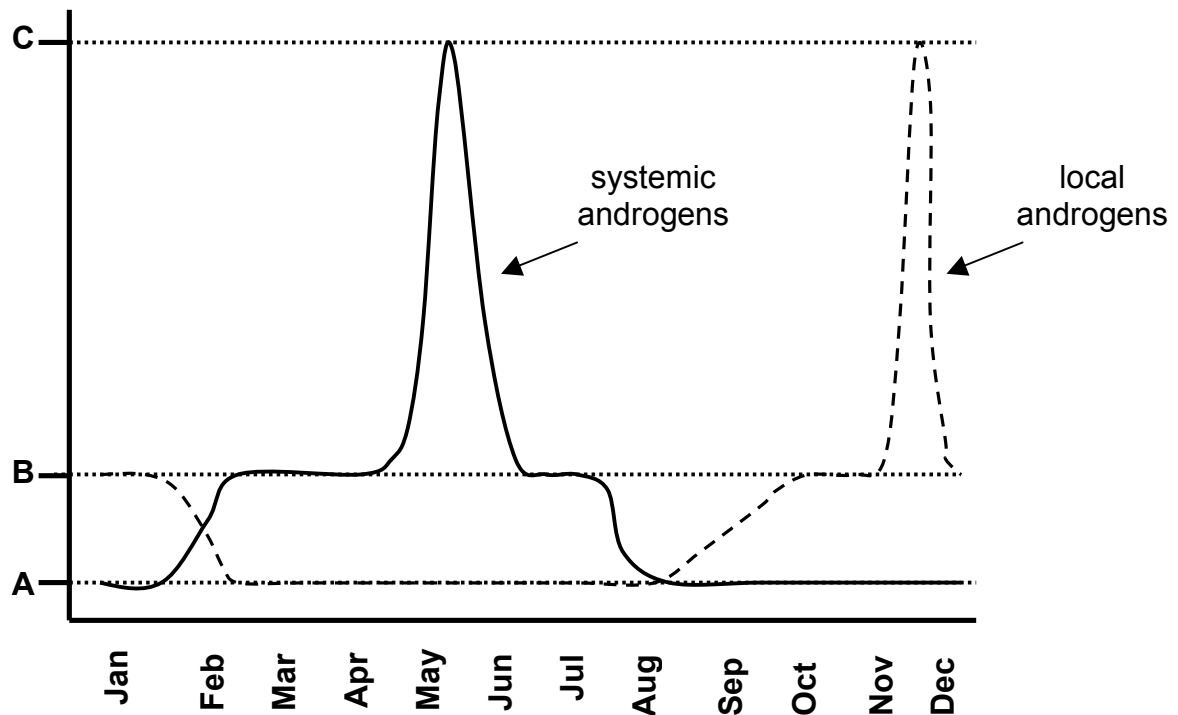
Patterns of systemic (solid line) and local (dashed line) levels of androgens in seasonally breeding male songbirds. The portion between levels A and B represents the approximate region for baseline androgen levels in the brain throughout the year. Level A, the non-breeding baseline of systemic androgens; Level B, the breeding baseline of systemic androgens; Level C, transient increases in systemic androgens during the breeding season, and transient increases in local androgens during the non-breeding season, in response to social challenge. The duration of increases can be highly variable, depending upon environmental and physiological factors (1). Dotted lines for A, B, and C represent approximate, rather than absolute levels.

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CONCLUSIONS AND GENERAL DISCUSSION

The goal of this thesis was to study the potential regulation of steroidogenic enzymes in the brain using an integrative approach. In the field, we manipulated the behavior of a songbird in its natural environment and collected tissue for neurochemistry analyses in the laboratory. The data from Experiment 1 provide an example of long-term regulation of plasma DHEA and T levels, as well as steroidogenic enzymes in different regions of the brain as a function of season. In Experiment 2, I studied animals from the non-breeding season only, to examine the short-term regulation of steroidogenic enzymes by male-male conspecific interactions. Previous studies had ruled out the role of sex steroids in regulating non-breeding aggression, based on their low levels in systemic circulation (1). By measuring activity of steroidogenic enzymes in the brain, I challenged the accepted notion. These results suggest a seasonal shift in the physiological mechanisms regulating behavior, from being primarily systemic in the breeding season, to primarily local during the non-breeding season (2).

In summary, regulation of brain 3β-HSD activity by social encounters is rapid and region-specific. As reported in previous studies, these data indicate that the endocrine environment in the brain is dynamic and probably involve multiple regulatory mechanisms (3). The conclusions about the neuroendocrine regulation of non-breeding aggression in song sparrows are limited. In vivo measurement of brain DHEA, T, and E_2 levels in discrete regions will complement studies on steroidogenic capacity of those tissues. We can predict that decrease in DHEA levels in STI stimulated birds will parallel with the present data suggesting increased metabolism, while the increase in levels of active androgens would parallel with their increased synthesis. It would also be useful to measure the regulation of

brain steroidogenic enzymes and steroid levels by social encounters during the breeding season.

In the present study, aromatase activity decreased in the telencephalon. This could be a reflection of the dynamic steroid environment and measuring additional timepoints earlier than 30 min may help understand the timecourse. Further, we decided to measure 3β-HSD activity by adding NAD^+ exogenously in all the brain tissues a priori, based on previous studies (4, 5). It would have been useful to divide the tissue appropriately to measure 3β-HSD activity in the absence of NAD^+ in other brain regions in as well. For example, the diencephalon is very important in the control of motivated behaviors and has been reported to have high aromatase activity.

Here, we performed gross dissections to study conversion of DHEA to AE via 3β-HSD in different brain regions. However, measurement of enzyme activity in large chunks of tissue may not be reflective of the true steroidogenic capacity of the specific region, due to dilution by non-steroidogenic tissue. Measuring 3β-HSD activity in minute amounts of brain tissue by using laser guided micro-dissection or Palkovits Punch technique will provide better spatial resolution.

Finally, ongoing studies must clarify the cellular mechanisms involved in the rapid regulation of 3β-HSD activity. These might involve compounds such as catecholamines, endozepines and neuropeptides, which are known to act as neuromodulators and neurotransmitters. We could also perform long-term social stimulation studies by performing multiple simulated territorial intrusions over days. These studies will be critical in understanding the regulation of DHEA metabolism in the adult brain.

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