The Neuroprotective Effects of Estradiol and Genistein in Zebra Finch Cerebellum

Renee E. Breaux

University of Mississippi

Follow this and additional works at: https://egrove.olemiss.edu/hon_thesis

Part of the Neurosciences Commons

Recommended Citation
https://egrove.olemiss.edu/hon_thesis/2715

This Undergraduate Thesis is brought to you for free and open access by the Honors College (Sally McDonnell Barksdale Honors College) at eGrove. It has been accepted for inclusion in Honors Theses by an authorized administrator of eGrove. For more information, please contact egrove@olemiss.edu.
THE NEUROPROTECTIVE EFFECTS OF ESTRADIOL AND GENISTEIN IN ZEBRA FINCH CEREBELLUM

by
Renee E. Breaux

A thesis submitted to the faculty of The University of Mississippi in partial fulfillment of the requirements of the Sally McDonnell Barksdale Honors College.

Oxford
April 2022

Approved By

_____________________________
Advisor: Dr. Lainy B. Day

_____________________________
Reader: Dr. Christopher Leary

_____________________________
Reader: Dr. Alberto Del Arco
ACKNOWLEDGEMENTS

I would like to begin by thanking Dr. Lainy Day for her incredible guidance during my time in her lab. Her dedication to her students and her passion for neuroscience are truly inspiring. I am forever grateful for my time in her lab, and she has provided me with much knowledge that I will surely use in the future. Second, I would like to thank my second and third readers for taking the time to share with me their thoughts on my project. I am very grateful for their feedback, and I am happy to share this experience with them. Third, I would like to thank my fellow members of the Day lab who have supported my research and me. Specifically, I would like to thank Belinda Bagwandeen, who spent countless hours training me and preparing me for my own role in this project. Fourth, I would like to thank the Sally McDonnell Barksdale Honors College for their tremendous support throughout my time at the University of Mississippi. From providing me with incredible educational opportunities to introducing me to amazing people, I will always remember the impact of my time with the Honors College. Finally, I would like to thank my friends and my family. Without them, I would not have had the opportunity and the drive to reach this point, and I will forever owe them for their impact on my life.
ABSTRACT

Renee E. Breaux: The Neuroprotective Effects of Estradiol and Genistein in Zebra Finch Cerebellum
(Under the Direction of Dr. Lainy B. Day)

The estradiol (E$_2$) synthetic pathway converts testosterone to E$_2$ via aromatase (AROM) and plays an important role in neuroplasticity. However, exogenous E$_2$ increases cancer risk and interferes with gonadal function. Phytoestrogens, plant-based estrogens, may provide neuroprotection without negative E$_2$ effects. Genistein (GEN), a soy phytoestrogen, preferentially binds to estrogen receptor beta (ER $\beta$), which is expressed at a relatively higher concentration than ER $\alpha$ in the cerebellum (CB). The songbird CB is an ideal model for steroid-mediated plasticity. Songbird brains are highly plastic and CB contains all steroid-synthetic enzymes. Previous studies in zebra finches (ZF) have shown that AROM and E$_2$ prevent post-injury secondary neurodegeneration. In our lab, we implanted adult male ZF with either E$_2$, GEN, or silastic vehicle (CON) and injected either saline (S) or an AROM inhibitor, letrozole (LET) during delivery of cerebellar puncture lesions to test hormonal effects on testes morphology, body weight, and neuroprotection. We previously reported that E$_2$, compared to GEN and control birds, had reduced testis mass, spermatozoa number, and laminarity. We also found that body mass, which often decreases in birds over the course of invasive experiments, decreased in E$_2$ and control birds but not GEN birds. This suggests GEN may influence fat metabolism as has been previously shown. In the present study, I determined the extent of neuroprotection by measuring the volume of the lesion as a consequence of secondary degeneration as labeled by Terminal Deoxynucleotidyl Transferase dUTP Nick End Labeling (TUNEL) for each group. We expected that subjects given an injection of E$_2$ + S and GEN + S would have the smallest lesion volume, as the E$_2$ and GEN should provide neuroprotection. We
expected that the size of the lesion would be somewhat increased in subjects given $E_2 + LET$ and $GEN + LET$, as the implanted $E_2$ and $GEN$ could counter local inhibition of AROM. We expected that subjects given $CON + S$ would have the second largest lesion volume, as there is no additional $E_2$ or $GEN$ to contribute to neuroprotection. Finally, we expected that subjects given $CON + LET$ would have the largest lesion volume, as LET blocks the production of $E_2$ by AROM and no additional $E_2$ or $GEN$ is present. Having measured 3-4 subjects per group, we did not detect a significant impact of local or systemic treatments on secondary degeneration. There was considerable individual variation within some groups that could be due to variation in circulating hormone levels that will be measured by high-performance liquid chromatography (HPLC) in the future. Power analyses indicate low power and the need for 18 additional subjects or 3 per group, which we have ready to be measured. Furthermore, we have performed a secondary assay that labels necrotic cells with Fluro-Jade (FJ) and measuring of lesion volumes in this complementary assay should help to refine our conclusions.
# TABLE OF CONTENTS

<table>
<thead>
<tr>
<th>Section</th>
<th>Page</th>
</tr>
</thead>
<tbody>
<tr>
<td>LIST OF TABLES AND FIGURES</td>
<td>vi</td>
</tr>
<tr>
<td>LIST OF ABBREVIATIONS</td>
<td>vii</td>
</tr>
<tr>
<td>INTRODUCTION</td>
<td>1</td>
</tr>
<tr>
<td>MATERIALS/METHODS</td>
<td>6</td>
</tr>
<tr>
<td>RESULTS</td>
<td>11</td>
</tr>
<tr>
<td>DISCUSSION</td>
<td>14</td>
</tr>
<tr>
<td>LIST OF REFERENCES</td>
<td>19</td>
</tr>
</tbody>
</table>
LIST OF FIGURES AND IMAGES

IMAGE 1 .................................................................................................................. 10

FIGURE 1 .................................................................................................................. 12

FIGURE 2 .................................................................................................................. 13

FIGURE 3 .................................................................................................................. 13
<table>
<thead>
<tr>
<th>Abbreviation</th>
<th>Description</th>
</tr>
</thead>
<tbody>
<tr>
<td>AROM</td>
<td>Aromatase</td>
</tr>
<tr>
<td>CB</td>
<td>Cerebellum</td>
</tr>
<tr>
<td>CON</td>
<td>Silastic Vehicle/Control</td>
</tr>
<tr>
<td>DH₂O</td>
<td>Distilled Water</td>
</tr>
<tr>
<td>E₂</td>
<td>Estradiol</td>
</tr>
<tr>
<td>ER</td>
<td>Estrogen Receptor</td>
</tr>
<tr>
<td>EtOH</td>
<td>Ethanol</td>
</tr>
<tr>
<td>FJ</td>
<td>Fluoro-Jade</td>
</tr>
<tr>
<td>GEN</td>
<td>Genistein</td>
</tr>
<tr>
<td>HPLC</td>
<td>High-Performance liquid Chromatography</td>
</tr>
<tr>
<td>LET</td>
<td>Letrozole</td>
</tr>
<tr>
<td>PPARγ</td>
<td>Peroxisome-Proliferator Activated Receptor-γ</td>
</tr>
<tr>
<td>S</td>
<td>Saline</td>
</tr>
<tr>
<td>TUNEL</td>
<td>Terminal Deoxynucleotidyl Transferase dUTP Nick End Labeling</td>
</tr>
<tr>
<td>ZF</td>
<td>Zebra Finch</td>
</tr>
</tbody>
</table>
Introduction

Steroidal pathways are involved in neuroprotection in the brain. Within one particular pathway, the enzyme AROM converts testosterone into the steroid E$_2$ (Azcoitia et al., 2010; Duncan et al., 2013; Duncan & Saldanha, 2020; Mirzatoni et al., 2010; Peterson et al., 2001; Saldanha et al., 2005; Saldanha et al., 2009; Spence et al., 2009). Recently, researchers have begun to link this steroid hormone to prevention of secondary degeneration following neural injury in a songbird model, the zebra finch (Taeniopygia Guttata) (Azcoitia et al., 2010; Duncan et al., 2013; Duncan & Saldanha, 2020; Mirzatoni et al., 2010; Peterson et al., 2001; Saldanha et al., 2005; Saldanha et al., 2009; Spence et al., 2009). Similar to E$_2$, the enzyme AROM is associated with protective and reparative functions. In the CB, constitutive expression of AROM is limited (Duncan & Saldanha, 2020; Saldanha et al., 2005; Saldanha et al., 2009). However, similar to previous studies where researchers found AROM increased within the hippocampus of birds and mammals following neural injury, an upregulation of AROM transcription and translation occurs within reactive glial cells of the ZF entopallium and Bergman glial cells of the CB in regions surrounding neural injury, leading to increased production of E$_2$ (Azcoitia et al., 2010; Duncan et al., 2013; Duncan & Saldanha, 2020; Mirzatoni et al., 2010; Peterson et al., 2001; Saldanha et al., 2005; Saldanha et al., 2009; Spence et al., 2009). When an AROM inhibitor is administered, the extent of secondary degeneration increases (Azcoitia et al., 2010; Duncan et al., 2013; Duncan & Saldanha, 2020; Saldanha et al., 2005; Saldanha et al., 2009). This suggests that E$_2$ is neuroprotective because when AROM is inhibited from producing E$_2$, the extent of damage worsens. Songbirds, like the ZF, are a useful model to examine the neuroprotective effects of E$_2$ and AROM as they have greater steroidogenesis than non-songbirds and many mammals (Brann et al., 2022) but low levels of AROM in CB (Azcoitia et al., 2010;
Duncan et al., 2013; Duncan & Saldanha, 2020; Mirzatoni et al., 2010; Peterson et al., 2001; Saldanha et al., 2005; Saldanha et al., 2009; Spence et al., 2009). While ZF AROM is constitutively low in CB scattered in only a few Purkinje cells, AROM and all other steroidogenic enzymes are produced in CB and several have been shown to be sensitive to neural insult (Mirzatoni et al., 2010; Peterson et al., 2001; Spence et al., 2009). Furthermore, few studies have linked upregulation of AROM or its inhibition with behavioral outcomes post-injury as has been demonstrated in the CB of ZF (Spence et al., 2009). When an AROM inhibitor is present, behavioral outcomes are impaired; and when E$_2$ was added, injury related deficits improved (Spence et al., 2009). Thus, the ZF CB is an ideal model for studying the effects of estrogens and phytoestrogens on neuroprotection.

Neuroprotection occurs by impeding a variety of processes pre and post injury. The processes of neuronal cell death occur in two distinct stages: primary degeneration and secondary degeneration (Duncan et al., 2013; Duncan & Saldanha, 2020). During the process of primary degeneration, cell death occurs at the direct site of contact with the source of injury. Secondary degeneration then follows primary degeneration and affects the cells surrounding the site of injury. This secondary degeneration results from the release of apoptotic signals by the cells that die during primary degeneration and occurs via apoptosis or necrosis. While necrosis occurs as a result of mechanical tissue damage, apoptosis occurs actively and requires the input of energy. Within the brain, AROM and E$_2$ are thought to function to protect the neurons by limiting these processes of apoptosis and secondary necrosis surrounding the site of injury.

While research shows that E$_2$ plays a role in neuroprotection, its potential for negative effects within the body complicate administration of E$_2$ to prevent secondary degeneration. In women E$_2$ is known to increase the risk of stroke and cancer when administered during
menopause (Jiang et al., 2013; Sites, 2008). In men E₂ is associated with a decrease in male reproductive functions (Halldín et al., 2005; Leavy et al., 2017; Rochester et al., 2010). Phytoestrogens, plant-derived estrogenic compounds, may potentially serve as a replacement for E₂ to decrease the extent of neural degeneration at the site of injury while avoiding the negative effects of E₂ (Corbitt et al., 2007). Both the structure and function of phytoestrogens within the brain are similar to that of E₂. Specifically GEN, a type of phytoestrogen classified as an isoflavone that is found in soy and fava beans, binds to estrogen receptors within the brain and has been shown to have neuroprotective functions by decreasing apoptosis during secondary degeneration in rodents (Corbitt et al., 2007; Jiang et al., 2013). While chronic administration of GEN is associated with reproductive harm within males, decreasing sperm count and quality, it is believed that acute administration of GEN will have few negative consequences on gonadal function (Eustache et al., 2009). Within tissues, two distinct types of estrogen receptors exist: estrogen receptor alpha (ER α) and estrogen beta (ER β) (Duncan & Saldanha, 2020). In the reproductive system of male rats, E₂ binds to both ER α and ER β (Ball et al., 2010). While GEN binds to both ER α and ER β, it is more potent when binding to ER β (Katzenellenbogen & Katzenellenbogen, 2000), the estrogen receptor more common in the ZF CB. The binding of E₂ or GEN to an ER is what activates genes and proteins that function in neuroprotection, showing that binding to an ER is essential for E₂-related neuroprotection (Azcoitia et al., 2010; Duncan et al., 2013; Duncan & Saldanha, 2020; Mirzatoni et al., 2010; Peterson et al., 2001; Saldanha et al., 2005; Saldanha et al., 2009; Spence et al., 2009).

Our lab examined the role of GEN and E₂ on male gonads and neuroprotection in zebra finches by implanting E₂ or GEN silastic hormones or silastic alone in addition to injecting LET and S at the site of cerebellar lesions. Thus far, we have examined the detrimental effects of E₂
on the male reproductive system and the possible protective effects of GEN on testes mass, sperm density, and laminarity (McFatridge, 2020). We found that E$_2$ significantly reduced testes mass relative to CON, while GEN maintained a mass that was not significantly different from CON. With regards to spermatozoa production and laminarity, we found that only 5/9 birds that received E$_2$ produced spermatozoa and birds that received E$_2$ experienced reduced sperm density and laminarity, while birds treated with GEN and CON showed normal levels of each. Thus GEN could serve as a replacement for E$_2$ if it provides neuroprotection while diminishing reproductive harm.

In our previous study, we found that GEN offered protection against the typical handling related decreases in body mass that were seen in E$_2$ and controls over the 15 days of the experiment (McFatridge, 2020). This maintenance of body weight may be attributed to the potential lipogenic effects of GEN. This ability to induce adipogenesis, however, is regulated by transcription factors and is found to be dosage-dependent (Dang, Audinot, Papapoulos, Boutin & Löwik, 2003). Specifically, GEN is known to bind to the peroxisome proliferator-activated receptor-γ (PPARγ), causing an increase in adipogenesis (Dang et al., 2003). While estrogens typically bind to ER α and β to induce lipolysis, the binding of GEN to PPARγ has anti-estrogenic effects, reducing lipolysis and increasing adipogenesis (Dang et al., 2003; Penza et al., 2006). The ability of GEN to induce adipogenesis, however, depends on the amount of GEN present. In one study, researchers found that a high dosage of GEN is needed to have anti-estrogenic effects and increase adipogenesis (Penza et al., 2006). While it is unclear with regards to ZF what dosage of GEN will increase adipogenesis or how many PPARγ receptors are present within the cerebellum, it is likely that the amount of GEN administered was high enough
to activate PPARγ, increasing adipogenesis and protecting against experimental handling related weight loss given our findings of weight maintenance in GEN implanted birds.

Ultimately, the purpose of my continuation of this study was to evaluate the neuroprotective effects of E2, as well as to test the potential neuroprotective effects of GEN. Based on previous studies, which suggest that E2 is neuroprotective, I hypothesized that groups administered E2 will experience a decrease in the extent of secondary degeneration relative to the CON. When LET is administered, I predicted that the extent of neurodegeneration would increase, as there was a lack of E2 present due to the inhibition of AROM. Similarly to the effects of E2, I expected that GEN would reduce the size of the lesion, providing neuroprotection. From the results of this study, I hoped to determine if GEN could serve as a neuroprotective replacement for E2.
Materials/Methods

Subjects

Male ZF (36) were bred at the University of Mississippi (IACUC protocol 13-024). Birds selected for this experiment were adults (6.5 months to 3 years old and weighing between 12 and 20.5 g. Following assignment to treatment groups, birds were housed 6 subjects per cage (L 40.6 cm, W 59.7 cm, H 40.6 cm). A 12 hour cycle of light and dark was maintained, and the temperature and humidity were maintained at ~70°F and ~37% humidity. Seeds and water were provided daily, along with egg and bread twice a week. A cuttle bone and perches were provided.

Materials

Rope implants made from medical grade silastic adhesive (Dow Corning, Auburn, MI) mixed 11:1 with ~500 μg of E2, as has previously been shown to raise E2 levels and provide neuroprotection in ZF (Saldanha et al., 2005). GEN was given at 40:1 with ~1000 μg of GEN, based on effective dosages and the weight of subjects in studies using mice and Japanese quail (Penza et al., 2006; Viglietti-Panzica et al., 2007), as well as unpublished data from our lab on the effects of three doses of GEN on behavior post-CB-lesion in ZF. There appear to be no published studies using silastic implants of GEN in ZF, and almost all studies of GEN effects in birds use dietary administration (Corbitt et al., 2007). Control implants contained silastic only (CON). Silastic mixtures were extruded from a 5 mL luer lok syringe onto a glass slide, allowed to dry, and sliced into 6 mm pieces weighing between 18.4 and 20.7 mg. They were matched to bird weight as much as possible. Intracerebral injections contained either 50 micrograms of 1% LET in saline or avian physiological saline (S).
Methods

Implants and Injections

On day 0, 12 days prior to surgery, silastic ropes (E2, GEN, or silastic control) were implanted. Each bird was provided brief isoflurane exposure for light anesthesia, and the rope was implanted subcutaneously within the back of the neck according to the assigned group. Opsite adhesive dressings were placed on top of the site of implantation, and implant adjustment and additional adhesive was applied if the implant moved out of its original location.

On post-implant day 12, implantation, ZF were deprived of food for 2 hours prior to surgery. Birds were anesthetized by injecting 15-20 μL of 1.5-2 mL/Kg equithesin IM into the pectoral muscle. This was followed by concentrated isoflurane inhalant delivered from a 50 mL conical tube until the birds were no longer reactive to plucking of feathers around the ears and were no longer affected by the ear bars, which were covered with EMLA cream (2.5% lidocaine/2.5% prilocaine, generic). A Kopf stereotax (Tujunga, CA) secured their beaks and allowed accurate lesion placement. A small tube was placed on the bottom side of the Kopf beak holder with only the ventral beak position modified to administer the isoflurane gas inhalant. Anesthesia was maintained with oxygen to isoflurane gas at 1-2% during surgery. Held ventral side down on a 37°C heating pad with circulating water, an injection of lidocaine/bupivacaine local analgesic solution (2 mg/kg) was administered under the skin of the head. A craniotomy was performed above the central sinus and CB and a unilateral puncture lesion (left and right counterbalanced within groups) was placed with a 26-gauge needle at LM +/−0.7, RC -2.4 and DV 4.5 relative to 0 set at the caudal aspect of the junction between the basilar artery and the caudal ramus. The needle was held in place for one minute and then backed out halfway from the lesion, and an intracerebral injection of 50-μg of 1% LET or S was administered within the
lesion site. The skin was then replaced, and the area was covered with Opsite dressing (Smith & Nephew, Memphis, TN) and the wound treated with neosporin + pain. Following surgery, the birds were placed in an incubator at 32°C, and offered droplets of water at the corner of their beaks after they began to ambulate. For two hours, the birds remained in isolation and were then returned to mixed experimental bird cages after confirming a lack of stress behaviors. The administration of the rope implant and intracerebral injection created six groups of subjects: GEN + S, GEN + LET, E₂ + S, E₂ + LET, CON + S, or CON + LET.

Tissue Preparation and Staining Procedures

Seventy-two hours following surgery the subjects were euthanized. This time period was selected based on previous studies, where researchers observed a maximal increase in AROM 72 hours after surgery (Mirzatoni et al., 2010; Peterson et al., 2001; Wynne & Saldanha, 2004). The brain of each bird was extracted, frozen on a dry ice acetone slurry, and stored at -80°C. Each brain was cut at 30μ into 3 series using a cryostat, slices were mounted on electrostatic slides, and stored at -80°C. The slides were then thawed for 30 minutes and baked in a 37 degree oven for 20 minutes. After baking, one series was used in a TUNEL assay and another was stained with Fluoro-Jade (FJ). TUNEL detects DNA breakage that occurs during apoptosis by labeling the exposed 3’-OH termini of DNA strands. TUNEL cells were labeled with VectorNovaRED HRP substrate. The tissue was counterstained with methyl green, then allowed to air dry. A second slide series was stained using FJ B, which identifies cells affected by secondary degeneration. Each slide was rinsed with distilled water (DH₂O), dehydrated using ethanol (EtOH), incubated in 0.06% KMnO₄, rinsed again in DH₂O, incubated in 0.001% FJ B solution for 30 minutes with
gentle shaking, then finally rinsed a third time with DH₂O before coverslipping. The third series of slides remains unstained.

Quantification of Neuroprotection

Lesion volume was measured using Stereologer software (Stereological Resource Center, St. Petersburg, FL) to determine the extent of secondary degeneration. Analyzed images were taken using an Imi Tech IMC-3145FT camera (Imi Tech, Seoul, South Korea) from a Zeiss light microscope (Zeiss AxioImager M1, Germany). A single hemisphere of each cerebellum was examined, as the lesion only appeared in one hemisphere. For most subjects, lesions were found in 10-15 sections. With sampling intervals varying between 270-450 μm apart depending on the length of the lesion from rostral to caudal to equate sampling density. Using 5x magnification, each lesion was identified, a rough outline of the lesion was circled, and a grid of Cavalieri points was placed over this outline. The points that overlapped with the lesion, including points located within the lesion path but excluding regions without TUNEL reactivity, were selected. The points on the grid represent an area of 15,000 μ².
Then, at 100x oil magnification, the slice thickness was measured along the Z-axis by locating the values at which the upper and lower aspects of the slice were focused. With this information, the overall volume of each lesion was calculated using the formula $V = T \cdot \Sigma A$, where $V$ is the region volume estimate, $T$ is the thickness, and $A$ is the sum of areas from included points (Mouton, 2011). The average coefficient error across all subjects, which accounts for variance and variation between samples, was 3.7% (Mouton, 2011). This process has been performed on a portion of the TUNEL-labeled subjects and will be performed on the FJ-labeled subjects in the near future.

**Image 1: TUNEL-labeled lesion with grid of Cavalieri points**
Results

We used univariate two-way analysis of variance (ANOVA) with the Type III Sum of Squares (SPSS 28, IBM Corp., Chicago) to compare differences between treatment groups. We confirmed homogeneity of variance among groups via Levene’s Test (all ps>0.52) and homoscedasticity of the error variance in our model via the F-Test (p=0.40). Residuals were normally distributed based on Q-Q plots. One possible concern is that despite statistical tests that ANOVA assumptions were met, the influence of injections and implants on secondary degeneration is unlikely to be independent. Thus, we also ran analyses using one-way ANOVA of our 6 groups, followed by post-hoc tests, as well as the nonparametric Kruskal-Wallis tests for completeness. Given trends for preferences in non-frequentists statistical approaches, we also examined equivalent Bayesian models and calculated effect sizes using JASP 16.1 (University of Amsterdam) and projected sample sizes needed to detect group differences via G*Power 3.1.9.6 (UCLA).

Contrary to expectations, $E_2$ and GEN treatment did not decrease secondary degeneration ($F(2,14)=4.6$, $p=.18$), nor did LET increase TUNEL labeled volume ($F(1,14)=3.08$, $p=.22$), with no detectable interaction ($F(2,14)=.24$, $p=.79$). Effect sizes were very low ($\omega$ from 0.00-0.01). Significance of these effects and interpretation did not change using One-Way ANOVA or nonparametric equivalents. Bayesian analysis also supported the null model using JASP default settings for posterior sample runs and prior distributions. The $BF_{10}$ values in favor of the null ranged from 0.32 to 0.61, and $R^2=0.05$ (95% credibility interval 0-0.27) suggesting great uncertainty about any effect size despite low error in the model for implant (0.02%) and injection (0.002%) and high error for the interaction (0.88%) regardless of using auto vs. 100,000 sample
run settings. Finally, G*Power analysis suggested a total of 18 subjects would be needed to obtain significance at p=0.05 and power of 0.8 given effect sizes found for partial $\eta^2$.

Figure 1: Mean and range of TUNE-labeled lesion volume for each treatment.
Figure 2: Model averaged posterior probability distributions for effects. The density is the likelihood of the variable falling at points on the curve showing posterior means (x-axis), and the lines above the curves are the 95% credibility range.
Discussion

Drug treatments did not appear to have the expected effects on the extent of secondary degeneration. Power analyses suggested that we need at least 18 more subjects (3/group) to detect differences. Fortunately, this number of subjects has already been run in this experiment, but the TUNEL reactive lesion volumes have not yet been measured for all subjects.

Understanding that differences were not statistically significant likely due to low sample size, we nonetheless discuss herein the pattern of results we saw and interpret these results in light of our hypotheses. As can be seen in Figure 1, relative to controls, the extent of degeneration numerically trended in a direction suggesting that GEN decreased the extent of secondary degeneration. In addition, a numerical trend was found that suggests that subjects locally injected with LET had a greater extent of secondary degeneration relative to birds injected with S (see Figure 1). Thus, acute, local AROM inhibition might prevent conversion of testosterone to E_2 and/or decrease reactive gliosis, ultimately leading to a decrease in neuroprotection. Contrary to expectations, we observed that the results for subjects treated with E_2 implants trended in an unexpected direction. While not statistically significant, the data collected so far for E_2 subjects suggested increasing secondary degeneration surrounding lesions similar to or greater than CON subjects, and possible reasons for this result will be discussed below.

Researchers have previously suggested that GEN has neuroprotective effects by decreasing apoptosis, inflammation, and antioxidants in rats following a stroke (Azcoitia et al., 2010; Jiang et al., 2013). In this study, I similarly found the mean values of the extent of degeneration in subjects that received a GEN implant trended in the direction that suggests GEN offers some neuroprotection post-cerebellar lesion in ZF, although not statistically significant.
These results were present in both subjects injected with S and those injected with LET. Acute local AROM inhibition, therefore, may not interfere with the ability of GEN to prevent secondary degeneration 3 days post-lesion.

In previous studies, researchers found that when AROM is inhibited, secondary degeneration increases (Azcoitia et al., 2010; Duncan et al., 2013; Duncan & Saldanha, 2020; Saldanha et al., 2005; Saldanha et al., 2009). Based on these studies and my own results from my current study, one could assume that when AROM is inhibited and unable to convert testosterone to E$_2$, there is a lack of neuroprotection, suggesting the neuroprotective effects of AROM-induced E$_2$.

Compared to the effects of GEN and LET on secondary degeneration, the mean values of secondary degeneration for subjects treated with E$_2$, although not significant, trended in an unexpected direction. Previous researchers have found that E$_2$ decreases the extent of degeneration following neural damage (Azcoitia et al., 2010; Duncan et al., 2013; Duncan & Saldanha, 2020; Mirzatoni et al., 2010; Peterson et al., 2001; Saldanha et al., 2005; Saldanha et al., 2009; Spence et al., 2009). It is possible that many factors may have contributed to this. First, it is possible that our results strayed from what was expected due to different mechanisms occurring within the cerebellum compared to studies in entopallium (Saldanha et al., 2005; Wynne & Saldanha, 2004). Specifically, rather than E$_2$, it is possible that AROM or other steroids and/or synthetic precursors that are upregulated within the CB post-lesions (Mirzatoni et al., 2010) provide neuroprotection. However, E$_2$ related improvement of CB-lesion induced deficits do not fit with this interpretation, unless behavioral improvements are not due to neuroprotection. In addition, given that acute, local LET seems to inhibit local AROM from upregulating E$_2$ due to the increase in secondary degeneration relative to the CON, E$_2$ may need
to be administered locally to compensate for the lack of local AROM. Using HPLC will allow us to quantify the amount of circulating E\(_2\) and GEN in subjects to determine what amounts of E\(_2\) and GEN were actually present. Regardless of the effects of E\(_2\) on secondary degeneration, it is clear that the amount of E\(_2\) present was sufficient to induce reproductive problems within our subjects (McFatridge, 2020). Given that similar levels of E\(_2\) are also known to protect against lesion induced behavioral deficits, it is possible that an increase in our sample size may support these previous results (Spence et al., 2009) or provide clarity of CB E\(_2\) mechanisms related to neuroplasticity.

Given that GEN seems to reduce the extent of secondary degeneration following injury while decreasing negative consequences, such as reproductive issues, it is possible that GEN may be used as a replacement for E\(_2\). Because previous studies have shown that E\(_2\) may protect against secondary degeneration following neural damage, such as ischemic strokes, it is possible that, as women approach menopause and experience a decrease in the amount of estrogen circulating throughout their bodies, there would be an increase in damage following neural injury (Duncan & Saldanha, 2020). Limitations exist, however, in the amount of estrogen supplementation that the body can tolerate before additional complications, such as breast cancer, can arise (Jiang et al., 2013; Katzenellenbogen & Katzenellenbogen, 2000; Sites, 2008). Because cancer cells typically contain ER \(\alpha\), an increase in E\(_2\) supplementation which binds to ER \(\alpha\) would lead to this increase in cell proliferation, increasing cancer risk. In contrast, because ER \(\beta\) is typically not found on cancer cells, the preferential binding of GEN to ER \(\beta\) would diminish cancerous effects (Jiang et al., 2013). Therefore, our results thus far suggest the possibility that GEN supplementation might be beneficial for neuroprotection in menopausal women while avoiding additional harmful effects that E\(_2\) could cause if added subjects continue
the current trend. In addition, men may benefit from GEN as well. We and others have shown that E2 leads to reproductive deficiencies, including a decrease in testis mass, spermatozoa number, and laminarity (Halldin et al., 2005; Leavy et al., 2017; McFatridge, 2020; Rochester et al., 2010). Therefore it is possible that the negative effects of taking an estrogen supplement following a brain injury may outweigh the positives. Furthermore, we have found that GEN maintains body mass throughout the course of an experiment, showing that it may have lipogenic effects, which may be beneficial in humans during recovery from brain damage (McFatridge, 2020). However, similarly to how our results for E2 differed from previous studies, the potential for different reactions to GEN supplements within the human brain compared to ZF CB still remains, and further research will need to be conducted to test these reactions.

In order to fully interpret the neuroprotective effects of E2 and GEN in the ZF cerebellum and make appropriate comparisons with human neurophysiology, we need to complete data collection in the current study. Because we currently have low power, we plan to continue our measurements of the extent of TUNEL reactive lesion volumes on at least 18 more subjects. We know that some subjects lost implants prior to the end of the experiment, and our initial analyses focused on only those subjects who completed the experiment with intact implants. Thus, it will be crucial to quantify the amount of circulating E2 and GEN using HPLC to adjust for any differences among individuals in these levels and to allow inclusion of subjects for which we could not locate implants, but may have levels of circulating E2 or GEN suggesting influence of these drugs at the time of blood sampling. We will also complete analyses of FJ-labeled slices to determine the extent of apoptosis surrounding lesions. Thus far FJ-labeled lesions appear slightly less extensive, but very similar in shape and size in the same individuals as the TUNEL reactive regions. Our studies focusing on the extent of secondary damage to the lesioned brain,
specifically in the CB, with its low constitutive AROM and E₂, offer a unique extension to previous research. A handful of studies have shown upregulation of molecules in the steroidogenic pathway or effects of AROM and E₂ manipulations on behavior post CB lesion (Duncan & Saldanha, 2020; Mirzatoni et al., 2010; Spence et al., 2009). Yet, none of these confirmed the effects of these changes on the damaged brain with regards to the extent of secondary degeneration. Further, the vast majority of research in the area of AROM and E₂ effects have been in brain regions like the hippocampus that are regularly steroidogenic, blurring the effects of neural reactivity to injury and normal steroidogenic effects (Saldanha et al., 2005; Saldanha et al., 2009). Thus, our research offers an essential focus on whether these steroidogenic pathways provide neuroprotection via local and systemic delivery and further extend these studies into the possible protective effects of a readily available phytoestrogen that might be administered as a subcutaneous implant or as a dietary supplement.
List of References


McFatridge, Emily; Chong, Sarah; Zadrozny, Ethan G.; and Blair, Estelle, "Neuroprotective Effects of Estradiol and Genistein in Zebra Finch Cerebellum" (2020). *Neuroscience Research Showcase. 6*. https://egrove.olemiss.edu/neuro_showcase/6


Sites, C. K. Bioidentical Hormones for Menopausal Therapy. *Women’s Health, 4*(2), 163-71. doi:http://dx.doi.org/10.2217/17455057.4.2.163


