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Corticosterone and carbenoxolone effects on neural cell proliferation in the adult songbird.

A thesis submitted in partial satisfaction of the requirements for the degree Master of Science in

Physiological Science

by

Rehan Karmali

2017

ABSTRACT OF THE THESIS

Corticosterone and carbenoxolone effects on neural cell proliferation in the adult songbird.

by

Rehan Karmali

Master of Science in Physiological Science

University of California, Los Angeles 2017

Professor Barnett Schlinger, Co-chair

Professor Christopher S. Colwell, Co-chair

Acute and/or chronic stress can negatively affect neurogenesis of mammals and birds via action of the stress hormone and glucocorticoid, corticosterone (CORT). The zebra finch ventricular zone (VZ) has persistent neurogenesis during adulthood. Our lab previously reported that the adult female VZ is protected from stress-induced declines in neurogenesis in contrast to males. 11-beta hydroxysteroid dehydrogenases (11β HSDs) regulate CORT, with the type 2 isoform de-activating it. In this study, we tested the hypothesis that inhibiting this enzyme with carbenoxolone will reduce neurogenesis in females and further decrease it males. We measured cell proliferation using the mitotic marker 5'-bromo-2'deoxyuridine (BrdU) and treated brain slices with CORT reflecting increasing stress levels with or without carbenoxolone. We found no

indicate that perhaps 11β HSD2 does not mediate the previously-observed sex differences in adult neurogenesis.

The thesis of Rehan Karmali is approved.

David Walker

Christopher S. Colwell, Committee Co-chair

Barnett Schlinger, Committee Co-chair

University of California, Los Angeles

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ACKNOWLEDGEMENTS

Introduction

Neurogenesis a conserved property across vertebrates and occurs in the region bordering the lateral ventricles known as the ventricular zone (Alvarez-Bullya et al.,1994; Eriksson et al., 1998; Gould et al., 1999; Lacar et. al. 2014). Neurogenesis is the process during which new neurons, astrocytes and glia are generated from undifferentiated neural progenitor cells. While it is widespread and critical during development, adult neurogenesis is important various functions including, but not limited to temporal and pattern separation as well as memory resolution (Aimone et. al. 2014). A hallmark of the adult songbird brain is persistent neural cell proliferation in distinct dorsal and ventral "hotspots" along the VZ (Goldman and Nottebohm, 1983; Garcia-Verdugo et al., 2002; DeWulf and Bottjer, 2005). These regions are comprised of pluripotent stem cells that differentiate into new neurons and radial glia (Alvarez- Buylla et al., 1998). Newly born neurons remain the VZ for up to 3 days after mitosis (Barami et al., 1995), before they migrate to specific brain regions and are incorporated into efferent motor pathways for song learning and vocalization as well as hippocampal-dependent spatial learning and navigation (Gould et al., 1999; Alvarez-Buylla and Garcia-Verdugo, 2002; Barnea, 2009). Hence, the songbird brain serves as an ideal model wherein one can study factors affecting adult neurogenesis.

Stress is one such factor. Typically, in response to a stressor, the hypothalamus releases trophic neurohormones which in turn increase levels of plasma glucocorticoids such as corticosterone (CORT) in songbirds or cortisol in mammals. This adrenal steroid signals the liver to release glucose, suppresses the immune system and mediates processing in limbic neuronal networks that are integral to memory, cognition and emotion (De Kloet et. al., 1998). Moreover, CORT negatively feeds back to the hypothalamus to restore baseline plasma CORT levels and

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protect various organs from overexposure. Given CORT's lipid-soluble structure, it can cross the blood-brain-barrier and affect stress physiology in the brain via neural glucocorticoid (GR) and mineralcorticoid receptors (MR) (Gould et al., 1992).

CORT effects on the brain do not display a simple inverse relationship. On one hand, regions like the mammalian hippocampus express high levels of GR and MR and binding of CORT to these receptors plays a key role in cognition and memory formation (Wurmser et. al. 2004). Furthermore, physical stressors such as exercise can boost adult neurogenesis as evidenced by wheel running activity in mice that enhances neuronal differentiation as well as elevates circulating glucocorticoids (Van Praag et. al. 1999, Droste et al. 2003). On the other hand, diminished neurogenesis and neuronal loss are among detrimental effects resulting from chronically elevated CORT (Sapolsky et al., 1985; Mirescu & Gould, 2006; Newman et al., 2010).

Interestingly, CORT effects on the brain highlight a sex-specific phenomenon. Various studies have documented how acute (Westenbroek et al., 2004; Shors et. al., 2007; Chan et a., 2017) or chronic (Falconer & Galea, 2003; Hillerer et al., 2013; Mekiri et al. 2017) stress decreases hippocampal cell proliferation in adult male rats, but not in females. Moreover, our lab reported that the VZ of adult male zebra finches respond to high CORT levels with a decrease in neurogenesis, while females are protected from similar stress-induced declines (Katz et. al, 2008) Hence, what then is protecting the adult female VZ from stress-induced declines in neurogenesis? Two key 11-beta hydroxysteroid dehydrogenases (11β HSDs) may mediate this sex difference. The type 1 isoform (11β HSD1) catalyzes the conversion of the inactive 11-dehydroCORT (11-DHC) to active CORT (11-OH-GC) (Tomlinson et al., 2004) such that CORT can bind to mineralcorticoid receptors (MR) at baseline levels and glucocorticoid receptors (GR)

at high levels (Reul and deKloet, 1985; Romero et al., 2004; Hodgson et al. 2007; Dickens et al., 2009; Shahbazi et al., 2011). 11β HSD1 is expressed in various regions of the adult mammalian brain and is implicated in regulating neurogenesis and spatial memory function (Moisan et al., 1990; Sakai et al., 1992; Rajan et al., 1996; Ajilore and Sapolsky, 1999; Sandeep et al., 2004). On the other hand, the type 2 isoform (11β HSD2) de-activates CORT by converting it from active CORT to 11-DHC, thereby impairing its ability to bind its receptor (Stewart and Krozowski, 1999). 11β HSD2 is expressed at low levels in the adult mammalian brain (Robson et al., 1998), but interestingly, it is expressed at high levels in the adult zebra finch brain (Katz et al., 2010; Rensel et al., 2014). More importantly, 11β HSD2 has been shown to protect fetal mammals from CORT overexposure as evidenced by its expression in placenta, body and brain. For instance, methylation of the 11β HSD2 gene results in reduced infant birthweight (Marsit et. al. 2012), while a brain-specific 11β HSD2 knockout results in depressive-like behaviors and cognitive impariments during adulthood (Wyrwoll et. al. 2015).

Hence, this study tests the hypothesis that sex differences in neurogenesis result from 11-BHSD2 de-activating CORT in the female zebra finch brain, but not the male. To test this hypothesis, we will measure cell proliferation in adult male and female brain slices using the mitotic marker 5'-bromo-2'deoxyuridine (BrdU). The slice culture media will contain CORT reflecting varying levels of stress with our without carbenoxolone (CBX), a reversible and competitive inhibitor of 11β HSD (Baker and Fanestil, 1991). We predict that female brain slices treated with CBX will show a reduction in neurogenesis along the VZ at high stress levels.

Experimental procedures and methods

<u>*Animals:*</u>

All research and protocols were approved by the University of California, Los Angeles Chancellor's Committee on Animal Care and used following NIH guidelines. Zebra finches were housed under 14/10 h light:dark cycle with ad libitum food and water. Adult non-breeding zebra finches (>90 days of age, n = 24) were obtained from our colony at the UCLA Life Sciences Vivarium and utilized for this study.

<u>*Tissue collection:*</u>

All birds were sacrificed by decapitation within three minutes of capture in order to minimize acute CORT effects due to handling. Plasma CORT has a significant diel rhythm and is lowest at midday while brain CORT does not have a robust diel rhythm (Rensel et. al. 2014). Hence, birds, were sacrificed between 08:00 and 14:00 of the subjective day to further minimize effects of endogenous CORT. Brains were extracted and tissue containing the ventricular zone (VZ) was isolated by removing the caudal 2 mm and rostral 5 mm of the telencephalon. The whole brain was mounted and glued on its flat caudal plain and submerged in ice-cold oxygenating artificial cerebral spinal fluid (aCSF) (Tam and Schlinger, 2007). The brain was sectioned coronally into 300-μM-thick slices and left and right brain slices readily separated during the sectioning process. 9–12 slices were produced from each hemisphere per bird, which were then placed in 12-well polystyrene plates containing aCSF. To reduce the effects of and to metabolize endogenous steroids present at the time of sacrifice, all slices were first incubated for one hour in a water bath set to 40°C for a recovery phase (Tam and Schlinger, 2007).

Slice culture

Experiment 1 - Female brain slices pre-treated with 100 nM CBX and then 100 nM CORT:

After the recovery phase, slices were transferred to a new plate containing fresh aCSF with or without 100 nM CBX, a competitive and reversible 11β HSD inhibitor and incubated for one hour in a water bath set to 40º C. Following the inhibitor phase, paired slices were either incubated with the mitotic marker 5'-bromo-2'deoxyuridine (BrdU) (40 ug/mL in aCSF) or BrdU (40 ug/mL in 100 nM CORT in aCSF) for 2 hours in a water bath set to 40º C. After 2 hours, the slices were transferred to fresh aCSF or aCSF + CORT for a 16-20 hour overnight incubation in the absence of BrdU in a water bath set to 40º C. Following the overnight incubation, slices were fixed in fresh 4% paraformaldehyde for 2 hours at room temperature and stored in 0.1M Phosphate Buffer (PB) in 24-well plates at 4ºC until immunocytochemistry was conducted. Slices were stored in 0.1MB for a period of 2 hours - 48 hours.

Experiment 2a and 2b - Male/Female brain slices pre-treated with 1uM CBX and subsequently 0nM, 5nM, 10nM, 20nM and 30nM CORT :

After the recovery phase, slices were transferred to a new plate containing fresh aCSF with or without 1 uM CBX, a competitive and reversible 11β HSD inhibitor and incubated for one hour in a water bath set to 40º C. Following the inhibitor phase, paired slices were incubated with either the mitotic marker 5'-bromo-2'deoxyuridine (BrdU) (40 ug/mL in aCSF) or BrdU (40 ug/mL in 0nM, 5nM, 10nM, 20 nM or 30nM CORT in aCSF) for 2 hours in a water bath set to 40º C. After 2 hours, the slices were transferred to fresh aCSF (control) or to the same respective dose of CORT in the absence of BrdU for a 16-20 hour overnight incubation in a water bath set to 40º C.

Experiment 3a - Male brain slices treated with 30nM CORT or aCSF only.

After the recovery phase, paired slices were incubated with either the mitotic marker 5'-bromo-2'deoxyuridine (BrdU) (40 ug/mL in aCSF) or BrdU (40 ug/mL in 30nM CORT in aCSF) for 2 hours in a water bath set to 40º C. After 2 hours, the slices were transferred to fresh aCSF or aCSF + 30 nM CORT in the absence of BrdU for a 16-20 hour overnight incubation in a water bath set to 40º C.

Experiment 3b – Male brain slices pre-treated with 1uM CBX only and then with 30nM CORT with or without 1 uM CBX:

After the recovery phase, slices were transferred to a new plate containing fresh aCSF with or without 1 uM CBX, a competitive and reversible 11β HSD inhibitor and incubated for one hour in a water bath set to 40º C. Following the inhibitor phase, paired slices were either incubated with the mitotic marker 5'-bromo-2'deoxyuridine (BrdU) (40 ug/mL in 30nM CORT in aCSF) or BrdU (40 ug/mL in 30nM CORT and 1 uM CBX in aCSF) for 2 hours in a water bath set to 40º C. After 2 hours, the slices were transferred to fresh 30 nM CORT in aCSF or 30 nM CORT + 1 uM CBX in aCSF in the absence of BrdU for a 16-20 hour overnight incubation in a water bath set to 40º C.

Immunocytochemistry:

Immunocytochemistry (ICC) for BrdU was performed on free-floating sections (300μm) based on the previously described staining method (Peterson et al., 2007) and adapted for use on brain slices (Katz et al., 2008, Mirzatoni et al., 2010). Slices were transferred from 24-well plates to 12-well net-well carriers and washed 2 x 15min in 0.1 M PB, for 30 min in 3N HCL to denature DNA followed by a 3 x 5 min wash in 0.1M PB, and then a 15 min wash in 0.036% H_2O_2 to neutralize endogenous peroxidases. After washing 3 x 15 min in 0.1 M PB, slices were

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transferred to 24-well plates containing 10% normal horse serum (Vector S-2000) in 0.3% Triton X-100 (Sigma) and incubated for 60 min at room temperature. Slices were then transferred to new 24-well plates containing 1:500 rat monoclonal antibody against BrdU (Abcam 6326) in 0.3% PBT and incubated at 48 h at 4°C.

Slices were then washed for 2 x 15min 0.1% PBT, incubated with 1:200 biotinylated anti-mouse secondary (Vector BA-2000) in 0.3% PBT for 60 min, then washed for 3 x 15min 0.1% PBT, incubated in 1:200 avidin–biotin complex (Vectastain PK-4002) in 0.3% PBT for 90 min at room temperature. After final 3 x 15min washes in 0.1% PBT, immunoproduct was visualized using an SG chromogen kit (Vector SK-4700). Following development, the tissues were mounted on gelatin-coated glass slides and allowed to dry for a period of 18-24 hours. Then, tissues were cleared 2 x 2 min in 70% ethanol, 2 x 2 min in 95 % ethanol, 3 x 2 min in 100% ethanol, 2 x 2 min in Hemo – DE (Fisher) and finally coverslipped using Permount.

Data Analysis:

Following BrdU-labeling, the ventricular zone was located using a compound light microscope (Fig. 1). BrdU-labeled cells were counted through the microscope lens at 40x magnification from all defined dorsal, medial, and ventral regions by two investigators blind to experimental treatments. The defining features of a BrdU labeled cell are a distinct dark round/elliptical shape with a diameter of about 1-2 microns at the given magnification. If cells appeared to show a sharp cleavage similar to one observed during cytokinesis, but two distinct cells were not observed, then this potentially dividing cell was still counted as single cell. Two experimenters performed counts on a sample of slices and were highly correlated (r = 0.993), thereby indicating the reliability of this counting technique. In order to analyze counts, we

compared means and utilized paired samples t-test and a linear mixed model as necessary. Detailed descriptions are included in the results.

Results

<u>*Experiment 1:*</u>

We utilized a paired samples t-test to compare the means of control slices treated with aCSF to the means of slices treated with CORT. We also assessed the means of another set of control slices treated with CORT to the means of paired slices treated with CORT + CBX. Since each bird receives all four treatments, the paired t-test is appropriate to compare pairwise means. The mean count for aCSF treated slices was 181.84 BrdU+ cells with a standard error of 40.94, while the mean count for CORT treated slices was 180.46 BrdU+ cells with a standard error of 32.06. The t-test statistic is -0.038 with 5 degrees of freedom. The p-value is 0.971 (Fig. 2). Hence, we fail to reject the null and conclude that there is not enough evidence to suggest that the mean number of BrdU+ cells in the CORT treated group is different from mean number of BrdU+ cells in the aCSF control group.

Additionally, the mean count for CORT + CBX treated slices was 254.16 BrdU+ cells with a standard error of 70.66, while the mean count for paired CORT treated slices was 233.90 BrdU+ cells with a standard error of 70.66. The t-test statistic is -1.232 with 5 degrees of freedom. The p-value is 0.273. (Fig. 2). Hence, we fail to reject the null and conclude that there is not enough evidence to suggest that the mean number of BrdU+ cells in the CORT + CBX treated group is different from mean number of BrdU+ cells in the CORT control group.

Experiment 2:

In this experiment, we had 12 different treatments with varying concentrations of CORT with or without CBX across 6 different female and male birds. We utilized a linear mixed model to assess differences in BrdU+ cell counts across treatments. Birds were treated as random effects and treatment as fixed effects. For females, the F-stat is 1.829 with 11 and 60 degrees of freedom with a p-value of 0.069 (Fig. 3a). For males, the F-stat is 0.864 with 11 and 60 degrees of freedom with a p-value of 0.579. (Fig. 3b). These results suggests that there is not a difference in BrdU+ cell counts across treatments for either males or females.

Experiment 3a :

We utilized a paired samples t-test to compare the means of control slices treated with aCSF to the means of slices treated with 30nM CORT. Since each bird receives both treatments, the paired t-test is appropriate for analyzing this data. The mean count for aCSF treated slices was 224.02 BrdU+ cells with a standard error of 68.12, while the mean count for CORT treated slices was 237.74 BrdU+ cells with a standard error of 74.68. The t-test statistic is -1.456 with 2 degrees of freedom. The p-value is 0.283 (Fig. 4). Hence, we fail to reject the null and conclude that there is not enough evidence to suggest that mean number of BrdU+ cells in the CORT treated group is different from mean number of BrdU+ cells in the aCSF control group.

Experiment 3b :

We utilized a paired samples t-test to compare the means of control slices treated with 30 nM CORT to the means of slices treated with 30nM CORT + 1uM CBX. Since each bird receives both treatments, the paired t-test is appropriate for analyzing this data. The mean count for CORT treated slices was 249.49 BrdU+ cells with a standard error of 32.96, while the mean count for CORT + CBX treated slices was 262.49 BrdU+ cells with a standard error of 24.56.

The t-test statistic is -0.627 with 2 degrees of freedom. The p-value is 0.595 (Fig. 4). Hence, we fail to reject the null and conclude that there is not enough evidence to suggest that mean number of BrdU+ cells in the CORT + CBX treated group is different from mean number of BrdU+ cells in the CORT control group.

Discussion

In this study, we investigated the effects of stress on adult neurogenesis. We treated brain slices with either corticosterone and/or carbenoxolone and used BrdU to label proliferating cells in the VZ. In experiment 1, treatment of brain slices with 100nM CORT did not decrease adult female VZ neurogenesis, which is consistent with our lab's previous findings (Katz et. al. 2008). In addition, co-treatment of slices with CORT and 100nM CBX did not produce decreased levels of neurogenesis in the same female birds (Fig. 2). In experiment 2, we increased the concentration of CBX to 1uM to test whether a higher concentration of the enzyme's inhibitor would produce different results in females, and included males to test for sex differences. In sections treated with CORT reflecting, baseline, low stress, medium stress and high stress levels with or without CBX, we found no effect of CORT or CORT paired with CBX on neurogenesis in either males or females (Fig 3a. and 3b.). Finally, in experiment 3 we treated only male brain slices with CORT reflecting high stress levels (30 nM) that were paired to contralateral control slices that only received aCSF. We also treated a separate set of male birds with 30 nM CORT with or without 1uM CBX. We found that treatment of brain slices with 30nM CORT did not decrease adult male VZ neurogenesis, which is in contrast with our lab's previous findings (Fig 4., Katz et. al. 2008). Moreover, treatment of brain slices with 1 uM CBX paired with 30 nM

CORT reflecting high stress levels did not decrease neurogenesis in a different set of male birds (Fig 4.).

From a methodological perspective, the lack of an observable effect in experiments 1 and 2 can be attributed to slices being treated with CBX for only one hour since CBX was not present in the incubation media overnight. Moreover, since CBX is a reversible inhibitor of 11β HSD2, it may have been metabolized and as a result, the enzyme 11β HSD2 could have de-activated CORT during the overnight incubation, thereby eliminating any observable effects on neurogenesis. Hence, for experiment 3 we used a high concentration of CBX at 1 uM, pre-treated treated brain slices with CBX prior to addition of any CORT and ensured that CBX was present in the media throughout the overnight incubation period. Our data did not reflect stress-induced declines in males as hypothesized (Fig. 4).

From a larger neurobiological perspective, the lack of an effect of CORT or CORT paired with CBX on either male or female neurogenesis points to the possibility that 11β HSD2 does not mediate the previously-observed sex differences in adult neurogenesis, where the female ventricular zone appeared to be protected from high stress levels (Katz. et. al. 2008). Given the unique gonadal hormone profiles of females compared to males, our lab has previously investigated the effects of estradiol (E2), testosterone (T) dehydroepiandrosterone (DHEA), progesterone (PROG), allopregnenolone, its sulfate derivatives and steroidogenic enzymes on neurogenesis. These neurosteroids and gonadal steroids were previously shown to induce neural proliferation in the mammalian brain (Karishma and Herbert, 2002; Keller et al., 2004; Mayo et al., 2005; Galea, 2008). However, we found that neither exogenously added gonadal steroids or steroids synthesized *de novo* definitively promoted or protected male or female neurogenesis in a brain slice culture model. (Mirzatoni et al., 2010)

Furthermore, while many studies have shown that elevated stress decreases adult neurogenesis (Dranovsky et. al., 2006, Schoenfeld et. al. 2012), other findings indicate that stress may not have an effect (Hanson et. al. 2011) and in some cases, even increase neurogenesis (Lyons et. al. 2010, Parihar et. al. 2011). Interestingly, chronic stress has been shown to decrease cell survival, but not hippocampal proliferation (Lee. et. al. 2006). Moreover, our lab previously reported that the female ventricular zone is susceptible to loss of newly born cells (Mirzatoni et. al. 2010). As such, this raises the question as to whether the lack of observable stress-induced effects on neurogenesis is a result of varying degrees of apoptosis and neuronal migration not being taken into consideration. As such, future studies measuring neurogenesis should utilize Doublecortin (DCX) as a marker to account for immature migrating neurons. These studies should also measure activated caspase, a key protease that facilitates apoptosis, via immunostaining to account for stress-induced changes in apoptotic activity.

In conclusion, the findings from this study challenge the general hypothesis that a stressor of high-enough intensity and duration negatively affects adult neurogenesis. We show here that the inhibiting corticosterone-inactivating enzyme 11β HSD2 under high-stress conditions does not reduce neurogenesis in the adult zebra finch ventricular zone for either males or females. Ongoing studies in our lab are exploring the regional distribution of this enzyme to help us draw further insights about corticosterone regulation in the adult avian brain.
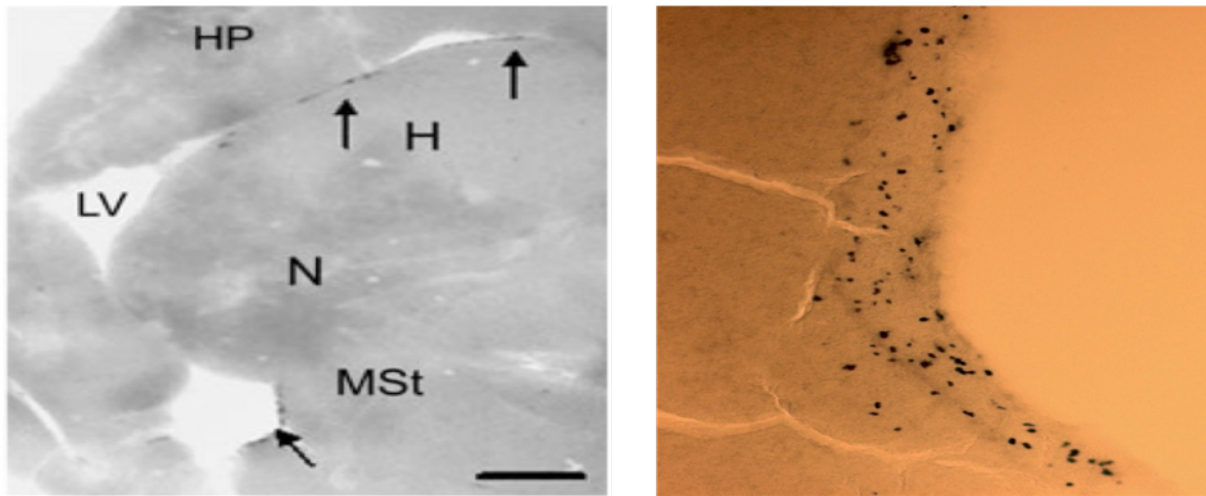
Figure 1 – Standard images of BrdU+ cells (dark nuclei) along the interior wall of adult zebra finch ventricular zone (VZ). **Left -** VZ of a 300-μM coronal slice (2.3× magnification) located ~ 6.5 mm and 3.5 mm from the rostral and caudal ends, respectively: arrows indicate dorsal (top) and ventral (bottom) 'hot spots' of cell proliferation. (bar = 1 mm). H, hyperpallium; HP, hippocampus; LV, lateral ventricle; MSt, medial striatum; N, nidopallium. **Right -** Magnification of counted BrdU+ cells (40× mag).
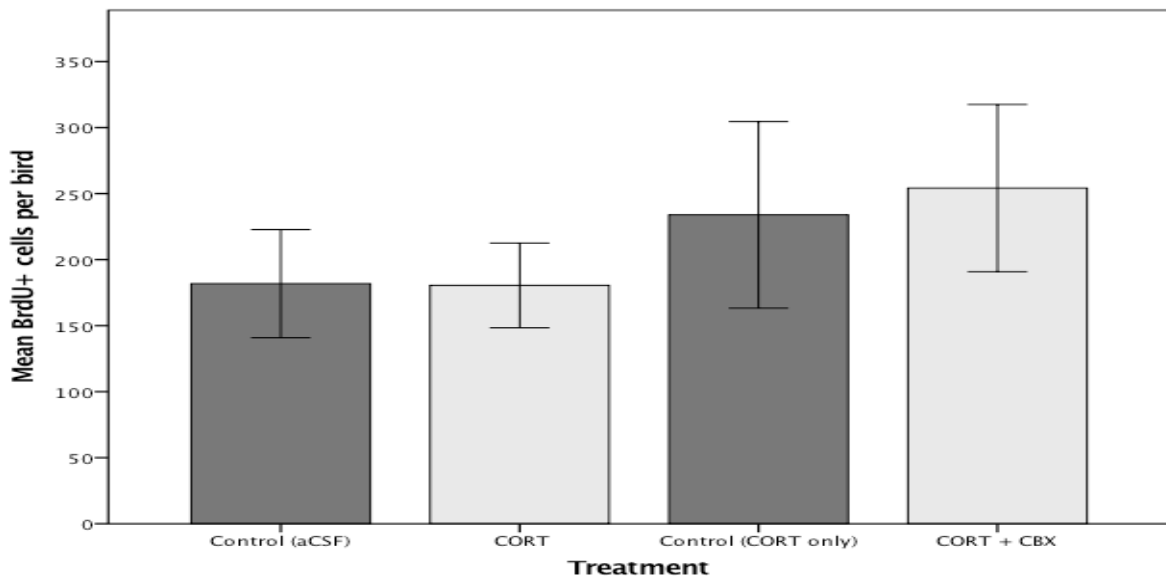

Figure 2 - The mean (±SEM) BrdU+ cells prepared from bisected brain hemispheres of adult females. One set of paired brain slices were incubated with 100nM Corticosterone (treatment) or with artificial cerebrospinal fluid (aCSF control) (n = 6 birds); $p > 0.05$ (0.971). A different set of paired slices were incubated with 100 nM of carbenoxolone (CBX) and then 100 nM of CORT (treatment) or only with 100nM CORT (control) (n = 6 birds); $p > 0.05$ (0.273). One hemisphere was treated and the contralateral hemisphere was the control.
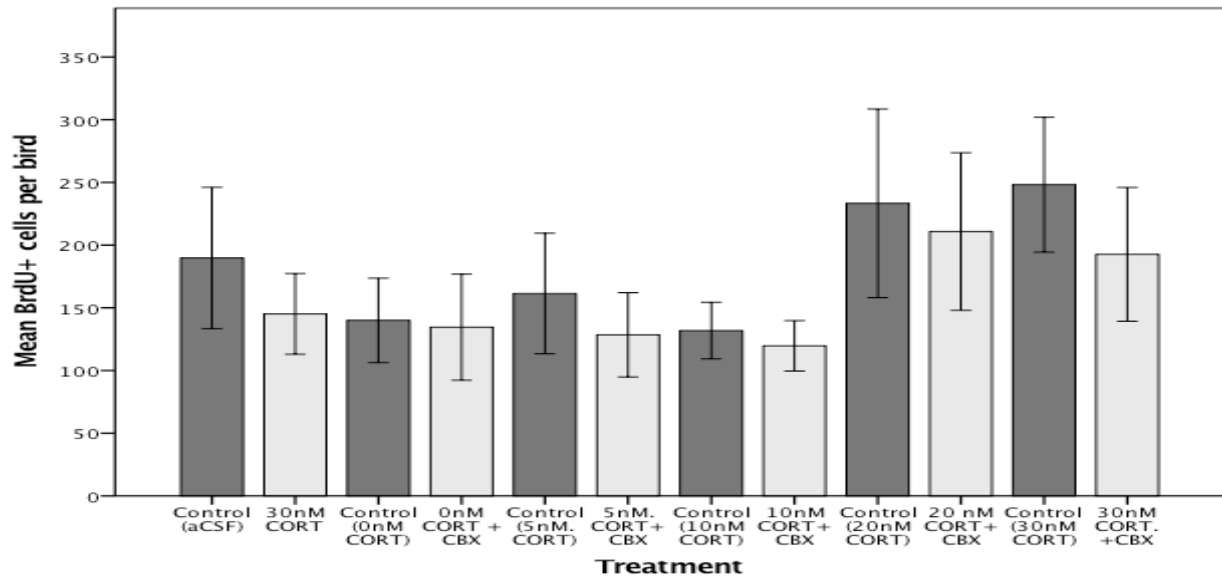
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Figure 3a - The mean (±SEM) BrdU+ cells prepared from bisected brain hemispheres of adult males. Paired brain slices were incubated with a solution 0nM, 5nM, 10nM, 20 nM or 30nM CORT and CBX (treatment) or without CBX (control). (n = 6 birds); $p > 0.05$ (0.069). One hemisphere was treated and the contralateral hemisphere was the control.

Figure 3b - The mean (±SEM) BrdU+ cells prepared from bisected brain hemispheres of adult females. Paired brain slices were incubated with a solution 0nM, 5nM, 10nM, 20 nM or 30nM CORT and CBX (treatment) or without CBX (control). (n = 6 birds); $p > 0.05$ (0.579). One hemisphere was treated and the contralateral hemisphere was the control.
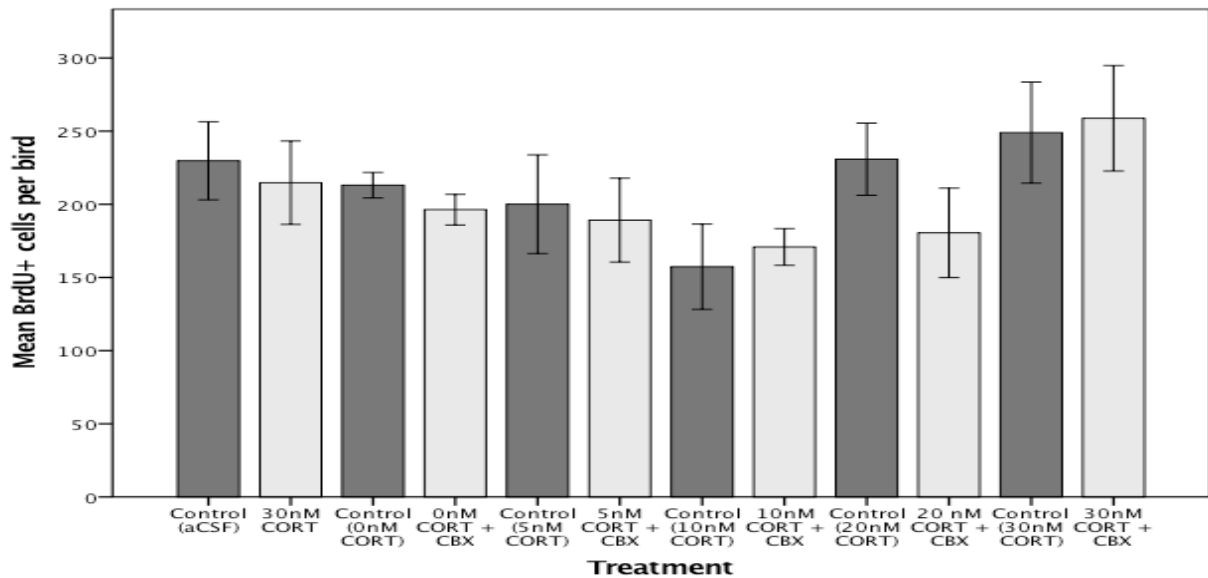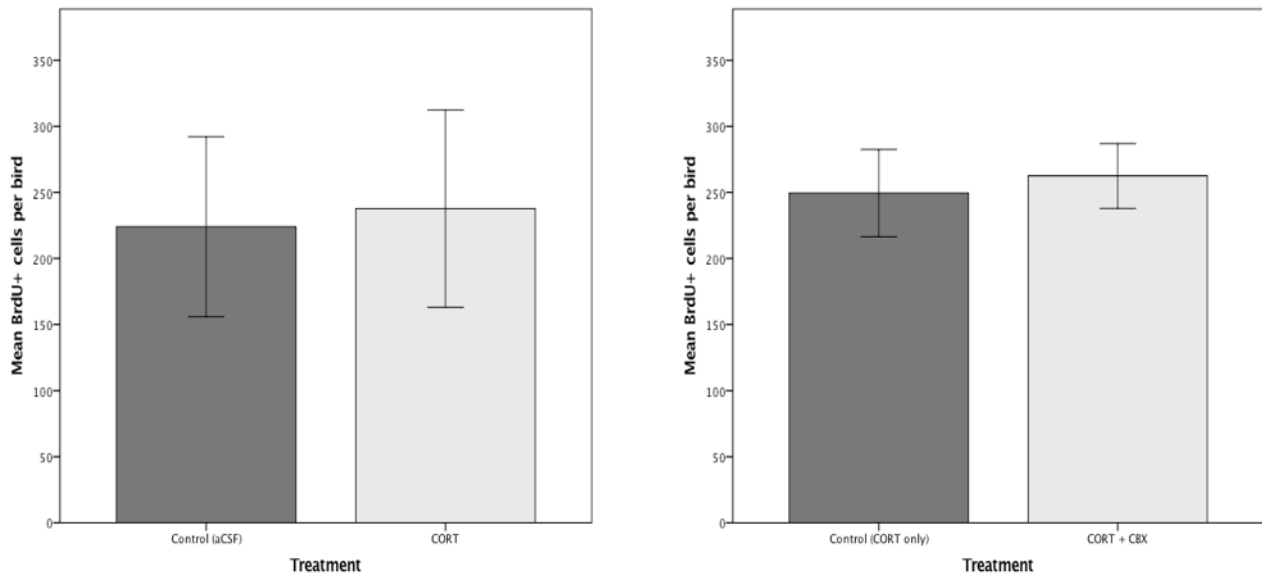
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Figure 4 – Left: The mean (±SEM) BrdU+ cells prepared from paired slices of bisected hemispheres of adult males treated with 30nM of Corticosterone (CORT) or without CORT (aCSF control). Slices from one hemisphere were treated with CORT and slices from the contralateral hemisphere served as the control. (n = 3 birds); p > 0.05 (0.283). **Right:** The mean (±SEM) BrdU+ cells prepared from paired slices of bisected hemispheres of adult males treated with a solution of 30nM corticosterone and 1uM carbenoxolone (CORT + CBX) or 30nM CORT only (control). Slices from one hemisphere were treated with CORT + CBX and slices from the contralateral hemisphere served as the control. (n = 3 birds); p > 0.05 (0.595).

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