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Rapid effects of an aggressive interaction on DHEA, testosterone, and estradiol levels in the male song sparrow brain: a seasonal comparison

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ABSTRACT

Across vertebrates, aggression is robustly expressed during the breeding season when circulating testosterone (T) is elevated, and T activates aggression either directly or after aromatization into 17β-estradiol (E_2) in the brain. In some species, such as the song sparrow, aggressive behavior is also expressed at high levels during the non-breeding season, when circulating T is non-detectable. At this time, the androgen precursor dehydroepiandrosterone (DHEA) is metabolized within the brain into T and/or E_2 to promote aggression. In the present study, we used captive male song sparrows to test the hypothesis that an acute agonistic interaction during the non-breeding season, but not during the breeding season, would alter steroid levels in the brain. Non-breeding and breeding subjects were exposed to either a laboratory simulated territorial intrusion (L-STI) or an empty cage for only 5 min. Immediately

following, the brain was rapidly collected and flash frozen. The Palkovits punch technique was used to microdissect specific brain regions implicated in aggressive behavior. Solid phase extraction followed by radioimmunoassay was used to quantify DHEA, T, and E_2 in punches. Overall, levels of DHEA, T, and E_2 were higher in brain tissue than in plasma. Local T and E_2 levels in the preoptic area, anterior hypothalamus, and nucleus taeniae of the amygdala were significantly higher in the breeding season than the non-breeding season and were not affected by the L-STI. Surprisingly, subjects that were dominant in the L-STI had lower levels of DHEA in AH and medial striatum in both seasons and lower levels of DHEA in TnA in the breeding season only. Taken together, these data suggest that local levels of DHEA in the brain are very rapidly modulated by social interactions in a context- and region-specific pattern.

INTRODUCTION

Aggressive behavior is one of the best-studied forms of social behavior. Aggression is present throughout the animal kingdom and occurs most frequently when two or more individuals compete for limited resources such as mates or food. Thus, despite the costs (e.g., time, energy, risk of injury), aggressive behavior is considered highly adaptive because it can increase survival and/or reproductive success. Male-male territorial aggression is typically highest during the breeding season when gonadal testosterone (T) is elevated in the circulation. In this context, plasma T promotes aggressive behavior via androgen receptor (AR) activation, as well as after aromatization to 17β-estradiol (E_2, Figure 1) within specific regions of the brain and subsequent estrogen receptor (ER) activation. Notably, this positive association between gonadal T and territorial aggression is prominent during periods of social instability or challenge, such as territory establishment in the early breeding season [1].

In several species of mammals and birds, territorial aggression persists outside of the context of breeding, when plasma T is very low or non-detectable. The neuroendocrine regulation of non-breeding aggression has been studied extensively in song sparrows (*Melospiza melodia*). Song sparrows are common throughout North America, and in the Pacific Northwest, males defend territories throughout the year

(except for a brief period during molt) [2]. During the non-breeding season, the testes of male song sparrows are completely regressed (<1mm in length) and castration has no effect on territorial behavior (reviewed in [3]). Remarkably, despite non-detectable circulating T, both acute and chronic administration of fadrozole hydrochloride (FAD, an aromatase inhibitor) decreases non-breeding aggression [4]. Further, the effect of FAD on non-breeding aggression is rescued by E_2 replacement [4]. These data suggest that neurally-synthesized steroids (neurosteroids), particularly E_2, regulate non-breeding aggression. Moreover, brain nuclei that regulate aggressive behavior express aromatase, ERα, and ERβ at high levels during the non-breeding season [5, 6]. Further, acute administration of E_2 rapidly increases aggressive behavior via non-genomic mechanisms in non-breeding, but not breeding, male song sparrows [7]. This is relevant because neurosteroids might be more likely to act via non-genomic mechanisms than circulating steroids [8, 9].

Dehydroepiandrosterone (DHEA) might be an indirect source of androgen substrate for brain aromatase during the non-breeding season. DHEA is a steroid prohormone that can be metabolized into androstenedione (AE), an aromatizable androgen, by 3β-hydroxysteroid dehydrogenase/Δ5-Δ4 isomerase (3β-HSD) (Figure 1). DHEA is synthesized in peripheral organs (adrenal glands, testes, liver) as well as in the brain, and several lines of evidence implicate DHEA in non-breeding aggression. First, DHEA is elevated in the circulation of non-breeding male song sparrows, and seasonal changes in plasma DHEA parallel seasonal changes in territorial aggression [10, 11]. Second, a 30-min simulated territorial intrusion (STI) during the non-breeding season increases DHEA levels in plasma collected from the jugular vein but not from the brachial vein [12]. This suggests that engaging in agonistic behavior increases DHEA synthesis in the brain. Third, DHEA treatment increases territorial singing in non-breeding song

sparrows [13]. Fourth, a 30min STI during the non-breeding season increases brain 3β-HSD activity [14]. Taken together, these data suggest that DHEA metabolism within the brain promotes the expression of aggressive behavior during the non-breeding season.

If DHEA metabolism plays a role in the neuroendocrine regulation of non-breeding aggression, then agonistic interactions should alter DHEA, T and/or E_2 levels in the brain. To test this hypothesis, captive non-breeding and breeding male song sparrows were exposed to a 5-min laboratory-STI (L-STI) or control (empty cage). Brain tissue was immediately collected and frozen, and DHEA, T, and E_2 levels were measured in multiple microdissected brain areas implicated in social behavior: medial striatum, (MSt), preoptic area (POA), anterior hypothalamus (AH), nucleus taeniae of the amygdala (TnA), caudomedial neostriatum (NCM), and midbrain.

METHODS

Subjects and housing

Adult male song sparrows were caught using conspecific song playback and mist nets in late October/early November (non-breeding season) and early April (breeding season) near Vancouver, British Columbia (49^0 12'N, 123^0 01'W). After capture, subjects were housed outdoors in individual wire cages (91cm x 47cm x 47cm) in the University of British Columbia Animal Care Centre Annex. While in captivity, subjects were exposed to the natural photoperiod (breeding season: $\bar{x}$ = 15.5L : 8.5D; non-breeding season: $\bar{x}$ = 9.5L : 14.5D) and natural temperature (breeding season: $\bar{x} = 11^0$C; non-breeding: $\bar{x} = 4.2^0$C). Each cage contained conifer branches and two wooden perches. Although housed individually, subjects were kept together in a colony pen and visually (but not acoustically) isolated, except during behavioral testing. Water and seed were provided *ad libitum* and one wax moth larva was provided daily. Protocols were

approved by the UBC Animal Care Committee and complied with the guidelines of the Canadian Council on Animal Care. Note that these are the same subjects that were used in a prior experiment, which examined the rapid effects of acute E_2 administration on agonistic behavior [7]. Importantly, the experiment described here occurred at least 4 weeks after the prior experiment [7] was completed, and all subjects were visually isolated during the time between the two experiments.

Behavioral testing

Subjects were assigned to one of two treatment groups: dyadic social encounter (DYAD; breeding: n = 14; non-breeding: n = 16) or empty cage control (CON; breeding: n = 7; non-breeding n = 7). Importantly, we ensured that the DYAD and CON groups both contained focal subjects and stimulus subjects from the prior experiment [7](REF). We also ensured that the subjects that comprised each dyad had not encountered each other in the prior experiment [7] (REF).

Approximately 20hr before behavioral testing, subjects were moved in their home cage from the colony pen to a behavioral testing pen. DYAD subject cages were placed immediately adjacent to each other and separated by an opaque partition. CON subject cages were placed immediately adjacent to an empty cage and separated by an opaque partition. Subjects were then allowed to habituate to their new surroundings overnight.

The next day, immediately prior to behavioral testing, a Canon Vixia AF 20 HD camcorder was placed on a tripod ~1m away from the subject cage (both cages were entirely visible in the recording) and a tie clip microphone was placed between the two cages. Then, video recording commenced, the opaque partition was removed, the experimenter quickly exited the behavioral testing pen, and subjects were allowed to interact with each other (or with an empty cage) for 5min. The L-STI paradigm (both

with and without conspecific song playback) has been used previously by several laboratories [6, 7, 15, 16].

Behavior quantification

The number of ***barrier contacts*** (operationally defined as when the subject made full contact [both feet] with the wire barrier separating the two cages) and ***proximity time*** (operationally defined as the number of seconds that the subject spent in the third of its cage that was adjacent to the wire barrier separating the two cages) during an L-STI are both clear and easily quantifiable measures of territorial aggression in captive male song sparrows [7, 15]. Barrier contacts, proximity time, and the number of contacts made with the opposite cage wall were quantified from the video recordings for both members of each dyad. As observed previously [7], one subject within each dyad always made more barrier contacts and had higher proximity time than the other subject. Based on prior research [7], it is likely that if dyads had been allowed to interact beyond the 5min behavioral test, then this pattern of behavior would have been stable for at least several days. Accordingly, socially dominant subjects (DOM) were operationally defined as the member of each dyad that made more barrier contact and had higher proximity time than the other member of the dyad (hereafter referred to as socially subordinate subjects (SUB)).

Tissue collection

Immediately following the 5-min behavioral test, subjects were captured and euthanized via rapid decapitation (mean latency to capture = 24sec). Brains were dissected from the skull and flash frozen on powdered dry ice (mean latency to dry ice = 4.5 min). Whole brains were stored at -80°C until sectioning. At the time of euthanasia,

trunk blood was collected into microcentrifuge tubes and kept on wet ice until centrifugation. Plasma was collected and stored at -20°C until radioimmunoassay (RIA).

The Palkovits punch technique was used to microdissect brain tissue from six brain areas: the medial striatum (MSt), preoptic area (POA), anterior hypothalamus (AH), nucleus taeniae of the amygdala (TnA), caudomedial neostriatum (NCM), and midbrain [17-19]. Brains were sectioned in the coronal plane at 300µm on a cryostat (-10^{0}C) using a plane of sectioning that closely matched a zebra finch atlas [20]. Sections were lightly thaw-mounted onto microscope slides, and specific sections containing the brain regions of interest were identified using the songbird brain atlas and major landmarks. A stainless steel sample corer (i.d. = 2mm, o.d. = 3mm; Fine Science Tools, catalog #18035) was used to microdissect brain tissue from each area. Specifically, four bilateral punches from the MSt were collected from two serial sections beginning 900µm rostral to the first appearance of the tractus septopalliomesencephalicus (TrSM). MSt punches included, but were not restricted to, the song control nucleus Area X. Two punches from POA were collected from two serial sections immediately caudal to the last section containing TrSM. Two punches from AH were collected from two serial sections immediately following POA sections. POA and AH punches were centered on the midline and top of the 3rd ventricle. AH punches did not include the ventromedial nucleus of the hypothalamus. Four bilateral punches from TnA were collected from two serial sections following the disappearance of the CoA and tractus occipito-mesencephalicus (OM) fiber path from the ventromedial telencephalon. Four bilateral punches from the NCM were collected from the same two serial sections as TnA. NCM punches did not include the song control nucleus HVC. Four punches of the midbrain were collected from 4 serial sections containing the posterior commissure (CP, a landmark for the central gray (GCt)) and the oculomotor nerve (NIII, a landmark for the

ventral tegmental area (VTA)). Midbrain punches were centered on the midline. Punches were expelled into microcentrifuge tubes cooled inside the cryostat. Tubes were stored at -80°C until processing.

Homogenization and solid phase extraction

Samples were homogenized as described previously [21]. All samples (~50 µL of plasma and ~1-3 mg of brain tissue) were homogenized in 2mL polypropylene microcentrifuge tubes with 90µL ice-cold de-ionized water and 400µL HPLC-grade methanol, using a bead homogenizer (Omni Bead Ruptor 24) [21]. Homogenates were left at 4°C overnight.

Solid phase extraction was conducted as described previously [22, 23]. Following centrifugation, 475µL of supernatant was added to 10mL de-ionized water and then loaded onto C18 columns (Agilent Bond-Elut OH, 500mg, cat # 12113045) that had been primed with 3mL HPLC-grade methanol and equilibrated with 10mL de-ionized water. Samples were then washed with 10mL 40% HPLC-grade methanol, and steroids were eluted with 5mL 90% HPLC-grade methanol. The eluted samples were dried at 40°C in a vacuum centrifuge (ThermoElectron SPD111V Speedvac) and stored at -20°C until assayed.

Radioimmunoassays

Samples were resuspended in PBSG (phosphate-buffered saline containing 0.1% gelatin) with absolute ethanol (1.0%) to aid resuspension (Newman et al., 2008). These resuspended samples were used to measure DHEA, testosterone, and estradiol using sensitive and specific radioimmunoassays (RIAs). All samples were run in singleton as described previously [21]. DHEA was measured using an assay kit (#DSL-8900) from

Beckman Coulter (inter-assay variation = 18.0%; intra-assay variation = 16.5%). Modifications to the DHEA kit instructions are detailed elsewhere [15, 24]. Briefly, 50µL of primary antibody was added to 100µL of standards and diluted samples and incubated for 30min at 37°C. Next, 250µL of tracer was added and incubated for 3hr at 37°C. Finally, 500µL of precipitating reagent was added and incubated for 20min at room temperature. Following centrifugation at 4°C and decanting, tubes were counted for 1min on a gamma counter. Final values were corrected for recovery, which was assessed for plasma and tissue [25] by comparing unspiked samples to samples spiked with a known amount of steroid (brain 90.0%; plasma 85.0%).

Testosterone was measured using an assay kit (#07189102) from MP Biomedicals (inter-assay variation = 8.5%; intra-variation = 7.4%). Modifications to the T kit instructions are detailed elsewhere [26]. Briefly, 200µL of primary antibody was added to 175µL (100µL resuspended sample + 75µL of PBSG) of standards and diluted samples and incubated for 4hr at room temperature. Next, 200µL of tracer was added and incubated for 3hr at 37°C in a hot water bath. Finally, 50µL of precipitating reagent was added and incubated for 60min at 37°C. Following centrifugation at 4°C and decanting, tubes were counted for 2min on a gamma counter. Final values were corrected for recovery as described above (brain 78.9%; plasma 90.2%).

E_2 was measured using an assay kit (#DSL-4800) from Beckman Coulter (inter-assay variation = 6.5%; intra-assay variation = 6.0%). Modifications to the E_2 kit instructions are detailed elsewhere [27]. Briefly, 100µL of diluted primary antibody was added to 300µL (150µL resuspended sample + 150µL of PBSG) of standards and diluted samples and incubated for 4hr at room temperature. Next, 100µL of tracer was added and incubated overnight at 4°C. Finally, 500µL of precipitating reagent was added and incubated for 20min at room temperature. Following centrifugation at 4°C and

decanting, tubes were counted for 1min on a gamma counter. Final values were corrected for recovery as described above (brain 81.6%; plasma 72.3%).

Protein and tissue mass calculation

The tissue pellets that remained after homogenization were resuspended in 0.2M NaOH. Protein was quantified using Coomassie Plus Bradford Assay Kit. As previously described [28], when using small brain samples, it is more accurate and reliable to take advantage of the tight correlation between tissue mass and tissue protein content in order to estimate tissue mass. Such small samples (1-2 mg) cannot be accurately measured on a microbalance. Here, we multiplied protein values by 9.08 to yield tissue mass. This conversion factor of 9.08 was determined using song sparrow brain samples of known mass. The samples ranged in mass from 4.75 to 24mg, and samples were weighed on a microbalance. There is a very tight linear correlation between the measured protein amount in mg (x) and brain sample mass in mg (y), yielding an equation of $y = 9.08x$ ($n=12$, $r^2=0.96$).

Data analysis

ANOVAs were used to test for a main effect of Season (breeding versus non-breeding), a main effect of Group (CON versus DOM versus SUB), and a Season × Group interaction effect on steroid levels and behavior. Fisher's LSD *post hoc* tests were used when significant interactions were observed. Data were analyzed using Statistica software (version 9. 1, StatSoft, Tulsa, OK). When appropriate, data were log transformed prior to statistical analysis in order the meet the assumptions of parametric statistical tests. All figures represent the mean and standard error of the mean (raw data). P values < 0.05 were considered significant.

Results

Behavior

There was a significant main effect of Group on barrier contacts ($F_{(2,40)} = 10.51$, $p < 0.01$, Figure 2A) and proximity time ($F_{(2,40)} = 36.59$, $p < 0.01$, Figure 2B). This is not surprising, given that differences in aggressive behavior were used to operationally define DOM and SUB males. As expected, *post hoc* comparisons revealed that SUB males made significantly fewer barrier contacts ($p < 0.01$, Figure 2A) and spent significantly less time in close proximity to the barrier ($p < 0.01$, Figure 2B) than DOM males. Surprisingly, *post hoc* comparisons also revealed that DOM and CON males were not significantly different from each other (barrier contacts, $p = 0.41$; proximity time, $p = 0.99$). Notably, there were no significant main effects of Season or interaction effects on either barrier contacts or proximity time. There were also no significant main or interaction effects on the number of contacts subjects made with the opposite wall ($p > 0.12$, Figure 2C), suggesting no differences in general activity.

Plasma steroids

There was a main effect of Season on plasma T ($F_{(1,40)} = 9.24$, $p < 0.01$, Figure 2A, Figure 3B). *Post hoc* comparison revealed that breeding males had significantly higher plasma T than non-breeding males ($p < 0.01$). There were no significant main or interaction effects on either plasma DHEA ($p > 0.66$, Figure 3A) or plasma E_2 ($p > 0.06$, Figure 3C).

Brain steroids

Medial striatum (MSt). There was a significant main effect of Group on DHEA levels in MSt ($F_{(2,40)} = 3.47$, $p = 0.04$, Figure 4A). *Post hoc* comparison revealed that DOM males had significantly lower levels of DHEA in MSt than SUB males ($p = 0.01$). There were no significant main or interaction effects on either T levels ($p > 0.26$, Figure 4B) or E_2 levels ($p > 0.87$, Figure 4C).

Preoptic area (POA). There was a significant Season × Group interaction effect on DHEA levels in POA ($F_{(2,40)} = 3.19$, $p = 0.05$, Figure 5A). *Post hoc* comparison revealed that, in the non-breeding season, CON males had significantly lower DHEA levels in POA than SUB males ($p = 0.02$). There was also a significant main effect of Season on T levels (breeding > non-breeding, $F_{(1,40)} = 8.38$, $p = 0.01$, Figure 5B) and E_2 levels (breeding > non-breeding, $F_{(2,40)} = 4.10$, p 0.05, Figure 5C).

Anterior hypothalamus (AH). There was a significant main effect of Group on DHEA levels in AH ($F_{(2,40)} = 4.68$, $p = 0.02$, Figure 6A). *Post hoc* comparison revealed that DOM males had significantly lower DHEA levels in AH than CON males ($p = 0.03$) and SUB males ($p < 0.01$). There was also a significant main effect of Season on T levels (breeding > non-breeding, $F_{(1,40)} = 5.28$, $p = 0.03$, Figure 6B) and E_2 levels (breeding > non-breeding, $F_{(1,40)} = 4.02$, $p = 0.05$, Figure 6C).

Nucleus taeniae of the amygdala (TnA). There was a significant Season × Group interaction effect on DHEA levels in TnA ($F_{(2,40)} = 4.24$, $p = 0.02$, Figure 7A). *Post hoc* comparison revealed that, in the breeding season, DOM males had significantly lower DHEA levels in TnA than CON males ($p < 0.01$) and SUB males ($p = 0.02$). There was also a significant main effect of Season on T levels (breeding > non-breeding, $F_{(1,40)} = 6.02$, $p = 0.02$, Figure 7B) and E_2 levels (breeding > non-breeding, $F_{(1,40)} = 5.59$, $p = 0.02$, Figure 7C).

Caudomedial nidopallium (NCM). There was a significant main effect of Season on T levels in NCM (breeding > non-breeding, $F_{(1,40)} = 4.11$, $p = 0.05$, Figure 8B). There were no significant main or interaction effects on either DHEA levels ($p > 0.19$) or E_2 levels ($p > 0.07$).

Midbrain. There were no significant main effects of Season or Group or interaction effects on DHEA ($p > 0.13$), T ($p > 0.06$), or E_2 ($p > 0.11$) levels in the midbrain.

DISCUSSION

This study demonstrates that a brief (5min) L-STI induces rapid changes in the concentrations of DHEA in the brain, but not in the general circulation, in both breeding and non-breeding male song sparrows. Remarkably, in some cases, the effects of this brief social interaction on brain DHEA levels were group-, region-, or season-specific. Unlike brain DHEA levels, brain T and E_2 levels were only modulated by season. Notably, in all regions examined, neural DHEA, T, and E_2 levels far exceeded plasma levels of these steroids. These data, taken together with past work, are consistent with the hypothesis that neurosteroidogenesis is involved in the regulation of social behavior, when circulating sex steroid levels are low as well as when they are high.

Effect of a short dyadic encounter on behavior

Field studies show that song sparrows in the Pacific Northwest aggressively defend territories in both the breeding and non-breeding seasons [29, 30]. The L-STI paradigm complements these field studies and is powerful tool to investigate the neuroendocrine basis of an important social behavior under more controlled conditions [6, 7, 15, 16]. Here, male song sparrow dyads were subjected to an L-STI for 5min. Quantitative analysis of barrier contacts and proximity time during this very short social encounter

revealed that one male (DOM) made more barrier contacts and had higher proximity time than the other male (SUB). Notably, the number of contacts made with the opposite wall did not differ between DOM and SUB males. A previous study utilizing the L-STI paradigm found that dominance status that is apparent in the first 5min of a dyadic social encounter will remain stable for at least the next 2 days [7].

Barrier contacts and proximity time were not significantly different between DOM and CON males. This unexpected but intriguing finding might be a consequence of subjects having prior experience with this behavioral testing paradigm. As stated above, subjects in this study were used in a prior experiment, which examined the rapid effects of E_2 on aggression during an L-STI [7]. While the two experiments were separated by at least 4 weeks, it is possible that the prior experiment [7] trained subjects to expect to interact with a conspecific when a partition separating its cage from another cage was removed. Accordingly, barrier contacts and proximity time exhibited by CON males might reflect the anticipation of an aggressive encounter rather than aggressive behavior *per se*. Alternatively, CON male barrier contacts and proximity time might simply reflect curiosity about a novel object in their immediate environment. Wild song sparrows experience territorial conflicts year-round and likely use contextual cues to anticipate aggressive encounters. Thus, while unexpected, we do not view CON male behavior in this experiment to be problematic because DOM males were engaged in a dynamic social encounter whereas CON males were not. By extension, the motivational and physiological state accompanying barrier contacts and proximity time were likely fundamentally different between DOM and CON males.

Effect of a short dyadic encounter on plasma steroids

Overall, plasma levels of DHEA, T, and E_2 observed in this study are similar to levels reported elsewhere (reviewed in [3]). There were no effects of season or the brief social interaction on plasma DHEA. These data are consistent with previous research showing no difference between breeding and non-breeding birds in plasma DHEA levels [12] and no effect of a 30min STI on systemic DHEA levels in non-breeding song sparrows [10]. Similarly, there was no effect of season or the social interaction on plasma E_2, which is consistent with previous reports [10]. As expected, plasma T was significantly higher in breeding males than in non-breeding males [3]. There is widespread evidence that elevated systemic T levels in the breeding season regulate territorial aggression [31, 32], and in song sparrows, an STI leads to a rapid increase in both aggression and circulating T [33, 34]. However, unlike this previous work, the present data do not show a significant effect of the social interaction on plasma T among breeding males (although there is a trend for CON < SUB < DOM males). This is most likely because the dyadic encounter used here (5 min) was shorter than the STIs used in field studies (10 to 30 min).

Effect of a short dyadic encounter on neurosteroids

Importantly, compared to plasma levels, the brain levels of DHEA, T and E_2 were approximately 5.8×, 1.4×, and 6.6× higher (respectively) in spring and 5.7×, 5.0×, and 5.0× higher (respectively) in winter. Further, in contrast to plasma DHEA levels, brain DHEA levels were rapidly modulated by the social interaction in both seasons (see below). The present data are consistent with previous work that shows that the songbird brain expresses all of the steroidogenic enzymes required to synthesize DHEA and to metabolize DHEA into T and E_2 [5, 35-39]. Thus it appears that the metabolism of

DHEA into active sex steroids could play a role in the neuroendocrine regulation of dominance and aggression throughout the year.

In support of this hypothesis, in both seasons, DHEA levels in the AH were significantly lower in DOM subjects than CON and SUB subjects. These data suggest that "winning" an aggressive interaction rapidly upregulates DHEA metabolism (or downregulates DHEA synthesis) in the AH. AH is a node within the social behavior network [40] and regulates multiple forms of social behavior, including dominance and aggression [41-44]. DHEA lacks a classical intracellular steroid receptor [45] but is converted by 3β-HSD into androstenedione, an active androgen and direct substrate for aromatase (Figure 1). *In situ* hybridization [35] and enzyme activity assays [38] show that 3β-HSD is present and active in this area of the brain in adult male songbirds. Further, 3β-HSD activity in song sparrows does not differ seasonally in the rostral diencephalon (which contains AH) [14]. Taken together, these data suggest that DHEA metabolites act within the AH to promote aggressive behavior and social dominance in both the breeding and non-breeding seasons (see below for discussion of T and E_2 levels in the brain). This interpretation is consistent with work by Newman et al. showing that a STI rapidly alters DHEA levels in the jugular vein, but not brachial vein, in breeding and non-breeding song sparrows [12]. Thus these data add to a growing body of evidence implicating neurosteroids in the regulation of aggressive behavior throughout various stages of the life history cycle.

The present data also suggest that winning upregulates DHEA metabolism (or downregulates DHEA synthesis) in TnA but, unlike AH, in the breeding season only. The TnA is also a component of the social behavior network [40], regulates agonistic behavior [44, 46], and expresses 3β-HSD at relatively high levels [35]. Thus, the present

data suggest that DHEA metabolites act within TnA to promote aggressive behavior specifically within the breeding season.

DHEA levels in MSt were significantly lower in DOM males than SUB males in both seasons. However, DHEA levels in MSt of CON males were not different from either DOM or SUB males in either season, which limits our ability to interpret these findings. DHEA metabolites might act in MSt to regulate behaviors associated with social dominance. Alternatively, engaging in behaviors associated with becoming socially subordinate either may either upregulate DHEA synthesis or downregulate 3β-HSD activity in MSt. The functional significance of these effects is also difficult to interpret because MSt punches were centered on the song control nucleus Area X yet no subjects sang during the L-STI. However, MSt punches also contained striatal tissue surrounding Area X. Evidence from bird species that do not sing implicate MSt in associative learning, behavior reinforcement, and the anticipation of reward [47-50]. Thus an alternative explanation for the effects seen in MSt here is that neurosteroidogenesis in medial striatum is involved in reinforcing behaviors associated with social dominance and/or subordination.

Although DOM subjects had lower DHEA levels in AH, TnA, and MSt, it is surprising that DOM subjects did not have higher T or E_2 levels in these brain areas. Previous research shows that estrogens increase aggressive behavior in the non-breeding season [4, 7, 51-54] and androgens and estrogens are strongly implicated in dominance and aggression that occurs in the context of breeding. Several factors may explain this pattern of results. First, we may have ended the behavioral trial before changes in T and/or E_2 were observable by our methods. This seems plausible given that 1) brain E_2 levels in brain punches have been shown to change following a 30min STI in free-living white-crowned sparrows [55] and 2) *in vivo* microdialysis studies show forebrain E_2

levels fluctuate rapidly during social interactions in zebra finches [56]. Second, locally-produced steroids might act more like neurotransmitters than blood-borne hormones. Thus, locally-produced T and E_2 might be produced, bind their receptors, and be subsequently catabolized on too rapid of a rapid timescale to be observable by our methods. Third, we did not measure androstenedione (AE) or estrone (E_1). DHEA is converted to AE by 3β-HSD. While AE is best known as a precursor to T, it also binds to androgen receptors with high affinity and can be aromatized to E_1. Thus, perhaps DHEA → AE → E_1 (rather than DHEA → AE → T → E_2) is a more relevant metabolic pathway for neurosteroidogenic regulation of the social behavior observed in this study.

Conclusions

The present data, taken together with past work, strongly suggest that the metabolism of DHEA into active sex steroids is important for the neuroendocrine regulation of social behavior throughout the year. Because brain levels of DHEA exceed circulating levels in the blood, it seems most likely that the metabolism of DHEA synthesized *de novo* from cholesterol (rather circulating DHEA) is critically involved in modulation of aggression and social dominance. The lack of an effect of dominance status on brain T and E_2 levels suggests that there are complex mechanisms regulating the synthesis and catabolism of neurosteroids, at least on an acute timescale. Future studies employing *in vivo* microdialysis followed by steroid profiling via mass spectrometry may be required in order to fully characterize the effect of aggressive behavior on neurosteroidogenesis.

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FIGURE LEGENDS

Figure 1. A simplified diagram of sex steroid synthesis. Steroids: PREG = pregnenolone; PROG = progesterone; DHEA = dehydroepiandrosterone; AE = androstenedione; T = testosterone; E_1 = estrone; E_2 = 17β-estradiol. Enzymes: P450scc = Cytochrome P450 side chain cleavage; P450c17 = Cytochrome P450 17α-hydroxylase/C17,20 lyase; 3β-HSD = 3β-hydroxysteroid dehydrogenase/isomerase; 17β-HSD = 17βhydroxysteroid dehydrogenase; Aromatase = Cytochrome P450 aromatase.

Figure 2. Bar graph (mean + SEM) representing a significant main effect of Group on (A) barrier contacts and (B) proximity time, and (C) no effects of Season, Group or Season x Group on contacts made with the opposite wall during the 5-min dyadic encounter.

Figure 3. Bar graph (mean + SEM) representing (A) no effects of Season, Group or Season x Group on plasma DHEA, (B) a significant main effect of Season on plasma T, and (C) no effects of Season, Group or Season x Group on plasma E_2.

Figure 4. Bar graph (mean + SEM) representing (A) a significant main effect of Group on DHEA levels and no effects of Season, Group or Season x Group on (B) T levels or (C) E_2 levels in brain punches collected from the medial striatum (MSt).

Figure 5. Bar graph (mean + SEM) representing (A) a significant Season x Group interaction effects on DHEA levels and a significant main effect of Season on (B) T levels and (C) E_2 levels in brain punches collected from the preoptic area (POA).

Figure 6. Bar graph (mean + SEM) representing (A) a significant main effect of Group on DHEA levels and a significant main effect of Season on (B) T levels and (C) E_2 levels in brain punches collected from the anterior hypothalamus (AH).

Figure 7. Bar graph (mean + SEM) representing (A) a significant Season x Group interaction effect on DHEA levels and a significant main effect of Season on (B) T levels and (C) E_2 levels in brain punches collected from nucleus taeniae of the amygdala (TnA).

Figure 8. Bar graph (mean + SEM) representing (A) no Season, Group or Season x Group interaction effects on DHEA levels, (B) a significant main effect of Season on T levels and (C) no Season, Group or Season x Group interaction effects on E_2 levels in brain punches collected from the caudomedial nidopallium (NCM).

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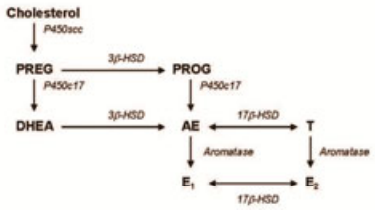
Figure 1



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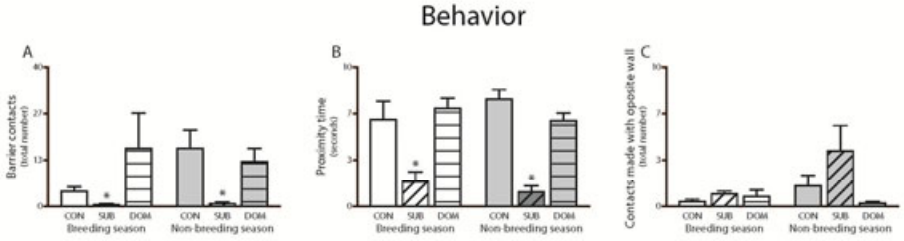

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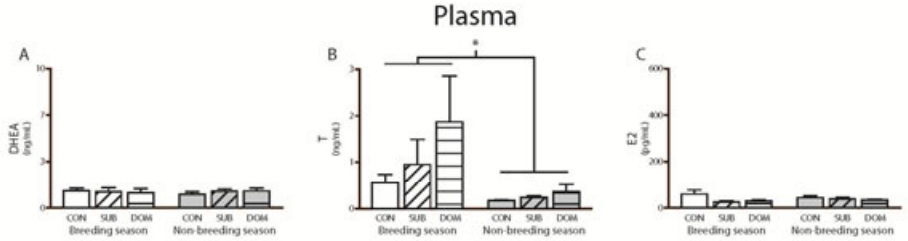

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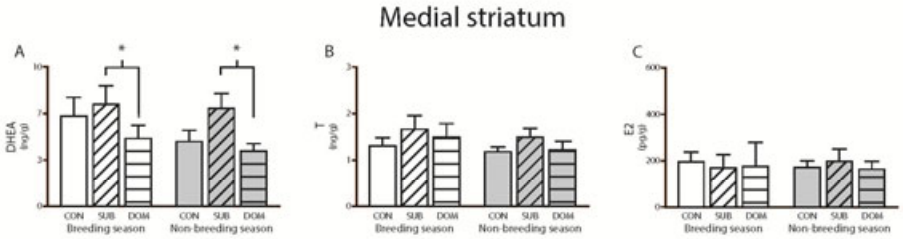

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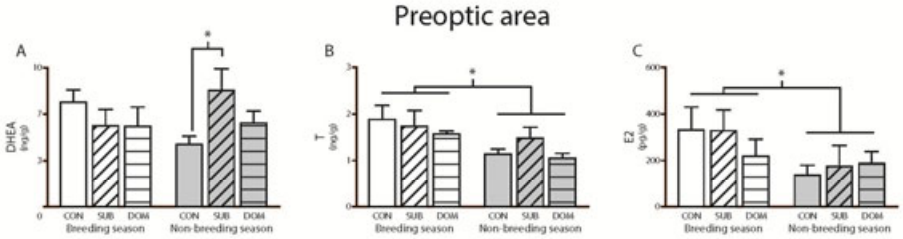

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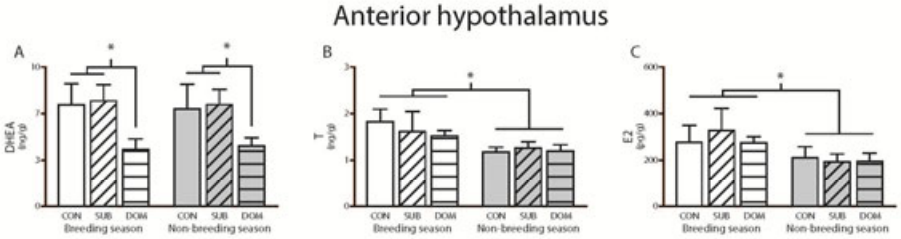

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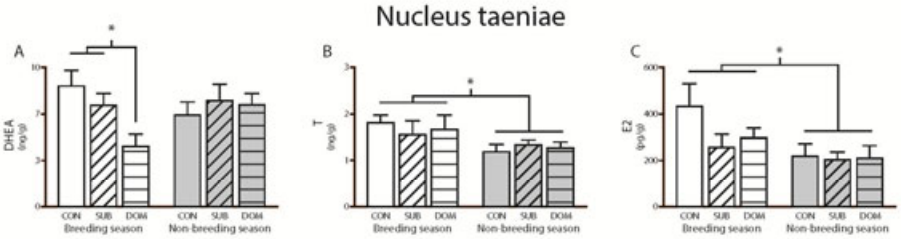

Figure 8