Published in final edited form as:

Eur J Neurosci. 2008 July ; 28(1): 99–106. doi:10.1111/j.1460-9568.2008.06303.x.

Sex differences in cell proliferation and glucocorticoid responsiveness in the zebra finch brain

Amnon Katz, Anahid Mirzaton, Yin Zhen, and Barney A. Schlinger

Department of Physiological Science and Laboratory of Neuroendocrinology, Brain Research Institute, University of California, Los Angeles, CA 90095, USA

Abstract

Neural proliferation is a conserved property of the adult vertebrate brain. In mammals, stress reduces hippocampal neuronal proliferation and the effect is stronger in males than in females. We tested the effects of glucocorticoids on ventricular zone cell proliferation in adult zebra finches where neurons are produced that migrate to and incorporate within the neural circuits controlling song learning and performance. Adult male zebra finches sing and have an enlarged song circuitry; females do not sing and the song circuit is poorly developed. Freshly prepared slices from adult males and females containing the lateral ventricles were incubated with the mitotic marker BrdU with or without steroid treatments. BrdU-labeled cells were revealed immunocytochemically and all labeled cells within the ventricular zone were counted. We identified significantly higher rates of proliferation along the ventricular zone of males than in females. Moreover, acute administration of corticosterone significantly reduced proliferation in males with no effects in females. This effect in males was replicated by RU-486, which appears to act as an agonist of the glucocorticoid receptor in the songbird brain. The corticosterone effect was reversed by Thiram, which disrupts corticosterone action on the glucocorticoid receptor. Sex differences in proliferation and responses to stress hormones may contribute to the sexually dimorphic and seasonal growth of the neural song system of songbirds.

Keywords

brain slices; cell proliferation; corticosterone; ventricular zone; zebra finch

Introduction

Neural proliferation has been recorded in adults animals of all vertebrate taxa and, with few exceptions, occurs in the cell layer surrounding the lateral ventricles, the ventricular zone (VZ) (Garcia-Verdugo *et al.*, 2002). In songbirds, VZ proliferation (Goldman & Nottebohm, 1983; DeWulf & Bottjer, 2005) is concentrated in ventral and dorsal 'hotspots' (Alvarez-Buylla *et al.*, 1990) with most, if not all, of the dividing cells classified as radial glia or neurons (Alvarez-Buylla *et al.*, 1998). Neural progenitor cells migrate into distinct brain areas where they differentiate into functional neurons (Alvarez-Buylla & Garcia-Verdugo, 2002; Taupin & Gage, 2002). In mammals these cells migrate largely to the hippocampus and olfactory bulb areas (Garcia-Verdugo *et al.*, 2002), whereas in songbirds many neurons migrate to nuclei of the efferent motor pathway for learned vocalization (Alvarez-Buylla & Garcia-Verdugo, 2002).

Steroids are among the factors affecting neural proliferation. Estradiol (E2), progesterone (PROG), dehydroepiandrosterone (DHEA), allopregnanolone and pregnenolone sulfate have all been shown to increase cell proliferation in one or more species and preparations (Karishma & Herbert, 2002; Keller *et al.*, 2004; Ghoumari *et al.*, 2005; Mayo *et al.*, 2005; Galea, 2008). In contrast, glucocorticoids (GC) secreted by the adrenal cortex in response to stress have degenerative effects on the brain (Gould *et al.*, 1992; Sapolsky, 1996; Tata *et al.*, 2006). Under basal conditions, GC act via mineralocorticoid receptors for proper maintenance of cellular metabolic rates and cellular function. When GC levels are elevated they act via glucocorticoid receptors (GR) (de Kloet *et al.*, 2005; Avital *et al.*, 2006). GC likely suppress cell proliferation (Gould *et al.*, 1992) through an increase in transcriptional activity of p53 (Crochemore *et al.*, 2002) and an increase in the ubiquitination of cyclin D1, an important regulator of G1 to S-phase (Sundberg *et al.*, 2006).

Stress or administration of corticosterone (CORT) to young zebra finches causes decrements in adult song and diminishes masculine growth of the neural song system (Spencer *et al.*, 2003; Buchanan *et al.*, 2004; Hodgson *et al.*, 2007). Although CORT has numerous documented effects on behavior in songbirds (e.g. Landys *et al.*, 2004; Cyr & Romero, 2007; Spencer & Verhulst, 2007), little is known about the neural impact of CORT in adult zebra finches. CORT could diminish neural proliferation as it does in mammals.

Manipulation of adrenal hormones in songbirds can be problematic because adrenalectomy is prohibitively difficult and handling can rapidly elevate endogenous levels of CORT (Breuner *et al.*, 2000). Therefore, to test the acute effects of CORT on cell proliferation in the adult zebra finch VZ we utilized a brain slice culture method (Williams *et al.*, 1999; Holloway & Clayton, 2001; Tam & Schlinger, 2007) that resembles the *in vivo* condition. These studies document higher rates of proliferation along the VZ of males as compared with females. Furthermore, acute administration of CORT significantly reduces proliferation in males but not in females. Sex differences in proliferation and responses to stress hormones may contribute to the sexually dimorphic and/or seasonal growth of the neural song system in these birds.

Methods

Animals

All zebra finches were obtained from our colony under the care of the UCLA Life Sciences vivarium. Birds were maintained in a 14/10 h light/dark cycle with food and water available *ad libitum*. Protocols were approved by the UC Chancellor's Committee on Animal Care and Use and follow the NIH Principles of Animal Care.

Brain slices culture

CORT increases within minutes of a stressful stimulus to zebra finches (Soma *et al.*, 2004). To limit acute effects of CORT due to handling, all birds were killed by decapitation within 3 min of capture and brains dissected immediately. Tissue containing the lateral ventricles of each hemisphere was isolated by removing the caudal 2 mm and rostral 5 mm of the telencephalon. The two hemispheres were separated along the midline, mounted and glued on their flat caudal edges, submerged in ice-cold oxygenated artificial cerebral spinal fluid (aCSF) (Tam & Schlinger, 2007) and sectioned coronally into 300- μ M-thick slices producing 9–12 slices from each hemisphere per bird. The slices were then placed in 12-well polystyrene plates containing aCSF. To reduce endogenous factors, particularly steroids that are rapidly metabolized in brain (Tam & Schlinger, 2007), all slices were incubated for 1 h in a shaking water bath set to 40°C and before being transferred to a new plate containing the mitotic marker 5'-bromo-2'-deoxyuridine (BrdU) (40 μ g/mL) (Sigma, Saint Louis, MO, USA) in aCSF. After 2 h, the slices were transferred to fresh aCSF in the absence of BrdU and fixed or the incubation

was continued for 16–20 h before fixation. Fixation involved immersion in fresh 4% paraformaldehyde for 2 h at room temperature after which sections were stored in 1 M PBS at 4°C until staining.

Treatments included adding one of the following substances to the 2-h or overnight incubations: pregnenolone (PREG), PROG (Steraloids, Newport, RI, USA), trilostane (gift of Micron Technologies), an inhibitor of 3 β HSD, the enzyme mediating PREG conversion into PROG (Ukena *et al.*, 1999), and CORT (Sigma). Some treatments with CORT included Thiram (Sigma), a compound capable of disrupting the bond between CORT and GR (Garbrecht *et al.*, 2006) or RU-486 (Sigma, Saint Louis, MO), a GR antagonist (Cadepond *et al.*, 1997). All compounds were dissolved in EtOH, except trilostane, which was dissolved in dimethyl sulfoxide (DMSO) (Sigma) as 10 mM stock solutions with aliquots of stock added to aCSF for treatments (see Table 1 for complete incubation design). The final concentration of EtOH or DMSO never exceeded 0.1%. For each experiment, all of the slices from one hemisphere received a single treatment with all the slices of the contralateral hemisphere serving as the matched controls. Left and right hemispheres served alternately as control or treatment.

We used aCSF as the incubation media in these experiments to limit acute actions of factors present in other media. However, we were concerned that proliferation might be impaired under these basal conditions. We performed two additional assays to evaluate this possibility. In the first assay ($n = 3$ males) we compared proliferation in slices incubated with aCSF or with the enriched Dulbecco's modified Eagle's medium (DMEM) (Mediatech Inc., Herndon, VA, USA) supplemented with 10% (v/v) dextran-treated bovine fetal serum (Sigma), 14 mM HEPES and 1% (v/v) penicillin/streptomycin (Invitrogen, Carlsbad, CA, USA). The second assay ($n = 4$ females) was conducted in DMEM devoid of fetal calf serum with or without the steroid PREG at concentration of 1 μ M and the results were compared with a similar assay conducted in aCSF (Table 1, assay 1).

BrdU label

Slices were stained as described previously (Peterson *et al.*, 2004). Briefly, free-floating slices (300 μ M) were washed in 0.1 M PB, pH 7.4, to eradicate excess aldehydes. After DNA denaturation in 3 N HCl followed by washing in 0.1 M PB, pH 7.4, slices were incubated in 0.036% H₂O₂ for 15 min to neutralize endogenous peroxidases. Slices were washed again in 0.1 M PB, pH 7.4, and treated with 10% normal horse serum (Sigma) in 0.3% Triton X-100 in 0.1 M PB, pH 7.4 (PBT), for 60 min at room temperature. For primary antibody treatment, sections were incubated in 1 : 500 rat anti-BrdU antibody (Abcam, Cambridge, MA, USA) in 0.3% PBT for 48 h at 4°C. After primary incubation, sections were washed with 0.1% PBT and treated with a 1 : 200 biotinylated horse anti-mouse IgG (Vector, Burlingame, CA, USA) in 0.3% PBT for 60 min. Additional washes were performed in 0.1% PBT, and the slices were incubated in 1 : 200 avidin–biotin complex (Vectastain) in 0.3% PBT for 90 min at room temperature. Following washing in 0.1% PBT, slices were transferred into 1 M PBS and immunoperoxidase was visualized using an SG chromagen kit (Vector).

Counting of BrdU-labeled cells

Following immunostaining, lateral ventricles were identified under a binocular scope (at 13.2 $\times$) and digitally photographed. Three regions of VZ proliferation were defined, dorsal, medial and ventral. Although cells were counted in all areas, the majority were found either in the dorsal or ventral 'hotspots'. BrdU-labeled cells were counted from all slices by an experimenter blinded to the treatment. These counts were then summed to give for each bird a single total BrdU-labeled cell count for treatment slices and a single total BrdU-labeled cell count for control slices. In one experiment, dorsal and ventral areas were directly compared in control and CORT (100 nM)-treated slices ($n = 7$) using two-way ANOVA (area $\times$ treatment). No

differences were found between area ($F = 0.98$, $P = 0.38$) and the CORT effect (see Results below) remained ($F = 4.32$, $P = 0.04$) with no interaction ($F = 0.15$, $P = 0.69$). Because dorsal and ventral regions of proliferation did not differ in their responsiveness we summed counts from all areas in all slices per hemisphere to give the total number of cells labeled over the course of this treatment. In addition, to ensure reliability of this counting method, a sample of slices was counted independently by two experimenters and these counts were found to be highly correlated ($r = 0.93$).

RNA extraction and RT-PCR

To confirm that GR are present in the songbird brain, we used RT-PCR analysis. Zebra finch total RNA was isolated from the rostral and caudal telencephalon, cerebellum, hypothalamus, optic tectum, liver and kidney from adult male and female zebra finches using Trizol reagent (Invitrogen). The same method was used to extract RNA from brain slices obtained as described above and incubated in aCSF at 40°C for 1 and 8 h. Following DNase (Promega, Madison, WI, USA) treatment of the RNA samples, first-strand cDNA was synthesized by Superscript II reverse transcriptase (Invitrogen). PCR was carried out in a 25- μ L mixture containing 0.375 mM each dNTP, 0.4 μ M each of each primer, 50 ng of template cDNA, 0.06 U/ μ L of the enzyme DNA *Taq* polymerase (Bioline, Randolph, MA, USA) and buffer. Primers for GR and the ribosomal 18S protein gene (loading control) were designed using the *Gallus* genome (Table 2). The PCR reaction was performed at 95°C for 5 min, then 40 cycle and 72°C for 1 min, followed by 72°C for 10 min. PCR products were separated by agarose gel electrophoresis and sequenced by the UCLA Sequencing Facility.

Statistical analysis

Data were analyzed using JMP 5.1 for windows (SAS Institute, Cary, NC, USA) and are presented as mean $\pm$ SEM. Within each steroid hormone treatment, statistical significance was determined using a paired *t*-test. For comparison between factors, one- or two-way ANOVAs were used following log-transformation.

Results

As expected, BrdU-labeled cells were observed only along the surface of the walls of the lateral ventricles (Fig. 1), concentrated in the well-described dorsal and ventral ‘hotspots’ (Fig. 1A). DMEM was not significantly advantageous over aCSF in promoting BrdU-labeling either when compared directly or when examined in the presence of PREG (see below). When half of the slices were incubated in DMEM and the other half in aCSF no significant differences were observed between the number of BrdU-labeled cells on slices incubated in aCSF (670 ± 29.3) compared with the number of labeled cells from slices incubated with DMEM (762 ± 122 ; $t = 0.99$; $P = 0.42$).

Although newly born cells along the VZ persist for several days before initiating migration (Goldman, 1998), we were concerned that BrdU-labeled cell counts from overnight incubations might reflect some cell death in addition to proliferation. We examined this possibility by replicating one experiment with CORT as the treatment but terminated the experiment at the conclusion of the 2 h of exposure to BrdU. As described below, although there were fewer cells labeled after 2 h, and these cells were stained more lightly, the effect of CORT was identical to that described for the overnight incubation. Given this absence of experimental difference but increased intensity of cell staining, we felt the overnight counts were more accurate and chose that time period for all subsequent experiments as a measure of proliferation *per se*.

Treatments with CORT in concentrations resembling acute stress levels in songbirds (Soma *et al.*, 2004; Rich & Romero, 2005) significantly decreased the number of BrdU-labeled cells along the VZ of adult male zebra finches by 55% at 30 nM ($t = 6.39$, $P = 0.023$) and by 40% at 100 nM ($t = 6.15$, $P < 0.001$; Fig. 2). We repeated the assay using 100 nM CORT but the experiment was terminated at the end of the 2 h of incubation with BrdU or BrdU/CORT. As little as 2 h of exposure to BrdU with CORT revealed the effect of CORT on VZ cell proliferation ($t = -3.34$, $P = 0.02$) (Fig. 2). The total number of labeled cells after 2 h of incubation was approximately half the number of labeled cells after overnight incubation (49 and 44% in control and treatment slices, respectively).

We observed no significant effect on BrdU-labeling of adult female slices treated with three concentrations of CORT (Fig. 2): 30 nM ($t = 1.19$, $P = 0.29$), 100 nM ($t = -2.11$, $P = 0.079$) and 1 μ M ($t = 1.98$, $P = 0.11$). Furthermore, we detected no significant effect on numbers of BrdU-labeled cells in males or in females after treatment with PREG (Fig. 3A), even at a concentration of 1 μ M (males: $t = -0.059$, $P = 0.95$; females: $t = -0.98$, $P = 0.36$). When female PREG-treated brain slices incubated in DMEM were compared with the above shown female PREG-treated slices incubated in aCSF, two-way ANOVA (treatment $\times$ medium) revealed no effect of either treatment ($F = 0.056$; $P = 0.81$) or medium ($F = 0.83$; $P = 0.37$) and there was no significant interaction between the two factors ($F = 0.17$; $P = 0.67$).

To determine if the effect of CORT in males involved an interaction with GR, we attempted to block the CORT (100 nM) effect by treating slices of male brains simultaneously with the GR-antagonist RU-486 in low (1 μ M) and high (50 μ M) concentrations. Surprisingly, RU-486 at both concentrations failed to reverse the CORT effect (Fig. 4). At the lower concentration (1 μ M), although the number of BrdU-labeled cells in CORT- plus RU-486-treated slices was 18% greater than the number in CORT-treated slices, this increase was not significant ($t = 0.63$, $P = 0.55$; Fig. 4). The higher concentration of RU-486 further decreased (44%) the number of BrdU-labeled cells as compared with CORT treatment alone ($t = -5.32$, $P < 0.001$; Fig. 4). Finally, we tested the effects of Thiram, a compound capable of dissociating the binding between CORT and GR (Garbrecht *et al.*, 2006). When Thiram (10 nM) was incubated together with CORT (100 nM), the number of BrdU-labeled cells was significantly increased by 38% compared with CORT alone ($t = 2.88$, $P = 0.027$; Fig. 4), an indication that CORT impacts proliferation via GR.

RU-486 might act as an agonist of GR, so we examined the effect on proliferation of RU-486 (50 μ M) by itself. Slices from males treated with RU-486 showed a significant decrease of 52% (593 ± 207.06 to 282.6 ± 121.02) in number of BrdU-labeled cells ($t = -2.95$, $P = 0.041$). Alternatively, RU-486 can act as an antagonist of the progesterone receptor (PR) so the effects of RU-486 on proliferation might be a PROG-mediated mechanism (Spitz, 2003; Ghoumari *et al.*, 2005). To test this possibility, we treated male and female slices with PROG at a concentration of 1 μ M (Fig. 3A) but observed no effect on BrdU-labeling (Fig. 3A). We considered the possibility that PROG could be synthesized *de novo* by the slice (London & Schlenger, 2007; Tam & Schlenger, 2007), obscuring the effect of added PROG. Therefore, we incubated slices with the 3 β HSD inhibitor trilostane at a concentration of 2 μ M to block formation of endogenous PROG. This treatment also had no effect on proliferation in either males or females (Fig. 3B).

To confirm that GR are indeed present in the songbird brain, we used RT-PCR analysis and amplified GR mRNA from five brain regions of both sexes, including the rostral telencephalon containing the VZ (Fig. 5A). In addition, we amplified GR mRNA from brain slices prepared identically to those used in all experiments described here with 1 and 8 h of incubation in aCSF (Fig. 5B). GR mRNA expression was detected in all brain areas tested as well as in peripheral controls. In incubated brain slices, GR mRNA remained detectable after 8 h of incubation.

While analyzing the data it became apparent that brain slices obtained from female brains had fewer BrdU-labeled cells. We analyzed the data from the four experiments described above (CORT 100 nM, CORT 30 nM, PREG 1 μ M and PROG 1 μ M) for controls and treatments separately ($n = 48$) using two-way ANOVA with sex and experiment as factors. In controls, male slices contained 43.5% more BrdU-labeled cells than female slices ($F = 7.61$, $P = 0.0087$). There was no effect of experiment ($F = 1.06$, $P = 0.37$) and there was no interaction between factors ($F = 0.81$, $P = 0.49$). By contrast, when only treated slices from the combined steroid experiments were analyzed, there were no significant differences between males and females ($F = 0.45$, $P = 0.5$). As expected, experimental condition had a significant effect ($F = 4.43$, $P = 0.0087$) and there was no interaction between the factors ($F = 1.7$, $P = 0.18$), further confirming the sex difference in response to CORT.

Discussion

Nuclei of the adult song system in zebra finches are strikingly sexually dimorphic with males having several nuclei that are larger than females, with more and larger neurons (Konishi & Akutagawa, 1985; Arnold *et al.*, 1986; Ball *et al.*, 1994). These sex differences arise developmentally, although the mechanisms generating these differences remain a matter of debate (Wade & Arnold, 2004). Sex differences in neural proliferation may account for some of the sexually dimorphic growth of the neural song system but differential cell survival and ultimate connectivity further establish the final masculine and functional song circuitry (Burek *et al.*, 1995). Nevertheless, neural proliferation persists throughout life in both males and females. A previous study identified sex differences between juvenile males and females *in vivo* (DeWulf & Bottjer, 2002). Such differences were found also in Bengalese finches (Zeng *et al.*, 2007) as well as in young of other non-songbird avian species, such as the quail (Nikolakopoulou *et al.*, 2006) and ring doves (Ling & Cheng, 1995). However, adult males and females generated cells equally along the adult VZ (DeWulf & Bottjer, 2002), suggesting a developmental loss of sexually dimorphic neural proliferation. Our studies indicate that adult males generate more new cells along the VZ than do adult females and this sex difference is revealed *in vitro* in the absence of added factors other than the minimal supplements of aCSF. Chronic exposure to sex-specific hormones or other factors may predispose neural progenitor cells to undergo mitosis differently *in vitro* in adult males and females. However, it is also possible that the sex difference in neural proliferation we document represents a constitutive property of the adult zebra finch brain, established early by sex hormones or by neural differences in sex-chromosome gene expression (Schlinger, 1998; Agate *et al.*, 2003).

Why then were sex differences in adult birds not detected previously (DeWulf & Bottjer, 2002)? Our results indicate the likelihood that in *in vivo* studies, stress-induced increases in secretion of CORT acutely blunt mitosis along the male VZ only, erasing the normal sex difference. In rats, acute (Falconer & Galea, 2003) or chronic (Westenbroek *et al.*, 2004) stress decreases hippocampal cell proliferation in males, but not in females. Thus, sex differences in neuroprogenitor responses to glucocorticoids appear to be a conserved property of the vertebrate brain.

Given the role of GC in the stress response and the effect of GC on cell proliferation, it is possible that CORT-induced decreases in size of song nuclei and song behavior (Buchanan *et al.*, 2004) result from CORT effects along the VZ (but see, Pravosudov & Omanska, 2005). Three major populations of cells have been described in the songbird VZ, namely types E, B and A cells. Of these, type B cells are the primary cells proliferating within the hotspots and the cells on which we believe CORT acts. These cells give rise to type A cells, which are neuroblasts that migrate into the high vocal center (HVC) and differentiate into functional neurons (Alvarez-Buylla & Kirn, 1997; Alvarez-Buylla & Garcia-Verdugo, 2002). High levels

of CORT may reduce the size and function of song nuclei by inhibiting post-mitotic events as well (Wong & Herbert, 2004, 2006).

Interestingly, males were sensitive to the effects of CORT whereas females were unresponsive to CORT at several doses tested. This sex difference in response to CORT may reflect a differential sensitivity to our experimental conditions and may not be a direct measure of the response to CORT. For example, the male VZ might be more energetically active than the VZ of females, and the addition of CORT may be a stressor that lowered rates of proliferation in males only. Use of media supplements did not promote higher rates of proliferation along the VZ of males as compared with females, suggesting no inherent sex difference in utilization of resources that would lead to stress of male tissue. We do not know the basis of the sex difference in tissue responsiveness to CORT, but such differences have been described previously in other tissues of different species (Young, 1996; Willet *et al.*, 1997; Hayward *et al.*, 2006). Gonadal steroids secreted differentially by adult males and females are obvious candidates to induce sex differences in adult responsiveness to GC. In non-neural tissues, estrogens have been shown to limit the actions of GC by regulation of 11 β -hydroxysteroid dehydrogenases (11 β HSD), the *in-situ* GC-metabolizing enzymes (Draper & Stewart, 2005). E2 substantially decreases mRNA expression and activity of the activating type 1 11 β HSD and increases expression and activity of the deactivating type 2 11 β HSD (Baggia *et al.*, 1990; Jamieson *et al.*, 1999; Gomez-Sanchez *et al.*, 2003), with estrogen-dependent regulatory elements on promoter regions of the latter (Baggia *et al.*, 1990). Given that these enzymes are phylogenetically conserved it is plausible that the same regulation of GC by estrogen occurs in the zebra finch brain. Estrogen-induced high levels of 11 β HSD2 and low levels of 11 β HSD1 may render females insensitive to the effects of CORT on cell proliferation. Although no direct effect of estrogens on cell proliferation was detected in the VZ of female canaries (Brown *et al.*, 1993), estrogens seem to promote VZ proliferation of female zebra finches following hippocampal injury (Lee *et al.*, 2007). Estrogens also impact neuronal incorporation in the neural song system by affecting post-mitotic processes (Hidalgo *et al.*, 1995; Williams *et al.*, 1999) and may do so, in part, by regulating enzymes of GC metabolism.

We considered the possibility that sex steroids such as PREG and PROG might impact neural proliferation. Steroidogenic factors (the cholesterol transporter StAR and the steroidogenic enzymes CYP11A1, 3 β -HSD and CYP17) are expressed along the lateral border of the lateral ventricles (London & Schlinger, 2007) at ages when proliferation is greatest and when sex differences in neural circuitry are forming (Konishi & Akutagawa, 1985; DeWulf & Bottjer, 2002). PREG or PROG had no effect on proliferation in adults, suggesting that these steroids are either never important or that they lose their effects in adults. Additional tests of these hormones in developing birds are required to evaluate this possibility.

In these experiments, RU-486 did not abolish the effect of CORT on proliferating cells as it did in rat neural stem cells (Sundberg *et al.*, 2006) or rat hippocampus (Mayer *et al.*, 2006; Oomen *et al.*, 2007), and in high concentration it even intensified the decrease in cell proliferation. It is possible that RU-486 acts as a GR agonist in the songbird brain to diminish proliferation, as has been shown in several non-neural cell lines (Zhang *et al.*, 2007). This idea finds support in a recent study showing RU-486 inhibition of cell proliferation of ovarian cancer cells (Goyeneche *et al.*, 2007), although it had no effect on neurogenesis in rat hippocampus (Oomen *et al.*, 2007). Indeed, when tested alone in our system, RU-486 showed a relatively robust reduction of VZ cell proliferation. In addition, we considered the possibility that the slices synthesized PROG *de novo* (see, London & Schlinger, 2007; Tam & Schlinger, 2007), obscuring effects of added PROG. Nevertheless, treatment of slices with trilostane to block the PROG synthesis enzyme 3 β -HSD had no effect, suggesting either that PROG is not synthesized by the slices or that locally formed PROG has no impact on proliferation. Two pieces of evidence support the conclusion that CORT achieves its inhibition of mitosis via

actions on the GR. First, not unexpectedly, we provide evidence for GR expression in brain, including in slices *in vitro*. Although GR have been studied in the brain of non-songbird species (Sandi & Rose, 1994; Bossis *et al.*, 2004; Kwok *et al.*, 2007), only one study has examined GC binding in the songbird brain (Breuner & Orchinik, 2001). As far as we are aware, the present study is the first to show GR mRNA expression in the zebra finch telencephalon. Secondly, Thiram, a specific inhibitor of the GC-inactivating enzyme 11 β HSD2 (Atanasov *et al.*, 2003; Katz *et al.*, 2008), can also dissociate the bond between GC and GR (Garbrecht *et al.*, 2006) and act as a GR inhibitor. Incubating brain slices in the presence of CORT together with Thiram decreased the CORT effect as expressed in a higher number of proliferating cells. If Thiram functioned as an 11 β HSD2 inhibitor, we would have expected to see an exacerbation of the CORT effect. The observed inhibition of CORT suppression of proliferation supports our belief that Thiram functioned to disrupt CORT interactions with GR.

The number of BrdU-labeled cells within and between assays showed high variability, and an understanding of the sources for this variability may shed light on factors underlying the sex differences we observe. Several factors influence the rate of cell proliferation in the VZ. For example, the number of dividing cells within the VZ is reduced as a function of age in both males and females (DeWulf & Bottjer, 2002) with neurogenesis declining in older zebra finches (Wang *et al.*, 2002). Although all birds used in this study were adults (90 days and older), age differences in this population may have created some of the variability that we see. Estrogens and non-estrogenic ovarian factors can also impact VZ proliferation (Hidalgo *et al.*, 1995; Lee *et al.*, 2007; Peterson *et al.*, 2007) so variability in female slices may result from differences in the ovulatory cycle of females when brains were collected (Ormerod & Galea, 2001). Future studies will examine these possibilities. Finally, it is important to recognize that studies of neural proliferation in adult male songbirds *in vivo* that do not take stress into account may underestimate the full capacity of VZ proliferation.

Acknowledgements

This study was supported by MH6061994.

Abbreviations

aCSF	artificial cerebral spinal fluid
BrdU	5'-bromo-2'-deoxyuridine
CORT	corticosterone
GC	glucocorticoids
GR	glucocorticoid receptors
HSD	hydroxysteroid dehydrogenases
PR	progesterone receptor
PREG	

pregnenolone

PROG

progesterone

VZ

ventricular zone

References

- Agate RJ, Grisham W, Wade J, Mann S, Wingfield J, Schanen C, Palotie A, Arnold AP. Neural, not gonadal, origin of brain sex differences in a gynandromorphic finch. *Proc Natl Acad Sci USA* 2003;100:4873–4878. [PubMed: 12672961]
- Alvarez-Buylla A, Garcia-Verdugo JM. Neurogenesis in adult subventricular zone. *J Neurosci* 2002;22:629–634. [PubMed: 11826091]
- Alvarez-Buylla A, Kirn JR. Birth, migration, incorporation, and death of vocal control neurons in adult songbirds. *J Neurobiol* 1997;33:585–601. [PubMed: 9369461]
- Alvarez-Buylla A, Theelen M, Nottebohm F. Proliferation “hot spots” in adult avian ventricular zone reveal radial cell division. *Neuron* 1990;5:101–109. [PubMed: 2369518]
- Alvarez-Buylla A, Garcia-Verdugo JM, Mateo AS, Merchant-Larios H. Primary neural precursors and intermitotic nuclear migration in the ventricular zone of adult canaries. *J Neurosci* 1998;18:1020–1037. [PubMed: 9437023]
- Arnold AP, Bottjer SW, Brenowitz EA, Nordeen EJ, Nordeen KW. Sexual dimorphisms in the neural vocal control system in song birds: ontogeny and phylogeny. *Brain Behav Evol* 1986;28:22–31. [PubMed: 3552110]
- Atanasov AG, Tam S, Rocken JM, Baker ME, Odermatt A. Inhibition of 11 beta-hydroxysteroid dehydrogenase type 2 by dithiocarbamates. *Biochem Biophys Res Commun* 2003;308:257–262. [PubMed: 12901862]
- Avital A, Segal M, Richter-Levin G. Contrasting roles of corticosteroid receptors in hippocampal plasticity. *J Neurosci* 2006;26:9130–9134. [PubMed: 16957069]
- Baggia S, Albrecht ED, Pepe GJ. Regulation of 11 beta-hydroxysteroid dehydrogenase activity in the baboon placenta by estrogen. *Endocrinology* 1990;126:2742–2748. [PubMed: 2328704]
- Ball GF, Casto JM, Bernard DJ. Sex differences in the volume of avian song control nuclei: comparative studies and the issue of brain nucleus delineation. *Psychoneuroendocrinology* 1994;19:485–504. [PubMed: 7938349]
- Bossis I, Nishimura S, Muchow M, Porter TE. Pituitary expression of Type I and Type II glucocorticoid receptors during chicken embryonic development and their involvement in growth hormone cell differentiation. *Endocrinology* 2004;145:3523–3531. [PubMed: 15070856]
- Breuner CW, Orchinik M. Seasonal regulation of membrane and intracellular corticosteroid receptors in the house sparrow brain. *J Neuroendocrinol* 2001;13:412–420. [PubMed: 11328450]
- Breuner CW, Jennings DH, Moore MC, Orchinik M. Pharmacological adrenalectomy with mitotane. *Gen Comp Endocrinol* 2000;120:27–34. [PubMed: 11042008]
- Brown SD, Johnson F, Bottjer SW. Neurogenesis in adult canary telencephalon is independent of gonadal hormone levels. *J Neurosci* 1993;13:2024–2032. [PubMed: 8478689]
- Buchanan KL, Leitner S, Spencer KA, Goldsmith AR, Catchpole CK. Developmental stress selectively affects the song control nucleus HVC in the zebra finch. *P Roy Soc B-Biol Sci* 2004;271:2381–2386.
- Burek MJ, Nordeen KW, Nordeen EJ. Initial sex differences in neuron growth and survival within an avian song nucleus develop in the absence of afferent input. *J Neurobiol* 1995;27:85–96. [PubMed: 7643078]
- Cadepond F, Ulmann MDA, Baulieu MDEE. RU486 (MIFE-PRISTONE): mechanisms of action and clinical uses. *Annu Rev Med* 1997;48:129–156. [PubMed: 9046951]
- Crochemore C, Michaelidis TM, Fischer D, Loeffler J, Almeida OFX. Enhancement of p53 activity and inhibition of neural cell proliferation by glucocorticoid receptor activation. *FASEB J* 2002;16:761–770. [PubMed: 12039857]

- Cyr NE, Romero LM. Chronic stress in free-living European starlings reduces corticosterone concentrations and reproductive success. *Gen Comp Endocrinol* 2007;151:82–89. [PubMed: 17280663]
- DeWulf V, Bottjer SW. Age and sex differences in mitotic activity within the zebra finch telencephalon. *J Neurosci* 2002;22:4080–4094. [PubMed: 12019327]
- DeWulf V, Bottjer SW. Neurogenesis within the juvenile zebra finch telencephalic ventricular zone: a map of proliferative activity. *J Comp Neurol* 2005;481:70–83. [PubMed: 15558733]
- Draper N, Stewart PM. 11{beta}-Hydroxysteroid dehydrogenase and the pre-receptor regulation of corticosteroid hormone action. *J Endocrinol* 2005;186:251–271. [PubMed: 16079253]
- Falconer EM, Galea LAM. Sex differences in cell proliferation, cell death and defensive behavior following acute predator odor stress in adult rats. *Brain Res* 2003;975:22–36. [PubMed: 12763590]
- Galea LAM. Gonadal hormone modulation of neurogenesis in the dentate gyrus of adult male and female rodents. *Brain Res Rev* 2008;57:332–341. [PubMed: 17669502]
- Garbrecht MR, Krozowski ZS, Snyder JM, Schmidt TJ. Reduction of glucocorticoid receptor ligand binding by the 11-[beta] hydroxysteroid dehydrogenase type 2 inhibitor, Thiram. *Steroids* 2006;71:895–901. [PubMed: 16857225]
- Garcia-Verdugo JM, Ferron S, Flames N, Collado L, Desfilis E, Font E. The proliferative ventricular zone in adult vertebrates: a comparative study using reptiles, birds, and mammals. *Brain Res Bull* 2002;57:765–775. [PubMed: 12031273]
- Ghousari AM, Baulieu EE, Schumacher M. Progesterone increases oligodendroglial cell proliferation in rat cerebellar slice cultures. *Neuroscience* 2005;135:47–58. [PubMed: 16054770]
- Goldman SA. Adult neurogenesis: from canaries to the clinic. *J Neurobiol* 1998;36:267–286. [PubMed: 9712309]
- Goldman SA, Nottebohm F. Neuronal production, migration, and differentiation in a vocal control nucleus of the adult female canary brain. *Proc Natl Acad Sci USA* 1983;80:2390–2394. [PubMed: 6572982]
- Gomez-Sanchez EP, Ganjam V, Chen YJ, Liu Y, Zhou MY, Toroslu C, Romero DG, Hughson MD, de Rodriguez A, Gomez-Sanchez CE. Regulation of 11 beta-hydroxysteroid dehydrogenase enzymes in the rat kidney by estradiol. *Am J Physiol Endocrinol Metab* 2003;285:E272–279. [PubMed: 12700160]
- Gould E, Cameron HA, Daniels DC, Woolley CS, McEwen BS. Adrenal hormones suppress cell-division in the adult-rat dentate gyrus. *J Neurosci* 1992;12:3642–3650. [PubMed: 1527603]
- Goyeneche AA, Caron RW, Telleria CM. Mifepristone inhibits ovarian cancer cell growth in vitro and in vivo. *Clin Cancer Res* 2007;13:3370–3379. [PubMed: 17545545]
- Hayward LS, Richardson JB, Grogan MN, Wingfield JC. Sex differences in the organizational effects of corticosterone in the egg yolk of quail. *Gen Comp Endocrinol* 2006;146:144–148. [PubMed: 16430891]
- Hidalgo A, Barami K, Iversen K, Goldman SA. Estrogens and non-estrogenic ovarian influences combine to promote the recruitment and decrease the turnover of new neurons in the adult female canary brain. *J Neurobiol* 1995;27:470–487. [PubMed: 7561828]
- Hodgson Z, Meddle S, Roberts M, Buchanan K, Evans M, Metzdorf R, Gahr M, Healy S. Spatial ability is impaired and hippocampal mineralocorticoid receptor mRNA expression reduced in zebra finches (*Taeniopygia guttata*) selected for acute high corticosterone response to stress. *Proc R Soc Lond B Biol Sci* 2007;274:239–245.
- Holloway CC, Clayton DE. Estrogen synthesis in the male brain triggers development of the avian song control pathway in vitro. *Nat Neurosci* 2001;4:170–175. [PubMed: 11175878]
- Jamieson PM, Nyirenda MJ, Walker BR, Chapman KE, Seckl JR. Interactions between oestradiol and glucocorticoid regulatory effects on liver-specific glucocorticoid-inducible genes: possible evidence for a role of hepatic 11 beta-hydroxysteroid dehydrogenase type 1. *J Endocrinol* 1999;160:103–109. [PubMed: 9854182]
- Karishma KK, Herbert J. Dehydroepiandrosterone (DHEA) stimulates neurogenesis in the hippocampus of the rat, promotes survival of newly formed neurons and prevents corticosterone-induced suppression. *Eur J Neurosci* 2002;16:445–453. [PubMed: 12193187]

- Katz A, Heiblum R, Meidan R, Robinson B. Corticosterone oxidative neutralization by 11-beta hydroxysteroid dehydrogenase in kidney and colon of chickens. *Gen Comp Endocrinol* 2008;155:814–820. [PubMed: 18022174]
- Keller EA, Zamparini A, Borodinsky LN, Gravielle MC, Fisman ML. Role of allopregnanolone on cerebellar granule cells neurogenesis. *Dev Brain Res* 2004;153:13–17. [PubMed: 15464213]
- de Kloet ER, Joels M, Holsboer F. Stress and the brain: from adaptation to disease. *Nat Rev Neurosci* 2005;6:463–475. [PubMed: 15891777]
- Konishi M, Akutagawa E. Neuronal growth, atrophy and death in a sexually dimorphic song nucleus in the zebra finch brain. *Nature* 1985;315:145–147. [PubMed: 3990816]
- Kwok AHY, Wang Y, Wang CY, Leung FC. Cloning of chicken glucocorticoid receptor (GR) and characterization of its expression in pituitary and extrapituitary tissues. *Poult Sci* 2007;86:423–430. [PubMed: 17234861]
- Landys MM, Wingfield JC, Ramenofsky M. Plasma corticosterone increases during migratory restlessness in the captive white-crowned sparrow *Zonotrichia leucophrys gambelli*. *Horm Behav* 2004;46:574–581. [PubMed: 15555499]
- Lee DW, Gowry Fernando R, Peterson S, Allen TA, Schlinger BA. Estrogen mediation of injury-induced cell birth in neuroproliferative regions of the adult zebra finch brain. *Dev Neurobiol* 2007;67:1107–1117. [PubMed: 17565708]
- Ling C, Cheng MF. Sex differences in cell proliferation in the ventricular zone of young ring doves. *Brain Res Bull* 1995;37:657–662. [PubMed: 7670892]
- London SE, Schlinger BA. Steroidogenic enzymes along the ventricular proliferative zone in the developing songbird brain. *J Comp Neurol* 2007;502:507–521. [PubMed: 17394140]
- Mayer JL, Klumpers L, Maslam S, de Kloet ER, Joels M, Lucassen PJ. Brief treatment with the glucocorticoid receptor antagonist mifepristone normalises the corticosterone-induced reduction of adult hippocampal neurogenesis. *J Neuroendocrinol* 2006;18:629–631. [PubMed: 16867184]
- Mayo W, Lemaire V, Malaterre J, Rodriguez JJ, Cayre M, Stewart MG, Kharoubi M, Rougon G, Le Moal M, Piazza PV, Abrous DN. Pregnenolone sulfate enhances neurogenesis and PSA-NCAM in young and aged hippocampus. *Neurobiol Aging* 2005;26:103–114. [PubMed: 15585350]
- Nikolakopoulou AM, Pappas A, Panagis L, Zikopoulos B, Dermon CR. Early post-hatching sex differences in cell proliferation and survival in the quail telencephalic ventricular zone and intermediate medial mesopallium. *Brain Res Bull* 2006;70:107–116. [PubMed: 16782501]
- Oomen CA, Mayer JL, de Kloet ER, Joels M, Lucassen PJ. Brief treatment with the glucocorticoid receptor antagonist mifepristone normalizes the reduction in neurogenesis after chronic stress. *Eur J Neurosci* 2007;26:3395–3401. [PubMed: 18052970]
- Ormerod BK, Galea LAM. Reproductive status influences cell proliferation and cell survival in the dentate gyrus of adult female meadow voles: a possible regulatory role for estradiol. *Neuroscience* 2001;102:369–379. [PubMed: 11166123]
- Peterson RS, Lee DW, Fernando G, Schlinger BA. Radial glia express aromatase in the injured zebra finch brain. *J Comp Neurol* 2004;475:261–269. [PubMed: 15211466]
- Peterson RS, Gowry F, Day L, Allen TA, Chapleau JD, Menjivar J, Schlinger BA, Lee DW. Aromatase expression and cell proliferation following injury of the adult zebra finch hippocampus. *Dev Neurobiol* 2007;67:1867–1878. [PubMed: 17823932]
- Pravosudov VV, Omanska A. Prolonged moderate elevation of corticosterone does not affect hippocampal anatomy or cell proliferation rates in mountain chickadees (*Poecile gambeli*). *J Neurobiol* 2005;62:82–91. [PubMed: 15389682]
- Reiner A, Perkel DJ, Mello CV, Jarvis ED. Songbirds and the revised avian brain nomenclature. *Ann NY Acad Sci* 2004;1016:77–108. [PubMed: 15313771]
- Rich EL, Romero LM. Exposure to chronic stress downregulates corticosterone responses to acute stressors. *Am J Physiol Regul Integr Comp Physiol* 2005;288:R1628–R1636. [PubMed: 15886358]
- Sandi C, Rose SPR. Corticosteroid receptor antagonists are amnesic for passive-avoidance learning in day-old chicks. *Eur J Neurosci* 1994;6:1292–1297. [PubMed: 7981871]
- Sapolsky RM. Stress, glucocorticoids, and damage to the nervous system: the current state of confusion. *Stress* 1996;1:1–19. [PubMed: 9807058]

- Schlinger BA. Sexual differentiation of avian brain and behavior: current views on gonadal hormone-dependent and independent mechanisms. *Annu Rev Physiol* 1998;60:407–429. [PubMed: 9558471]
- Soma KK, Alday NA, Hau M, Schlinger BA. Dehydroepiandrosterone Metabolism by 3{beta}-hydroxysteroid dehydrogenase/{Delta}5-{Delta}4 isomerase in adult zebra finch brain: sex difference and rapid effect of stress. *Endocrinology* 2004;145:1668–1677. [PubMed: 14670998]
- Spencer KA, Verhulst S. Delayed behavioral effects of postnatal exposure to corticosterone in the zebra finch (*Taeniopygia guttata*). *Horm Behav* 2007;51:273–280. [PubMed: 17196201]
- Spencer KA, Buchanan KL, Goldsmith AR, Catchpole CK. Song as an honest signal of developmental stress in the zebra finch (*Taeniopygia guttata*). *Horm Behav* 2003;44:132–139. [PubMed: 13129485]
- Spitz IM. Progesterone antagonists and progesterone receptor modulators: an overview. *Steroids* 2003;68:981–993. [PubMed: 14667991]
- Stokes TM, Leonard CM, Nottebohm F. Telencephalon, diencephalon, and mesencephalon of canary, *Serinus canaria*, in stereotaxic coordinates. *J Comp Neurol* 1974;156:337–374. [PubMed: 4609173]
- Sundberg M, Savola S, Hienola A, Korhonen L, Lindholm D. Glucocorticoid hormones decrease proliferation of embryonic neural stem cells through ubiquitin-mediated degradation of cyclin D1. *J Neurosci* 2006;26:5402–5410. [PubMed: 16707792]
- Tam H, Schlinger BA. Activities of 3[beta]-HSD and aromatase in slices of developing and adult zebra finch brain. *Gen Comp Endocrinol* 2007;150:26–33. [PubMed: 16919626]
- Tata DA, Marciano VA, Anderson BJ. Synapse loss from chronically elevated glucocorticoids: relationship to neuropil volume and cell number in hippocampal area CA3. *J Comp Neurol* 2006;498:363–374. [PubMed: 16871536]
- Taupin P, Gage FH. Adult neurogenesis and neural stem cells of the central nervous system in mammals. *J Neurosci Res* 2002;69:745–749. [PubMed: 12205667]
- Ukena K, Honda Y, Inai Y, Kohchi C, Lea RW, Tsutsui K. Expression and activity of 3 beta-hydroxysteroid dehydrogenase Delta(5)-Delta(4)-isomerase in different regions of the avian brain. *Brain Res* 1999;818:536–542. [PubMed: 10082843]
- Wade J, Arnold AP. Sexual differentiation of the zebra finch song system. *Ann N Y Acad Sci* 2004;1016:540–559. [PubMed: 15313794]
- Wang NG, Hurley P, Pytte C, Kirn JR. Vocal control neuron incorporation decreases with age in the adult zebra finch. *J Neurosci* 2002;22:10864–10870. [PubMed: 12486180]
- Westenbroek C, Den Boer JA, Veenhuis M, Ter Horst GJ. Chronic stress and social housing differentially affect neurogenesis in male and female rats. *Brain Res Bull* 2004;64:303–308. [PubMed: 15561464]
- Willet KE, Jobe AH, Ikegami M, Polk D, Newnham J, Kohan R, Gurrin L, Sly PD. Postnatal lung function after prenatal steroid treatment in sheep: effect of gender. *Pediatr Res* 1997;42:885–892. [PubMed: 9396574]
- Williams S, Leventhal C, Lemmon V, Nedergaard M, Goldman SA. Estrogen promotes the initial migration and inception of NgCAM-dependent calcium-signaling by new neurons of the adult songbird brain. *Mol Cell Neurosci* 1999;13:41–55. [PubMed: 10049530]
- Wong EYH, Herbert J. The corticoid environment: a determining factor for neural progenitors' survival in the adult hippocampus. *Eur J Neurosci* 2004;20:2491–2498. [PubMed: 15548194]
- Wong EYH, Herbert J. Raised circulating corticosterone inhibits neuronal differentiation of progenitor cells in the adult hippocampus. *Neuroscience* 2006;137:83–92. [PubMed: 16289354]
- Young EA. Sex differences in response to exogenous corticosterone: a rat model of hypercortisolemia. *Mol Psychiatry* 1996;1:313–319. [PubMed: 9118357]
- Zeng SJ, Song K, Xu N, Zhang XW, Zuo MX. Sex difference in cellular proliferation within the telencephalic ventricle zone of Bengalese finch. *Neurosci Res* 2007;58:207–214. [PubMed: 17493701]
- Zhang SM, Jonklaas J, Danielsen M. The glucocorticoid agonist activities of mifepristone (RU486) and progesterone are dependent on glucocorticoid receptor levels but not on EC50 values. *Steroids* 2007;72:600–608. [PubMed: 17509631]

Fig. 1.

Typical images of BrdU-labeled cells (black nuclei) along the adult male zebra finch VZ after slices were incubated with aCSF with BrdU for 2 h followed by aCSF with no BrdU for an additional 16–20 h before fixation. (A) VZ of a 300- μ M-thick coronal slice (2.3× magnification) located approximately 6.5 mm and 3.5 mm from the rostral and caudal ends, respectively: arrows indicate dorsal (top) and ventral (bottom) 'hot spots' of cell proliferation. (bar = 1 mm). (B) Magnification at which BrdU-labeled cells were counted (13.2×); (bar = 1 mm). (C) Photomicrograph of BrdU-labeled cell nuclei (100×; bar = 0.1 mm). H, hyperpallium; HP, hippocampus; LV, lateral ventricle; MSt, medial striatum; N, nidopallium (Stokes *et al.*, 1974; Reiner *et al.*, 2004).

Fig. 2.

BrdU-labeled cells counts (mean $\pm$ SEM) from slices prepared simultaneously from bisected brain hemispheres of male and female zebra finches treated with 30 nM, 100 nM or 1 μ M CORT (right bar in each pair) as compared with the opposite hemisphere Control slices (left bar in each pair); $n = 3-9$ birds per treatment. In each bird, all sections from one hemisphere per bird were assigned to a treatment, all sections from the contralateral hemisphere serving as the control. Paired t -tests: * $P = 0.02$; ** $P < 0.05$, *** $P = 0.005$.

Fig. 3.

BrdU-labeled cells counts (mean $\pm$ SEM) from slices prepared simultaneously from bisected brain hemispheres of male and female zebra finches ($n = 6-7$ birds per treatment). In each bird, all sections from one hemisphere per bird were assigned to a treatment, all sections from the contralateral hemisphere serving as the control, treated with (A): 1 μ M pregnenolone (PREG)- (solid grey bars) or progesterone (PROG)-treated (solid black bars) as compared with the opposite hemisphere Control slices treated with EtOH only (dotted bars in each pair). (B) Trilostane (2 μ M; striped bars) as compared with the opposite hemisphere Control slices treated with the vehicle DMSO only (white bars); $n = 6-7$ birds per treatment. In each bird, all sections from one hemisphere per bird were assigned to a treatment, all sections from the contralateral

hemisphere serving as the control. Paired t -tests: no significant effects of treatments were found.

Fig. 4.

BrdU-labeled cells counts (mean $\pm$ SEM) from slices prepared simultaneously from bisected brain hemispheres of male zebra finches with treatment (patterned bar in each pair) with 1 μ M RU-486, 50 μ M RU-486 or 10 nM Thiram compared with the control slices from the opposite hemisphere (white bar in each pair) supplied with 100 nM CORT + vehicle: $n = 5-10$ birds per treatment. In each bird, all sections from one hemisphere per bird were assigned to a treatment, all sections from the contralateral hemisphere serving as the control. Paired t -tests: * $P < 0.0012$; ** $P = 0.027$. In paired t -test analysis significance was achieved although standard error bars may appear overlapping.

Fig. 5.

(A) Glucocorticoid receptor (GR) mRNA expression and a loading control (18S) in tissues of adult female and male zebra finches after amplification using RT-PCR (a representation of six independent replicates). TC, telencephalon (caudal); TR, telencephalon (rostral); CER, cerebellum; HYP, hypothalamus; OT, optic tectum; L, liver; K, kidney. (B) GR mRNA expression and a loading control (18S) in adult male zebra finch 300-μM brain slices immediately after slice preparation (0 h) and after 1 and 8 h of incubation in aCSF (a representation of three independent replicates).

Table 1

List of assays used

	Assay	Sex/ <i>n</i>	Treatment	Control
1	PREG	M/6 F/6	PREG, 1 μ M	Vehicle
2	PROG	M/7 F/6	PROG, 1 μ M	Vehicle
3	Trilostane	M/6 F/6	Trilostane, 2 μ M	Vehicle
4	CORT (a)	M/3 F/5	CORT, 30 nM	Vehicle
5	CORT (b)	M/9 F/7	CORT, 100 nM	Vehicle
6	CORT (c)	F/5	CORT, 1 μ M	Vehicle
7	CORT (d)	M/5	CORT, 1 μ M	Vehicle
8	RU-486 (a)	M/5	RU-486, 1 μ M+ CORT, 100 nM	CORT 100 nM + vehicle
9	RU-486 (b)	M/10	RU-486, 50 μ M+ CORT, 100 nM	CORT 100 nM + vehicle
10	RU-486 (c)	M/5	RU-486, 50 μ M	Vehicle
11	Thiram	M/5	Thiram, 10 nM+ CORT, 100 nM	CORT 100 nM + vehicle

M, males; F, females; CORT, corticosterone; PREG, pregnenolone; PROG, progesterone.

Table 2

List of primers used

Genbank accession number	Gene	Sequence	Product length (bp)
AF173612	18S	F: GCGCAAGACGAACTAAAGC R: CTCACTAAACCATCCAATCGG	758
DQ227738	GR	F: GCCTCAAGTGAAAACGGAAA R: TTGCAGCTGCCACAAGTAAG	501

F, forward; R, reverse; GR, glucocorticoid receptor; 18S, ribosomal 18S protein gene.