Steroid profiling reveals widespread local regulation of glucocorticoid levels during mouse development

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Glucocorticoids (GCs) are produced by the adrenal glands and circulate in the blood to coordinate organismal physiology. In addition, different tissues may independently regulate their local GC levels via local GC synthesis. Here, we find that in the mouse, endogenous GCs show tissue-specific developmental patterns, rather than mirroring GCs in the blood. Using solid-phase extraction, HPLC and specific immunoassays, we quantified endogenous steroids and found that in tissues of female and male mice, 1) local GC levels can be much higher than systemic GC levels, 2) local GCs follow different age-related patterns than systemic GCs, and 3) local GCs have different identities from systemic GCs. For example, while corticosterone is the predominant circulating adrenal GC in mice, high concentrations of cortisol were measured in neonatal thymus, bone marrow, and heart. The presence of cortisol was confirmed with liquid chromatography-tandem mass spectrometry (LC-MS/MS). In addition, gene expression of steroidogenic enzymes was detected across multiple tissues, consistent with local GC production. Our results demonstrate that local GCs can differ from GCs in circulating blood. This suggests that steroids are widely used as local (paracrine or autocrine) signals, in addition to their classical role as systemic (endocrine) signals. Local GC regulation may even be the norm, rather than the exception, especially during development.

Traditionally, endocrinology has focused on hormonal changes at the systemic level, in which endocrine organs secrete hormones into the circulating blood (1, 2). This results in regulation of whole-body hormone levels and functions to coordinate organismal physiology. More recently, a growing body of evidence has shown multiple mechanisms by which hormone-sensitive tissues may independently regulate their local hormone concentrations (3–5). However, the extent to which these mechanisms differentially affect specific tissues is not well understood.

Glucocorticoids (GCs) are a particularly interesting example to examine tissue-specific regulation. Under control of the hypothalamic-pituitary-adrenal (HPA) axis, circulating GCs have pleiotropic effects, orchestrating immune, cardiovascular, metabolic, and neural function (6). GCs act on nearly every cell of the body, and are critical effectors of development (7), homeostasis (6, 8), and disease (4, 9, 10). While GCs have varied effects on different cell and tissue types, these effects are widely thought to follow systemic GC patterns. As such, measurements of the predominant adrenal GC in the blood (eg, cortisol in humans, corticosterone in rats and mice) are widely used to understand how changes in systemic GC levels regulate these processes (1, 2, 11, 12).

Although GC levels in tissues are thought to parallel GC levels in the blood, a variety of tissues express cellular machinery by which local GC levels could be regulated independently of systemic GC levels. GC synthesis de novo from cholesterol may occur in extra-adrenal tissues, such as the thymus (3). Cultured murine thymus converts cholesterol into corticosterone, the predominant murine GC (13, 14), and this activity was especially high in thymi of

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Abbreviations:

young mice (13). Similarly, GC-synthetic activity was found in cultured chicken thymus and other lymphoid organs (15). Interestingly, while corticosterone is the predominant circulating avian GC, as in mice and rats, lymphoid organs of chickens synthesized cortisol, suggesting the possibility of distinct adrenal and extra-adrenal GCs (15). We measured glucocorticoid levels in songbirds and indeed found high cortisol (but not corticosterone) levels in developing lymphoid organs and low levels of both GCs in blood (16). We also found high local levels of upstream precursors, coinciding with high local levels of cortisol (Taves et al, unpublished data). Taken together, these data suggest that tissue-specific regulation of GCs occurs in vivo and that the identities of systemic and local GCs can differ. However, in mice, little is known regarding the local levels of GCs in vivo and their identities.

Here, we quantify endogenous GCs in a variety of GC-sensitive tissues to look for local GC regulation in vivo over murine development. In addition to measuring the predominant murine GC, corticosterone, we also quantify, cortisol and the precursors 11-deoxycorticosterone (deoxycorticosterone), 11-deoxycortisol (deoxycortisol), and progesterone, which can also bind mineralocorticoid and glucocorticoid receptors (MR and GR) (17–20). We also measured gene expression of steroidogenic enzymes using qPCR. Early development is especially well suited for examining local GC regulation. In altricial species, whose offspring are completely dependent on their parents, the adrenal glands undergo a period of quiescence in early neonatal life, resulting in low or nondetectable glucocorticoid levels in blood (the stress hyporesponsive period, SHRP) (21). The reduction in adrenal GCs is thought to promote neural development and body growth (7, 22), but might deprive other organs of GCs where they are beneficial, such as the thymus (23), heart (24), and liver (25). Thus, these and other developing organs are good candidates for examining locally elevated GC levels, especially during the SHRP.

Materials and Methods

Mice. Samples were collected from C57BL/6 mice at embryonic day 16.5 (E16.5) and postnatal days (PND) 1, 5, 15, and 60 (n = 8, 14, 12, 12, and 13, respectively), with PND0 defined as the first day pups were present in the cage. PND5 was specifically selected because this age is well within the SHRP, when the predominant murine adrenal GC, corticosterone, is extremely low in circulating blood (26). Mice were housed in a specific pathogen-free colony in the Wesbrook Animal Unit at the University of British Columbia, with corn cob bedding, under a 12:12 light: dark cycle, with free access to water and food (LabDiet 5021 for breeding parents, and LabDiet 5010 after weaning at PND20). Samples were collected in the morning (from 0900 to 1100 hours) to reduce diel variation in steroid levels. Protocols were approved by the UBC Animal Care Committee (A07–0417) and were in compliance with regulations established by the Canadian Council on Animal Care.

Tissue collection. Postnatal mice were deeply anesthetized with isoflurane delivered in oxygen (<1 minute) and euthanized by decapitation. Trunk blood was collected into two heparinized tubes and immediately placed on wet ice. Blood collection was completed within 3 minutes of initial disturbance (2.42 ± 0.05 minutes) to avoid any rise in circulating GC levels. Spleen, liver, thymus, and brain were dissected and immediately frozen on dry ice. The heart was briefly blotted to remove blood prior to freezing on dry ice. Femurs were isolated, ends cut off, and also frozen on dry ice. For collection of embryonic tissues, the pregnant dam was deeply anesthetized with isoflurane and euthanized, after which embryos were euthanized by decapitation and chilled on wet ice as tissues were collected. Spleen and bone marrow could not be obtained from embryos. Genomic DNA was extracted from tail clips and sex determined as previously described (27).

Steroid extraction. Steroids were extracted from all samples (plasma, whole blood, thymus, bone marrow, heart, liver, spleen, and brain) using solid phase extraction (SPE) with C_{18} columns as previously described (28, 29). Organs were weighed, homogenized in 84% methanol with a bead homogenizer (Omni Bead Ruptor), and supernatants were loaded onto SPE columns (Agilent #12113045). For PND1 mice, whole femurs containing marrow were weighed and homogenized, while marrow from older mice was flushed from femurs with ice-cold water before being weighed and homogenized. Samples were washed with 10 ml of 40% methanol to remove conjugated (glucuronidated and sulfated) steroids and interfering substances (30), and unconjugated steroids were eluted with 5 ml of 90% methanol and dried at 60°C in a vacuum centrifuge (ThermoElectron SPD111V).

Steroid separation. Samples were further processed using reversed-phase HPLC (Gilson 322) with a Waters SymmetryShield C_{18} column (4.6 × 250 mm, 5 μm silica particles) kept at 40°C with a column heater (Torrey Pines #CO20), using 30% acetonitrile, 0.01% formic acid in water as mobile phase A (MPA) and 100% acetonitrile, 0.01% formic acid as mobile phase (MPB). Dried steroid residues were resuspended in 475 μl MPA, centrifuged at 16 000 g for 5 minutes, and supernatants transferred to HPLC vials. 400 μl of each sample was loaded onto the HPLC column using an autoinjector (Gilson 234). The gradient profile started at 0% MPB for 20 minutes, ramped to 100% MPB over 25 minutes, returned to starting conditions in 0.5 minutes, and held for 15 minutes to re-equilibrate the column. The total run time was 60.5 minutes. Samples were eluted at a flow rate of 1.0 ml/min, and steroid fractions were obtained using 3-minute collection windows (Supplemental Figure 1) with a fraction collector (Gilson FC 204). We established elution times of other steroids to determine if they coeluted with our steroids of interest (Supplemental Table 1). Cortisone coeluted with cortisol, but this is highly unlikely to affect our cortisol measurements, due to minimal cross-reactivity of cortisone with our anticortisol antibody (0.13%, Supplemental Table 2). Fractions were dried at 60°C in a vacuum centrifuge.

Immunoassays. Steroids were measured in duplicate using specific and sensitive immunoassays. Assay details and specificities are given in Supplemental Table 2. Corticosterone was quantified using a radioimmunoassay (RIA) following the manufacturer's instructions. The lowest standard was further diluted to allow detection of lower corticosterone quantities (16). Cortisol quantification was performed by enzyme immunoassay (EIA) following the manufacturer's instructions (16). Deoxycorticosterone and deoxycortisol were each quantified using specific antibodies, steroid standards, and tritiated steroids with charcoal-dextran separation of free and bound steroids (18). Briefly, 100 μl of resuspended sample (with 2.5% ethanol to aid in resuspension) was added to 100 μl of assay buffer containing tracer (~6000 cpm) and antibody (antideoxycorticosterone, 1:14,000 final dilution; antideoxycortisol, 1:3333 final dilution). Tubes were incubated overnight at 4°C after which 500 μl of dextran-coated charcoal was added. Tubes were incubated for 15 minutes on ice and centrifuged at 1455 g for 12 minutes at 4°C. Supernatants were decanted, mixed with 5 ml of scintillant, and counted for 5 minutes in a scintillation counter. Progesterone was quantified using a double-antibody ^{125}I RIA that we modified to increase sensitivity. 60 μl of tracer was added to 390 μl of resuspended sample (with 1% ethanol to aid resuspension), followed by 250 μl of primary antibody. Samples were incubated for 1 hour in a 37°C water bath. 500 μl of precipitant (secondary antibody) was added, and tubes centrifuged at 1000 g for 20 minutes at 4°C. Supernatants were decanted, and pellet radioactivity counted for 1 minute in a gamma counter. Recovery was estimated by spiking tissue homogenates with known amounts of steroids and comparing these samples with unspiked samples from the same pools.

Liquid chromatography-tandem mass spectrometry (LC-MS/MS). Although we used a combination of SPE, HPLC separation with a long run time, and specific immunoassays, LC-MS/MS is useful for definitive steroid identification. We used a nonquantitative method with pooled samples (one pool of each tissue type, at each age) to optimize steroid detection. We spiked pools with deuterated internal standards (d4-cortisol, d8-corticosterone), and extracted and separated steroids with SPE and HPLC as described above. Dried HPLC fractions were resuspended in 150 μl of 22% Optima-grade methanol and transferred into a 96-well autoinjector sample plate. 50 μL of resuspended sample was injected into a Shimadzu Prominence LC20AC and separated on a Phenomenex Gemini NX-C_{18} column (100 × 2.1 mm, 3.5 μm) in a 55°C column oven using 2 mM ammonium acetate in water as mobile phase A (MPA), and 2 mM ammonium acetate in methanol as mobile phase B (MPB). The gradient profile started at 20% MPB for 1 minute, ramped to 70% MPB for 4 minutes, held for 1 minute, ramped to 90% MPB for 0.5 minutes, held for 1 minute, and returned to starting conditions in 0.1 minute and held for an additional 2.4 minutes to re-equilibrate the column. The total run time was 10 minutes. Steroids were detected with multiple reaction monitoring (MRM), with two MRM transitions each for corticosterone (m/z 347.4 → 121.0, m/z 347.4 → 97.1) and cortisol (m/z 363.4 → 121.1, m/z 363.4 → 97.1) and 1 MRM transition for each internal standard (corticosterone-d8 m/z 355.4 → 125.2, cortisol-d4 367.2 → 121.2), acquired on an AB SCIEX 5500Qtrap triple quadrupole tandem mass spectrometer in positive electrospray ionization mode. None of the endogenous steroids tested interfered with the LC-MS/MS cortisol assay (Supplemental Table 3). Product ion spectra for cortisol in a representative standard and sample were obtained by acquiring product ion scans on the cortisol parent (Q1) mass of 363.4, using the same liquid chromatography parameters as described for the MRM method.

Real-time quantitative PCR (qPCR). Tissue samples from PND5 and PND15 mice (n = 3 mice at each age) were collected and snap-frozen on dry ice as described above. Samples were then homogenized in ice-cold isol-RNA lysis reagent (5 Prime #2302700), and RNA was extracted with chloroform, precipitated with isopropanol, and washed with 75% ethanol. Next, cDNA was synthesized from 4 μg of total RNA using a Maxima First Strand synthesis kit (Thermo #K1641) according to the manufacturer's instructions. Then qPCR for gene expression of steroidogenic enzymes was performed using SYBR Green assays (using SsoFast EvaGreen supermix, Bio-Rad #172–5201) with previously published primers (31) (Supplemental Table 4). Assays were run at 95°C for 3 minutes, followed by 50 cycles of 95°C for 10 seconds, 60°C for 15 seconds on a Bio-Rad CFX96 real-time PCR system. Product specificity was examined by dissociation curve analysis and gel electrophoresis. For two genes, Cyp21a1 and Cyp11b1, SYBR Green assays gave nonspecific results, so we used instead previously validated 5′ nuclease probe-based assays (using Brilliant III Ultra-Fast QPCR master mix, Agilent #600880) to quantify expression of these genes (Supplemental Table 5). These assays were run at 95°C for 3 minutes, followed by 45 cycles of 95°C for 5 seconds, 60°C for 10 seconds. The ribosomal gene *Rps29* was used as a reference gene to normalize sample loading (32), and expression of steroidogenic enzymes was expressed relative to PND5 thymus, as the developing thymus is known to express mRNA, protein, and activity of the full suite of glucocorticoid-synthetic enzymes (13, 14, 31, 33). Negative controls (no RNA, no reverse transcription) were always nondetectable.

Statistical Analysis. For immunoassays, nondetectable samples (below the lowest standard on the standard curve and lower than the average water blank + two SD) were set to zero. Log-transformed data were analyzed with linear mixed-effects models, using R and Prism 5. The E16.5 subjects were analyzed separately, as several tissues could not be obtained at this age. Concentrations of all steroids, each analyzed separately, varied with tissue type ($P < .0001$) and showed age × tissue interactions ($P < .0001$). The relationships between tissue and circulating steroid levels were similar in females and males, so sexes were pooled for further analyses. At each age we compared organ steroid levels with whole blood (hereafter "blood") steroid levels, as blood is more reflective of circulating steroid levels than plasma (30, 34). However, plasma steroids were also quantified to allow comparison with previously published data (Supplemental Figure 2), and observed patterns were similar whether organs were compared with plasma or blood. We conducted planned pairwise comparisons, using paired t-tests or Wilcoxon tests as appropriate. As we were looking specifically for local elevation of steroid concentrations in different tissues (compared with concentrations in blood), tests were one-directional. Gene expression of steroidogenic enzymes was compared between PND5 and PND15 mice using unpaired two-directional t-tests. Significance set at $\alpha = 0.05$.

Results

Tissue and blood steroid levels

To assess the evidence for or against tissue-specific steroid regulation, we measured steroids (Figure 1) in organs of interest, and compared these tissue steroid levels with corresponding steroid levels in circulating blood. When steroid concentrations in an organ are higher than concentrations in blood, this indicates that the organ is actively increasing its local steroid content.

Corticosterone and cortisol are locally elevated in the embryonic and neonatal thymus. Corticosterone, the major circulating GC in mice, was present at higher concentrations in the thymus than in circulating blood of embryos and neonates to PND5 (Figure 2C). Corticosterone in blood was only detectable in 25% of subjects at PND5 (Supplemental Table 6), consistent with this age being during the SHRP. At later ages, corticosterone levels were similar in thymus and blood. Cortisol, a GC that is not (or minimally produced by mouse adrenals (1, 35, 36), was locally elevated in the thymus of embryonic and PND1 mice (Figure 2E). Cortisol levels in the embryonic thymus were more than 30-fold higher than levels in blood, and decreased thereafter with age. At PND5, cortisol concentrations were 3.67-fold greater than corticosterone concentrations in the thymus (Supplemental Table 7). Consistent with minimal cortisol secretion by the adrenals, circulating cortisol was nondetectable in most subjects (Supplemental Table 6). Both of the GC precursors, deoxycorticosterone and deoxycortisol, followed similar local patterns as their respective downstream GCs. Uniquely, progesterone was locally elevated in the thymus into adulthood (Figure 2A, B, D).

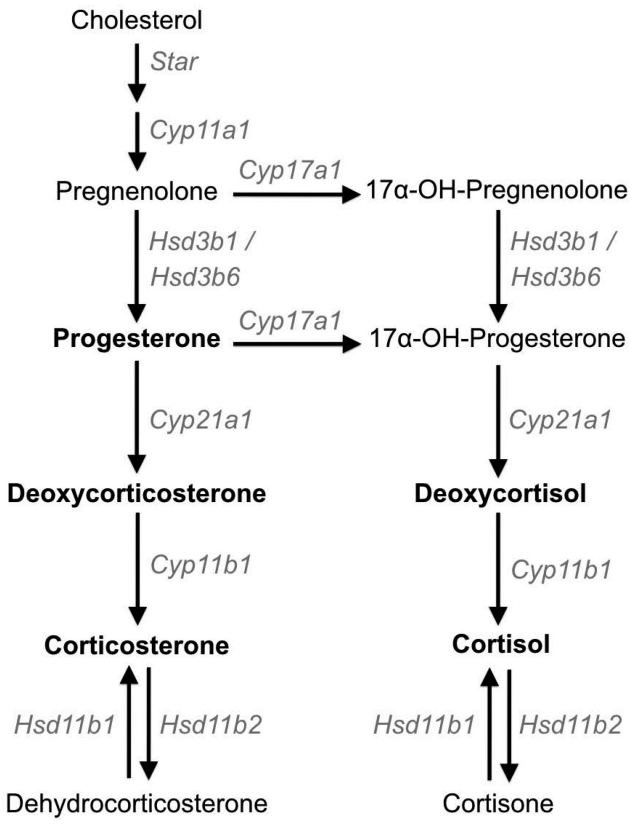

Figure 1. Simplified glucocorticoid-synthetic pathway, with steroid names in black and steroidogenic enzyme gene names in gray italics. Steroids in bold were quantified in this study.

Corticosterone and cortisol are locally elevated in neonatal bone marrow. Corticosterone and cortisol were both locally elevated in the bone marrow at PND5 (Figure 2H, J). Cortisol levels in bone marrow were approximately 1000-fold higher than cortisol levels in blood, and more than 35-fold higher than corticosterone levels in bone marrow (Supplemental Table 7), suggesting that at this age, cortisol might be considered the predominant GC in bone marrow. The GC precursors deoxycorticosterone and deoxycortisol were both elevated in PND1 and PND5 bone marrow (Figure 2G, I), while progesterone again remained locally elevated into adulthood (Figure 2F), as in the thymus.

Deoxycorticosterone is locally elevated in the developing and adult heart. Deoxycorticosterone was locally elevated in the heart at all ages except PND5, when there was a nonsignificant trend for local elevation ($P = .057$) (Figure 2L). Mean deoxycorticosterone levels in the heart were always more than double the levels in blood. Its precursor, progesterone, had a nearly identical pattern of local elevation in the heart (Figure 2K). Cortisol and deoxycortisol were elevated at PND5 (Figure 2N, O), while corticosterone was locally elevated at PND1 and PND5 (Figure 2M).

Corticosterone is locally elevated in the liver, spleen, and brain. Corticosterone levels were locally elevated in the liver of postnatal and adult mice (Figure 3C), while its precursors deoxycorticosterone and progesterone were locally elevated in the developing but not adult liver (Figure 3A, B). Corticosterone was also elevated in the spleen at PND1 and PND5 (Figure 3H), and in the brain at PND5 (Figure 3M). Cortisol and deoxycortisol were not significantly locally elevated in liver (Figure 3D, E), spleen (Figure 3I, J), or brain (Figure 3N, O) at any age, and cortisol and deoxycortisol concentrations were always much lower than those of corticosterone (Supplemental Table 7).

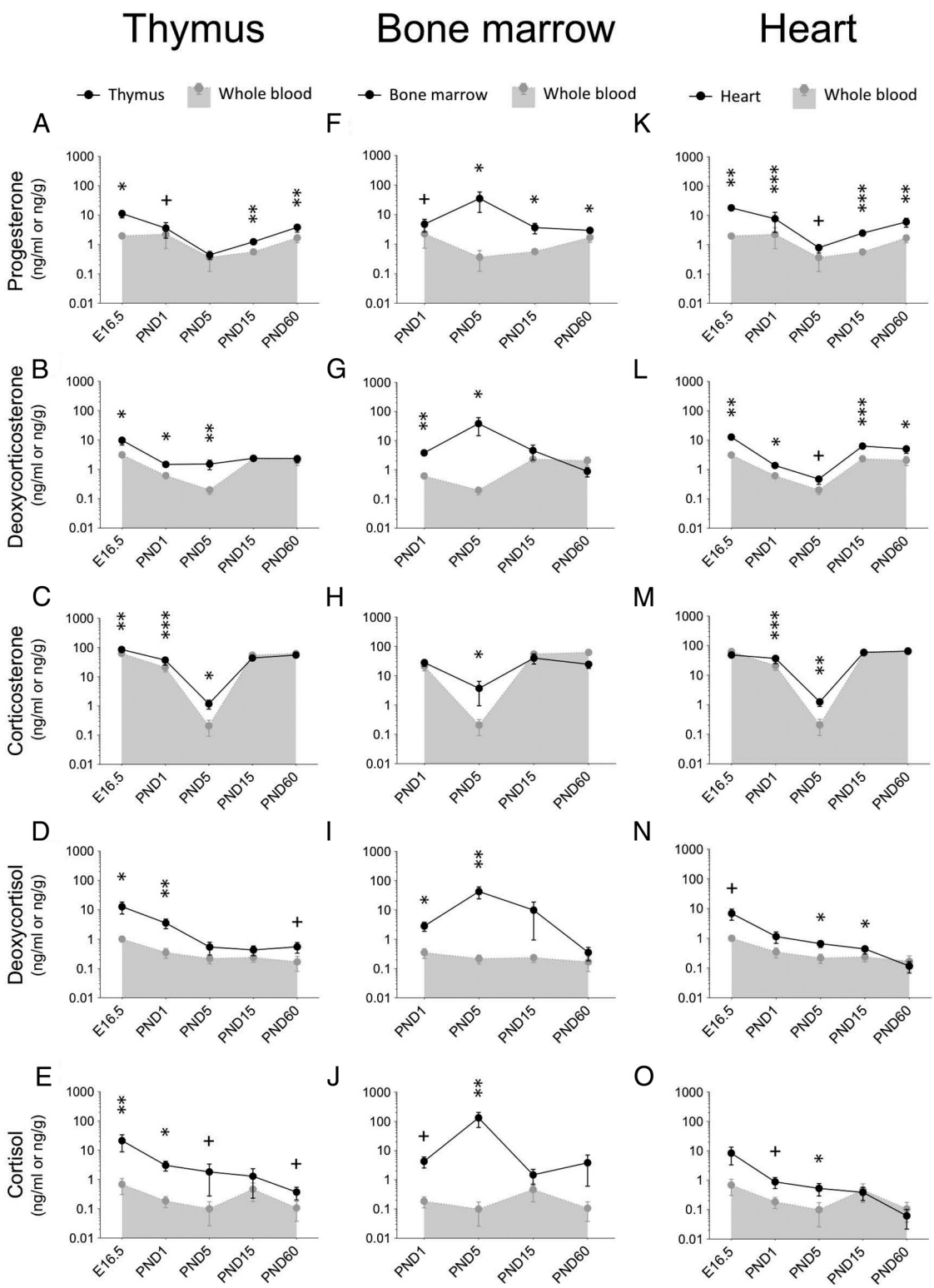

Figure 2. Steroid concentrations in thymus, bone marrow, and heart (, ng/g) relative to whole blood (, shaded region, ng/ml) of mice at embryonic day 16.5 (E16.5) and postnatal day (PND) 1, PND5, PND15, and PND60. Data are presented as mean ± SEM, and tissue steroid concentrations greater than blood steroid concentrations are indicated at each age as follows, + $P \leq .10$, * $P \leq .05$, ** $P \leq .01$, *** $P \leq .001$. Bone marrow was not obtained from embryos. Furthermore, we were unable to flush marrow from femurs at PND1, and we thus quantified steroids in whole femur, including solid bone. Steroid concentrations in PND1 bone marrow may thus be higher than shown here.

LC-MS/MS detection of cortisol

As cortisol is an unexpected steroid in the mouse, we used nonquantitative LC-MS/MS to confirm the presence of both cortisol and corticosterone as endogenous steroids in mouse tissues. We pooled tissue from several developing mice and several adult mice, and after SPE and HPLC

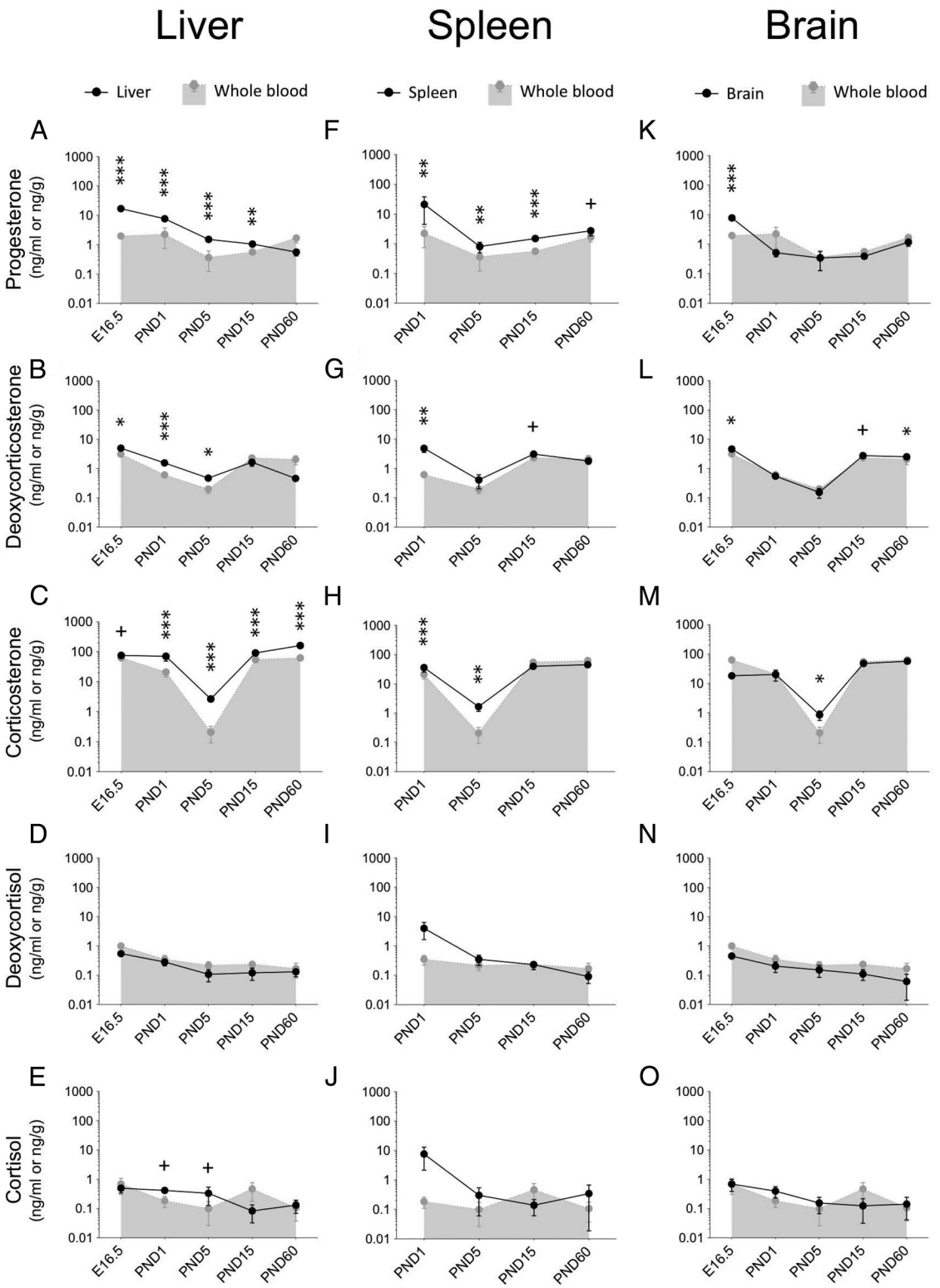

Figure 3. Steroid concentrations in liver, spleen, and brain (, ng/g) relative to whole blood (, shaded region, ng/ml) of mice at embryonic day 16.5 (E16.5) and postnatal day (PND) 1, 5, 15, and 60. Data are presented as mean ± SEM, and tissue steroid concentrations greater than blood steroid concentrations are indicated at each age as follows, + $P \leq .10$, * $P \leq .05$, ** $P \leq .01$, *** $P \leq .001$. Spleens were not obtained from embryos.

separation, used LC-MS/MS to detect corticosterone and cortisol. Ion chromatograms of MRMs specific to corticosterone or cortisol showed peaks at identical retention times to spiked corticosterone or cortisol, respectively, and exhibited the same positive fragmentation ion mass spectra as spiked standards. Specifically, corticosterone was present in all tissue types in both developing and adult mice, as expected (data not shown). We also confirmed the presence of cortisol in the developing whole femur (including bone marrow) and brain, and the adult thymus and heart (representative product ion scans from prepared standards or thymic extracts obtained at the elution time of cortisol shown in Figure 4A, B; MRM detection shown in Figure 4C, D and Supplemental Figure 3). However, cortisol was not detected in plasma, whole blood, liver, or spleen (Supplemental Figure 3), consistent with immunoassay findings.

Gene expression of steroidogenic enzymes

To test whether the enzymes needed for glucocorticoid synthesis are present in these tissues, we next looked at gene expression of these enzymes in mouse tissues during and after the SHRP (in PND5 neonates and PND15 juveniles, respectively). We found that transcripts for the upstream GC-synthetic enzymes *Star, Cyp11a1, Hsd3b6, Cyp21a1, and Cyp11b1* were widely expressed across tissues, in both PND5 and PND15 mice (Figure 5A-F).*Hsd3b1* expression was also detected, although at very low levels (usually detectable after 40 qPCR cycles for tissues other than thymus and liver). Negative controls

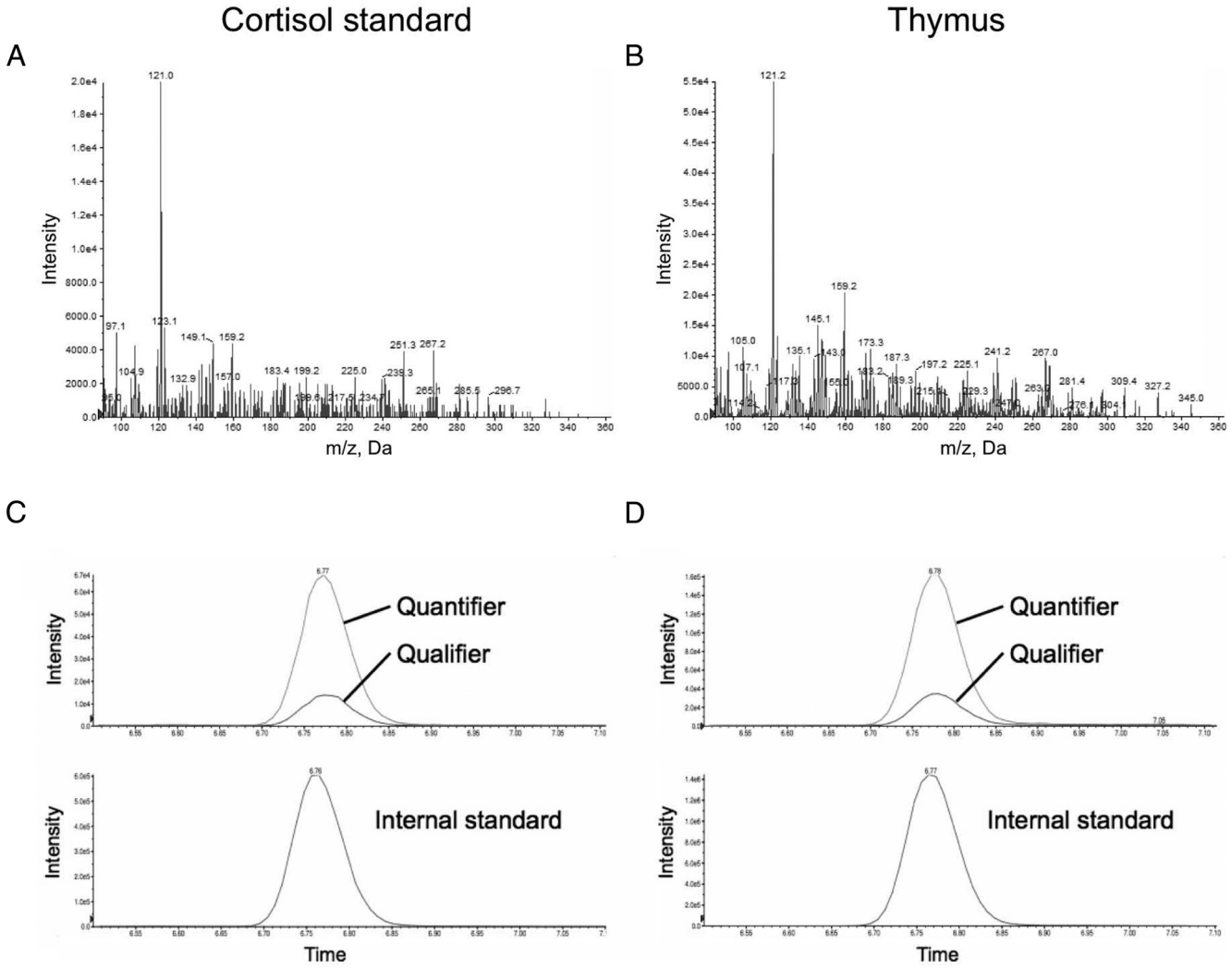

Figure 4. Detection of cortisol by liquid chromatography-tandem mass spectrometry (LC-MS/MS). The fragmentation pattern of the cortisol standard (**A**) matches that from pooled adult thymus (**B**). Cortisol was also detected with multiple reaction monitoring (MRM), with two MRM transitions for cortisol (quantifier m/z 363.4 → 121.1, qualifier m/z 363.4 → 97.1) (**C, D**, top panels) and one MRM transition for the cortisol-d4 internal standard (m/z 367.2 → 121.4) (**C, D**, bottom panels). Cortisol was also detected using LC-MS/MS in developing femur (containing bone marrow), developing brain, and adult heart (Supplemental Figure 3), but cortisol was not detected in plasma, whole blood, liver, or spleen.

were all nondetectable. *Cyp21a1* and *Cyp11b1* expression were lower at PND5 than PND15 in the bone marrow. Importantly, transcripts of *Cyp17a1*, which is necessary for the production of cortisol, were widely expressed in mice (Figure 5G). In the thymus, *Cyp17a1* transcripts were present at similar levels in PND5 and PND15 mice, while expression decreased with age in bone marrow and possibly heart (Figure 5G), Liver expression of *Hsd3b1*, *Hsd3b6*, and *Cyp21a1* was very high (Figure 5C-E). Gene expression varied in the brain; *Star* expression decreased with age, while *Cyp11a1* and *Cyp11b1* increased with age. Overall, we found the full suite of steroidogenic enzyme genes to be expressed in each of the tissues examined.

In tissues where GCs were locally elevated in the absence of elevated GC precursors, *Hsd11b1* reactivation of inactive GC metabolites (Figure 1) could be a major contributor to endogenous GC levels. Thus, we also examined *Hsd11b1* gene expression, and found that transcript levels were very high in the liver, and increased with age in the thymus, brain, and potentially heart (Figure 5H).

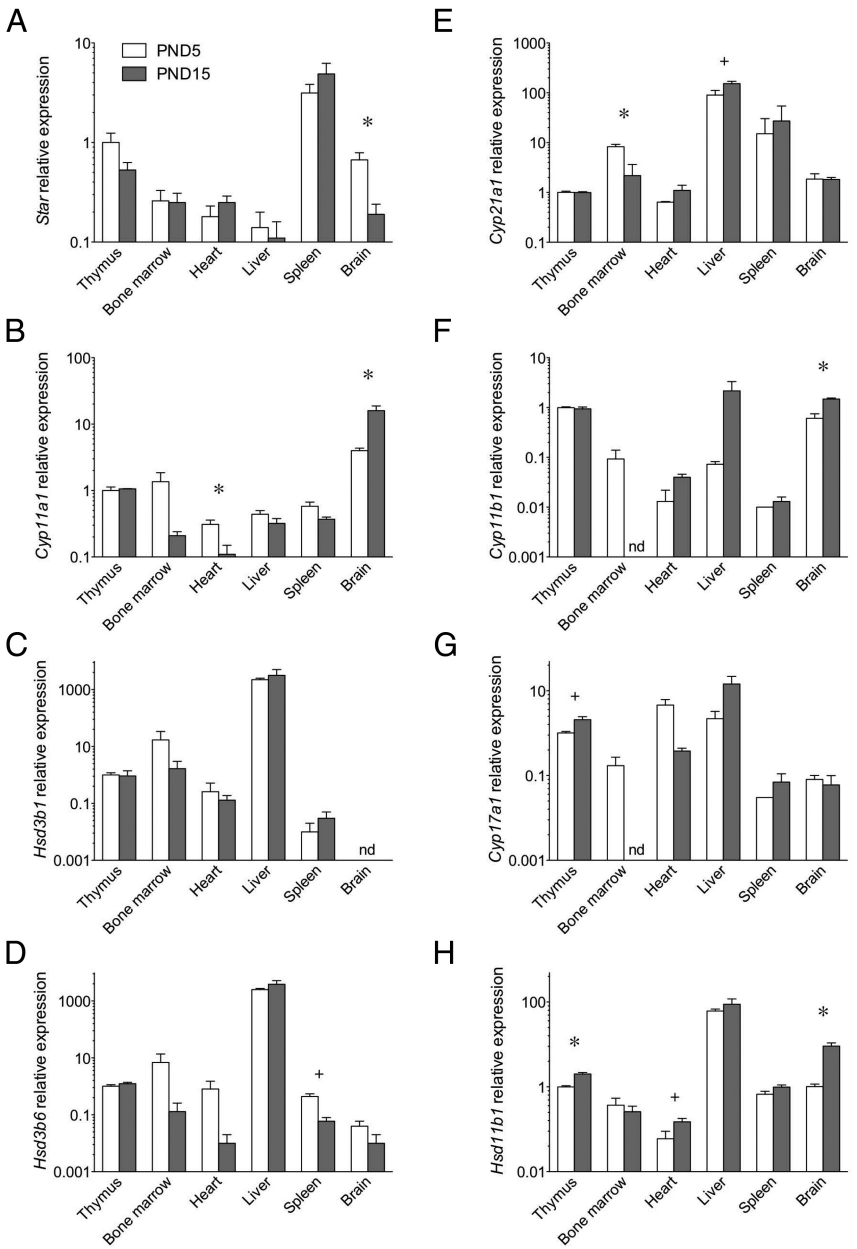

Figure 5. Gene expression of steroid synthetic enzymes (**A**) *Star*, (**B**) *Cyp11a1*, (**C**) *Hsd3b1*, (**D**) *Hsd3b6*, (**E**) *Cyp21a1*, (**F**) *Cyp11b1*, (**G**) *Cyp17a1*, and (**H**) *Hsd11b1* in thymus, bone marrow, heart, liver, spleen, and brain. Relative expression was corrected using Rps29 as a reference gene, and is presented as relative abundance compared to PND5 thymus, in which these transcripts (31, 33) and their corresponding enzymatic activities (13, 15) have been shown. Data are presented as mean ± SEM, and differences in transcript levels between PND5 and PND15 tissues are indicated for each tissue as follows, + $P \leq .10$, * $P \leq .05$, ** $P \leq .01$, *** $P \leq .001$. nd = nondetectable.

Discussion

Here we present evidence that different tissues are able to regulate their local GC levels in vivo, independent of GC patterns in circulating blood. Specifically, we quantified endogenous GCs and precursors over multiple developmental timepoints and in multiple organs where GCs have important actions. Strikingly, each organ had a unique pattern of elevated GC (and GC precursor) concentrations over development, and several organs had elevated levels of GCs that are unexpected in mice (eg, deoxycortisol, cortisol). These results demonstrate that local regulation allows organisms to maintain high steroid concentrations at specific locations where they are beneficial. Local regulation also allows steroid levels in different locations to follow distinct age-related patterns. Finally, local regulation allows tissues to utilize different steroid ligands from those in systemic circulation (eg, cortisol in thymus, bone marrow, and heart, deoxycorticosterone in heart vs corticosterone in blood). These distinct local steroids may have different mechanisms of action and effects than systemic steroids.

Local GC regulation is widespread across organs, and may even be the norm rather than the exception. Such autonomous regulation allows the organism to limit high GC concentrations to where they are needed, while keeping systemic GC concentrations low to avoid the detrimental effects of chronic GC exposure. This strategy may be beneficial in reducing GC-induced "wear and tear," or allostatic load (8). Furthermore, it explains how the distinct GC requirements of individual organs (such as the thymus, heart, and liver) can be met when circulating GCs are minimal, such as during the SHRP, or variable, as they are in later development and in adulthood.

Local regulation could occur via different mechanisms. Previous work has shown that several organs have the enzyme activities needed for synthesis of GCs from cholesterol or upstream steroids (thymus, 33–36; heart, 37,38 but see 39; brain, 40,41). Additionally, studies have shown widespread activity of 11β-hydroxysteroid dehydrogenase type 1 (coded by the *Hsd11b1* gene), which converts inactive metabolites (11-dehydrocorticosterone, cortisone) back into active GCs (corticosterone, cortisone) (liver, 42; spleen, 43; brain, 11, (37); heart, (38)). GCs can also be preferentially imported into (or out of) cells and tissues by ATP-binding cassette (ABC) transporters, potentially resulting in local accumulation of specific GCs that are present in circulating blood (12, 44). Finally, GCs may be sequestered by high-affinity binding to membrane and cytoplasmic proteins, such as corticosteroid-binding globulin (39) and glutathione S-transferase (40, 41). Such proteins could affect access of GCs to their cognate receptors. Differential expression of receptors might also affect tissue GC distributions.

Multiple mechanisms likely contribute to our observations. GC synthesis from upstream precursors might be the most important during the SHRP, as circulating GCs are minimal. Our detection of widespread gene expression of GC-synthetic enzymes is consistent with this possibility, and in the thymus, heart, and brain, these gene expression data are consistent with previous demonstrations of enzyme activity in these tissues (13, 14, 42, 43). In many cases, we also found that GC precursors had similar patterns of elevation as their downstream GCs, which is also consistent with local synthesis. Outside of the SHRP, and especially in adulthood, reactivation of circulating GC metabolites and binding of circulating GCs to high-affinity intracellular proteins likely play increased roles. This is consistent with increased expression of *Hsd11b1* in certain tissues after the SHRP and with the absence of elevated GC precursors, such as in the adult liver.

In the thymus, corticosterone, cortisol, and their precursors were locally elevated in the embryo and neonate. The thymus produces T cells, a critical arm of adaptive immunity, and most T cells are produced during embryonic and early postnatal development, coincident with locally elevated thymic GC levels. GCs promote T cell immunocompetence, ensuring development of a functional immune system (23), and local GC synthesis may ensure this when circulating GCs are low (13, 14, 33). The presence of cortisol and *Cyp17a1* mRNA in the thymus is intriguing, as cortisol is often considered to be minimal or absent in the mouse (1, 35, 36). While the embryonic adrenal expresses *Cyp17a1* transcripts before birth (44) and may produce cortisol in early life (45), circulating cortisol was usually nondetectable in this study. Murine T cell activation is regulated more potently by cortisol than corticosterone (46, 47), local and systemic GCs could differentially regulate T cells, especially at PND5 when thymic cortisol nearly four times higher than corticosterone. Interestingly, a highly similar pattern is seen in altricial songbirds, where corticosterone is the major circulating GC but thymus has higher cortisol levels (16). Progesterone, in contrast, remained elevated in the adult mouse thymus; progesterone may regulate T cell differentiation (48, 49) or thymic involution (50).

Similar to the thymus, bone marrow corticosterone and cortisol levels were locally elevated in early postnatal development, with cortisol and GC precursor levels far higher than those in blood. GCs were quite high in the bone marrow during the SHRP, when they were usually nondetectable in the blood. These data, together with gene expression of the full suite of GC-synthetic enzymes, provide the first indication of local steroid synthesis in the bone marrow. Bone marrow hematopoiesis, which peaks in the neonate and decreases thereafter (51), parallels the neonatal peak and drop in local GC levels and GC-synthetic enzymes with age. Immature B cells express especially high levels of the glucocorticoid receptor (52) and are more sensitive to cortisol than corticosterone (53), suggesting a role for local GCs (especially cortisol) in B cell development. Local elevation of cortisol is also seen in the corresponding avian site of B cell development, the bursa of Fabricius, contrasting with adrenal production of corticosterone (15, 16). Furthermore, cortisol (but not corticosterone) specifically binds to membrane GC receptors in the bursa of Fabricius (54). Membrane GC receptors are also present in mammalian B cells (55) and could be a mechanism for cortisol-specific signaling. Locally regulated GCs could also promote production of innate immune cells (56) and erythrocytes (57).

Heart levels of deoxycorticosterone and progesterone were locally elevated in development and adulthood, while corticosterone and cortisol were also elevated in the neonatal heart. The timing of cortisol, corticosterone, and *Cyp11a1* mRNA elevation correspond with the require-

ment of GCs for murine cardiac maturation (24). Clinical findings (58) have motivated a search for local mineralocorticoid synthesis in the adult heart, and aldosterone, which is downstream of corticosterone, is the major mineralocorticoid in rodents and humans. Some studies have found aldosterone-synthetic activity in heart tissue (eg, (42), while others have only found expression of deoxycorticosterone-synthetic enzymes (eg, (59–61)). Further, aldosterone levels are extremely low or absent in the rat heart after adrenalectomy (62). While often dismissed as an inactive precursor, deoxycorticosterone functions as a mineralocorticoid (17), and our results suggest that locally-elevated deoxycorticosterone could bind to mineralocorticoid receptors in the heart to regulate cardiac remodeling in heart failure.

The liver had elevated corticosterone and precursor levels during development, consistent with expression of upstream steroidogenic enzymes. Corticosterone however remained elevated in the adult liver, potentially by high levels of *Hsd11b1* (63) and binding to high levels of corticosteroid-binding globulin (5) and glutathione S-transferase (40). Locally elevated corticosterone in the liver and also spleen coincides with and could promote erythropoiesis (64) and clearance of toxic free hemoglobin (65).

Brain levels of deoxycorticosterone and progesterone were elevated in the embryo, and corticosterone was elevated at PND5. Elevated corticosterone and high *Cyp11b1* expression during the SHRP was surprising, as GCs generally impede neural growth, and the SHRP is believed to minimize circulating GCs to facilitate brain development (7, 22). However, brain regulation of GCs is heterogeneous and likely occurs at some locations while levels are low elsewhere (43, 66). Future steroid measurements, in brain and other tissues, would benefit from the use of techniques with greater spatial resolution (30).

Taken together, these data show that tissue-specific regulation of local GC levels occurs widely in the developing mouse. Local regulation may involve widespread expression of steroid metabolic enzymes, and further work should test for enzyme activity in tissues such as bone marrow where this has not been shown. This heterogeneity of endogenous GC levels is likely important to understanding GC functions in health and disease (67). For example, it is often thought that low systemic GCs are beneficial for some organs, such as the thymus, which atrophies in response to chronic elevation of adrenal GCs. However, the presence of locally elevated GCs in multiple developing organs of the mouse suggests that physiological (intermediate) levels of GCs, including different GCs than those in circulating blood, may have diverse and unsuspected roles in a variety of organs (67). This is clearly illustrated by the thymus, which requires GCs for production of immunocompetent lymphocytes (23). Furthermore, while the measurement of one circulating GC (corticosterone or cortisol) is widely employed (1, 2, 11, 12), such single measurements are often misleading with respect to GC levels in different organs. Instead, different organs display a remarkable variety of GC patterns, and GC functions in different tissues should therefore be evaluated in the context of local, not just systemic, GC levels.

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