Testosterone and Corticosterone in the Mesocorticolimbic System of Male Rats: Effects of Gonadectomy and Caloric Restriction

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Steroid hormones can modulate motivated behaviors through the mesocorticolimbic system. Gonadectomy (GDX) is a common method to determine how steroids influence the mesocorticolimbic system, and caloric restriction (CR) is often used to invigorate motivated behaviors. A common assumption is that the effects of these manipulations on brain steroid levels reflects circulating steroid levels. We now know that the brain regulates local steroid levels in a region-specific manner; however, previous studies have low spatial resolution. Using ultrasensitive liquid chromatography tandem mass spectrometry, we examined steroids in microdissected regions of the mesocorticolimbic system (ventral tegmental area, nucleus accumbens, medial prefrontal cortex). We examined whether GDX or CR influences systemic and local steroids, particularly testosterone (T) and steroidogenic enzyme transcripts. Adult male rats underwent a GDX surgery and/or CR for either 2 or 6 weeks. Levels of T, the primary steroid of interest, were higher in all brain regions than in the blood, whereas corticosterone (CORT) was lower in the brain than in the blood. Importantly, GDX completely eliminated T in the blood and lowered T in the brain. Yet, T remained present in the brain, even 6 weeks after GDX. CR decreased both T and CORT in the blood and brain. Steroidogenic enzyme (Cyp17a1, 3β–hydroxysteroid dehydrogenase, aromatase) transcripts and androgen receptor transcripts were expressed in the mesocorticolimbic system and differentially affected by GDX and CR. Together, these results suggest that T is synthesized within the mesocorticolimbic system. These results provide a foundation for future studies examining how neurosteroids influence behaviors mediated by the mesocorticolimbic system. **(*Endocrinology* 159: 450–464, 2018)**

Steroids influence behavior, neural activity, and neural cytoarchitecture via multiple pathways (1). Neuroactive steroids include sex steroids, such as testosterone (T) and 17β-estradiol (E_2), as well as glucocorticoids, such as corticosterone (CORT). These steroids have traditionally been considered to be produced in the gonads or adrenals. Steroids that are locally synthesized in the brain are referred to as neurosteroids, such as dehydroepiandrosterone (DHEA) and pregnenolone (2). Recent evidence suggests that T, E_2, and CORT can also be produced in the brain (2–5). For example, Hojo *et al.* (4) found that T was synthesized in hippocampal slices when the precursors androstenedione or androstenediol were added. The enzymes necessary for neurosteroid synthesis are present in the mammalian nervous system (6–8) (Fig. 1). However, most studies have examined

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Abbreviations: AL, *ad libitum*; CORT, corticosterone; Cq, cycle of quantification; CR, caloric restriction; DHEA, dehydroepiandrosterone; E_2, 17β-estradiol; GDX, gonadectomy; HPC, hippocampus; HPLC, high-performance liquid chromatography; HSD, hydroxysteroid dehydrogenase; LC-MS/MS, liquid chromatography tandem mass spectrometry; MPB, mobile phase B; mPFC, medial prefrontal cortex; mRNA, messenger RNA; NAc, nucleus accumbens; POA/HYP, preoptic area/hypothalamus; SHAM, sham surgery; T, testosterone; VTA, ventral tegmental area.

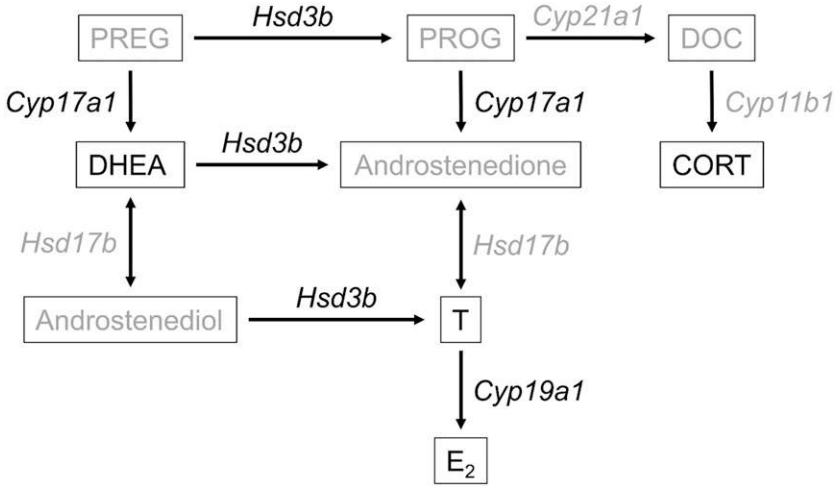

Figure 1. Simplified androgen, estrogen, and glucocorticoid synthetic pathways. Steroid names are in boxes, whereas gene nomenclature for steroid-synthetic enzymes are italicized. Steroids and enzymes in black were quantified in the brain in this study, whereas the ones in gray were not. The isoform numbers were omitted for *Hsd3b* and *Hsd17b* because of the multiple isoforms that exist for these enzymes. *Hsd3b1* was assayed in this study. DOC, 11-deoxycorticosterone; PREG, pregnenolone; PROG, progesterone.

steroids and steroidogenic enzymes in gross anatomical regions (*e.g.*, cerebral cortex) or large neuroanatomical structures [*e.g.*, hippocampus (HPC)] (9).

Neurosteroid synthesis in the mesocorticolimbic system has been largely overlooked. This system is comprised of dopamine-producing neurons in the ventral tegmental area (VTA) that sends efferents to the nucleus accumbens (NAc) and medial prefrontal cortex (mPFC), which feed back to the VTA (10). This system integrates salient sensory cues and assigns valence to contextually relevant stimuli associated with initially unconditioned incentives. The mesocorticolimbic system then uses this information to guide motivated behaviors and decision-making (11).

Steroids influence the mesocorticolimbic system (12, 13) either directly by binding to steroid receptors within this system (14, 15) or indirectly through secondary nodes that have abundant steroid receptors (16). Steroids influence neurochemical and electrophysiological activity in the mesocorticolimbic system in response to artificial rewards such as drugs of abuse (16, 17) and intrinsic natural rewards (18). Moreover, steroids influence numerous motivated behaviors that are controlled by the mesocorticolimbic system (19, 20). Depression, anxiety, and schizophrenia, which are marked by changes in motivation and dysfunction within the mesocorticolimbic system, are also modulated by steroid hormones (21).

Data on local steroid concentrations in these regions are lacking and the source of these steroids is unknown. Further, nearly all studies that have examined neurosteroid concentrations or neural steroidogenic enzymes have not examined both steroids and steroidogenic enzymes in the same subjects (4, 9, 22, 23). There is limited evidence that androgenic and estrogenic enzymes are present in the mesocorticolimbic system in rodents (8, 24) and humans (25), suggesting that sex steroids are synthesized in these regions. The functional significance of neurosteroids in the mesocorticolimbic system remains in question because of the lack of data on neurosteroids in these regions. T is of particular interest because most studies examining behavioral correlates of the mesocorticolimbic system use male rodents. Moreover, T microinjected into the brain affects motivated behaviors (26) and decreases dopamine release in the NAc (27). However, it still remains unclear whether steroid concentrations differ between mesocorticolimbic nodes and the circulation, and if steroids are locally produced in these regions.

The current study examined the effects of a short-term (2-week) or long-term (6 week) gonadectomy (GDX) and/or mild caloric restriction (CR) on systemic and local steroids and steroid-synthetic enzymes in mesocorticolimbic nodes. We were principally interested in T, but also measured CORT, via ultrasensitive liquid chromatography tandem mass spectrometry (LC-MS/MS). The 2- and 6-week time points were selected to examine potential upregulation of neural T synthesis following long-term, but not short-term, GDX. Evidence for this hypothesis comes from prostate cancer research, which shows that xenografts in mice locally produce T 1 month after GDX (28). Moreover, in male song sparrows (*Melospiza melodia*), brain androgen synthesis is upregulated ~1 month after the breeding season ends and plasma T levels naturally decline (29). Finally, we examined the effects of mild CR because it is widely used to stimulate responding to food incentives when investigating motivated behaviors mediated by the mesocorticolimbic system. CR decreases circulating T levels (30), but its effects on brain T levels are unknown.

Materials and Methods

Animals

Adult male Long-Evans rats (postnatal days 60 to 65, 210 to 260 g; N = 80; Charles River Laboratories, Stone Ridge, NY) were pair housed and maintained on a 12-hour:12-hour light/dark cycle (lights on at 7 AM) in a temperature-controlled room (22°C; 40% to 50% relative humidity) in a conventional facility. Animals were housed in clear plastic cages with aspen-chip bedding and a small polyvinylchloride tube for enrichment. Rats had *ad libitum* (AL) access to rat chow (Rat Diet 5012; LabDiet, Land O'Lakes, Inc., St. Louis, MO; isoflavones

content 555 ± 129 μg/g, personal communication with the manufacturer) for a 2-week acclimation period and always had AL access to water. All animals were handled and weighed every 2 to 3 days during acclimation. Procedures were approved by the University of British Columbia Committee on Animal Care and were in accordance with the standards of the Canadian Council on Animal Care.

Experimental design and surgeries

To assess the effects of short-term (2-week) and long-term (6-week) GDX and CR, subjects were randomly assigned to GDX (n = 40) or sham surgery (SHAM; n = 40) at 2 weeks or 6 weeks before euthanasia (timeline in Supplemental Fig. 1). All surgeries were done under isoflurane anesthesia (Fresenius Kabi Canada Ltd, Richmond Hill, ON, Canada) using aseptic surgical protocols. A small incision was made along the midline of the ventral scrotum to expose the tunic. Another small incision was made in the ventromedial portion of the tunic to expose the testis. For GDX subjects, each testicle was excised by a cut through the cremaster muscle just rostral to the head of the epididymis. The SHAM groups underwent the same procedure with the exception of the excision of the testes. For GDX subjects, complete removal of testes was confirmed after euthanasia.

After surgery, subjects in the long-term (6-week) treatments were single housed, handled, and weighed every day. Animals were allowed 3 days to recover and then subjects were randomly assigned to AL food or CR. CR subjects were fed with the food wrapped in a paper towel, whereas AL rats were given an empty paper towel packet. CR rats were fed between 11 AM and 1 PM. Because the rats had not reached an age where their weight was stable, and because GDX can attenuate normal weight gain (31), we compared CR subjects to the respective AL subjects. The goal of CR was to reach and maintain a weight of 85% to 90% of the respective AL subjects (Supplemental Table 1). This weight restriction range is similar to studies that use CR to increase motivation to engage in operant conditioning (32, 33).

Tissue collection

All animals were euthanized at approximately postnatal day 120. On the day of euthanasia, CR rats were fed between 11 AM and 1:30 PM and were euthanized 3 hours later (2 PM to 4:30 PM). Animals were removed from the colony room, exposed to isoflurane until they were no longer responsive to a toe or tail pinch, and were euthanized via rapid decapitation. Trunk blood was collected and placed on dry ice within 3 minutes of removing the animal from the colony room. Whole blood (hereafter referred to as "blood") was stored at –80°C until processing for steroid analysis. Blood (rather than serum) was examined because T and CORT levels in serum overestimate T and CORT levels in circulation (5, 34).

Brains were rapidly extracted, snap frozen on crushed dry ice, and stored at –80°C until sectioning. Brains were sectioned in 300-μm coronal sections using a MicroHM525 cryostatic microtome (Thermo Fisher Scientific Inc., Waltham, MA) at –14°C and mounted on microscope slides. Frozen brain regions were microdissected using the Palkovits punch technique with a brain punch tool (0.94-mm cannula inner diameter; 0.208 mg/punch; Stoelting Co., Wood Dale, IL) (34, 35). Microdissected punches for steroid analysis were collected from a randomly selected hemisphere in each animal, and punches for gene expression analysis were collected from the contralateral hemisphere. Brain regions included the medial preoptic area/medial hypothalamus (POA/HYP), dorsal HPC, medial VTA, NAc (shell/core border), and mPFC (infralimbic/prelimbic border). The POA/HYP and HPC were considered "positive controls" as the presence of steroidogenic enzymes within these regions is well established (8, 36). Microdissected unilateral brain punches of the POA/HYP (wet weight: 3.48 ± 0.04 mg), HPC (3.64 ± 0.67 mg), VTA (1.62 ± 0.02 mg), NAc (1.59 ± 0.03 mg), and mPFC (1.70 ± 0.02 mg) as well as blood (2 μL) were placed in 2-mL polypropylene microtubes (Sarstedt AG & Co., Nümbrecht, Germany) and stored at –80°C until steroid extraction. Sections were counterstained using toluidine blue, and placement of microdissected extracts was verified microscopically (Supplemental Fig. 2).

Sample preparation for steroid analysis

To track recovery for each sample, we added 50 μL deuterated internal standards (corticosterone-d_8, DHEA-d_6, 17β-estradiol-d_4, testosterone-d_5; C/D/N Isotopes Inc., Pointe-Claire, Canada) in 50:50 high-performance liquid chromatography (HPLC)–grade methanol:HPLC-grade H_2O diluted from a stock solution to each sample. Steroids were extracted by adding 500 μL acetonitrile with 0.01% formic acid. Three zirconium ceramic oxide beads (1.4-mm diameter) were added to each microtube, and tissue was homogenized using a bead mill homogenizer at 5 m/s for 30 seconds (Omni International Inc., Kennesaw, GA). Proteins were precipitated by a 1-hour incubation at –20°C. Samples were centrifuged for 5 minutes (16,100×g), and 400 μL of the supernatant was transferred to a clean glass vial. Then, 400 μL acetonitrile with 0.01% formic acid was added to the remaining sample, vortexed (~5 seconds), and centrifuged as before. Again, 400 μL of the supernatant was transferred to the glass vial containing the initial 400 μL. Samples were dried in a vacuum centrifuge (60°C for 30 minutes; ThermoElectron SPD111V). The pellets were reconstituted with 0.5 mL HPLC-grade methanol.

Samples were run in tandem with blanks and calibration curves. Calibration curves made from certified reference standards of T, CORT, E_2, and DHEA (Cerilliant Co., Round Rock, TX) were prepared in the extraction solvent. The calibration curve range was 0.2 to 1000 pg for T, 0.4 to 1000 pg for E_2 and CORT, and 8 to 10,000 pg for DHEA (Fig. 2). Steroids were extracted using solid phase extraction with C_{18} columns (Agilent, Santa Clara, CA; catalog no. 12113045) using a method adapted from Weisser *et al.* (37). The sorbent was conditioned with 3 mL HPLC-grade hexane and then 3 mL HPLC-grade acetone and equilibrated with 3 mL HPLC-grade methanol. Samples were loaded onto the column, eluted, and collected. Any remaining steroids in the column were eluted with 2 mL HPLC-grade methanol and collected. Samples were vacuum dried as previously stated.

Steroid analysis by LC-MS/MS

Dried eluates were resuspended in 50 μL 50% HPLC-grade methanol in MilliQ water and transferred into a refrigerated autoinjector (15°C). Then, 45 μL of resuspended sample was injected into a Nexera X2 UHPLC system (Shimadzu Corp., Kyoto, Japan), passed through a Poroshell 120 HPH C_{18} guard column (2.1 mm) and separated on a Poroshell 120 HPH C_{18} column (2.1 × 50 mm; 2.7 μm; at 40°C) using

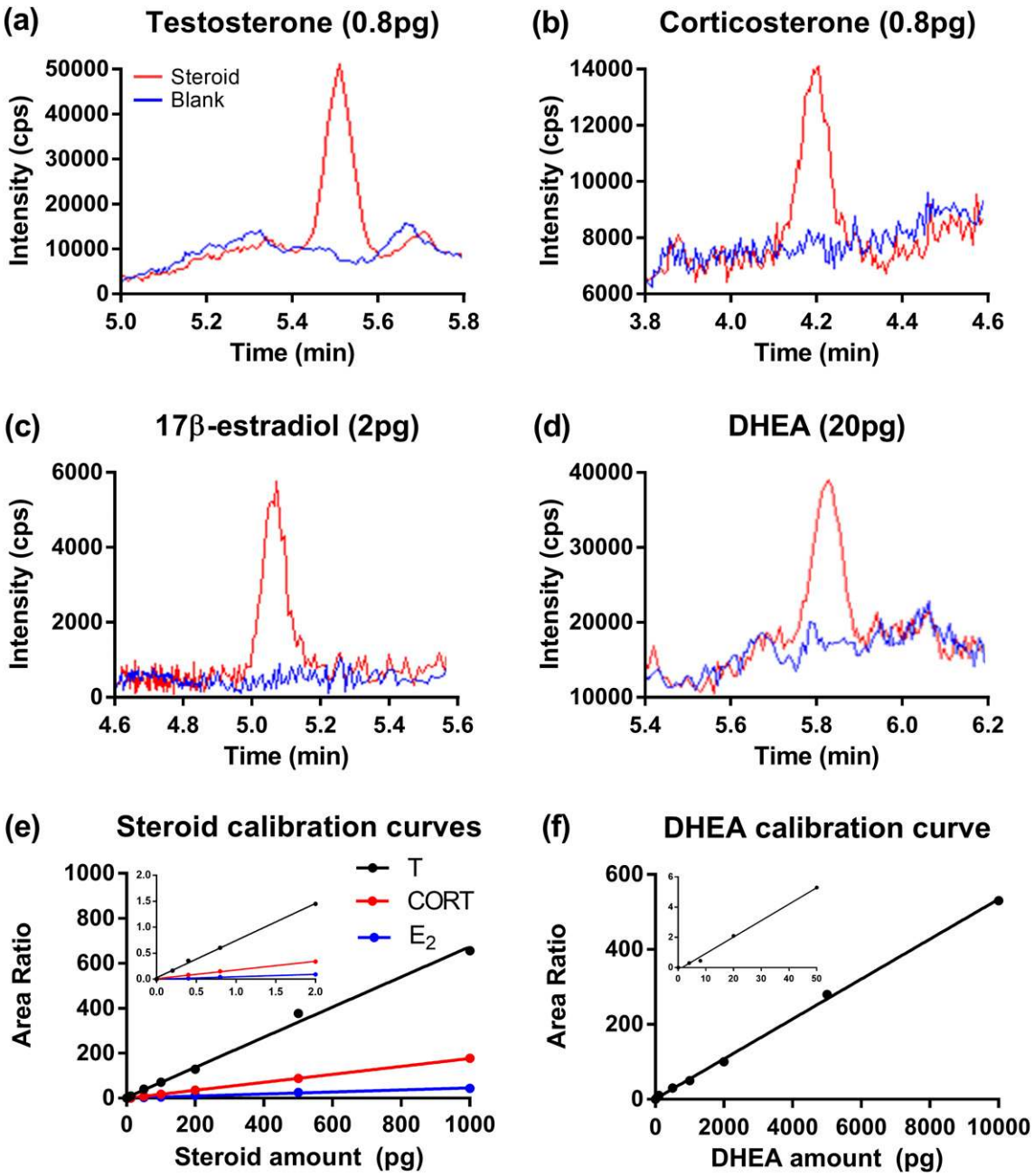

Figure 2. Detection of low levels of neuroactive steroids by LC-MS/MS. (a–d) Representative chromatograms of the steroid (red) over blank (blue) in a neat solution. Calibration curves of (e) T, CORT, and E_2, and of (f) DHEA, with insets displaying the lowest standards on the curve. The area ratio is calculated by dividing an analyte peak area with its deuterated internal standard peak area in the same sample. Calibration curve range was 0.2 to 1000 pg for T, 0.4 to 1000 pg for E_2 and CORT, and 8 to 10,000 pg for DHEA. Samples were set to 0 if they were below the lowest standard on the calibration curve. cps, counts per second.

0.1 mM ammonium fluoride in MilliQ water as mobile phase A and HPLC-grade methanol as mobile phase B (MPB). The flow rate was 0.4 mL/min. During loading, MPB was at 10% for 0.6 minutes, then began the gradient profile at 40% MPB, which was ramped to 60% MPB in 5.4 minutes, and finally a column wash at 98% MPB for 1.6 minutes. The MPB was then returned to starting conditions for 1.3 minutes. Total run time was 9 minutes. The needle was rinsed externally before and after each sample injection with 100% isopropanol.

Steroids were detected with scheduled multiple reaction monitoring with two multiple reaction monitoring transitions each for CORT, T, DHEA, and E_2, and one scheduled multiple reaction monitoring transition for each internal standard (Supplemental Table 2). Steroid concentrations were acquired on an AB Sciex 6500 Qtrap triple quadrupole tandem mass spectrometer (AB Sciex LLC, Framingham, MA) in positive electrospray ionization mode for CORT, T, and DHEA and in negative electrospray ionization mode for E_2. All water blanks were below the lowest standard on the calibration curve. Assay linearity and matrix effects are presented in Supplemental Table 3. Assay accuracy and precision are in Supplemental Table 4.

Real-time quantitative polymerase chain reaction

Total RNA was extracted from microdissected brain tissue using TRIzol (Life Technologies Corporation, Carlsbad, CA). With the exception of the addition of glycogen (2 μL) to the isopropanol RNA precipitation step and a 1-hour incubation in isopropanol at –20°C to enhance RNA precipitation, samples were extracted as per the manufacturer's instructions. RNA quality and quantity were determined with a NanoDrop 2000 spectrophotometer (Thermo Fisher Scientific Inc., Waltham, MA). Complementary DNA was reverse transcribed from 300 to 600 ng of total RNA with a High-Capacity cDNA Reverse Transcription Kit (Applied Biosystems, Foster City, CA).

Relative expression levels of reference genes and genes of interest were assessed using 5′-nuclease probe-based quantitative polymerase chain reaction assays (Integrated DNA Technologies, Inc., Coralville, IA). The quantitative polymerase chain reaction was performed using PrimeTime® gene expression master mix (Integrated DNA Technologies, Inc.) and run at 50°C for 2 minutes and 95°C for 10 minutes, followed by 40 cycles of 95°C (15 seconds) to 60°C (1 minute) on an ABI Prism 7000 Sequence Detection System (Applied Biosystems, Foster City, CA) with ROX as a reference dye. All samples were run in triplicate. Three reference genes (*Hprt1*, *Actb*, and *Ppia*) were measured in each sample, and their geometric mean was used as the reference cycle of quantification (Cq; Supplemental Table 5). The use of at least three reference genes greatly increases the reliability of relative expression quantification (38). The geometric mean of the reference genes was not influenced by the experimental manipulations in any brain region. Relative expression of the genes of interest (*Cyp17a1*, *Hsd3b1*, *Cyp19a1*, and *Ar*; Supplemental Table 5) was calculated and graphed to be relative to the expression levels of the 2-week/SHAM/AL group. Amplification efficiencies of all primers were comparable (88.9% to 108.4%). Negative controls (no template, no reverse transcription) confirmed the specificity of the assays.

Data analyses

For steroid analyses, a value was considered nondetectable if it was below the lowest standard on the calibration curve and was set to 0. To make a comparison between tissue types, we assumed that 1 mL of blood weighs 1 g (34, 39).

For gene expression data, the geometric mean of the three reference genes was used to calculate the ΔCq for the reference genes (38). Statistics were run using the $\Delta\Delta$Cq, whereas the graphs are presented as relative expression levels (relative to the 2-week/SHAM/AL group) using the $2^{-\Delta\Delta Cq}$ transformation to aid in visual interpretation (40). Given the very low levels of *Hsd3b1* in the brain regions examined (Cq = 37 to 40), the transcript was considered detectable if two of three technical replicates had a quantifiable amplification curve. For all other transcripts, all technical replicates were quantifiable. Relative expression of genes of interest in different brain regions are graphed relative to *Cyp17a1* in the POA/HYP, because *Cyp17a1* had the lowest and consistently detectable expression levels and the POA/HYP is a "positive control" region here (Supplemental Fig. 3).

A three-way analysis of variance was used to examine the main effects of surgery, food, time, and second- and third-order interactions. Second-order interactions were evaluated in the absence of a significant third-order interaction. *Post hoc* tests were performed using a general linear hypothesis test with Tukey contrasts in the presence of third- or second-order interactions. Significance criterion was set at $\alpha \leq 0.05$. Analyses were performed on log-transformed data if the nontransformed data did not exhibit homoscedasticity. Bivariate correlation coefficients with one-tailed tests of significance were calculated to determine the relationship between steroid concentrations and steroidogenic enzymes and *Ar* mRNA expression. All graphs are presented using the nontransformed data. All statistics were conducted using IBM SPSS Statistics for Windows version 23 (IBM Corp., Armonk, NY), GraphPad Prism version 6.00 (GraphPad Software, La Jolla, CA), and R version 3.3.2 (2016-10-31; Sincere Pumpkin Patch).

Results

GDX and CR attenuated body mass gain

At time of euthanasia, all CR groups weighed 84% to 90% of the matched AL group (Supplemental Table 1). GDX also attenuated increases in body mass in the AL groups relative to the SHAM/AL groups, which was evident within 1 week after surgery.

T levels in blood and brain regions

T was always nondetectable in the blood after GDX (2 or 6 weeks). Moreover, there was a significant surgery × food interaction [$F_{1,71}$ = 5.563; P = 0.021; Fig. 3(a)]; blood T levels were lower in SHAM/CR vs SHAM/AL subjects at 6 weeks ($P < 0.01$). Importantly, in GDX subjects, T levels in all brain regions were significantly lower, but still detectable, irrespective of time. In the POA/HYP and HPC, there were significant surgery × food interactions [POA/HYP: $F_{1,68}$ = 5.587, P = 0.021; HPC: $F_{1,68}$ = 9.214, P = 0.003; Fig. 3(b) and 3(c)]. Specifically, GDX significantly decreased T at both time points and CR significantly decreased T at both time points in the SHAM subjects.

In mesocorticolimbic nodes, in both SHAM and GDX subjects, T levels were higher in the brain than the blood, irrespective of time. In the VTA, there was a significant surgery × food interaction [$F_{1,69}$ = 12.721; P = 0.001; Fig. 3(d)]. In AL subjects, GDX significantly decreased T at both time points ($P < 0.01$) and CR significantly decreased T in SHAM rats at 6 weeks ($P < 0.001$). In SHAM/AL subjects, T concentrations were highest in the VTA compared with all other brain regions. In the NAc, GDX decreased T levels [$F_{1,68}$ = 32.581; $P < 0.001$; Fig. 3 (e)], but there were no main effects of food or time or an interaction. Finally, in the mPFC, GDX decreased T levels [$F_{1,67}$ = 24.458; $P < 0.001$; Fig. 3(f)], and CR decreased T levels ($F_{1,67}$ = 6.214; P = 0.015).

Not all GDX animals had detectable T in every brain region. The percentage of GDX subjects with detectable T in various brain regions ranged from 22.2% to 60.0% with a mean of 41.0% ± 2.75% (Supplemental Table 6). Analyses of *only* samples that had detectable T indicated

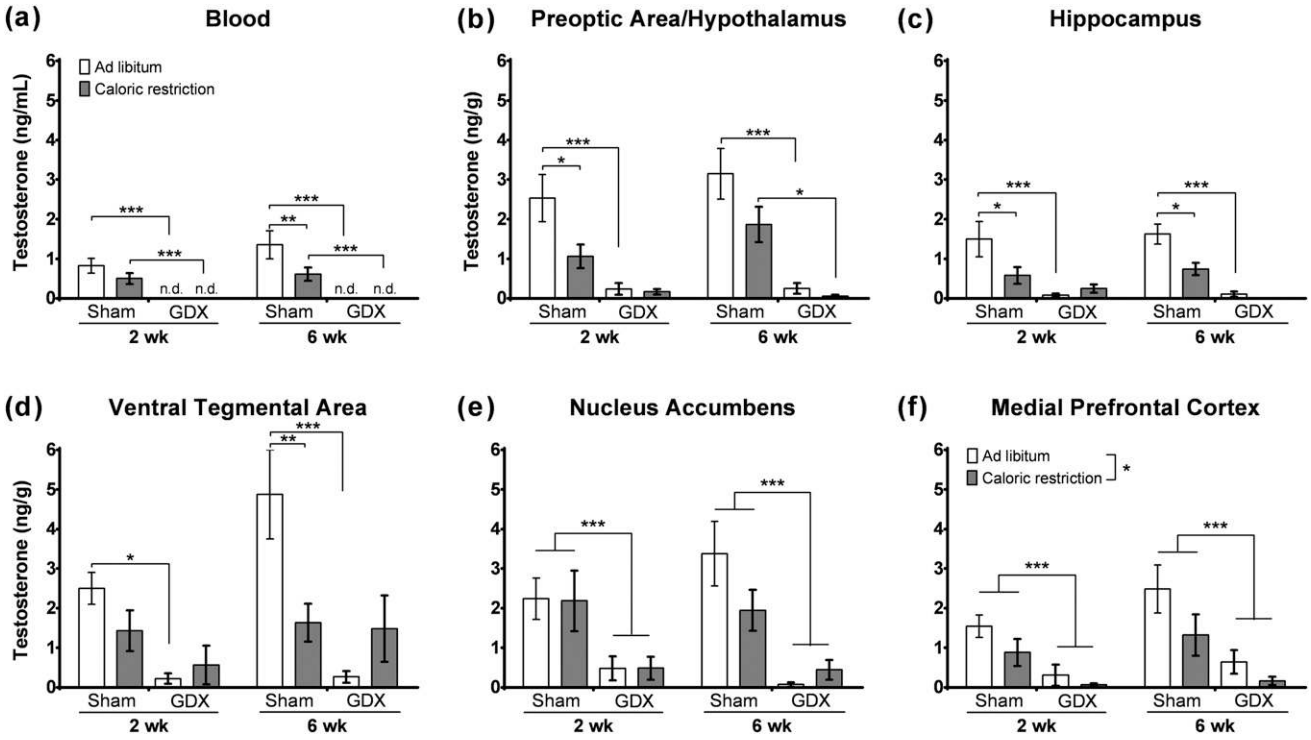

Figure 3. The effects of GDX and CR for 2 weeks or 6 weeks on T concentrations as quantified by LC-MS/MS. Bar graphs representing T concentrations in (a) the whole blood (ng/mL) and brain regions (ng/g), which include the (b) POA/HYP, (c) dorsal HPC, (d) VTA, (e) shell/core border of the NAc, and (f) mPFC. Values are expressed as mean ± standard error of the mean; $*P < 0.05$, $**P < 0.01$, $***P < 0.001$. n.d., not detectable.

that, as expected, GDX decreased T levels in the POA/HYP ($F_{1,48}$ = 20.689; $P < 0.001$), HPC ($F_{1,44}$ = 11.803; P = 0.001), and mPFC ($F_{1,49}$ = 17.959; $P < 0.001$). In the NAc, GDX decreased T levels in AL subjects but not CR subjects (surgery × food: $F_{1,45}$ = 5.229; P = 0.017). In VTA, there was no effect of GDX ($F_{1,49}$ = 2.098; P = 0.154).

In SHAM subjects only, T levels in the brain vs the blood were also examined (hereafter referred to as the T-ratio) (Fig. 4). GDX subjects were excluded from this analysis because blood T levels were always nondetectable. A ratio of >1 of brain T/blood T indicates higher T levels in the brain. All regions had a T-ratio > 1, with the highest T-ratio found in the NAc of the SHAM/CR groups [Fig. 4(d)] and the lowest T-ratio in the HPC [Fig. 4(b)]. In the VTA, there was a trend for CR to decrease the T-ratio [$F_{1,34}$ = 4.010; P = 0.053; Fig. 4(c)], whereas in the NAc, there was a trend for CR to increase the T-ratio [$F_{1,35}$ = 2.893; P = 0.098; Fig. 4(d)]. There were no effects of time or food on T-ratios in the POA/HYP, HPC, or mPFC [Fig. 4(a), 4(b), and 4(e)].

CORT levels in blood and brain regions

CORT was 3 to 10 times higher in the blood than in the brain, depending on the region (Fig. 5). In the blood, there was a trend for CR to decrease CORT levels [$F_{1,75}$ = 3.648; P = 0.060; Fig. 5(a)]. In the brain, GDX significantly

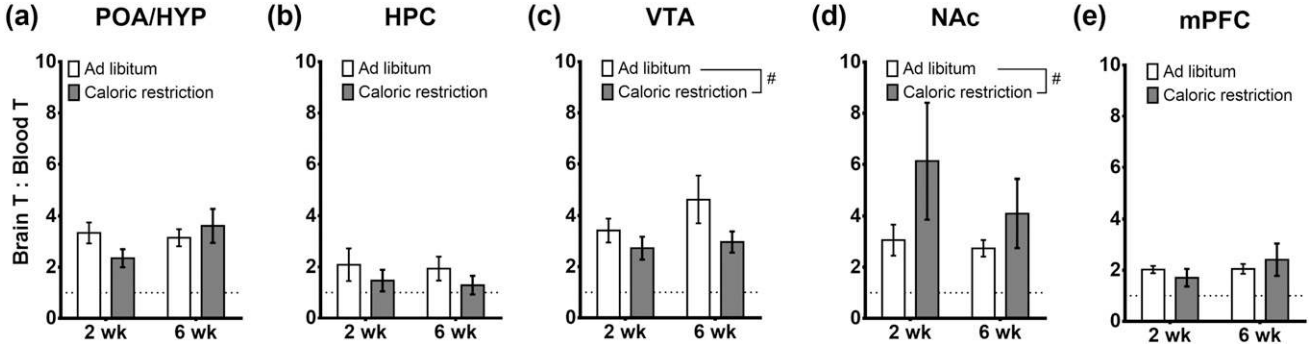

Figure 4. In SHAM subjects, T levels were higher in the brain than the whole blood (T ratio > 1) in the (a) POA/HYP, (b) HPC, (c) VTA, (d) NAc, and (e) mPFC. GDX subjects were excluded from the analysis because blood T levels were nondetectable. Values are expressed as mean ± standard error of the mean; $^{\#}P < 0.10$.

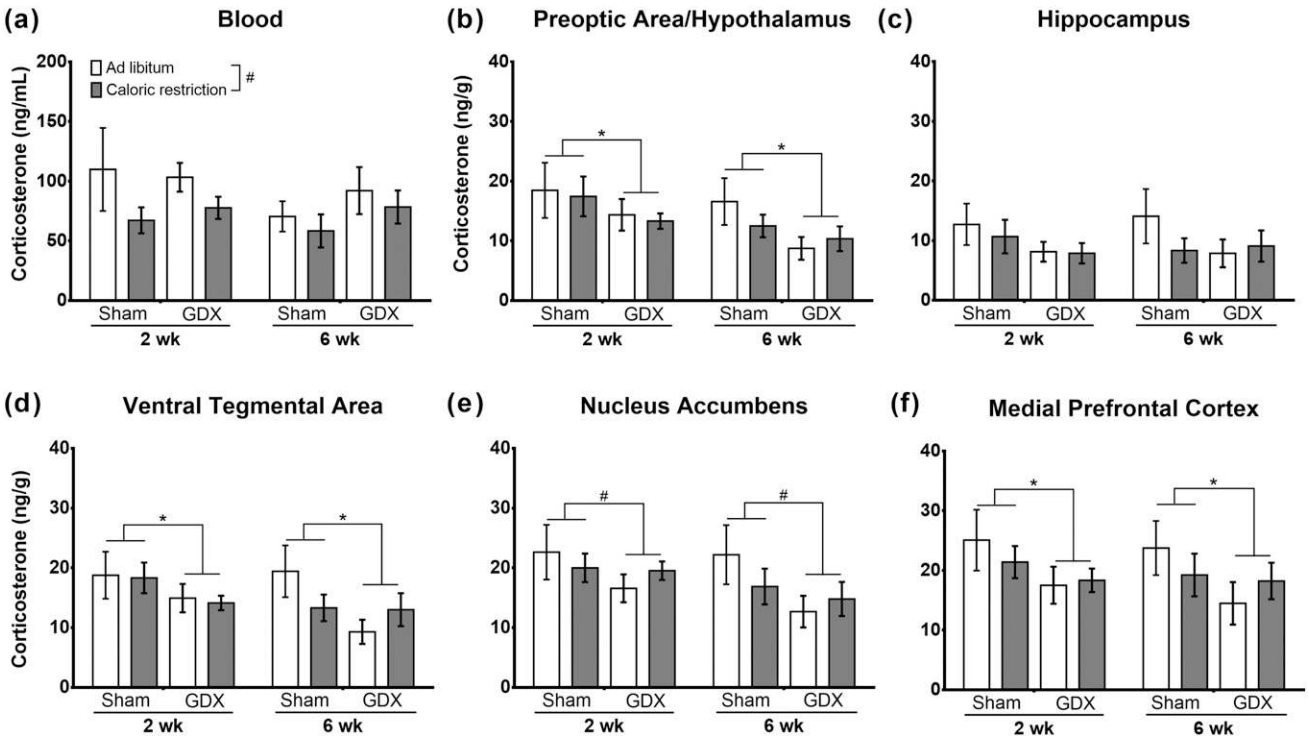

Figure 5. The effects of GDX and CR for 2 weeks or 6 weeks on CORT concentrations as quantified by LC-MS/MS. Bar graphs representing CORT concentrations in the (a) whole blood (ng/mL) and brain regions (ng/g), which include the (a) POA/HYP, (b) dorsal HPC, (c) VTA, (d) NAc, and (e) mPFC. Values are expressed as mean ± standard error of the mean; $^{*}P < 0.05$, $^{\#}P \leq 0.06$.

decreased CORT in the POA/HYP [$F_{1,70}$ = 4.789; P = 0.032; Fig. 5(b)], VTA [$F_{1,70}$ = 5.387; P = 0.023; Fig. 5(d)], and mPFC [$F_{1,68}$ = 4.081; P = 0.047; Fig. 5(f)]. There was also a trend for GDX to decrease CORT in the NAc [$F_{1,70}$ = 3.867; P = 0.053; Fig. 5(e)]. There were no effects of time on CORT concentrations.

GDX decreased the CORT-ratio (brain CORT/blood CORT) in the POA/HYP [$F_{1,70}$ = 37.047; $P < 0.001$; Fig. 6(a)], HPC [$F_{1,68}$ = 10.301; P = 0.002; Fig. 7(b)], NAc [$F_{1,70}$ = 29.660; $P < 0.001$; Fig. 6(d)], and mPFC [$F_{1,68}$ = 36.991; $P < 0.001$; Fig. 6(e)]. In contrast, CR increased the CORT-ratio in the POA/HYP [$F_{1,70}$ = 10.232; P = 0.002; Fig. 6(a)] and NAc [$F_{1,70}$ = 10.569; P = 0.002; Fig. 6(d)]. In the VTA, there was a significant surgery × food interaction [$F_{1,70}$ = 5.421; P = 0.023; Fig. 6(c)] on the CORT-ratio.

E_2 and DHEA were not detected in the blood or brain

E_2 was nondetectable in the blood, HPC, VTA, NAc, and mPFC. In the POA/HYP, only 3 of 10 subjects in one group (6-week/SHAM/AL) had detectable E_2. DHEA was not detectable in the blood or brain of any subject.

Cyp17a1 transcripts were decreased in the VTA after GDX and CR

Cyp17a1 messenger RNA (mRNA) was present in all brain regions [Fig. 7(a)]. In the POA/HYP, CR significantly decreased *Cyp17a1* mRNA [F(1,66) = 4.646; P = 0.035]. In the VTA, GDX significantly decreased *Cyp17a1* expression ($F_{1,69}$ = 8.975; P = 0.004) and there was a trend for a surgery × food interaction ($F_{1,66}$ = 3.818; P = 0.055), which was driven by a decrease in *Cyp17a1* mRNA in the 2-week/GDX/CR group. *Cyp17a1* expression in other brain regions was not affected by surgery, food, or time.

Hsd3b1 transcripts were detectable in the POA/HYP and VTA, and levels were unaffected by experimental treatments

Hsd3b1 mRNA was detectable in 64.0% of POA/HYP samples and 89.7% of VTA samples, but not detectable in the NAc or mPFC [Fig. 7(b)]. In the POA/HYP, there was a trend towards a surgery × food interaction [F(1,41) = 3.472; P = 0.070]. There were no significant main effects of surgery, food, or time or interactions.

Cyp19a1 transcripts decreased in response to GDX in the POA/HYP and increased after long-term GDX and CR in the mPFC

Cyp19a1 mRNA was present in all brain regions. In the POA/HYP, GDX decreased *Cyp19a1* mRNA [$F_{1,66}$ = 24.726; $P < 0.001$; Fig. 7(c)]. In the mPFC, there was a food × time interaction ($F_{1,70}$ = 8.496; P = 0.005). No changes in *Cyp19a1* mRNA levels in the VTA and NAc were detected.

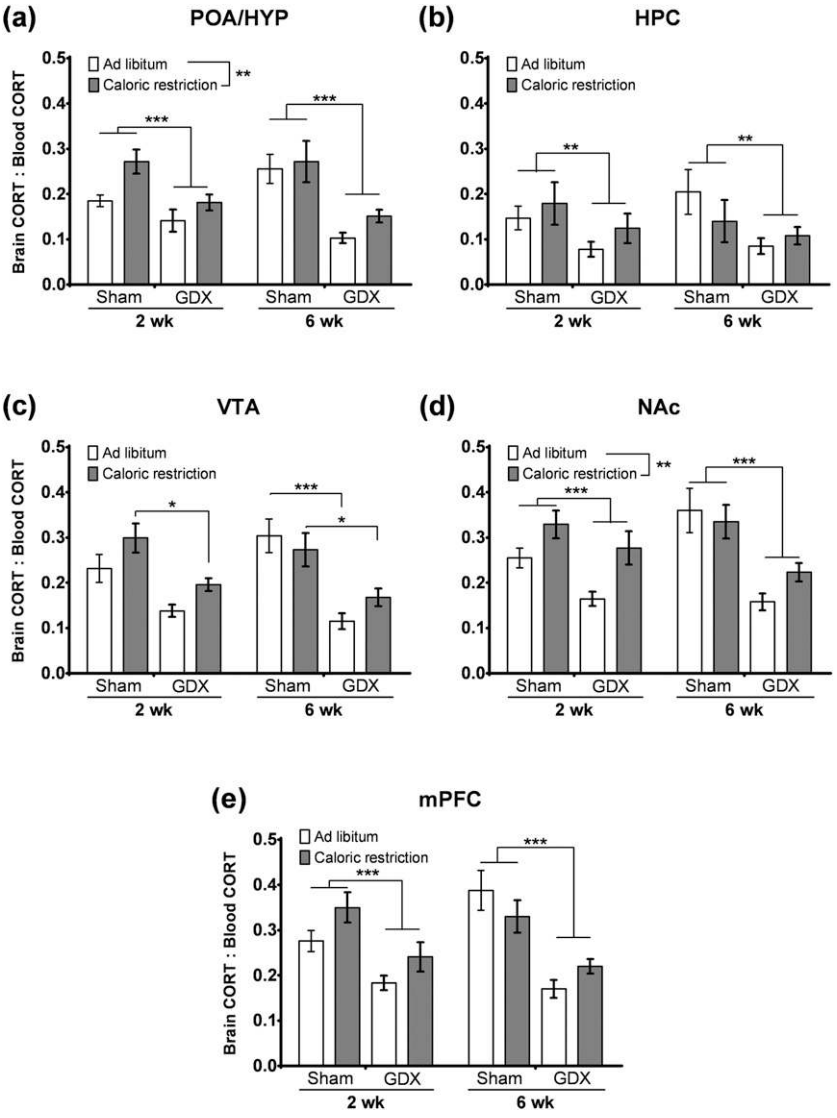

Figure 6. CORT levels were significantly lower in the brain than in the whole blood (CORT ratio <1). (a–e) Bar graphs representing the effects of GDX and CR on the CORT ratio in the (a) POA/HYP, (b) HPC, (c) VTA, (d) NAc, and (e) mPFC. Values are expressed as mean ± standard error of the mean; $*P < 0.05$, $**P < 0.01$, $***P < 0.001$.

Ar transcripts were decreased by GDX in the POA/HYP and increased in the NAc in response to long-term CR

As expected, GDX decreased *Ar* mRNA in the POA/HYP [$F_{1,65} = 10.352$; $P = 0.002$; Fig. 8(a)]. GDX did not affect *Ar* mRNA levels in mesocorticolimbic regions [Fig. 8(b)–8(d)]. In the NAc, CR increased *Ar* mRNA [$F_{1,68} = 7.420$; $P = 0.008$; Fig. 8(c)].

Brain T levels were correlated with blood T, *Hsd3b1*, and *Ar*

Correlations were run on SHAM subjects and GDX subjects independently because T was not detectable in the blood of GDX subjects (Supplemental Table 7). In SHAM subjects, T levels in the POA/HYP, VTA, NAc, and mPFC were significantly correlated with blood T levels [POA/HYP: $r_{(36)} = 0.782$, $P < 0.001$; VTA: $r_{(36)} = 0.807$, $P < 0.001$; NAc: $r_{(37)} = 0.576$, $P < 0.001$; mPFC: $r_{(36)} = 0.800$, $P < 0.001$]. In contrast, T levels in the HPC showed a trend to be correlated with blood T levels [$r_{(35)} = 0.274$, $P = 0.051$]. Expression of steroidogenic enzymes and *Ar* did not correlate with brain T levels, except in the VTA and NAc. In the VTA, T levels showed a trend to be correlated with both *Cyp19a1* mRNA [$r_{(35)} = 0.229$, $P = 0.087$] and *Ar* mRNA [$r_{(35)} = 0.256$, $P = 0.063$]. In the NAc, T levels were significantly correlated with *Ar* mRNA [$r_{(35)} = 0.424$, $P = 0.004$].

In GDX subjects, T levels in the VTA were significantly correlated with *Hsd3b1* mRNA [$r_{(31)} = 0.316$, $P = 0.004$]. Moreover, POA/HYP T trended toward a correlation with *Ar* mRNA [$r_{(35)} = 0.240$, $P = 0.077$].

Brain CORT levels were correlated with blood CORT levels

CORT levels in all regions were correlated with blood CORT levels [POA/HYP: $r_{(76)} = 0.718$, $P < 0.001$; HPC: $r_{(74)} = 0.553$, $P < 0.001$; VTA: $r_{(76)} = 0.651$, $P < 0.001$; NAc: $r_{(76)} = 0.680$, $P < 0.001$; mPFC: $r_{(73)} = 0.724$, $P < 0.001$; Supplemental Table 8].

Discussion

The current study provides unprecedented spatial resolution of T and CORT levels in brain regions that regulate motivated behaviors and indicates that these regions actively regulate local T and CORT levels. First, in SHAM subjects, T levels are higher in the brain compared with the blood, particularly in the VTA and NAc, and CORT levels are lower in the brain compared with the blood. Second, in GDX subjects, T is present in the brain but not in the blood 6 weeks after GDX. When examining only samples with detectable T, the levels of T were comparable in the VTA of SHAM and GDX subjects. Third, androgenic enzymes are expressed in the mesocorticolimbic nodes. Fourth, mild CR, which is often used to invigorate motivated behaviors, profoundly affects T and CORT levels in the mesocorticolimbic system of SHAM subjects. Taken together, these data provide fundamental insights into local steroid regulation in the mesocorticolimbic system.

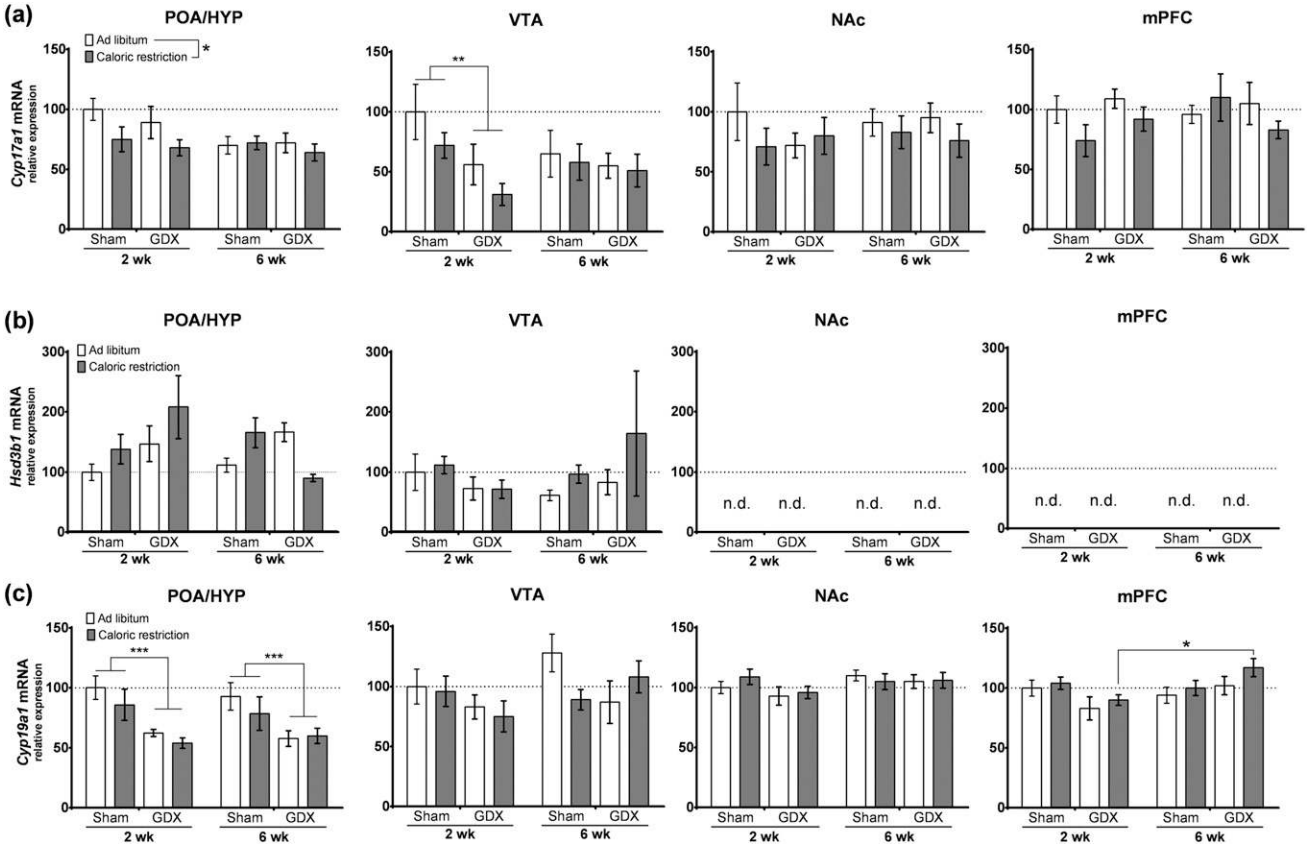

Figure 7. Relative expression of steroidogenic enzyme mRNA in the POA/HYP and mesocorticolimbic system. Bar graphs representing (a) *Cyp17a1* mRNA, (b) *Hsd3b1* mRNA, and (c) *Cyp19a1* mRNA expression in the POA/HYP, VTA, NAc, and mPFC. *Hsd3b1* mRNA was not detectable (n.d.) in the NAc or mPFC. Values are expressed as mean ± standard error of the mean relative to the 2-week/SHAM/AL group; $*P < 0.05$, $***P < 0.001$.

Development of steroid extraction and quantification protocols

The unique steroid extraction and LC-MS/MS protocols developed here enabled the precise and accurate measurement of T and CORT in 2 μL of blood and 1 to 3 mg of tissue from discrete mesocorticolimbic nodes, even after GDX. This is the first study in rodents, to our knowledge, to measure T in such small amounts of microdissected brain tissue. The steroid extraction method is simple, effective, consistent, and rapid, whereas other protocols have often been more complicated (41, 42). Moreover, this extraction method limits sample exposure to water, which can lead to auto-oxidation of cholesterol into DHEA (7). This method also limits contact with plastics, which can introduce interference or adsorb steroids (34, 43). Finally, the extraction method also effectively reduces brain lipids and thus decreases matrix effects.

After extraction, the LC-MS/MS protocol provided specific, sensitive, accurate, and precise measurements of T and CORT. LC-MS/MS is generally equal to or better than radioimmunoassay for sensitivity (44), but has greater analytical specificity, accuracy, and precision (45), and allows for simultaneous measurement of multiple steroids within the same sample (46). For this reason, we were not only able to measure T, the steroid of primary interest here, but also CORT. This LC-MS/MS protocol was sensitive enough to measure very low levels of T and CORT without requiring steroid derivatization (47). Nevertheless, we were not able to detect DHEA or E_2, which have been measured in the brain using much larger tissue samples (4, 9, 41, 48). Current work is refining the measurement of DHEA and E_2, as well as adding other steroid analytes to the panel.

Local regulation of steroids in the mesocorticolimbic system

Studies across vertebrates indicate that local regulation of steroids occurs in a variety of tissues (5, 28, 49–51). Steroid levels in the brain can be locally regulated by *de novo* synthesis from cholesterol, synthesis from circulating precursors, inactivation into metabolites, or regeneration from circulating metabolites (39). The enzymes necessary for steroid synthesis are present in the brain (6, 23, 52). Local control of steroids is evolutionarily conserved and allows organisms to regulate specific cells or regions without exposing all cells to circulating steroids (53).

In the blood, as expected, GDX and CR decreased circulating levels of T (30, 54, 55). In contrast, the fact that CR trended to decrease blood CORT levels was

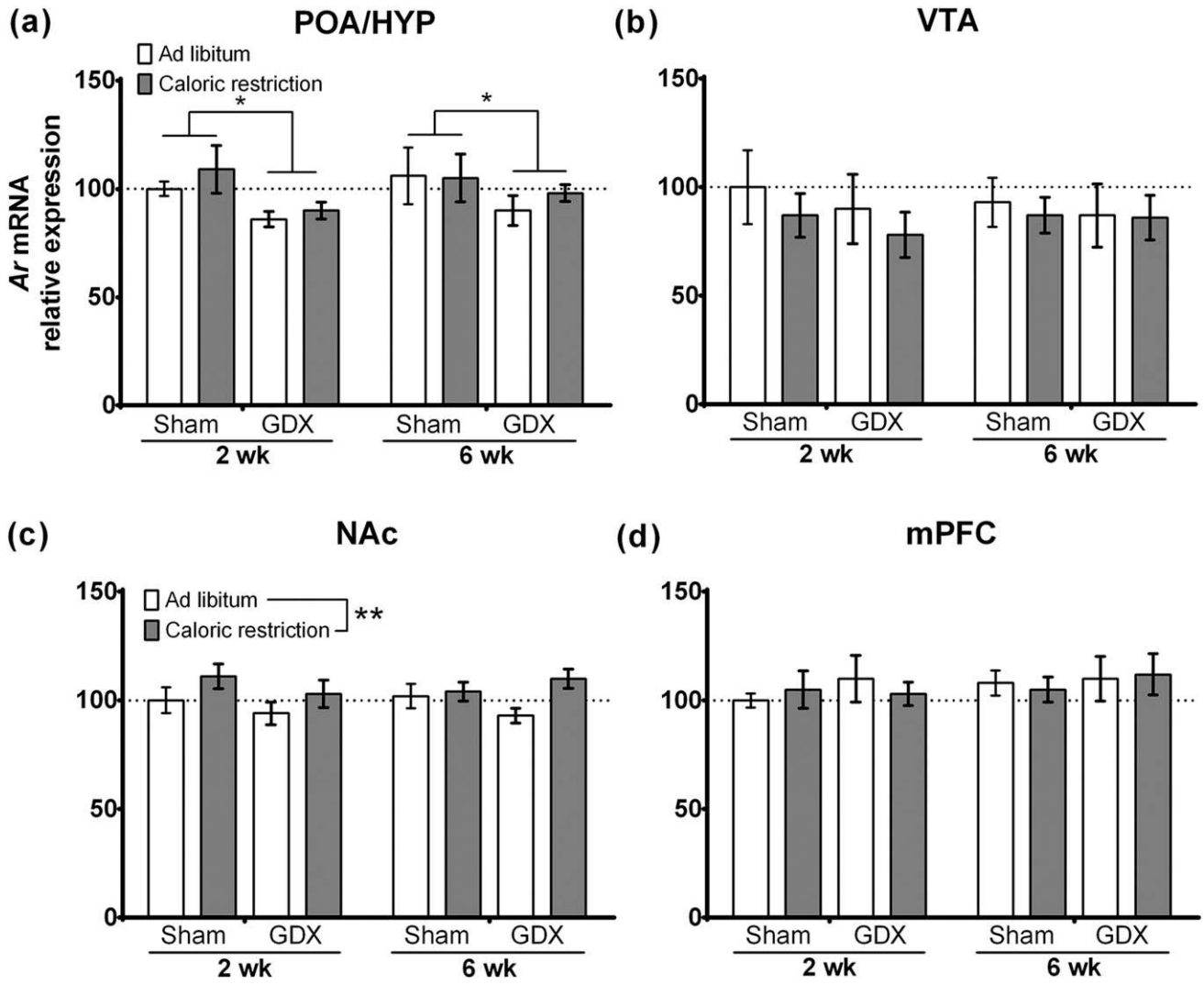

Figure 8. Bar graphs representing relative expression of *Ar* (androgen receptor) mRNA in the (a) POA/HYP, (b) VTA, (c) NAc, and (d) mPFC. Values are expressed as the mean ± standard error of the mean relative to the 2-week/SHAM/AL group; $*P < 0.05$, $**P < 0.01$.

surprising, because CR increases or has no effect on CORT levels in prior studies (56–58). Previous work has found that GDX does not have an effect on circulating basal or stress-induced CORT levels (58, 59), but other studies have shown that GDX increases (60) or decreases (61) CORT levels. These discrepancies may be due to differences in time of sample collection in relation to diurnal pulses (58), time of sample collection in relation to presentation of food (56), duration of GDX or CR (62), or severity of CR (63).

The effects of GDX and CR on brain levels of T and CORT, on the other hand, have not been thoroughly investigated. As expected, GDX clearly decreased T throughout the brain. This is consistent with previous studies that examined the effect of GDX on T levels in the blood and large brain samples (64, 65).

However, GDX did not completely eliminate brain T in any of the regions examined, even after 6 weeks (Fig. 3; Supplemental Table 6). In AL subjects, T levels were the highest in the VTA in both SHAM and GDX subjects, suggesting that T is preferentially sequestered or synthesized in the VTA [Fig. 3(d)]. This is remarkable considering that mean T levels in GDX subjects, which included the nondetectable samples set to 0, were within the range of SHAM subjects [see 2-week/GDX/CR and 6-week/GDX/CR subjects in Fig. 3(d)]. Furthermore, when examining only samples with detectable T, the T levels in the VTA were not significantly different in SHAM and GDX subjects, even after long-term GDX. This is contrary to what would be expected if all brain T were simply sequestered from the blood. Robel *et al.* (66) reported that T was nondetectable in the whole rat brain 3 days after GDX, which may not have been sufficient time for an upregulation of local synthesis. More recent studies were able to detect T in the HPC and frontal cortex 7 days or more after GDX (4, 9). This study is, to our knowledge,

the first to show that short- and long-term GDX does not eliminate T in the POA/HYP, VTA, or NAc, while providing greater spatial resolution for the frontal cortex (*i.e.*, mPFC). T was also higher in all brain regions than in the blood (T-ratio > 1), suggesting that T is synthesized in these regions in SHAM subjects. At the same time, brain T levels were strongly correlated with circulating T levels in SHAM subjects, except in the HPC. Brain T levels, therefore, likely reflect a combination of sequestration and synthesis in intact animals, with synthesis being the major driving force in GDX animals.

CR decreased T levels in the POA/HYP, HPC, VTA, and mPFC, but not in the NAc. Surprisingly, CR caused a more pronounced decrease in brain T levels in SHAM subjects than GDX subjects. T levels were also the same or higher in CR/GDX subjects than AL/GDX subjects in the HPC, VTA, and NAc. These results suggest that CR decreases circulating T levels, leading to lower T levels in mesocortical regions in SHAM subjects. In contrast, CR likely does not affect local T synthesis in these nodes.

Similar to T levels, CORT levels in the brain decreased in response to GDX. In particular, GDX decreased CORT in the POA/HYP, VTA, NAc, and mPFC. The strong correlation between brain CORT levels and blood CORT levels (Supplemental Table 8) suggests that these brain levels are mediated by circulating CORT. The ratio of brain-to-blood CORT levels gives a different perspective (Fig. 5). The low CORT-ratio (<1) suggests that a small amount of circulating CORT is transported into the brain, and thus not synthesized. However, either by regulating transportation, sequestration, or rapid metabolism, these brain regions seem to actively regulate CORT in response to GDX and CR. GDX decreased the CORT-ratio in all brain regions, but CR increased the CORT-ratio in the POA/HYP and NAc. Likewise, brain CORT levels decrease while plasma CORT levels increase in male mice in response to a stressor (67), but this is, to our knowledge, the first report to demonstrate that GDX decreases and CR increases CORT in the brain compared with the blood. Taken together, these data suggest that CORT is actively regulated, but not synthesized, in the brain.

Steroidogenic enzymes in the mesocorticolimbic system

To better understand the mechanisms of T synthesis in the mesocorticolimbic system, we examined androgenic enzymes and androgen receptors. Major enzymes responsible for T synthesis include cytochrome P450c17 (*Cyp17a1*), 3β–hydroxysteroid dehydrogenase (HSD) (*Hsd3b1*), and 17β-HSD, whereas aromatase (*Cyp19a1*) metabolizes T into E_2 (Fig. 1). The presence of androgenic enzymes in several brain regions has been established (6, 8, 23, 42, 52), but this is, to our knowledge, the first study to show that *Cyp19a1* is expressed in the rodent VTA and that *Cyp17a1* is expressed in the mesocorticolimbic system in adult male rodents. Transcripts for *Cyp17a1* and *Cyp19a1* were present in all brain regions examined, whereas *Hsd3b1* mRNA was not detected in the NAc or mPFC.

The prediction that long-term absence of circulating T would lead to an increase in androgenic enzyme levels in these regions was not supported. However, levels of enzyme transcripts were altered by both GDX and CR in a region-specific manner. In the VTA, GDX decreased *Cyp17a1* mRNA, but only in short-term CR subjects. Moreover, in the VTA of GDX subjects, the positive correlation between *Hsd3b1* mRNA and T suggests that this enzyme is a rate-limiting step in local T synthesis. The lack of other correlations between enzyme expression and neural T could be taken to suggest that the presence of T in these regions is due to sequestration rather than synthesis. Nevertheless, considering that many brain regions maintain measurable T long after GDX and depletion of systemic T, and that androgenic enzymes are present in the mesocorticolimbic system, it is likely that a large proportion of brain T in GDX subjects is explained by local synthesis. Gene transcript levels are clearly useful but do not give a complete picture of enzyme activity (68). Future studies can examine protein levels and enzyme activities in these brain regions.

Furthermore, steroid biosynthesis pathways can vary depending on tissue-specific enzyme expression, catalytic cofactor levels, and steroid substrate availability. It remains possible that the brain does not exclusively use the classical pathway for T synthesis (28) or that different brain regions use different pathways. For example, we did not detect *Hsd3b1* mRNA in the NAc or mPFC, but these regions might express the isoform *Hsd3b2* or convert circulating progesterone to androstenedione via P450c17.

Steroid regulation of the mesocorticolimbic pathway and functional implications

T and CORT can have dramatic effects on the mesocorticolimbic system, and these results will inform future studies that examine the roles of neurosteroids in motivated behaviors. For instance, microinjections of T or its metabolites into the NAc elicit conditioned place preference (26), which is attenuated by a dopamine receptor antagonist (69). In addition, GDX affects dopamine-synthesizing cells in the VTA and dopamine-sensitive cells in the NAc and mPFC (70). T is a modulatory hormone, and thus GDX will not always eliminate T-sensitive behaviors. For example, male consummatory sexual behavior (*i.e.*, ejaculation), a classic T-sensitive behavior mediated by the POA/HYP, is maintained in ~25% of GDX subjects (71). Male appetitive sexual

behaviors (*i.e.*, mounting and intromission) are also maintained in over 50% of GDX subjects at 6 weeks. The VTA and NAc directly mediate these appetitive sexual behaviors (72). These data raise the hypothesis that local T synthesis in the VTA or NAc modulates male appetitive sexual behavior after GDX.

The effects of GDX on behavior also depend on tissue sensitivity to T. In the current study, *Ar* transcripts were present in the mesocorticolimbic nodes, which is consistent with previous studies (6, 14, 23). GDX decreased *Ar* mRNA in the POA/HYP, as expected. In SHAM subjects, there was a significant negative correlation between *Ar* mRNA and T levels in the NAc. This negative correlation suggests that, in the NAc, T negatively feeds back on AR expression, limiting the effects that T has on the NAc. Moreover, the only region where CR affected *Ar* mRNA expression was in the NAc, whereby CR increased *Ar* mRNA. This increase in *Ar* mRNA in the NAc is important to note because the NAc was the only region that CR did not cause a significant decrease in T. Therefore, the NAc might compensate for the decrease in systemic T caused by CR, by both synthesizing T locally and increasing AR levels.

Even in mesocorticolimbic nodes that did not increase in *Ar* mRNA after GDX (*i.e.*, mPFC and VTA), the locally elevated levels of T may still have an influence on cells. T may be metabolized into E_2 and act through estrogen receptors (73) or may act through membrane AR (74). Given the extremely low levels of E_2 in the male rat brain and the large amount of tissue required for E_2 detection (22, 48, 75), it remains difficult to test this hypothesis in the discrete neural nodes that were examined in this study. Another possibility is that local T in the POA/HYP may indirectly influence the mesocorticolimbic system via T-sensitive projections to the VTA (16, 76). Thus, in GDX subjects, locally synthesized T may alter the mesocorticolimbic system via multiple mechanisms.

CORT also modulates the mesocorticolimbic system (77) and motivated behaviors (78). GDX decreased CORT in all brain regions except the HPC and decreased the CORT-ratio in all regions. The ability to maintain low levels of brain CORT is important, because chronic high levels of CORT can have detrimental effects on nerve cells (79). In contrast, transient increases in local CORT levels may promote foraging during food scarcity without increasing allostatic load on the rest of the brain. Here, CR increased the CORT-ratio in the VTA and NAc, but not in the mPFC. Limiting the increase to mesolimbic regions may increase ambulatory behavior (80) by increasing dopamine release in the NAc (12) without affecting regions important for higher-order functioning (*i.e.*, mPFC).

Conclusions

These data support the hypothesis that the mesocorticolimbic system regulates local levels of T and CORT. The unique LC-MS/MS protocol allows for sensitive and specific measurement of steroids in microdissected brain regions. Using this protocol, we were able to determine that many male rats continue to have detectable levels of brain T long after GDX, particularly within the VTA, NAc, and mPFC. Androgenic enzymes were also expressed in these regions, suggesting the ability to produce androgens *de novo* from cholesterol or from circulating precursors. In addition, a common CR regimen used to enhance instrumental learning differentially alters steroid levels in the blood and mesocorticolimbic system. Studies examining the activational effects of steroid hormones on motivation generally assume that steroid levels in the brain reflect steroid levels in the blood. However, these data indicate that this assumption may not be correct. Furthermore, these data provide a foundation for future behavioral studies that will examine the role of neurosteroids in the mesocorticolimbic system. These results have important implications for understanding steroid-sensitive diseases, including type 2 diabetes (36), Alzheimer's disease (81), depression (82), and substance abuse (83).

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