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The stressed brain: regional and stress-related corticosterone and stress-regulated gene expression in the adult zebra finch (*Taeniopygia guttata*)

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Abstract

Glucocorticoids (hereafter CORT) are well-known as important regulators of behavior and cognition at basal levels and under stress. However, the precise mechanisms governing CORT action and functional outcomes of this action in the brain remain unclear, particularly in model systems other than rodents. Here we investigated the dynamics of CORT regulation in the zebra finch, an important model system for vocal learning, neuroplasticity, and cognition. We tested the hypothesis that CORT is locally regulated in the zebra finch brain by quantifying regional and stress-related variation in total CORT across brain regions. In addition, we used an *ex vivo* slice culture system to test whether CORT regulates target gene expression uniquely in discrete regions of the brain. We documented a robust increase in brain CORT across regions after 30min of restraint stress, but interestingly, baseline and stress-induced CORT levels varied between regions. In addition, CORT treatment of brain slice cultures differentially affected expression of 3 CORT target genes: it up-regulated expression of FKBP5 in most regions and SGK1 in the hypothalamus only, while GILZ was unaffected by CORT treatment across all brain regions investigated. Whereas the specific mechanisms producing regional variation in CORT and CORT-dependent downstream gene expression remain unknown, these data provide additional support for the hypothesis that the songbird brain employs regulatory mechanisms that result in precise control over the influence of CORT on glucocorticoid-sensitive neural circuits.

Keywords

Glucocorticoids; Local Regulation; Stress-Induced Gene Expression; *Taeniopygia guttata*

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The authors of this manuscript have no conflicts of interest to declare.

Introduction

Glucocorticoid hormones (GCs, hereafter CORT) regulate a diverse array of physiological and behavioral functions necessary for energy balance, maintenance of allostasis, and ultimately fitness, such as memory, immunity, metabolism, and reproduction[1,2]. The effects of CORT, particularly on the brain, are of special interest to many, as glucocorticoids are implicated in aging, neurodegenerative disease, learning and memory, developmental origins of adult disease, and life history transitions[3-9].

The complexities of CORT function and regulation in the brain are apparent in its often contradictory and highly context-dependent effects. For instance, stress can enhance or suppress memory, based on the timing of glucocorticoid elevation, stage of learning/memory, sex, and memory type[4,10,11]. Therefore, CORT effects in brain reflect a dynamic, multi-factorial, and potentially region-specific suite of CORT-regulatory mechanisms that function solo and in concert to determine the impact of CORT on neural circuits and associated behaviors.

Glucocorticoid regulation in the brain may occur via a number of interacting mechanisms, including differential expression of CORT receptors (glucocorticoid and mineralocorticoid receptors; GR and MR) in brain tissue, regulation of CORT passage from the periphery to the central nervous system via corticosteroid binding globulins (CBGs), local enzymatic metabolism via the 11β-hydroxysteroid dehydrogenases (either CORT-inactivating or regenerating), or local synthesis. All of these mechanisms have been identified in rodent models[12-17], although they are rarely considered together in the same study.

While the nature of central CORT regulation has been widely explored in mammals, particularly rodents, less is known about these same mechanisms in other taxa. However, recent research suggests that songbirds are a useful model for exploring the impacts of environmental, behavioral, and developmental conditions on CORT regulation in the brain[18,19]. For instance, in song sparrows (*Melospiza melodia*), seasonal CORT patterns are apparent in the circulation, but *not* in the brain, suggesting local regulation of brain CORT[20]. In addition, peripheral diel and stress-induced CORT variation in adult zebra finches (*Taeniopygia guttata*) is not necessarily matched by measures in brain[21]. These results suggest that the songbird brain differentially regulates central CORT, but many gaps in our knowledge remain. For instance, how do methods of brain CORT quantification (free or extracellular vs. total CORT) affect these results, and what does this tell us about the mechanisms governing CORT access to neural receptors? In addition, we know of no studies in songbirds that investigate the downstream effects of central CORT binding nor do we appreciate how genes are regulated by CORT in discrete brain regions. While much of the CORT-regulatory machinery is highly conserved, there are important differences in patterns of CORT-metabolic enzyme expression[22], ligand specificity of CBGs[23], and the abundance and ligand specificity of MR between birds and mammals[24]. As the songbird is a useful model for exploring the dynamics of neurogenesis, vocal learning, and cognition, processes that are all sensitive to CORT, these are pressing questions to address.

The goal of this study is to extend our previous work to elucidate the nature of CORT regulation in the adult songbird brain. Specifically, we hypothesized that the songbird brain is capable of central CORT regulation such that peripheral and central CORT levels may become dissociated. In addition, we hypothesized that expression of genes regulated by CORT differs across regions in parallel with CORT regulatory mechanisms. To test these hypotheses, we first asked whether total brain CORT varies across brain regions and in response to stress. Using *in vivo* microdialysis, we previously found that free, extracellular CORT does not vary on a diel basis or in response to stress in two brain regions of the adult zebra finch [21]. However, recent work suggests that CBGs are expressed in the brain[25,26], which would prevent the measurement of bound CORT using microdialysis. In addition, measurements of extracellular CORT likely exclude CORT that is bound by membrane or intracellular receptors. To offset these complications, we quantified total (extracted) CORT in 3 brain regions of adult zebra finches, the diencephalon (DIEN), hippocampus (HP), and cerebellum (CER), both at baseline and after restraint stress.

Variation in brain CORT levels may be determined by the differential activity of 11β-hydroxysteroid dehydrogenase type 2 (11βHSD2). 11βHSD2 metabolizes CORT into inactive metabolites which are incapable of binding to GR or MR[27]. Although previous work failed to identify 11βHSD2 in the chicken brain[28], this enzyme is expressed and actively metabolizes CORT in the zebra finch brain[29], suggesting a potentially important role in songbirds. Moreover, we recently documented regional variation in 11βHSD2 expression in the adult zebra finch brain, with relatively higher levels in CER than in DIEN or HP[22]. If 11βHSD2 regulates CORT in the zebra finch brain, we predicted that brain CORT levels would be highest in regions expressing the least amounts of the enzyme, DIEN and HP, and lowest in CER, which expresses relatively higher levels of 11βHSD2[22].

In the second experiment, we asked whether CORT regulates neural expression of genes that are upregulated in the presence of CORT in mammals, and whether that expression is related to levels of 11βHSD2 or GR (which binds CORT at stress-induced levels[24]). We used an *ex vivo* slice culture technique to expose micro-dissected sections of adult zebra finch brain (CER, HP, caudal telencephalon (cTEL), and hypothalamus (HYP)) to physiological stress levels of CORT, then quantified expression of FKBP5, GILZ, and SGK1, three CORT-regulated genes in mammals[30-32]. CORT treatment mimicking acute stress levels should up-regulate expression of all three genes, with the greatest degree of up-regulation in those regions expressing the most GR, namely the CER and cTEL, based on our previous work[22]. However, these regions also express the most 11βHSD2, which would in theory prevent CORT access to GR and subsequent downstream signaling[22]. Therefore, we predicted that CORT treatment would more potently increase downstream gene expression in those regions with lower 11βHSD2, albeit with lower GR (HYP and HP).

Methods

Animals

Adult, non-breeding male and female zebra finches were obtained from our colony at UCLA. Finches in the colony are maintained on a 14L:10D light cycle and housed in large same-sex flight cages (up to 35 individuals per cage). Feed includes ad-libitum millet seed

supplemented with vitamins and egg mix, and grit, cuttle bone, and fresh water are available at all times. All procedures in this study involving animals were approved by the UCLA Chancellor's Animal Research Committee in alignment with the United States Public Health Service Policy on Humane Care and Use of Laboratory Animals, the USDA Animal Welfare Act, and the Guide for the Care and Use of Laboratory Animals. In addition, we avoided subjective bias in allocation of animals to treatment groups by capturing individuals in the dark, where individual identity was unknown to the researcher.

Experiment 1: Regional and Stress-Related Variation in Brain CORT

Sample Collection: Variation in brain CORT across region and in response to acute stress was examined by collecting plasma and brain between 0830 and 1130h from 16 adult zebra finches (n = 4 per sex per treatment group). Individuals assigned to the baseline group were captured and bled from the brachial vein within 3min of lights off in the colony room, followed by rapid decapitation. Individuals assigned to the stress group were similarly captured and bled within ~3min, then placed in an opaque cloth bag for 30min to induce a stress response. At 30min, an additional brachial blood sample was collected, followed by rapid decapitation. We took care to minimize disturbance and habituation of the colony with this procedure by capturing only 1-2 birds on a given day from the entire colony room. If 2 birds were caught on the same day, they were captured and processed simultaneously, randomly allocating one bird to the baseline group and one to the stress group. To achieve a final sample size of 16 birds, 3 birds were captured on consecutive days, followed by a 10-day gap in sampling and then another 7 consecutive days of sampling.

After sacrifice, brains were rapidly dissected on a petri dish situated on wet ice. The HP, CER, and DIEN (including the HYP) were collected as described in[22], frozen on dry ice within approximately 20min of decapitation, and stored in a −80°C freezer. Note that finer-scale dissections of the HYP were conducted in the slice culture experiment detailed below. Blood samples were centrifuged at 10,000g for 10min, the plasma portion saved, and samples frozen at −80°C until assay.

Solid Phase Extraction & ELISA—To quantify CORT, all brain and plasma samples were extracted prior to ELISA using a solid phase extraction (SPE) protocol adapted from[33]. Brain samples were carefully weighed, immediately transferred to 10x75mm culture tubes filled with 500µl 0.1M PB, and homogenized. The average mass of samples from each brain region (± 1 S.E.) were as follows: HP, 7.9 ± 0.5mg; DIEN, 37.9 ± 1.6mg; CER, 47.8 ± 1.1mg. Homogenate was decanted into a 16x100mm tube, followed by a rinse of the initial tube with 200µl 0.1M PB to recover as much sample as possible. Samples were mixed with 1.35ml HPLC grade methanol and vortexed for 60min in a multi-tube vortexer (Fisher Scientific), followed by refrigeration overnight at 4°C. The following day, samples were vortexed for 60min, then centrifuged at 1027g for 10min at 4°C. The supernatant was decanted into a fresh tube, and the volume brought up to ~10ml with ultrapure water prior to loading on columns. Plasma samples were prepared for extraction by thawing, vortexing, and diluting in 10ml ultrapure water (6.5µl plasma per sample) prior to column loading.

All samples were extracted using 6ml C18 cartridges (Thermo Scientific Hypersep; 500mg bed weight) situated in an extraction manifold. Columns were primed with 3ml HPLC grade methanol, followed by equilibration with two rounds each of 5ml ultrapure water. Sample (two rounds x 5ml) was next loaded onto columns, followed by 2 interference washes with 5ml ultrapure water. After vacuum drying the columns, steroids were eluted with 5ml HPLC-grade methanol into 13x100mm tubes and stored at −20°C. To prepare for the ELISA, the methanol was evaporated under a gentle stream of nitrogen in a 37°C water bath, and samples were resuspended in assay buffer after brief initial resuspension in ethanol (5% of total resuspension volume[33]). Total resuspension volume was 130μl for plasma samples and 250μl for brain samples.

Plasma and brain CORT were quantified using a Cayman Chemical Corticosterone ELISA kit, according to the manufacturer's instructions (catalog no. 501320). Cross-reactivity of this assay is as follows: 11-deoxycorticosterone: 15.8%; prednisolone: 3.4%; 11-dehydrocorticosterone: 2.9%; cortisol: 2.5%. Briefly, the 8-point standard curve ranged from ~8-5000pg/ml, and samples were run alongside extracted water blanks as well as extracted and un-extracted standards (417pg/ml). CORT concentrations in extracted standards were divided by un-extracted standard values to yield recovery estimates. The lowest standard in this assay is 8.2pg/ml CORT; however, the limit of sensitivity is 30pg/ml (Cayman Chemical). All sample values were above 30pg/ml (mean plasma CORT = 401pg/ml; mean brain CORT = 376pg/ml). Extracted water blanks from the 2 plasma CORT assays were higher than 30pg/ml (average = 78pg/ml), suggesting potential contamination. We therefore subtracted the blank values from all plasma sample values prior to analysis and confirmed that our plasma CORT measures were very similar to previously published baseline and stress-induced CORT values in zebra finches (see citations below). In addition, 1 of 3 water blanks during brain sample extraction was > 30pg/ml (at 57pg/ml). Patterns of statistical significance remained the same if these samples (n = 8 out of 48) were excluded from the analysis or left in after subtracting the blank value (results presented below). Before extracting and assaying experimental samples, a lack of assay interference in SPE-extracted zebra finch plasma and brain was confirmed by creating serial dilutions of extracted brain and plasma samples, then confirming that subsequent dilutions were within 80-120% agreement with each other. A total of 4 assays were run (2 for brain CORT and 2 for plasma CORT), with an average inter-assay CV of 6.6%. Average recovery and intra-assay CV based on 3 CORT standards run in duplicate in each assay were 82% and 8.7%, respectively.

Experiment 2: CORT-Mediated Gene Expression in Brain

Chemicals—CORT stock solution (10mM) was prepared by dissolving crystalline CORT (Sigma) in absolute ethanol followed by further dilution in aCSF into intermediate and working solutions of 10μM and finally 30nM (aCSF: NaCl (199mM), $NaHCO_3$ (26.2mM), Glucose (11mM), KCl (2.5mM), NaH_2PO_4 (1mM), $MgSO_4$ (1.3mM), and CaCl (2.5mM) (pH 7.4;[29,34]). We chose 30nM CORT as the working solution because it aligns most closely with typical stress-induced plasma CORT concentrations in zebra finches[21,35,36].

Sample Collection and Slice Culture—To obtain brain tissue, 6 birds (3 males/3 females) were sacrificed by rapid decapitation within 45sec of turning off the overhead light

in the colony room (sampling occurred between ~0800 and 1100h). The head was immediately buried in wet ice and transported to the lab for removal of the brain. Upon removal of the skull, the cerebellum was separated from the rest of the brain and both were submerged in ice-cold aCSF with gently bubbling carbogen (95% O_2, 5% CO_2). The average elapsed time from sacrifice to submersion in aCSF was 9-10min. Coronal brain slices were obtained with an NVSL Vibroslice (World Precision Instruments). Briefly, the whole brain (except CER) was mounted rostral side up on the stage with super glue, then submerged in the tissue bath filled with ice-cold, carbogen-bubbled aCSF. Starting at the first appearance of the hippocampus, 500μM slices were collected and transferred to 15mm mesh inserts (Corning Netwell) floating in ice-cold, oxygenated aCSF. The CER was mounted and sectioned separately. Next, sections were visualized one at a time on a frozen petri dish situated under a dissecting microscope. We used a carbon steel micro knife (FST) to take micro-sections of desired regions (HP, HYP (including the pre-optic area (POA) and ventromedial nucleus (VMN)), caudomedial nidopallium (NCM), caudal nidopallium (cNp) [21], and CER). The cNp and NCM were pooled together for RNA extraction and will hereafter be referred to as caudal telencephalon (cTEL). The number of slices sampled for each tissue was as follows: HP, 6-7 slices; HYP, 3-4 slices; NCM and cNp, 4-5 slices each; and CER, 4-5 slices. All regions except for the CER were bilateral; therefore, for each brain slice, the two micro-sections were split into two treatment groups to enable a paired design (see below). The brain side (left or right) that was allocated to each treatment group was unknown to the researcher as the slices were free floating in aCSF prior to placement on the petri dish for micro-sectioning. However, as tissue for each brain region was taken from multiple brain slices, it is unlikely that there was a side bias between the two treatment groups. For CER, alternating slices were assigned to each of the two treatment groups. Telencephalic tissue that remained after obtaining micro-sections was also collected, incubated, and utilized to generate quantitative RT-PCR standard curves (see below).

After micro-dissection of desired brain regions, all tissue from a given region and treatment group was collected into one mesh insert and situated in a 12-well Netwell carrier immersed in oxygenated aCSF. Tissues were incubated at 40°C for 3 hours with carbogen to enable recovery (D. Pradhan, unpublished data). After the 3-hour recovery period, Netwells were transferred to 12-well polysterene plates containing either 4ml of CORT (30nM) per well or 4ml of aCSF (vehicle) and incubated in a 40°C water bath for 6h with gentle shaking. We chose a 6h incubation period to enable time for CORT to affect gene expression, but to avoid mimicking chronic stressors, which may alter GR and/or MR expression[37]. Upon termination of incubation, tissues were immediately frozen on dry ice and stored at −80°C until further processing.

RNA Extraction and cDNA Synthesis—We followed the manufacturer's guidelines to extract RNA from slice culture tissues using the RNeasy mini kit (Qiagen), including an on-column DNase treatment to remove genomic DNA from samples (RNase-free DNase set; Qiagen). RNA concentration and purity were assessed with a Nanodrop ND-1000 spectrophotometer, where A260/280 ratios ≥ 1.80 were deemed acceptable. Samples with a concentration less than 12.5ng/μl or a A260/280 ratio < 1.80 were purified and concentrated with the RNeasy MinElute Cleanup Kit (Qiagen).

We reverse-transcribed 100ng RNA into cDNA using the Superscript III First-Strand cDNA Synthesis System (Invitrogen). To confirm that RNA was not degraded and was successfully reverse-transcribed to cDNA prior to quantitative PCR, we used PCR with a highly expressed gene (GR, MR, or GAPdH; see primer sequences below), followed by gel electrophoresis, to confirm the presence of a single, bright amplification signal at the predicted molecular weight. Water blanks and no-RT controls were also run to confirm the absence of DNA contamination.

Quantitative RT-PCR—The expression of each of the 5 target genes (Table 1) was quantified in each sample and normalized to expression of a reference gene, GAPdH, using the SYBR Green method and an Applied Biosystems 7300 Real Time PCR System. GAPdH was chosen as a reference because of its stability in songbird brain[38]. Primer concentrations were optimized prior to quantification by creating and assessing efficiency and linearity of standard curves and assessing variation among technical replicates run in triplicate at several primer concentrations (Table 1). To run the assay, individual samples were run in duplicate at a 1:10 dilution, with a reaction volume of 25μl/well. Technical replicates differing by more than 0.5 CT[39] were either re-run alongside their treatment pair or excluded from analysis. A 4-fold standard curve prepared from pooled rostral telencephalic tissue incubated in parallel with experimental tissues was run on each plate. Reaction conditions were as follows: 1) 2min at 50°C, 2) 10min at 95°C, 3) 15sec at 95°C, 4) 1min at 60°C, repeat steps 3 and 4 40 times, 5) 15sec at 95°C, 6) 1min at 60°C, 7) 15sec at 95°C, and 8) 15sec at 60°C. No template controls were run to confirm the absence of reaction mixture contamination, and no-RT controls confirmed lack of double-stranded DNA contamination. A single CER sample was chosen as a plate calibrator and run on every plate to enable comparison across plates. After completion of each assay, dissociation curves were visualized to ensure the presence of a single peak (indicating a single amplification product). Reaction efficiency (90-110%) and linearity (>0.98) were determined from the standard curve. We confirmed the specificity of GR and MR primers in zebra finch brain tissue in a previous study[22]. For this study, SGK1, FKBP5, and GILZ primers were designed with Ensembl or the NCBI Primer Design tool. We sequenced a subset of qPCR products obtained with these genes and used BLAST (NCBI) to compare sequences with the zebra finch genome. All qPCR amplification products matched their predicted sequences.

Sample mRNA expression values for SGK1, GILZ, GR, and MR were calculated using the $2^{-\Delta\Delta CT}$ method, where ΔΔCT = [CT (sample gene of interest) – CT (sample GAPdH)]-[CT (calibrator gene of interest) – CT (calibrator GAPdH)], after confirming that reference gene and gene of interest efficiencies were within ~5% of one another in each assay. Efficiencies for GAPdH and FKBP5 were not always within 5% of each other, so we utilized the Pfaffl method to calculate relative expression levels[40]. In addition, technical replicates within 0.5CT of each other were difficult to obtain for FKBP5, ultimately leading to sample loss for CER (we report results for 3 of the original 6 birds below in which samples met these criteria). Results for FKBP5 were qualitatively similar when using the $2^{-\Delta\Delta CT}$ and the Pfaffl methods.

Statistics

We used a 2-way ANOVA to assess the impact of restraint stress on plasma CORT, with treatment group (baseline or stress) and sex as fixed factors. Plasma CORT values were natural log transformed prior to analysis to satisfy requirements for normality and equality of variances. Region and stress-dependent changes in brain CORT were assessed using a general linear mixed model, with bird identity as a random factor to control for repeated samples from different brain regions, and sex, treatment group, and brain region as fixed factors. All 2-way interaction terms were included in the initial model, and non-significant interaction terms were removed in a stepwise fashion to achieve the final model. To further explore the relationship between circulating CORT and brain CORT, we used a simple linear regression to compare plasma CORT to CORT for each brain region (separately for baseline and stress conditions).

To determine whether *ex vivo* CORT treatment affected expression of FKBP5, GILZ, SGK1, GR, or MR in slice cultures, for each brain region and gene, we used a paired t-test to compare relative expression between vehicle and CORT-treated sections. As there was a maximum of 3 individuals per sex (n = 6 total birds), we did not explicitly test for sex differences. Because we hypothesized *a priori* that FKBP5, GILZ, and SGK1 would be up-regulated in the presence of CORT, we utilized a 1-tailed test for these analyses. We utilized a 2-tailed test for analysis of GR and MR expression. We also verified that the reference gene, GAPdH, was unaffected by CORT treatment by conducting a paired t-test for each assay within each brain region (n = 5 assays per region to quantify 5 genes of interest). All paired t-tests for GAPdH were non-significant at $P > 0.05$ (HP assays: mean $P = 0.75$; cTEL assays: mean $P = 0.56$; CER assays: mean $P = 0.76$; HYP assays: mean $P = 0.16$).

All analyses were conducted in SPSS v25 and GraphPad Prism. Data in figures are presented as means ± 1 standard error. The data that support the findings of this study are available from the corresponding author upon reasonable request.

Results

Regional and Stress-Related Variation in Brain CORT

Brain CORT ranged from approximately 1-5ng/g (Fig. 1). These concentrations are very similar to those reported in a previous study that quantified CORT in adult zebra finch CER, telencephalon, and DIEN[35]. Brain CORT varied significantly between regions ($F_{2,41} = 30.1$; $P < 0.001$) and increased in response to stress ($F_{1,41} = 16.3$; $P < 0.001$; Fig 1). CORT variation across brain regions also differed depending on treatment group (region * treatment group: $F_{2,41} = 5.7$; $P = 0.007$), with significant differences among regions in both baseline and stress-induced samples (baseline: $F_{2,41} = 19.4$; $P < 0.001$; stress-induced: $F_{2,41} = 16.4$; $P < 0.001$). At baseline, HP CORT was significantly higher than DIEN or CER CORT (both $P < 0.01$), while DIEN and CER CORT did not differ ($P = 0.25$). However, in response to stress, HP and CER CORT were similar ($P = 0.80$) and both significantly higher than DIEN CORT (both $P < 0.01$; Fig 1). Within each brain region, stress-induced CORT was significantly higher than baseline CORT (HP: $F_{1,41} = 5.3$; $P = 0.026$; DIEN: $F_{1,41} = 8.0$; $P = 0.007$; CER: $F_{1,41} = 26.9$; $P < 0.001$; Fig 1). Brain CORT levels were unaffected by sex or

the interactions between sex, region, and treatment group (sex: $F_{1,41} = 0.06$; $P = 0.81$; region*sex: $F_{2,39} = 0.70$; $P = 0.50$; treatment group *sex: $F_{1,38} = 0.40$; $P = 0.53$).

Peripheral Stress Response and Relationship to Central Response

Circulating plasma CORT was elevated in the restraint stress group ($F_{1,13} = 6.35$; $P = 0.026$), with a roughly 157% increase in CORT at 30min post-capture relative to baseline (Fig 1). Both baseline and stress-induced plasma CORT levels were very similar to previous studies[21,35]. There was no effect of sex or interaction between sex and treatment group (sex: $F_{1,13} = 0.003$; $P = 0.96$; sex*treatment group: $F_{1,12} = 0.33$; $P= 0.58$).

The percentage increase in brain CORT between baseline and stress-induced samples varied by region, with a ~42% increase in HP CORT, ~161% increase in DIEN CORT, and ~207% increase in CER CORT. Baseline plasma CORT was not correlated with baseline brain CORT among individuals in any region (HP: $F_{1,6} = 1.38$; $P = 0.28$; DIEN: $F_{1,6} = 0.53$; $P = 0.50$; CER: $F_{1,6} = 1.1$; $P = 0.34$; Fig 2). One CER sample was an outlier with lower CORT than other samples; the relationship between baseline plasma and CER CORT was unchanged by removal of this sample from the analysis ($F_{1,5} = 0.09$; $P = 0.78$). There was, however, a strong positive correlation between stress-induced plasma CORT and stress-induced HP CORT ($F_{1,6} = 120.8$; $P < 0.001$; $R^2 = 0.95$) and DIEN CORT ($F_{1,6} = 133.7$; $P < 0.001$; $R^2 = 0.96$). One HP and one DIEN sample were high outliers in these analyses, but the relationships remained highly significant when these samples were excluded (HP: $F_{1,5} = 23.3$; $P = 0.005$; $R^2 = 0.82$; DIEN: $F_{1,5} = 29.3$; $P = 0.003$; $R^2 = 0.85$). The relationship between stress-induced plasma and brain CORT was also significant in CER ($F_{1,6} = 6.2$; $P = 0.047$; $R^2 = 0.51$; Fig 3). However, one plasma CORT value was more than 2 standard deviations higher than the mean; with this outlier removed, stress-induced plasma CORT was still highly positively correlated with HP ($F_{1,5} = 23.3$; $P = 0.005$; $R^2= 0.82$) and DIEN CORT ($F_{1,5} = 29.3$; $P = 0.003$; $R^2 = 0.85$), but not CER CORT ($F_{1,5} = 1.6$; $P = 0.23$; $R^2 = 0.27$).

CORT-Mediated Gene Expression in the Brain

CORT treatment increased FKBP5 expression in HP ($t = 2.6$; $P = 0.025$), HYP ($t = 4.4$; $P = 0.006$), and cTEL ($t = 2.4$; $P = 0.032$), but not CER ($t = 2.3$, $P = 0.075$), although the sample size for this region was low (n = 3 individuals). GILZ expression was unaffected by CORT treatment in all brain regions, although there was a non-significant trend towards increased expression in HYP ($t = 2.1$; $P = 0.065$; all other regions $P > 0.2$). SGK1 expression was significantly elevated with CORT treatment in HYP ($t = 12.0$; $P = 0.0001$), trended towards significant elevation in HP ($t = 2.0$; $P = 0.053$), and was unaffected by CORT in cTEL and CER ($P > 0.2$). Finally, GR and MR expression were unaffected by CORT treatment across all regions (all $P > 0.1$; Fig 3).

Discussion

While an abundance of CORT-regulatory mechanisms have been identified, few studies have assessed the functional outcomes of these dynamic and complex actions. In this study we tested the hypothesis that the songbird brain regulates CORT in a region-specific manner by

quantifying total CORT concentrations across different brain regions. In addition, we explored the possibility that central CORT exposure regulates expression of putative CORT-dependent genes and determined whether patterns of CORT-dependent expression varied according to regional differences in CORT binding or CORT-metabolic enzyme expression. While CORT regulates a vast number of genes in the brain[31], we know of no studies to assess the conservation of these pathways in songbirds, which are important models for learning and memory, speech, and neurodegeneration (e.g.,[41-44]).

Regional and Stress-Related Variation in Brain CORT

We quantified CORT in the HP, which plays a role in learning and memory[45], the DIEN, which includes hypothalamic nuclei critical to the regulation of the hypothalamic-pituitary adrenal (HPA) axis[46], and the CER, which regulates motor function and cognition[47]. Our results support the hypothesis that CORT access to and activity in the songbird brain is locally regulated. For example, the percentage increase in brain CORT from baseline to stress-induced conditions differed markedly across regions, with approximate increases of 40% in HP, 160% in DIEN, and 200% in CER. At the same time, plasma CORT increased by about 150%. In addition, CORT concentrations differed across brain regions. At baseline, HP CORT was significantly higher than CER or DIEN CORT, while after 30min of restraint, HP and CER CORT were equivalent, yet higher than DIEN CORT (Fig 1). Finally, individuals' baseline plasma CORT levels were not correlated with brain CORT in any region, while stress-induced plasma CORT was highly correlated with CORT in the HP and DIEN, but not CER (Fig 2). Together, these dissimilarities between plasma CORT and brain CORT dynamics at baseline and under acute stress support the hypothesis that CORT is locally regulated in the zebra finch brain.

Previous work in our lab suggests that 11βHSD2, a CORT-inactivating enzyme, may regulate CORT levels in the adult songbird brain[21,22,29]. This enzyme is expressed and exhibits CORT-inactivating activity throughout the songbird brain. In addition, in two brain regions we previously examined, 11βHSD2 expression was inversely correlated with extracellular CORT: the cNp had significantly higher 11βHSD2 mRNA than the HP whereas CORT levels were significantly lower in the cNp than the HP[21]. With these results in mind, in this experiment we expected that total CORT levels in the HP, DIEN, and CER would also inversely relate to local 11βHSD2 expression levels, where HP and DIEN 11βHSD2 levels are lower than CER[22]. While baseline CORT in the HP was indeed higher than in the CER, levels of CORT in the DIEN were relatively low. Similarly, at stress-induced levels, HP and CER CORT concentrations were roughly equal, though we expected CER CORT levels to be lower[22]. Thus, these correlative data do not support a singular role for 11βHSD2 in regulating local CORT levels in the zebra finch brain. More study is needed to fully reject a role for neural 11βHSD2, including assessing if protein levels of this enzyme match expression levels and studies at a finer analytic scale that may prove to be more accurate.

Additional regulatory mechanisms may operate in the brain, perhaps in combination with 11βHSD2, to regulate local CORT concentrations. Local CORT synthesis is thought to produce region-specific patterns in total brain CORT in rodents[48,49], and the entire suite of CORT-synthetic enzymes is expressed in the rodent brain [17]. In addition, the rodent brain

expresses significant amounts of 11βHSD1 which regenerates CORT from peripheral 11-DHC[14]. It is unlikely that local CORT synthesis via steroidogenesis or 11-DHC regeneration produced our results, however. First, 11βHSD1 appears to be absent from the adult zebra finch brain, in stark contrast to rodents[22]. Second, if local synthesis were occurring in brain, brain levels could be found to exceed plasma levels[16], which we did not observe (Fig 1). Similar results were seen in wild adult song sparrows, where baseline and stress-induced plasma CORT levels always exceeded brain CORT levels[20]. Interestingly, this latter study found no regional differences in brain CORT, except during molt, when CORT levels in the HP were undetectable after stress. Another analysis of song sparrow CORT in the non-breeding season similarly found no differences between brain regions[18]. These differences across species warrant the investigation of additional songbirds to determine whether general patterns in brain CORT regulation exist.

Differential expression and occupation of CORT receptors may explain the regional brain CORT variation we observed. Upon binding to high affinity MR under basal and early stress conditions and low-affinity GR under stressed conditions, the intracellular CORT-receptor complexes translocate to the nucleus, where they act as transcription factors regulating numerous target genes in brain[31]. While it is unclear how long the CORT-receptor complex remains in the nucleus, it is possible that this “sequestering” of hormone could influence regional total CORT concentrations. Support for this hypothesis was found in rats, where regional brain CORT levels were correlated with GR binding in adrenalectomized rats receiving CORT replacement[50]. If this is true in our system, then stress-induced CORT levels would be highest in regions with the highest GR expression (CER), an imperfect proxy for GR binding[22]. At first glance, this hypothesis is not supported, as we found equally high stress-induced CORT in HP and the CER, yet the zebra finch HP expresses far lower levels of GR[22]. However, the HP expresses high levels of MR compared to the rest of the brain[22], consistent with mammalian studies that suggest a role for the HP in modulating daily activity of the HPA axis via baseline CORT interaction with MR[51]. Baseline brain CORT was highest in HP in this study, with only a small increase during stress (~50%). Therefore, this regional difference at baseline may reflect consistent occupancy of MR in the HP at both baseline and under stress, a possibility noted previously[50]. In summary, the regional brain CORT patterns we observed are potentially consistent with differences in CORT receptor expression and presumed occupancy (at basal or stress-induced levels).

At baseline, plasma and brain CORT levels were not correlated, despite measurable variation in both (Fig 2). Under stress, however, plasma CORT was highly correlated with DIEN and HP CORT, but not CER CORT. Peripheral CBG action may partially explain these results, as CBGs regulate CORT access to tissues, including the brain[15]. Under basal conditions, much of circulating CORT is bound by CBGs[36] which, according to the free hormone hypothesis, limits CORT access to tissues[52]. In zebra finches, stress causes a decline in peripheral CBG binding, increasing free CORT levels that theoretically could then cross the blood brain barrier to access the brain[36,53]. This may explain the correlation between total HP/DIEN and plasma CORT under acute stress. However, this reasoning does not hold for CER, and instead points to additional regulatory mechanisms in this region producing a mismatch between the brain and periphery. Regardless of the mechanism, these data suggest

that the cognitive and motor functions regulated by the CER are both sensitive to and require some degree of buffering from peripheral CORT levels under stress.

A simple increase in circulating free CORT reaching the brain cannot explain a key discrepancy between this study and our previous work, however. We previously demonstrated a lack of a central free CORT stress response for up to 1.5h post-stressor in the extracellular fluid using *in vivo* microdialysis in the adult zebra finch HP and cNp[21]. In the current study, we documented a stress response within 30min in all regions when measuring total CORT (a combination of extracellular and intracellular CORT as well as free and bound CORT). Therefore, it appears that additional central regulatory mechanisms restrict free CORT elevations in the brain, while not necessarily affecting total CORT measures. While hypothetical, the local expression of CBGs in brain may account for this discrepancy. CBGs are widely expressed in rodent and human brain[25,54-56], and while their function is still unclear, they may serve as cellular mediators of CORT action in the absence of CORT receptors or as mechanisms enhancing CORT transport to classical receptors[26,57]. The songbird CBG sequence has recently been identified[58] and our very preliminary work suggests that the protein is expressed, albeit at quite low levels, in the zebra finch brain (Rensel, unpublished data), indicating that it is at least possible that CBG binds CORT in the songbird brain. However, despite expressing central CBG, previous microdialysis experiments in rodents have documented free CORT stress responses in the brain (e.g., [59-61]). The reasons for this discrepancy between birds and mammals is unknown, although it is of note that songbird CBG is not identical to the mammalian protein, with structural differences and distinct patterns of affinity to additional steroid hormones[23,58]. However, if CBG is expressed and active in the songbird brain and regulates central CORT, it could potentially limit or prevent a free CORT response, much as CORT bound to CBGs would be unable to pass through a microdialysis probe. Experiments investigating the expression and dynamics of CBG activity in the songbird brain are necessary to adequately test this hypothesis.

Corticosterone-Mediated Gene Expression in the Brain

CORT acts as a transcription factor when bound to GR and/or MR and can up- or down-regulate a large number of genes involved in CORT feedback, cellular metabolism, growth, immunity, and more[31]. To assess CORT-regulated gene expression in the songbird brain, we measured expression levels of SGK1, FKBP5, and GILZ (also known as Tsc22d3). All three of these genes are consistently up-regulated by CORT in the mammalian brain[31]. We asked if these genes were expressed in the songbird brain and if they were also subject to regulation by CORT. SGK1 and FKBP5 both act as short feedback regulators of CORT action in the cell. SGK1 (serum and glucocorticoid-inducible kinase) expression in brain is implicated in neurogenesis, depression, and pre-frontal cortex-mediated working memory[62-65]. In particular, SGK1 mediates the CORT-induced down-regulation of GR expression[66], but enhances the movement of GR-bound CORT to the nucleus and increases GR activity through phosphorylation. Conversely, the CORT chaperone FKBP5 (FK506 binding protein 5) reduces phosphorylation of GR and reduces nuclear translocation[67,68]. FKBP5 has been of intense recent interest for its involvement in psychiatric illness[69].

Finally, GILZ (glucocorticoid-induced leucine zipper) is a widely expressed protein thought to regulate neurodegeneration, cellular differentiation, and inflammation[32].

In this study, FKBP5 expression was significantly upregulated by CORT in the HP, cTEL and HYP but not in the CER, with the strongest effect in HYP (Fig 3). Previous studies have found widespread increases in FKBP5 in response to stress or to CORT, including in the mouse amygdala and HYP 4 hours after 30min of restraint stress[70] and the mouse HP and HYP after 4 weeks of CORT ingestion[71]. While FKBP5 was up-regulated by CORT exposure across multiple brain regions, SGK1 was only up-regulated in the HYP, and strongly so. Given the central role of the HYP in negative feedback regulation of the HPA axis, the strong effects of CORT on FKBP5 and SGK1 expression in this region are not surprising. The lack of an effect of CORT on SGK1 in HP and CER differs from previous studies in mammals, however, where 3 hours of CORT exposure increased SGK1 expression in human HP progenitor cells[62] and mouse and rat HP[63]. Nevertheless, another study reported no increase in HP SGK1 in response to acute or chronic stress in rats[72]. SGK1 is commonly investigated in HP because of its role in neurogenesis and depression. However, less is known about the role of SGK1 in the HYP, where we observed a robust increase in expression following CORT exposure. In mice, social isolation increases HYP SGK1 expression despite reducing circulating CORT levels[73]. HYP SGK1 may also regulate energy balance as shown previously in mice[74]. Finally, although GILZ expression in rodent brain is regulated by stress or CORT-ingestion in rats[75,76], this gene was unaffected by CORT treatment in adult zebra finch brain slices.

In the absence of CORT metabolism by 11βHSD2, one prediction is that CORT would most strongly regulate gene expression in those regions expressing the most abundant GR[22], in our case, the cTEL and CER. Our data do not support this hypothesis, as SGK1 expression was not elevated in cTEL or CER, and FKBP5 expression was elevated to a lesser degree in cTEL than in HYP and HP. Instead, our results indirectly support the hypothesis that 11βHSD2 reduces CORT-dependent GR activation, as FKBP5 expression was up-regulated most in the HYP and HP, regions with relatively lower 11βHSD2 expression[22]. In addition, SGK1 expression was strongly upregulated in the presence of CORT, but only in HYP (this same relationship approached significance in HP). It is important to note that while we previously reported lower GR expression in HYP and HP[22], GR is widely expressed across the brain and therefore it is unsurprising that CORT exposure would up-regulate downstream gene expression in these regions.

There are two important limitations to this study. First, our design did not allow us to explicitly test for effects of gene lateralization because we did not track the sides of the brain that were allocated into each treatment group, although the fact that we took samples from multiple brain sections makes it unlikely that there was a side bias. Lateralization of immediate early gene expression, which were not the focus of this study, has been detected in the zebra finch visual system[77-79], but not the hippocampus[80]. Future work should investigate whether the CORT targets we investigated are expressed in a side-dependent manner. Second, we chose to incubate brain slices in CORT concentrations mimicking those in plasma under acute physiological stress (~30ng/ml). However, as the results of our first experiment highlight, brain CORT levels are much lower than circulating levels, even during

stress. What remains unknown is *when* these lower levels are achieved: are levels lower because of reduced passage through the blood brain barrier due to systemic regulatory mechanisms, or is brain CORT rapidly metabolized by neural regulatory mechanisms? These questions warrant further investigation.

In summary, our results reveal regional differences in CORT levels and action that suggest the presence of CORT-regulating machinery in the zebra finch brain. In addition, the putative CORT-dependent genes FKBP5 and SGK1 appear to be regulated by physiological stress levels of CORT in the adult zebra finch brain, perhaps revealing important signaling pathways for neural CORT action in songbirds. As the zebra finch is a popular model system in the fields of cognition, behavior, and neurodegenerative disease, it is vital that researchers consider the local regulatory mechanisms that mediate the neural and behavioral effects of stress in this model system. Therefore, this analysis of regional CORT variation and CORT-regulated genes opens the doorway to further explorations of the complexities of CORT dynamics in the songbird brain and may assist in further developing songbirds as useful model systems.

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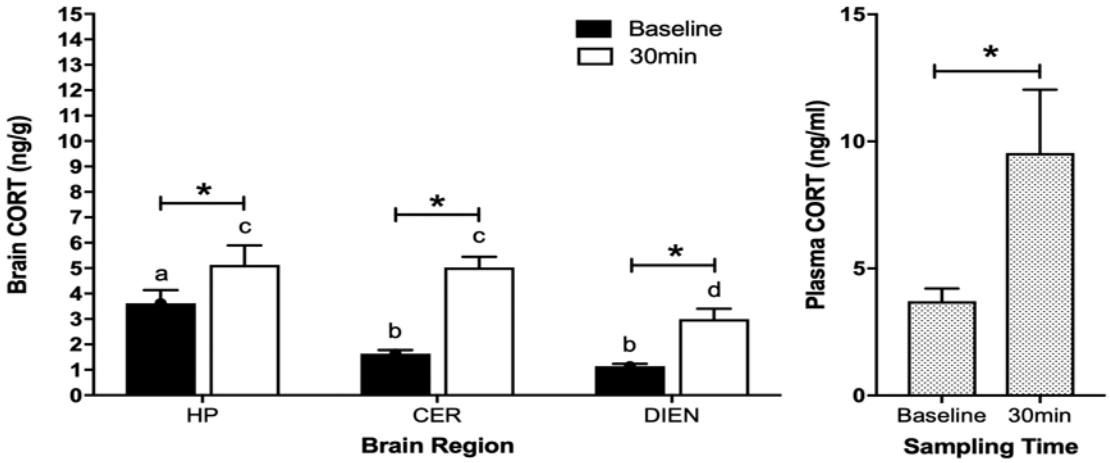

Figure 1. Brain and plasma CORT levels in adult zebra finches sacrificed at baseline (within 3min of capture) or after 30min of restraint stress.

n = 8 individuals per time point. CORT at 30min was significantly elevated over baseline in all brain regions and in plasma (*, $P < 0.05$). In addition, at baseline, HP CORT was significantly higher than CER or DIEN CORT, while at 30min, HP and CER CORT were significantly higher than DIEN CORT (significance indicated by letters above bars within each time period). HP = hippocampus; CER = cerebellum; DIEN = diencephalon.

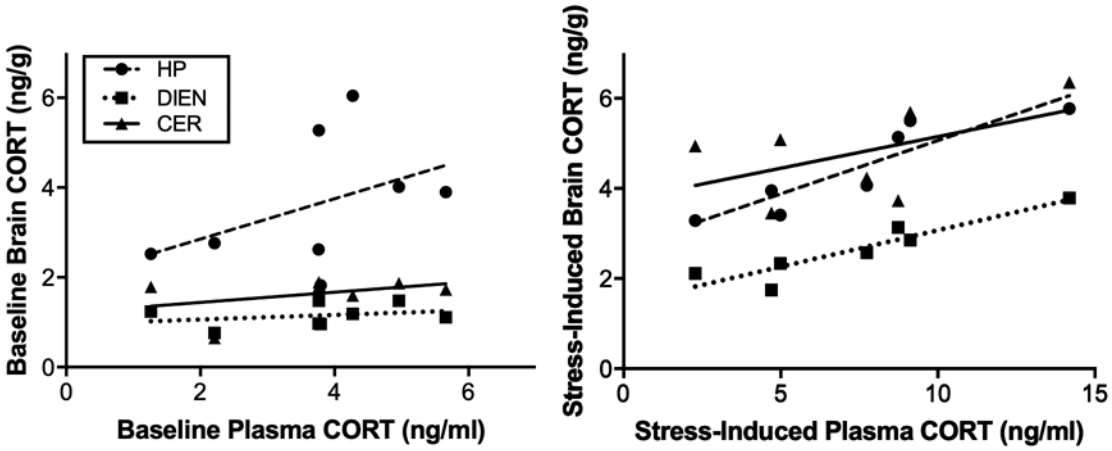

Figure 2. Relationship between individuals' brain and plasma CORT levels at baseline (left panel) and after 30min of stress (right panel).

Plasma and brain CORT were not correlated at baseline but were correlated under stressed conditions in the DIEN and HP, but not the CER (this relationship was non-significant when a single high plasma CORT outlier was excluded; figure shows data without the outlier). HP = hippocampus; CER = cerebellum; DIEN = diencephalon.

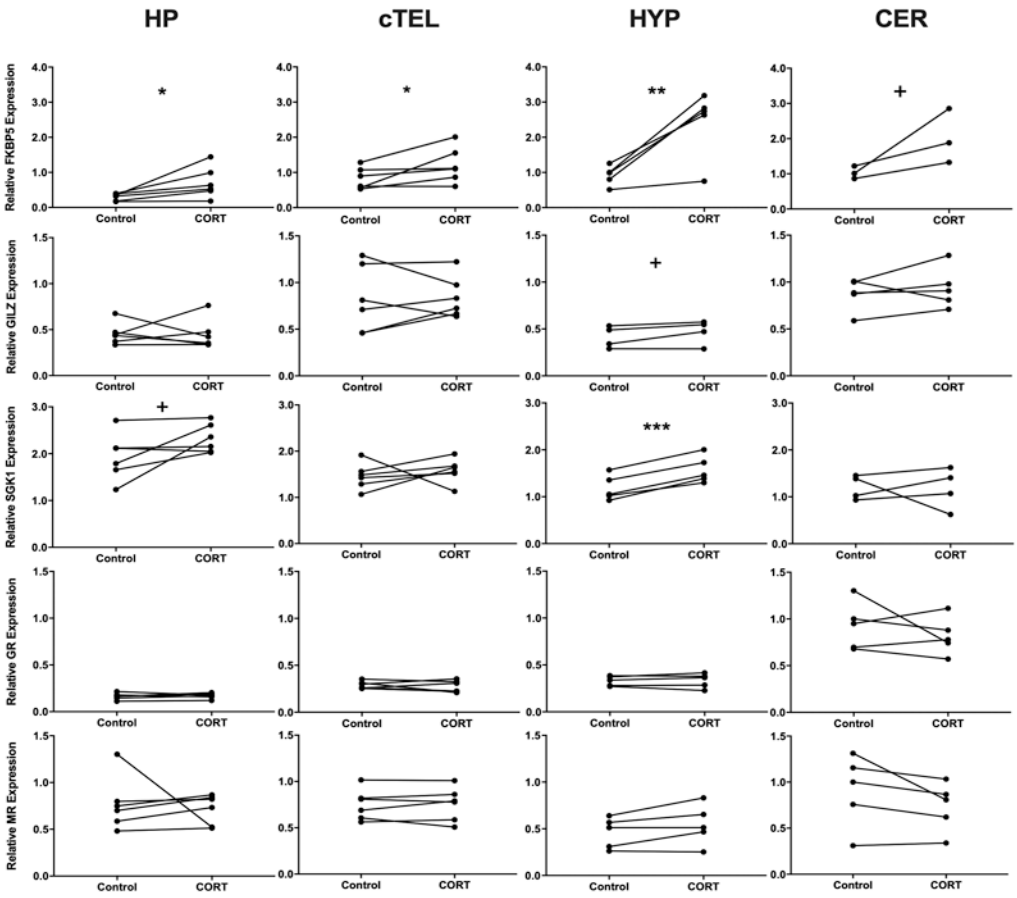

Figure 3. Relative expression of potential CORT-regulated genes in micro-dissected brain region slice culture.

After a 3h recovery period, sections were incubated with control media (aCSF) or CORT (30nM) for 6 hours, followed by rapid freezing, RNA extraction, cDNA synthesis, and quantitative PCR. Expression levels were determined relative to GAPdH for each sample, then normalized to a single CER sample run in every assay; n = 4-6 paired samples per assay (minus CER FKBP5, where n = 3 due to sample depletion). +, $P < 0.1$; *, $P < 0.05$; **, $P < 0.01$; ***, $P < 0.001$. HP = hippocampus; cTEL = caudal telencephalon; HYP = hypothalamus; CER = cerebellum.

Table 1.

Quantitative PCR Primer Details

Gene	Primer Sequence	[Primer](μM)†	Average Efficiency (%)
SGK1	F: AGGCGTCTGGTCCTACCTTA R: TGAACTTCAGGGTGCTTGCAT	0.6	95.8
GILZ	F: CTGCAACAGGAACATCGACC R: TTTTTCACAAGATCCATCGCCT	0.3	96.7
FKBP5	F: GGCAAGGGCCAGGTAATCAA R: CCTCAAACAAGTCCTCGCCT	0.3	94.7
GR	F: TGCAGTACTCCTGGATGTTCC R: GAGCATGTGTTTGCATTGTTC	0.6	96.1
MR	F: AAGAGTCGGCCAAACATCCTTGTTCT R: AAGAAACGGGTGGTCCTAAAATCCCAG	0.3	96.7
GAPdH	F: TGACCTGCCGTCTGGAAAA R: CCATCAGCAGCAGCCT	0.3	95.2

† per reaction