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BEHAVIORAL NEUROSCIENCE

Corticosterone and dehydroepiandrosterone in songbird plasma and brain: effects of season and acute stress

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Abstract

Prolonged increases in plasma glucocorticoids can exacerbate neurodegeneration. In rats, these neurodegenerative effects can be reduced by dehydroepiandrosterone (DHEA), an androgen precursor with anti-glucocorticoid actions. In song sparrows, season and acute restraint stress affect circulating levels of corticosterone and DHEA, and the effects of stress differ in plasma collected from the brachial and jugular veins. Jugular plasma is an indirect index of the neural steroidal milieu. Here, we directly measured corticosterone and DHEA in several brain regions and jugular plasma, and examined the effects of season and acute restraint stress (30 min) (n = 571 samples). Corticosterone levels were up to 10× lower in brain than in jugular plasma. In contrast, DHEA levels were up to 5× higher in brain than in jugular plasma and were highest in the hippocampus. Corticosterone and DHEA concentrations were strongly seasonally regulated in plasma but, surprisingly, not seasonally regulated in brain. Acute stress increased corticosterone levels in plasma and brain, except during the molt, when stress unexpectedly decreased corticosterone levels in the hippocampus. Acute stress increased DHEA levels in plasma during the molt but had no effects on DHEA levels in brain. This is the first study to measure (i) corticosterone or DHEA levels in the brain of adult songbirds and (ii) seasonal changes in corticosterone or DHEA levels in the brain of any species. These results highlight several critical differences between systemic and local steroid concentrations and the difficulty of using circulating steroid levels to infer local steroid levels within the brain.

Introduction

Chronic elevations in glucocorticoids can have adverse effects on the brain, such as hippocampal atrophy (Sapolsky, 2000). Glucocorticoids are synthesized *de novo* from cholesterol by the adrenal glands and also by the brain itself (Davies & MacKenzie, 2003; Gomez-Sanchez *et al.*, 2005; Ye *et al.*, 2008). Local steroid synthesis could increase neural glucocorticoid concentrations independently of systemic glucocorticoid concentrations. Local glucocorticoid levels are regulated, in part, by 11β-hydroxysteroid dehydrogenase isozymes, which catalyse the conversion between active glucocorticoids and their inactive metabolites (Pelletier *et al.*, 2007). Although numerous studies have measured systemic glucocorticoid levels in plasma or serum, very few studies have measured local glucocorticoid levels in the brain (Thoeringer *et al.*, 2007; Droste *et al.*, 2008).

Dehydroepiandrosterone (DHEA), a sex-steroid precursor, can also be synthesized by the adrenals and brain (Baulieu, 1998; Labrie *et al.*, 2005). DHEA has anti-glucocorticoid properties (Kalimi *et al.*, 1994) and reduces the neurodegenerative effects of elevated glucocorticoids on the rat hippocampus (HP) *in vitro* (Kimonides *et al.*, 1999) and *in vivo* (Karishma & Herbert, 2002). Moreover, DHEA levels in the rat brain are increased by acute stress (Corpechot *et al.*, 1981) and ACTH treatment (Torres & Ortega, 2003). DHEA may regulate local corticosteroid levels via effects on 11β-hydroxysteroid dehydrogenase isozymes (Apostolova *et al.*, 2005; Balazs *et al.*, 2008). Together, these data suggest that neural DHEA is regulated by stress and modulates the actions of glucocorticoids.

In a wild songbird, the song sparrow (*Melospiza melodia*), acute restraint affects corticosterone and DHEA levels in plasma from the brachial and jugular veins (Newman *et al.*, 2008a). Brachial plasma is an index of systemic steroid levels, and jugular plasma is enriched with neurally-synthesized steroids and is an indirect index of neural steroid levels (Schlinger & Arnold, 1992, 1993). During the breeding season, stress does not affect brachial DHEA levels but decreases jugular DHEA levels. During the molt, stress does not affect brachial DHEA levels but increases jugular DHEA levels. Also during the molt, stress increases corticosterone levels to a greater extent in jugular plasma than brachial plasma. A specific change in jugular steroid levels suggests a change in neurosteroid synthesis or metabolism. However, jugular plasma is an indirect index of neural steroid levels.

Here, we directly examined the effects of acute restraint stress on corticosterone and DHEA concentrations in jugular plasma and brain of wild song sparrows under natural conditions. Hypothalamic–pituitary–adrenal axis activity varies across seasons (Romero, 2002; Pyter *et al.*, 2007) and thus we examined animals from three distinct

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seasons of the year: breeding season, molt and non-breeding season. This design allowed us to examine the effects of acute stress and season, as well as compare plasma and brain steroid concentrations within individuals. We were particularly interested in the HP, which is a major glucocorticoid target in the brain.

Materials and methods

Subjects

Subjects were wild adult male song sparrows ($n = 46$ total). Song sparrows are an excellent model for studying seasonal plasticity in hormones and the adult brain (Tramontin & Brenowitz, 2000; Soma *et al.*, 2008). This species shows large seasonal changes in circulating corticosterone and DHEA (Soma & Wingfield, 2001; Newman *et al.*, 2008a). Song sparrows, unlike laboratory rats or mice, have relatively high levels of plasma DHEA. Furthermore, a physiological dose of DHEA has pronounced effects on neuroanatomy and behavior (Soma *et al.*, 2002). DHEA is metabolized to androstenedione in the songbird brain and this metabolism is affected by acute stress (Soma *et al.*, 2004; Pradhan *et al.*, 2008, Schlinger *et al.*, 2008). The songbird brain might also synthesize DHEA *de novo* (London *et al.*, 2006).

Song sparrows were captured during three seasons: (i) breeding (May 9–17, 2006; baseline, $n = 8$; stressed, $n = 9$); (ii) molt (August 12–18, 2006; baseline, $n = 5$; stressed, $n = 6$) and (iii) non-breeding (January 3–11, 2006; baseline, $n = 9$; stressed, $n = 9$). Subjects were captured near Vancouver, British Columbia (49°12′N, 123°01′W). This population is sedentary and males maintain territories year-round (Wingfield & Hahn, 1994; Soma, 2006). During the breeding season, plasma testosterone levels are elevated and males aggressively defend territories. During the molt, when song sparrows are replacing their feathers, plasma testosterone levels are undetectable and territorial aggression is reduced. During the non-breeding season, when testes are regressed, plasma testosterone is undetectable but territorial aggression is high. Protocols were approved by the UBC Committee on Animal Care and complied with the guidelines of the Canadian Council of Animal Care.

Blood collection

Subjects were captured using conspecific playback (3.50 ± 0.53 min) and mist nets. A baseline blood sample (~150 μL) was collected within 3 min of capture (2.05 ± 0.07 min) from the jugular vein with a heparinized syringe (Newman *et al.*, 2008a). Subjects were killed by rapid decapitation within 3 min of capture (2.60 ± 0.16 min) or restrained for 30 min in a cloth bag. After restraint, another blood sample was collected from the jugular vein and subjects were killed (32.71 ± 0.23 min after capture). Blood was kept on wet ice until centrifuged. Plasma was stored at −20°C.

Tissue collection

After cooling on wet ice for 2–3 min, the brain was dissected into six regions: (i) rostral diencephalon (rDIEN); (ii) caudal diencephalon (cDIEN); (iii) HP; (iv) caudal medial nidopallium (NCM); (v) dorsal telencephalon (dTEL) containing the song nucleus HVC; and (vi) central medial telencephalon (cmTEL) containing the septum, bed nucleus of the stria terminalis and nucleus accumbens (Goodson *et al.*, 2004; Montagnese *et al.*, 2004).

The dissection protocol closely followed Soma *et al.* (1999). Briefly, the hippocampi were isolated by making two parallel cuts approximately 1.5 mm lateral to the midline and 1 mm deep. The songbird HP is located on the dorsomedial surface of the telencephalon and lies directly above the lateral ventricle (Saldanha *et al.*, 1998). Next, we removed a triangular piece of the dorsal telencephalon from the area lateral to the HP, designed to match the location of HVC (Reiner *et al.*, 2004). The NCM was then removed from directly under the excised HP by removing a rectangular piece of tissue 1.5 mm lateral to the midline and approximately 1.5 mm deep. The brain was then removed from the cranium and placed on its dorsal surface on a Petri dish resting on wet ice. The optic lobes and hindbrain (to the level of the mammillary bodies) were then removed. The diencephalon was removed to the depth of the anterior commissure and split into rostral diencephalon and caudal diencephalon. The remaining telencephalon was divided into three regions (rostral, central and caudal). The central telencephalon was further split into medial and lateral sections. All brain regions were divided into left and right halves; one half was used to measure steroid levels (present study) and the other half will be used to measure steroidogenic enzymes (to be presented separately). Left or right halves of brain tissues were chosen at random for steroid measurement. The body was kept on wet ice until dissected. We collected a piece of pectoral muscle, a piece of liver, the testes and the adrenals. The entire dissection took < 30 min. Tissues were stored separately in microcentrifuge tubes and frozen immediately on dry ice. In the laboratory, tissue was stored at −80°C.

Steroid measurement

Steroids were extracted from tissue and plasma samples ($n = 571$ total) using solid-phase extraction with C_{18} columns, as previously described (Newman *et al.*, 2008b). This extraction procedure results in high and consistent steroid recoveries and effectively removes interfering substances from plasma and lipid-rich brain tissue (Newman *et al.*, 2008b). We have thoroughly validated this procedure with both plasma and brain tissue for corticosterone and DHEA assays (e.g., serial dilutions, recovery of radiolabeled and radioinert steroids) (Newman *et al.*, 2008a,b; Schmidt & Soma, 2008). In addition, here we examined recovery of 30 pg of exogenous corticosterone and 35 pg of exogenous DHEA that were added to a song sparrow plasma pool ($n = 6$ replicates) and a brain tissue pool ($n = 6$ replicates) prior to extraction. Corticosterone and DHEA concentrations in these plasma and tissue samples were compared with concentrations in plasma and tissue samples from the pools that did not have exogenous steroid added ($n = 6$ each). Recovery of exogenous corticosterone was 94.3% from plasma and 73.9% from brain. Recovery of exogenous DHEA was 73.8% from plasma and 70.8% from brain.

We used sensitive and specific radioimmunoassays to measure corticosterone (ImmuChem 07-120103; MP Biomedicals, Orangeburg, NY, USA) and DHEA (DSL 8900; Diagnostic Systems Laboratories, Webster, TX, USA) (Newman *et al.*, 2008a,b; Schmidt & Soma, 2008). The corticosterone antibody has very low cross-reactivities to cortisol (0.05%), 11-deoxycorticosterone (0.34%), dehydrocorticosterone (0.50%), progesterone (0.02%) and other steroids (Schmidt & Soma, 2008). The DHEA antibody has very low cross-reactivities to androstenedione (0.73%), testosterone (0.28%), progesterone (0.05%), DHEA sulfate (0.02%) and other steroids (Boonstra *et al.*, 2008; Newman *et al.*, 2008b). Dried eluates were resuspended in 250 μL phosphate-buffered saline with gelatin. We used absolute ethanol (5% of resuspension volume) to aid the resuspension of steroids (Newman *et al.*, 2008a) and 100 μL (× 2) was

assayed for DHEA. Of the remaining 50 μL resuspension, 30 μL was removed, brought up to 100 μL with phosphate-buffered diluent provided with the corticosterone radioimmunoassay and 50 μL (× 2) was assayed for corticosterone.

The lowest points on the corticosterone and DHEA standard curves were 3.12 pg corticosterone and 2 pg DHEA (per tube). For corticosterone, intra-assay variation was 4.1% and inter-assay variation was 8.0% (low control) and 6.8% (high control) ($n = 10$ assays). For DHEA, intra-assay variation was 5.9% and inter-assay variation was 10.1% (low control) and 7.9% (high control) ($n = 12$ assays). Variability was low in replicates of the 'unspiked' plasma and brain tissue pools (see above). For corticosterone, the coefficient of variation was 4.08% for plasma and 4.16% for brain ($n = 6$ replicates each). For DHEA, the coefficient of variation was 8.93% for plasma and 6.95% for brain ($n = 6$ replicates each). For corticosterone, all water blanks were undetectable (< 3.12 pg; $n = 12$). For DHEA, 11 of 12 water blanks were undetectable (< 2 pg) and one was slightly above the lowest standard. Thus, the steroid measurement protocol functions reliably for both plasma and brain tissue.

Statistics

Undetectable samples (below the lowest point on the standard curve) were set to zero, as previously done for small tissues (Schmidt & Soma, 2008). Data were corrected for recovery and transformed [log ($x + 1$)] to reduce heteroscedasticity prior to analysis with JMP IN 5.1 (SAS, Cary, NC, USA).

For plasma, the effects of season and stress were tested using a two-factor ANOVA and Tukey's honestly significant difference (HSD) *post hoc* tests. For brain tissue, we used mixed-design three-factor ANOVA to test the effects of season, stress and brain region on corticosterone and DHEA levels. Season and stress were between-subject factors and brain region was a within-subject factor. Significant interactions were broken down using two-factor ANOVA tests within each season to assess the effects of stress and brain region. If the two-factor ANOVA revealed significant differences, we used Tukey's HSD tests for *post hoc* comparisons. For peripheral tissues, the effects of season and stress on steroid levels were examined using two-factor ANOVA tests and significant differences were analysed using Tukey's HSD *post hoc* tests.

Steroid levels in plasma and two specific brain regions (cmTEL and HP) were directly compared using mixed-design two-factor ANOVA tests, in which season was a between-subject factor and sample type (plasma or brain tissue) was a within-subject factor. The septum (in the cmTEL) and HP readily bind tritiated corticosterone (McEwen *et al.*, 1968) and are sensitive to restraint stress (Goodson *et al.*, 2004). Furthermore, both regions contain steroidogenic enzymes (Hojo *et al.*, 2004; Soma *et al.*, 2004; Tsutsui *et al.*, 2006). Lastly, in the cmTEL, corticosterone and DHEA were detectable in most samples and steroid concentrations were similar to those in other brain regions (except the HP).

The ratio of corticosterone to DHEA was calculated in jugular plasma, cmTEL and HP. This ratio has been informative in studies of patients with stress-related psychiatric diseases (Ritsner *et al.*, 2004). The corticosterone : DHEA ratio was calculated by dividing the corticosterone concentration (in pg/mL) by the DHEA concentration (in pg/mL). We previously presented a similar analysis for brachial and jugular plasma (Newman *et al.*, 2008b), although in that study we presented log-transformed data. We considered results significant for $P \leq 0.05$. Data are presented as mean ± SEM.

Results

Jugular corticosterone

We used a two-factor ANOVA to examine the effects of season and stress on jugular corticosterone (Fig. 1). There were significant main effects of season ($F_{2,67} = 148.89$, $P < 0.0001$) and stress ($F_{1,67} = 299.02$, $P < 0.0001$), and the interaction between season and stress was significant ($F_{2,67} = 109.90$, $P < 0.0001$). *Post hoc* tests revealed that baseline corticosterone was highest during the breeding season and similarly low during the molt and non-breeding season (Tukey's HSD, $P \leq 0.05$). Stress increased jugular corticosterone in all seasons but the effect of stress was greatest during the breeding season and similar during the molt and non-breeding season (Tukey's HSD, $P \leq 0.05$).

Brain corticosterone

In a mixed-design three-factor ANOVA examining the effects of season, stress and brain region on corticosterone, the main effects of stress and region were significant but there was no significant main effect of season (Fig. 1, Table 1). Also, the season × region, stress × region and season × stress × region interactions were significant (Table 1).

To break down the three-way interaction, we used a mixed-design two-factor ANOVA within each season. During the breeding season, there was a significant main effect of stress on corticosterone ($F_{1,100} = 115.87$, $P < 0.0001$). There was no effect of region ($F_{5,100} = 0.92$, $P = 0.48$) and no interaction between stress and region ($F_{5,100} = 0.45$, $P = 0.81$). During the molt, there was a significant interaction between stress and region ($F_{5,64} = 7.96$, $P < 0.0001$). *Post hoc* tests revealed that stressed corticosterone was higher than baseline corticosterone levels in all brain regions except in the HP, where stressed corticosterone was significantly lower than baseline corticosterone levels (Tukey's HSD, $P < 0.05$ in all cases). These data suggest that stress reduces corticosterone in the HP during the molt. During the non-breeding season, there was a significant effect of stress ($F_{1,105} = 21.13$, $P < 0.0001$) but no effect of region ($F_{5,105} = 0.88$, $P = 0.50$) or interaction between stress and region ($F_{5,105} = 0.65$, $P = 0.66$).

Jugular dehydroepiandrosterone

We used a two-factor ANOVA to examine the effects of stress and season on jugular DHEA (Fig. 2). There was a significant main effect of season ($F_{2,67} = 4.61$, $P < 0.002$) and no main effect of stress ($F_{1,67} = 2.08$, $P = 0.15$) but the interaction between season and stress was significant ($F_{2,67} = 3.83$, $P = 0.03$). *Post hoc* tests revealed that baseline DHEA levels were lower during the molt than during the breeding and non-breeding seasons and that stress significantly increased jugular DHEA during the molt (Tukey's HSD, $P < 0.05$).

Brain dehydroepiandrosterone

In a mixed-design three-factor ANOVA examining the effects of season, stress and brain region on DHEA, there was a significant main effect of region but no effects of stress or season (Fig. 2, Table 1). The HP had higher levels of DHEA than other regions (Tukey's HSD, $P < 0.05$).

Corticosterone and dehydroepiandrosterone concentrations in jugular plasma vs. brain

We used mixed-design two-factor ANOVA tests to examine the effects of season and sample type (plasma vs. cmTEL vs. HP) on

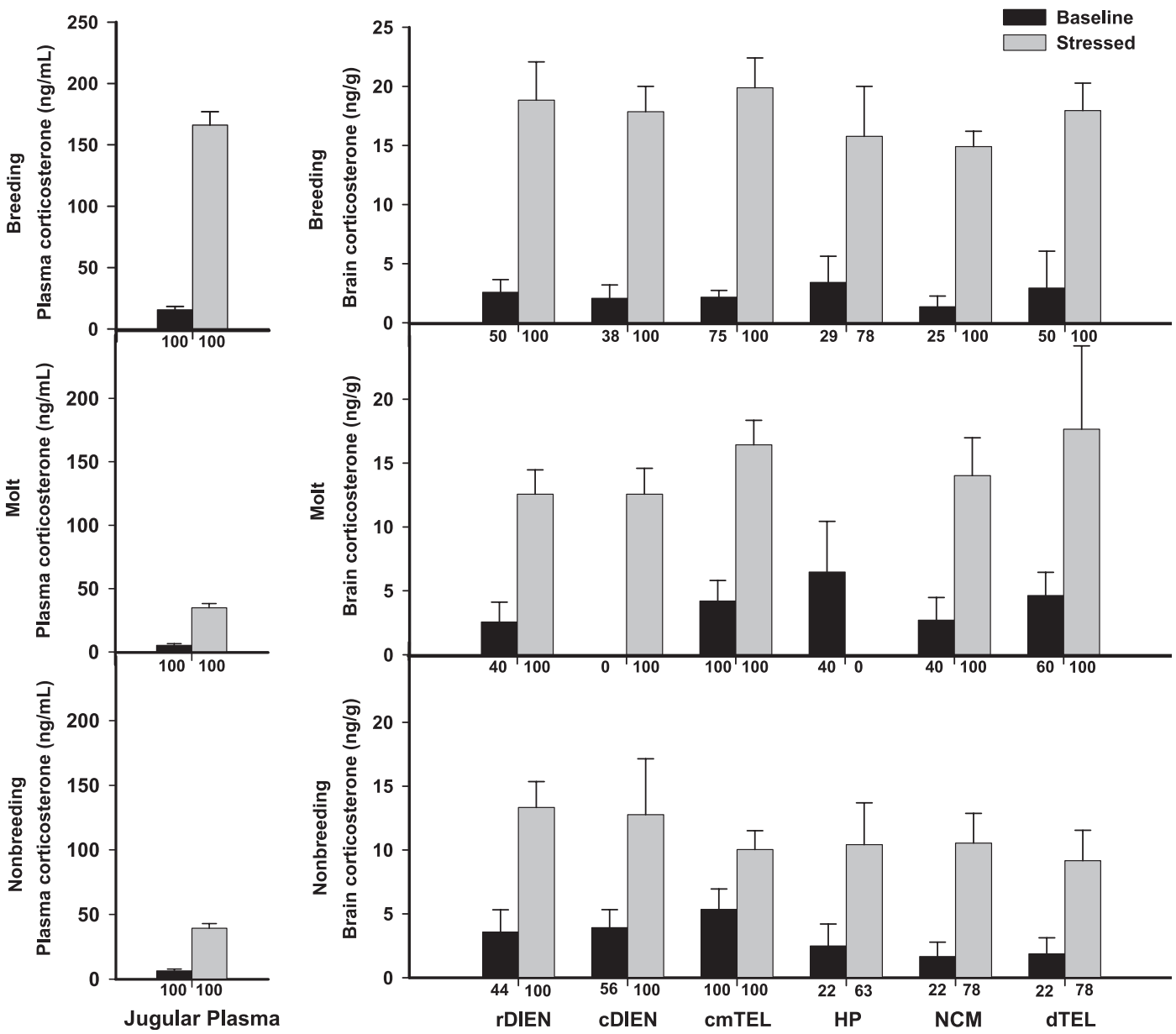

FIG. 1. Effects of season and stress on corticosterone levels in plasma (left) and brain (right). Corticosterone was regulated by season in jugular plasma but not in brain. Acute stress increased corticosterone in plasma and brain, except during molt, when stress decreased corticosterone levels in HP. rDIEN, rostral diencephalon; cDIEN, caudal diencephalon; dTEL, dorsal telencephalon. Numbers below bars indicate the percent of detectable samples.

corticosterone levels at baseline and after stress. At baseline, there was a significant main effect of season and an interaction between season and sample type (Fig. 3A, Table 2). During the breeding and non-breeding seasons, baseline corticosterone concentrations were greater in plasma than in cmTEL or HP (Tukey's HSD, $P < 0.05$). During the molt, there was no difference in baseline corticosterone concentrations in plasma, cmTEL and HP (Tukey's HSD, $P > 0.05$). After stress, the main effects of season and sample type were significant, as was the interaction between season and sample type (Fig. 3B, Table 2). During the breeding and non-breeding seasons, stressed corticosterone concentrations were greater in plasma than in cmTEL or HP (Tukey's HSD, $P < 0.05$). During the molt, stressed corticosterone concentrations were greater in plasma than in HP (Tukey's HSD, $P < 0.05$) but levels in cmTEL were not different from either plasma or HP (Tukey's HSD, $P > 0.05$).

We used the same approach to examine the effects of season and sample type on DHEA levels in plasma, cmTEL and HP. At baseline and after stress, there was a significant main effect of sample type only (Fig. 3C and D, Table 2). At baseline, DHEA concentrations were significantly lower in plasma than in cmTEL, and DHEA concentrations were highest in the HP (Tukey's HSD, $P < 0.05$). After stress, DHEA concentrations were significantly lower in plasma than in HP, and DHEA concentrations in cmTEL were intermediate and not different from either plasma or HP (Tukey's HSD, $P < 0.05$).

Ratio of corticosterone : dehydroepiandrosterone in jugular plasma vs. brain

At baseline and after stress, we used a mixed-design two-factor ANOVA to examine the effects of season and sample type on the

TABLE 1. Effects of season, stress and brain region on corticosterone and DHEA in the brain

	Three-way mixed-design ANOVA					
	Corticosterone			DHEA		
Variable	d.f.	*F*-ratio	*P*-value	d.f.	*F*-ratio	*P*-value
Season	2,271	1.39	0.25	2,271	1.22	0.30
Stress	1,271	115.41	< 0.0001	1,271	0.15	0.70
Season × stress	2,271	2.85	0.06	2,271	0.78	0.46
Region	5,271	6.67	< 0.0001	5,271	4.18	0.0012
Season × region	10,271	1.93	0.04	10,271	1.08	0.38
Stress × region	5,271	4.68	0.0005	5,271	0.28	0.92
Season × stress × region	10,271	2.21	0.02	10,271	1.09	0.37

DHEA, dehydroepiandrosterone; d.f., degrees of freedom. *P*-values ≤ 0.05 were considered significant.

corticosterone : DHEA ratio (Supporting information, Fig. S1). At baseline, there was no effect of season ($F_{2,81} = 0.60$, $P = 0.55$) but there was a significant effect of sample type ($F_{2,81} = 12.48$, $P < 0.0001$). The corticosterone : DHEA ratio was greater in plasma than in cmTEL or HP (Tukey's HSD, $P < 0.05$). After stress, the main effects of season and sample type were significant ($F_{2,68} = 7.50$, $P < 0.002$; $F_{2,68} = 51.54$, $P < 0.0001$, respectively), as was the interaction between season and sample type ($F_{4,68} = 7.72$, $P < 0.0001$). The corticosterone : DHEA ratio was greater in plasma than in cmTEL or HP and, in plasma, the ratio was greater during the breeding season (Tukey's HSD, $P < 0.05$).

Effects of season and stress on steroids in peripheral tissues

We used two-factor ANOVAs to examine the effects of season and stress on corticosterone concentrations in peripheral tissues (Table 3). In the adrenal glands, there were no effects of season or stress. In the gonads, there was a significant effect of season, with lowest corticosterone concentrations during the non-breeding season. Note that the adrenals in all seasons and the gonads in the non-breeding season were small and high steroid concentrations may be, in part, a result of the small tissue mass. In both liver and pectoral muscle, there was an interaction between season and stress, and stress increased corticosterone levels to a greater extent during the breeding season.

We also used two-factor ANOVAs to examine the effects of season and stress on DHEA concentrations in peripheral tissues (Table 3A and B). In the adrenals and gonads, there were no effects of season or stress on DHEA levels. Again, the adrenals and regressed gonads were small, possibly affecting calculated steroid concentrations. In liver, DHEA levels tended to change seasonally ($P = 0.06$). Overall high DHEA concentrations in liver suggest that the liver synthesizes DHEA, as in developing rats (Katagiri *et al.*, 1998). In pectoral muscle, DHEA levels were low and changed seasonally.

Discussion

Our results reveal dramatically different regulation of steroids in brain tissue vs. plasma. First, corticosterone levels were up to 10× lower in brain than in plasma, whereas DHEA levels were up to 5× higher in brain than in plasma. Second, we found strong seasonal changes in baseline corticosterone and DHEA levels in plasma but not in brain. Third, acute stress increased corticosterone levels in plasma but decreased corticosterone levels in the HP during molt. Also, during molt, acute stress increased DHEA levels in jugular plasma but had no effect on DHEA levels in the brain. This is the first study to measure (i) corticosterone or DHEA levels in the brain of adult songbirds and (ii) seasonal changes in corticosterone or DHEA levels in the brain of any species. Moreover, our results are from wild animals sampled under natural conditions.

Corticosterone levels were typically lower in brain than in plasma. However, the differences between plasma and brain concentrations varied with season, brain region and stress. During the breeding season, baseline and stressed corticosterone levels were up to 10× greater in plasma than in brain. The difference between plasma and brain was most pronounced for NCM at baseline, whereas after stress, the difference was similarly large for HP, NCM and dorsal telencephalon. During the molt and non-breeding season, the differences between plasma and brain were less pronounced, particularly at molt when corticosterone concentrations were only 2× greater in plasma. There are several possible explanations for these patterns. First, corticosteroid binding globulin binds ∼ 90% of corticosterone in plasma and may restrict its entry into brain tissue (Hammond, 1990; Breuner & Orchinik, 2002). We measured total (free + bound) corticosterone in the plasma and our measures of corticosterone in brain may primarily reflect free corticosterone in plasma. Plasma corticosteroid binding globulin levels are reduced at molt (Breuner & Orchinik, 2001; Romero *et al.*, 2006), which may reduce the difference between plasma and brain corticosterone levels. Second, brain levels of 11β-hydroxysteroid dehydrogenase type 2 (11β-HSD2), which converts corticosterone to inactive dehydrocorticosterone (Holmes & Seckl, 2006), could vary seasonally. Third, corticosterone synthesis in the brain could change seasonally and be up-regulated at molt (Newman *et al.*, 2008a). The rodent brain expresses the requisite enzymes (Davies & MacKenzie, 2003; Gomez-Sanchez *et al.*, 2005) and neural 11β-hydroxylase expression is up-regulated when systemic corticosterone levels are low (Ye *et al.*, 2008).

In contrast to corticosterone, DHEA levels were higher in brain than in plasma. During the breeding and non-breeding seasons, DHEA levels were up to 3× higher in brain than in plasma, across brain regions and stress conditions. During the molt, baseline DHEA concentrations in HP, NCM and dorsal telencephalon were 5–10× higher than plasma concentrations. Overall, DHEA levels were highest in the HP, consistent with hippocampal synthesis of DHEA. In the male rat HP, Hojo *et al.* (2004) detected the mRNA and activity of P450c17, which synthesizes DHEA. P450c17 and other steroidogenic enzymes are also expressed in the avian brain, suggesting that birds have the capacity to synthesize DHEA *de novo* in the nervous system (London *et al.*, 2006; Tsutsui *et al.*, 2006). It is unlikely that circulating DHEA is sequestered in brain tissue because seasonal changes in plasma DHEA were not reflected in the brain (in contrast to the liver and pectoral muscle). It is also unlikely that lipoidal or fatty acid esters of DHEA in the brain affected our measures of free DHEA because our extraction protocol used 90% methanol for elution and this is selective for free steroids (Liere *et al.*, 2004).

Plasma corticosterone levels showed dramatic seasonal changes, in stark contrast to brain corticosterone levels. Baseline and stressed corticosterone levels in plasma were elevated during the breeding season and reduced during the molt and non-breeding season, as in previous studies (Romero, 2002; Newman *et al.*, 2008a). In other species, seasonal changes in plasma corticosterone levels are due in part to changes in adrenal responsivity to ACTH and pituitary responsivity to CRH and arginine vasotocin (Romero, 2006). Remarkably, in the brain, there were small or no seasonal changes in baseline or stressed corticosterone levels. Seasonal regulation of

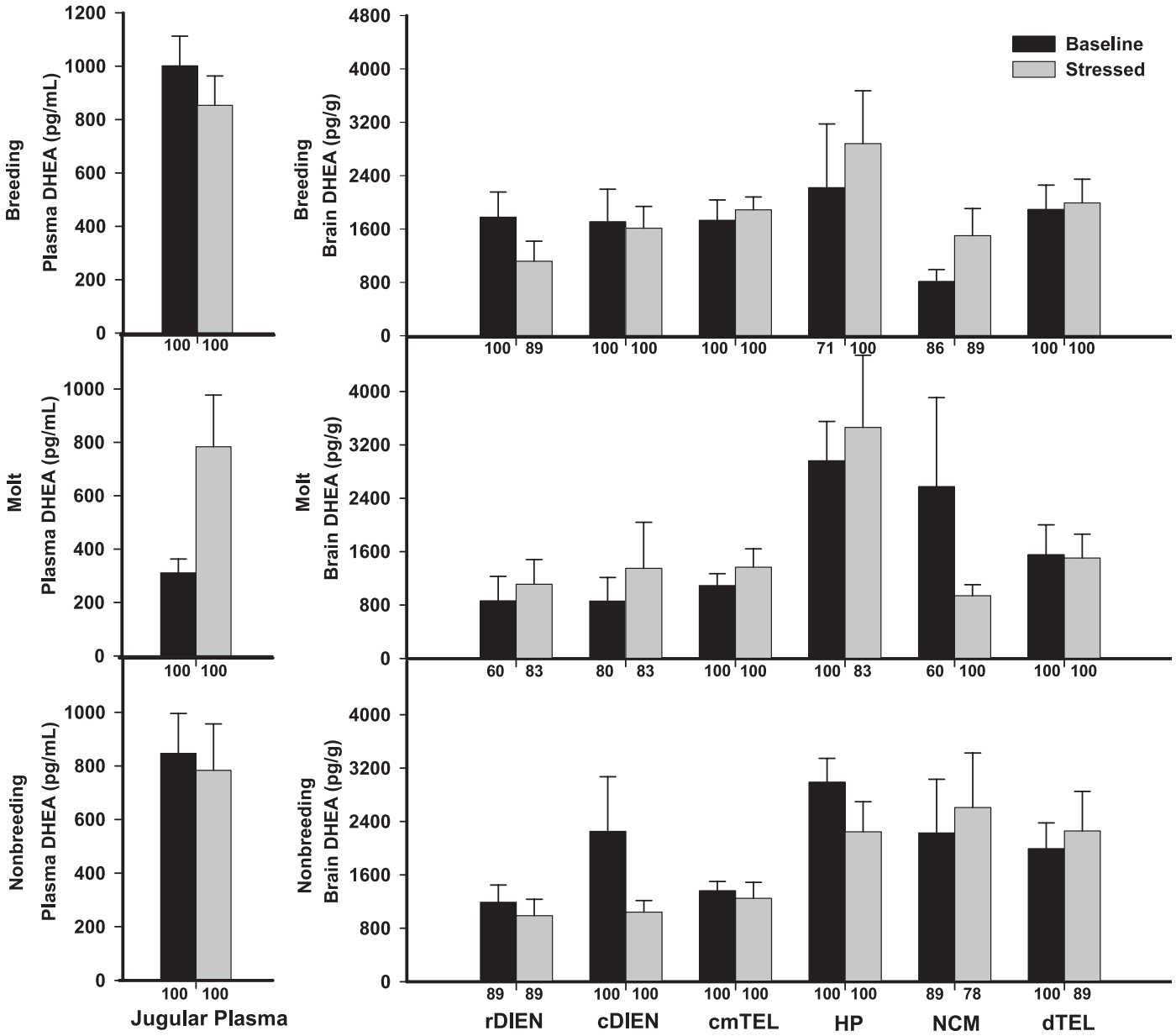

FIG. 2. Effects of season and stress on DHEA levels in plasma (left) and brain (right). DHEA was regulated by season in jugular plasma but not in brain. Acute stress increased plasma DHEA during the molt. Acute stress did not affect DHEA levels in brain. Overall, DHEA levels were highest in the HP. Numbers below bars indicate the percent of detectable samples. rDIEN, rostral diencephalon; cDIEN, caudal diencephalon; dTEL, dorsal telencephalon.

plasma corticosteroid binding globulin levels may account for the lack of seasonal changes in brain corticosterone levels. In house sparrows (*Passer domesticus*), corticosteroid binding globulin and total corticosterone levels in plasma change in parallel across the seasons. Thus, estimated free corticosterone levels in plasma (predicted using the equation of Barsano & Baumann, 1989) remain constant across seasons (Breuner & Orchinik, 2001; Romero *et al.*, 2006). If brain corticosterone levels are primarily determined by plasma free corticosterone levels, then the absence of seasonal changes in brain corticosterone levels may reflect the absence of seasonal changes in plasma free corticosterone levels. Thus, our empirical measurements of corticosterone in brain are consistent with estimates of free corticosterone in plasma.

Like corticosterone, DHEA levels also changed seasonally in plasma. Baseline plasma DHEA levels are elevated during the breeding and non-breeding seasons and reduced at molt. DHEA levels in the brain, however, did not change seasonally. These data suggest that neural DHEA synthesis is not seasonally regulated or even up-regulated during seasons when peripheral DHEA synthesis is down-regulated. Synthesis of local steroids and systemic steroids can be regulated independently (Schmidt *et al.*, 2008). High DHEA levels in the brain may facilitate year-round territorial behavior (Soma *et al.*, 2008). It is possible that brain DHEA sulfate changes seasonally. In the brain of an amphibian (*Rana nigromaculata*), levels of pregnenolone remain constant across the seasons, whereas levels of pregnenolone-S change seasonally (Takase *et al.*, 1999). Future studies should examine DHEA sulfate levels in songbirds, as already performed in mammals (Schumacher *et al.*, 2008).

Acute stress increased corticosterone in plasma and most brain regions. In molting song sparrows, acute stress increases corticoste-

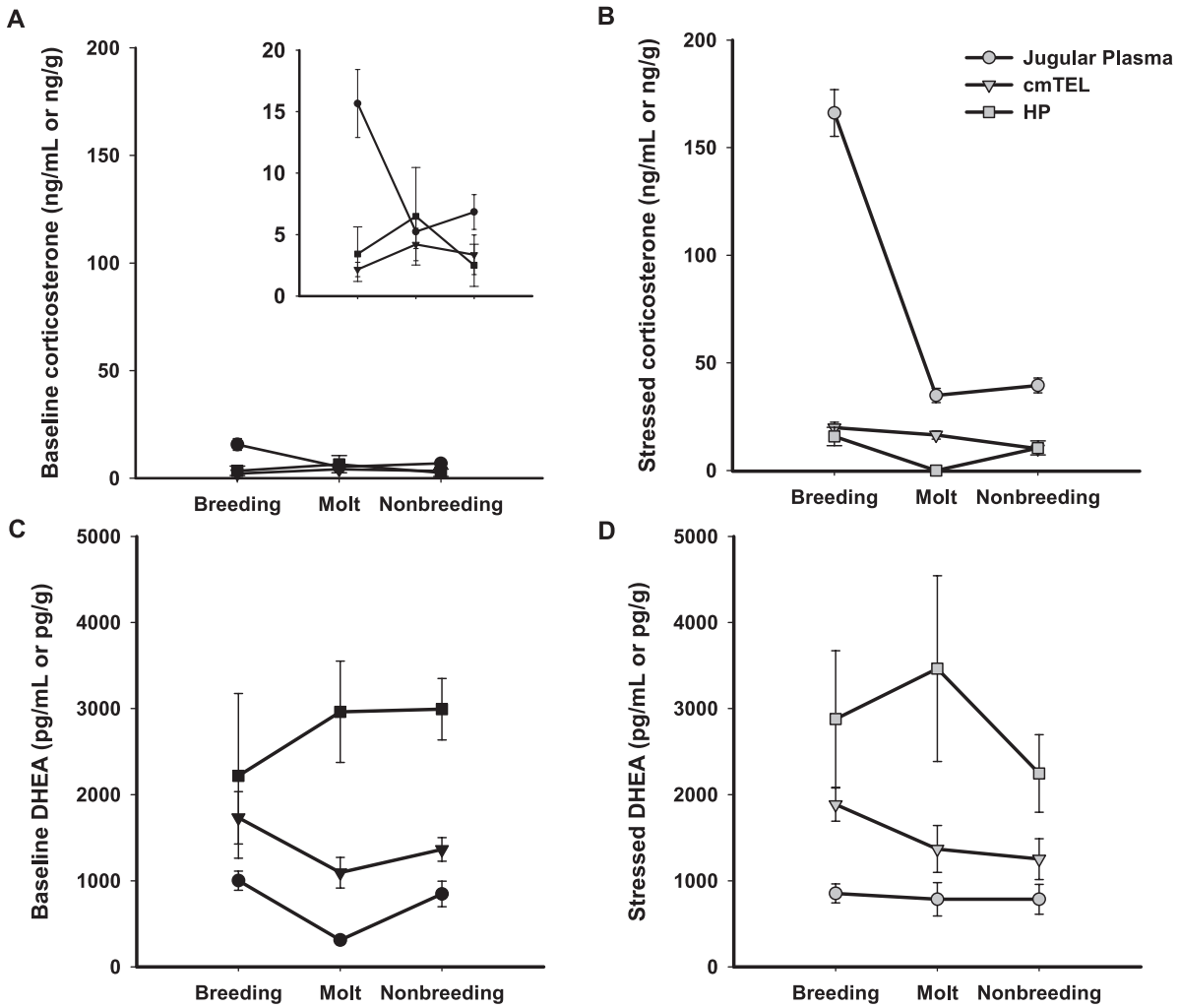

FIG. 3. Baseline and stressed corticosterone (A and B) and DHEA (C and D) in plasma, cmTEL and HP across seasons. Baseline and stressed corticosterone levels were higher in plasma than in cmTEL or HP. Inset: baseline corticosterone levels on an expanded *y*-axis scale. Baseline and stressed DHEA levels were lower in plasma than in cmTEL or HP.

TABLE 2. Effects of season and sample type on corticosterone and DHEA levels

	Two-way mixed-design ANOVA					
	Baseline			Stressed		
Steroid and variable	d.f.	*F*-ratio	*P*-value	d.f.	*F*-ratio	*P*-value
Corticosterone						
Season	2,85	0.25	0.78	2,69	12.81	< 0.0001
Sample (plasma, cmTEL, HP)	1,85	14.19	< 0.0001	1,69	62.57	< 0.0001
Season × sample	2,85	3.22	0.02	2,69	3.96	0.009
DHEA						
Season	2,85	0.95	0.40	2,70	0.79	0.46
Sample (plasma, cmTEL, HP)	1,85	13.46	< 0.0001	1,70	3.03	0.03
Season × sample	2,85	2.31	0.08	2,70	0.38	0.82

cmTEL, central medial telencephalon; DHEA, dehydroepiandrosterone; d.f., degrees of freedom. HP, hippocampus. *P*-values < 0.05 were considered significant.

rone levels to a greater extent in jugular plasma than in brachial plasma (Newman *et al.*, 2008a), suggesting that stress rapidly stimulates brain corticosterone synthesis during molt. However, brain corticosterone levels after 30 min of restraint were not greater at molt than at other seasons. Neurosteroids show very rapid and transient fluctuations (Balthazart & Ball, 2006; Remage-Healey *et al.*, 2008) due in part to rapid changes in steroid-synthesizing and -metabolizing enzymes. In addition, neurosteroids can passively diffuse or be actively transported into the bloodstream (Pariante, 2008). Future studies should examine earlier time-points (e.g., 5 and 10 min; Croft *et al.*, 2008). Alternatively, stress may increase corticosterone synthesis in a brain region not studied here.

Surprisingly, stress decreased corticosterone levels in the HP during molt, as in mice (Croft *et al*., 2008). The HP has the highest density of glucocorticoid receptors in the brain and is particularly vulnerable to stress (McEwen, 2001). One possible mechanism to reduce exposure of the HP to glucocorticoids is via 11β-HSD2, which rapidly inactivates corticosterone to dehydrocorticosterone (Holmes & Seckl, 2006; Klusoňová *et al.*, 2008). Acute restraint stress (45 min) rapidly up-regulates 11β-HSD2 activity in the rat placenta to protect the fetus

TABLE 3A. Effects of season and stress on corticosterone and DHEA levels in peripheral tissues: data from peripheral tissues

	Adrenal	Gonad	Liver	Muscle
(A) Data from peripheral tissues				
Corticosterone (ng/g)				
Breeding				
Baseline	12501.2 ± 5041.9 (100)	0.83 ± 0.34 (50)	3.95 ± 0.57 (100)	2.88 ± 0.65 (88)
Stressed	9118.4 ± 642.7 (100)	5.22 ± 1.49 (100)	19.08 ± 2.62 (100)	25.66 ± 3.21 (100)
Molt				
Baseline	12582.3 ± 834.9 (100)	6.74 ± 6.74 (20)	2.99 ± 0.30 (100)	3.17 ± 0.92 (100)
Stressed	8748.9 ± 1254.6 (100)	7.52 ± 5.60 (33)	6.64 ± 0.30 (100)	11.32 ± 2.54 (83)
Non-breeding				
Baseline	17738.6 ± 635.1 (89)	0 ± 0 (0)	4.08 ± 0.60 (100)	2.82 ± 1.06 (75)
Stressed	12276.4 ± 1978.0 (100)	0 ± 0 (0)	9.32 ± 1.41 (100)	10.88 ± 2.03 (89)
DHEA (pg/g)				
Breeding				
Baseline	5099.5 ± 1210.1 (88)	1100.9 ± 207.7 (100)	2538.3 ± 343.5 (100)	796.9 ± 171.4 (100)
Stressed	7975.7 ± 1976.5 (78)	1647.4 ± 330.6 (100)	3203.9 ± 313.1 (100)	788.8 ± 160.3 (100)
Molt				
Baseline	19380.3 ± 9924.7 (100)	2546.1 ± 1209.5 (60)	2477.4 ± 444.6 (100)	396.0 ± 107.3 (100)
Stressed	2215.6 ± 1105.1 (50)	3467.1 ± 1404.9 (67)	2146.6 ± 462.9 (100)	439.7 ± 62.8 (100)
Non-breeding				
Baseline	15793.6 ± 5138.0 (89)	42468.0 ± 7335.5 (57)	3550.8 ± 532.3 (100)	488.1 ± 103.9 (88)
Stressed	13255.7 ± 3447.5 (89)	53591.0 ± 18577.7 (88)	3622.0 ± 597.7 (100)	418.3 ± 93.8 (100)

Table 3B. Effects of season and stress on corticosterone and DHEA levels in peripheral tissues: two-way ANOVA analysis

	Adrenal			Gonad			Liver			Muscle		
Variable	d.f.	*F*-ratio	*P*-value	d.f.	*F*-ratio	*P*-value	d.f.	*F*-ratio	*P*-value	d.f.	*F*-ratio	*P*-value
Corticosterone (ng/g)												
Season	2,45	1.88	0.17	2,41	5.66	0.008	2,45	7.99	0.0012	2,45	8.12	0.001
Stress	1,45	0.62	0.44	1,41	2.89	0.10	1,45	71.56	< 0.0001	1,45	52.85	< 0.0001
Season × stress	2,45	0.29	0.75	2,41	1.63	0.21	2,45	4.86	0.012	2,45	8.28	0.001
DHEA (pg/g)												
Season	2,45	2.17	0.13	2,41	1.55	0.23	2,45	3.01	0.06	2,45	5.15	0.011
Stress	1,45	2.68	0.11	1,41	1.35	0.25	1,45	0.10	0.75	1,45	0.01	0.92
Season × stress	2,45	2.66	0.08	2,41	0.55	0.58	2,45	0.44	0.64	2,45	0.09	0.91

Numbers in parentheses indicate the percentage of tissue samples with detectable levels. *P*-values ≤ 0.05 were considered significant.

from high levels of maternal glucocorticoids (Welberg *et al.*, 2005). Future work will determine whether 11β-HSD2 activity is rapidly up-regulated by stress in the songbird HP. During molt, decreased adrenal reactivity may be coupled with increased hippocampal 11β-HSD2 reactivity to protect the HP from high levels of corticosterone.

Acute stress increases jugular, but not brachial, DHEA levels in molting song sparrows (Newman *et al.*, 2008b). These data suggest that stress also stimulates neural DHEA synthesis during molt, when peripheral DHEA synthesis is reduced. Here, we also found that stress increased jugular DHEA concentrations during molt. However, acute stress did not affect neural DHEA levels at molt or at other seasons. As with corticosterone, DHEA levels in jugular plasma may reflect DHEA that is synthesized in the brain but diffuses away before being rapidly metabolized. Alternatively, stress may increase DHEA synthesis in a region of the brain not examined here. Ongoing studies are examining the effects of season and stress on DHEA metabolism throughout the brain.

In conclusion, this study of wild animals under natural conditions identified regional variation in steroid concentrations and dynamic changes with season and acute stress. There are pronounced differences in the regulation of corticosterone and DHEA in plasma and brain, especially during molt. These data are consistent with accumulating evidence that neurosteroids may function more like neuromodulators or neurotransmitters than hormones (Balthazart & Ball, 2006; Schmidt *et al.*, 2008). Further, these results highlight the difficulties associated with using circulating steroid levels to infer local steroid levels within the brain. This is one of the first comprehensive comparisons between brain and systemic steroid concentrations, and the results lay the foundation for future work examining the cellular and molecular mechanisms of neurosteroid regulation.

Supporting Information

Additional supporting information may be found in the online version of this article:

Fig. S1. Effects of season and stress on the baseline and stressed corticosterone : DHEA ratio in jugular plasma, cmTEL and HP.

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Abbreviations

cDIEN, caudal diencephalon; cmTEL, central medial telencephalon; DHEA, dehydroepiandrosterone; dTEL, dorsal telencephalon; HP, hippocampus; HSD, honestly significant difference; 11β-HSD2, 11β-hydroxysteroid dehydrogenase type 2; NCM, caudal medial nidopallium; rDIEN, rostral diencephalon.

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