Oxidative and Endoplasmic Reticulum Stress in Response to Reproduction in the Female Mouse Brain

by

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Abstract

Reproduction is associated with a significant increase in energetic demand, particularly among small female mammals. When these demands are high, or an animal is under stress, the cost of reproduction can reduce future reproductive performance and longevity. An increase in reactive oxygen species (ROS) levels during reproduction has been proposed to drive this relationship. While empirical tests of this theory have been equivocal, relatively few studies have evaluated changes in oxidative stress within the brain and its impact on longevity. Prolonged exposure to excessive levels of ROS has been shown to impair cognitive ability, and correlations have also been found in humans between the number of offspring women have produced and the risk of developing neurological disorders. Endoplasmic reticulum stress has also been linked to oxidative stress and neurodegenerative disorders. These correlations suggest that there is a link between parity and brain damage over a lifetime. The goal of this study was to determine how reproductive experience impacted the brain by comparing mitochondrial density, oxidative damage, antioxidants and the linked, endoplasmic reticulum stress in the cerebrum, brainstem, and cerebellum of 3 groups of ICR lab mice. These age-matched mice include 1) a group of non-reproductive mice, 2) a group of mice that had one reproductive event, and 3) a group of mice that had four reproductive events. There were no significant differences found with oxidative damage markers or markers of endoplasmic reticulum induced apoptosis between reproductive groups in all brain regions, suggesting that the brain is highly resistant to oxidative stress, even under conditions of high reproductive demand. However, there was a decrease in mitochondrial density in the cerebrum of all animals that reproduced and a reduction in GPX1 levels in four-bout versus one-bout females in the cerebrum and cerebellum.

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List of Abbreviations

4-HNE	4-hydroxynonenal
BiP	Binding Immunoglobulin Protein
CHOP	CCAAT/Enhancer Binding Protein Homologous Protein
ER	Endoplasmic Reticulum
GPX1	Glutathione Peroxidase 1
ROS	Reactive Oxygen Species
UPR	Unfolded Protein Response
XBP1	X-box Binding Protein 1

Introduction

An individual's survival is impacted by its ability to allocate energy to key physiological processes. The physiological processes that underlie somatic maintenance play an influential role in allowing an animal to achieve its maximum lifespan. According to life history theory, natural selection is predicted to favor the allocation of resources away from maintenance when doing so improves an individual's fitness (Zera and Harshman 2001; Catoni et al. 2008). For example, when reproductive demands are high, a female may reduce energy allocated to repairing cellular damage and redirect that energy to support the production of offspring (Finkel, Toren; Holbrook 1951; Zera and Harshman 2001). As a result of the inability to reduce or correct damage incurred, longevity and future reproductive value maybe be curtailed (Williams 1966; Alonso-Alvarez et al. 2004; Speakman 2008; Zhang and Hood 2016). One process that has been proposed to contribute to the tradeoffs between reproduction and longevity is the production of reactive oxygen species (ROS).

The oxidative cost of reproduction hypothesis suggests that the high energetic demands of reproduction should lead to an increase in ROS production and an accumulation of oxidative damage that could reduce a female's future reproductive potential and her longevity (Alonso-Alvarez et al. 2004; Monaghan et al. 2009; Metcalfe and Alonso-Alvarez 2010; Hood et al. 2018). Support for this theory has been mixed (Garratt et al. 2011; Stier et al. 2012; Speakman and Garratt 2014; Blount et al. 2016; Zhang and Hood 2016). This is not surprising given that an increasing amount of data suggests the intercellular response to ROS is varies, where modest levels of ROS are beneficial, but high levels of ROS are damaging (Tapia 2006; Ristow and

Schmeisser 2014; Yun and Finkel 2014). To date, testing of this hypothesis has largely been evaluated by quantifying oxidative stress in tissues such as blood, liver, and muscle (Blount et al. 2016). Yet, the response of the most critical regulatory region in the body, the brain, has largely been ignored. The brain may be more susceptible to oxidative damage than other organs because it has a high concentration of peroxidizable unsaturated fatty acids, high consumption of oxygen per unit mass, and scarcity of antioxidant defense systems (Poon et al. 2004). Prolonged exposure to ROS has also been shown to impair cognitive function, potentially due to neurodegeneration (Malhotra and Kaufman 2007).

The incidence of several neurodegenerative diseases increases with reproduction output in females (Launer et al. 1999; Colucci et al. 2006a; Jarvik et al. 2008); this suggests that the impact of reproduction on the brain may play an important role in animal performance. Neurodegeneration is thought to be caused by both increased ROS levels and by endoplasmic reticulum (ER) stress (Tajiri et al. 2006; Sokka et al. 2007; Raghubir et al. 2011; Yin et al. 2017). There is evidence to suggest that protein folding, ER stress, and production of ROS are deeply connected to one another (Cullinan and Diehl 2006; Malhotra and Kaufman 2007; Zhang 2010; Bhandary et al. 2013; Chaudhari et al. 2014). Thus, it is feasible that either ROS production, ER stress, or both are upregulated in the brain during reproduction. The ER stress response is a response to unfolded proteins that either stimulates protein repair or initiates apoptosis when levels of unfolded proteins are high (Ron et al. 2000; Rutkowski et al. 2006; Ron and Walter 2007). Proper protein folding and the formation of disulfide bonds are dependent on the redox status maintained in the ER lumen (Malhotra and Kaufman 2007). This redox state is disrupted

when ER stress, oxidative stress, or both are high within the cell. This disruption of the redox state will reduce glutathione (GSH) and increase protein thiol levels (a product of protein oxidation) in the ER (Malhotra and Kaufman 2007). GSH depletion allows for the formation of ROS, as GSH is used to reduce the formation of unstable and improper disulfide bonds during ER stress. At a specific threshold, the increase in ROS production will cause calcium ions to leak from the lumen of the ER (Chaudhari et al. 2014). This increased calcium initiates a positive feedback loop, stimulating greater ROS production from the electron transport system. Calcium leakage also opens up pores within the ER membrane, which may cause GSH to leak out. This depletion of reducing equivalents, increase in calcium, and increase in ROS production can lead to cell death.

ER stress stimulates the release of transducers, including PERK, ATF6, and IRE1 from BiP (also known as GRP78). For this reason, measurements of BiP are considered a strong indicator of ER stress (Lee 2005). Once ATF6 has been released from BiP, ATF6 activates XBP1; in turn, XBP1 is sliced by IRE1. Once spliced, XBP1 activates the unfolded protein response (UPR) (Yoshida et al. 2001), which will upregulate the amount of BiP within the ER. When a protein is misfolded within the ER, it either stays within the ER or is degraded. An accumulation of unfolded proteins will trigger the UPR, and chronic activation of UPR signaling will induce apoptosis. Unfolded proteins cannot perform their designated functions and the accumulation of unfolded proteins has been shown to further increase levels of ROS already present within the ER from normal protein folding (Moreira et al. 2007). While this process will not always lead to apoptosis, it is unknown how much stress a cell must experience for apoptosis

to be triggered. There are processes to reverse this cascade of events. However, the exact mechanisms are not yet known.

Neurodegenerative disorders, such as Alzheimer's and Parkinson's disease, have been associated with UPR and misfolded proteins (Bubber et al. 2005; Moreira et al. 2007; Resende et al. 2008; Raghubir et al. 2011). Another proposed mechanism for increased neurodegeneration is CHOP. CHOP is an ER stress-induced, pro-apoptotic transcription factor that stands for CCAAT/enhancer binding protein homologous protein. The activation of CHOP occurs when BiP disassociates from PERK, which will activate apoptotic signaling cascades. CHOP deletion in cells is also neuroprotective by reducing hypoxia-induced apoptosis (Tajiri et al. 2006). CHOP works by activating the transcription of genes that may potentiate apoptosis. Due to the proposed linkages between ER stress, oxidative stress, and neurodegeneration, markers for BiP, XBP1, and CHOP were examined in this study.

Thus, the overarching goal of this study is to evaluate the impact of reproduction on both oxidative stress and ER stress in the brain. This study was designed to determine if age-matched female mice with differences in reproductive effort experienced different levels of oxidative damage and ER stress within the brain. This question was evaluated relative to the number of reproductive events (none, one, and four) and the number of pups produced (in four-bout females only). Due to evidence of a trade-off between reproduction, longevity, and neurodegenerative diseases (Zera and Harshman 2001; Mariani et al. 2005; Colucci et al. 2006a; Speakman 2008; Garratt et al. 2013; Zhang and Hood 2016), I predicted that reproduction would have the greatest impact on the brain of females with four reproductive events and of females that produced more

young. This impact would include lower mitochondrial density and greater oxidative and ER stress.

Further, I was interested in whether or not the impact of reproduction on the brain would vary with region. When an organism is forced to allocate a finite amount of energy to multiple processes, it must determine which processes will get the most resources. This selection is thought to follow priority rules, where physiological processes or organs that are most important to immediate survival are preferentially supported over less important processes or organs (Zera and Harshman 2001). Therefore, I expect the brainstem to be most protected, due to its control of major bodily functions necessary for immediate survival, such as breathing, heart rate, and consciousness. The cerebellum, responsible for the regulation of fine movement, is also expected to be highly protected. In contrast, the cerebrum, responsible for cognitive thinking, would be the least protected. Variation in oxidative damage and the ER stress response will be compared between the three parity groups in each of these regions of the brain.

Materials and Methods

Animal Care

All animal husbandry and experimental procedures followed the approved protocol outlined in the Institutional Animal Care and Use Committee (PRN 2017-3168). Outbred ICR

laboratory mice were used for this investigation (Envigo, Prattville, AL). Mice were maintained on a ventilated rack in 11.5" x 7.5" x 5" rodent boxes with a standard wire hopper and water bottle, and kept on a 14:10 hour light:dark cycle to provide the appropriate light stimulus for mating. Food and water were also provided *ad libitum* to all mice. Non-reproductive females were housed in pairs and reproductive females were housed with a male; once a female was determined to be pregnant, the male was removed and placed in a separate box. Pups from reproductive females were removed at 21 days of age.

Experimental Design

To evaluate the impact of parity on oxidative stress and the ER stress response, adult female mice were divided into three groups of ICR mice: non-reproductive females (n=12), females that experienced one full reproductive bout (n=13), and females that experienced four full reproductive bouts (n=12). The mating procedure for each of these females is described in Park et al. (2018). Due to one case of stillborn births and one case of cannibalism within two of the females within the four-bout group, a bout of reproduction was defined by successful weaning. Therefore, two of the females from the four-bout group gave birth to fives litters. However, due to the high energetic demand from lactation, these litters that resulted in stillbirth births and cannibalism were not considered one of the four bouts for each female. Females that weaned four litters were euthanized 14 days after their last litter was weaned to allow them to return to a non-reproductive state. An age-matched one-bout female and a non-reproductive female were assigned to one four-bout female upon arrival at our facility. These mice were euthanized at the same time as the assigned four-bout female, ensuring that the three treatment groups were age-matched (Figure 1). Mice were briefly anesthetized with isoflurane then

decapitated with a rodent guillotine. Once euthanized, the head for each was placed on ice and brains were rapidly extracted, separated into the cerebrum, brainstem, and cerebellum and flash frozen using 2-methylbutane and dry ice. These samples were then placed in labeled 2ml tubes and stored in a -80°C freezer for future analysis. The number of pups at weaning was recorded for each reproductive event.

Mitochondrial Density

A common method used to determine mitochondrial density within a cell is citrate synthase activity (Larsen et al. 2012; Spinazzi et al. 2012). In the Krebs cycle, citrate synthase is the enzyme used to convert acetyl-CoA and oxaloacetate into citrate. In the assay to measure citrate synthase, brain region homogenate was used. Each tissue was homogenized in a 1:10 (wt/vol) ratio with 5 mmol/L Tris HCl (pH 7.5), 5 mmol/L EDTA (pH 8.0), and protease inhibitor cocktail (14224- 396, VWR, Radnor, PA) and was centrifuged at 1,500g for 10 minutes at 4°C. Protein content of the supernatant was quantified by Bradford assay (Bradford 1976). Citrate synthase was measured as a function of the increase in absorbance from 5,5'-dithiobis-2-nitrobenzoic acid reduction.

Oxidative and ER Stress Proteins

Using an established protocol (Mowry et al. 2016), western blots were used to quantify protein levels of 4-HNE, OxyBlot, GPX1, BiP, XBP1, and CHOP. Proteins from tissue homogenates were separated by electrophoresis via 12% Criterion TGX precast gels (Bio-Rad, Hercules, CA). Proteins were transferred to polyvinylidene difluoride (PVDF) membranes following separation. Nonspecific sites were blocked in phosphate-buffered saline (PBS)

solution containing 0.1% Tween-20 and 5% nonfat milk. Membranes were then rocked for 1 hour at room temperature with primary antibodies for 4-hydroxynonenal, protein carbonyls (OxyBlot), Binding Immunoglobulin Protein, X-box Binding Protein 1, CHOP, and glutathione peroxidase 1 (4-HNE: GTX40953, OxyBlot: s7150, EMD Millipore, Cillerica, MA; BiP: GTX113340, XBP1: GTX32974, GADD153 (CHOP): GTX112827, GPX1: GTX116040; GeneTex, Irvine, CA). Membranes were washed (five minutes x3) with PBS-Tween and incubated with secondary antibodies for 1 hour at room temperature. After another round of washing with PBS-Tween, a chemiluminescent system was used to detect labeled proteins (GE Health-Care, Buckinghamshire, UK). Each membrane was stained by Ponceau and was used as the loading and transfer control. Images of the membranes were captured and analyzed using the ChemiDocIt Imaging System (UVP, LLC, Upland, CA).

Statistics

All comparisons were made using GraphPad Prism version 7.02 for Windows (Graphpad Software, La Jolla California, USA) and RStudio 1.1.463 (RStudio, Boston, MA, USA). Correlations were done in RStudio to confirm that the variables tested display any expected interactions. One-Way ANOVA was used to compare the effects of parity on 4HNE, OxyBlot, GPX1, XBP1, BiP, CHOP, and citrate synthase. Significance was established at $\alpha = 0.05$ for all statistical analyses. Normality was also confirmed for each ANOVA test. To test significant differences between groups, Tukey's post hoc test was used. To further examine the impact of reproductive performance on oxidative and ER stress, linear regressions were used to examine each variable against the number of offspring each four-bout female had over a lifetime.

Results

Correlations were confirmed between several of the variables tested. The ER stress markers BiP and XBP1 were both found to be positively correlated in within the cerebrum and cerebellum (Figures 2 and 4). Protein carbonyls and GPX1 were found to be positively correlated in all regions of the brain (Figures 2-4). Within the cerebrum, GPX1 and XBP1 were found to be positively correlated (Figure 2). Within the brainstem, 4HNE and CHOP, as well as XBP1 and CHOP, were found to be positively and negatively correlated respectively (Figure 3). In the cerebellum, 4HNE and BiP were found to be positively correlated (Figure 4). Protein carbonyls and BiP, as well as XBP1 and CHOP were negatively correlated in the cerebellum (Figure 4).

Mitochondrial density was compared between groups as an indicator of the capacity to produce ATP. Mitochondrial density, measured with citrate synthase, was varied between treatment groups in the cerebrum (Figure 5). In the cerebrum, citrate synthase was lower in one-bout females ($P<0.001$, Figure 5) and four-bout females ($P=0.003$, Figure 5) compared to non-reproductive female mice.

Oxidative damage and antioxidant protein levels were compared between treatment groups and regions of the brain. Neither of the markers of oxidative damage, 4HNE or protein carbonyls, varied with parity or region of the brain (Table 1). The antioxidant, GPX1, did not vary with parity in all regions of the brain (Table 1). However, within the four-bout female, GPX1 was negatively correlated with the number of offspring produced in the cerebrum ($P=0.008$,

$Y=-0.035*X+2.88$, Figure 6A) and in the brainstem ($P=0.03$, $Y=-0.056*X+4.49$, Figure 6B).

Discussion

The purpose of this study was to evaluate the impact of the number of reproductive events that a female experienced on oxidative damage and ER stress across various regions of the brain. I predicted that age-matched females that experienced more reproductive events and females that produced more young would have lower mitochondrial density, higher levels of oxidative damage, and elevated levels of ER stress within all brain regions. While there was no impact on the number of reproductive events or total number of pups produced on oxidative damage for any of the three major regions of the brain, I did detect a reduction in mitochondrial density for females that bred in the cerebrum, and a negative relationship between reproductive effort and the antioxidant, GPX1, in the cerebrum and brainstem, suggesting that females that allocate more to reproduction may bear the cost of that effort in the brain due to a reduction in the antioxidant GPX1.

Correlations were confirmed between protein carbonyls and GPX1 in all regions of the brain. It was expected that correlations would also occur between 4HNE and GPX1 (Halliwell and Gutteridge 2007). However, no correlations were found between these two variables in any of the brain regions. Each brain region had variations in what variables were correlated, contrary to what was predicted. Correlations between XBP1 and BiP were expected, due to increases in XBP1 stimulating the generation of more BiP within the ER (Ron et al. 2000; Yoshida et al. 2001; Lee 2005).

In the cerebrum, I found that mitochondrial density was impacted by a female's reproductive effort. Specifically, females that produced one and four litters had significantly fewer mitochondria per mg of tissue than non-reproductive females. Mitochondrial density was reduced within the one-bout females in comparison to the non-reproductive group, and

mitochondrial density stayed reduced within the four-bout group. This suggests that reproduction, regardless of the number of reproductive bouts, will reduce the number of mitochondria within the cerebrum.

Mitochondria are critical to neuronal performance, as it is one of the main sources of ATP production that drive neurons' highly polarized morphology (Suzuki et al. 2018). However, changes in the cerebrum are common in breeding females across multiple species (Hillerer et al. 2014). One study performed by Hoekzema et al. (2017) found reductions in grey matter within the cerebrum and brainstem immediately post-reproduction in humans, with the largest reductions being in the cerebrum. After 2 years, there was a partial recovery of grey matter within the brainstem, but no improvements in grey matter in the cerebrum. Reductions in grey matter are also a hallmark of Alzheimer's disease (Frisoni et al. 2007; Kunst et al. 2019). Along with correlations with an increased risk of Alzheimer's with reproduction (Colucci et al. 2006b), it can be speculated that these changes to the cerebrum during reproduction will leave a female more susceptible to future detrimental stressors that could impact brain functioning. Interestingly, it has been argued that these changes in the cerebrum are thought to be important in changing the morphology of the brain to be primed for maternal care of offspring, thus increasing the amount of maternal care given to offspring in mammals (Hillerer et al. 2014; Hoekzema et al. 2017).

While levels of GPX1 within all brain regions were not significantly different between reproductive groups, correlations between offspring numbers over a lifetime in the four-bout females and GPX1 levels within the cerebrum (Figure 6A) and brainstem (Figure 6B). This suggests a trade-off between reproductive output and antioxidant defenses necessary for the reduction of ROS. Potentially, this could lead to the cerebrum and brainstem being susceptible to

damage by ROS with an increase in offspring over a lifetime. In a two-part study, it was shown that several species of mice do not begin to show the impacts of reproduction until after their eighth litter (Newman et al. 1985a, 1985b). This could explain the lack of changes in oxidative damage and ER stress markers between reproductive groups.

While this study did reveal a negative impact of reproduction on mitochondrial density in the brain, we also show that the brain is highly resistant to oxidative damage during reproduction. The brain is also resistant to apoptosis induced from the accumulation of unfolded proteins, shown by no significant changes in CHOP, the ER stress marker that indicates cellular apoptosis.

Conclusions

This study shows that reproductive experience does have persistent impacts on mitochondrial density in the brain. In particular, the negative impact that parity had on mitochondrial density in the cerebrum and that total reproductive output has on antioxidants in the brainstem suggests that future performance could be impacted by reproduction. Future studies should consider the impact of reproduction on cognitive function, particularly those that are relative to the survival probability of mice, or other species under investigation. It should also be noted that this experiment was conducted on laboratory mice under optimal conditions. The costs of reproduction are often more pronounced when an animal is under stress (Monaghan et al. 2009; Stier et al. 2012; Costantini et al. 2016). It would be particularly interesting to see if the brain bears a greater burden of reproduction when it is coupled with exogenous stressors, such as reduced food availability and increased population density.

Figures

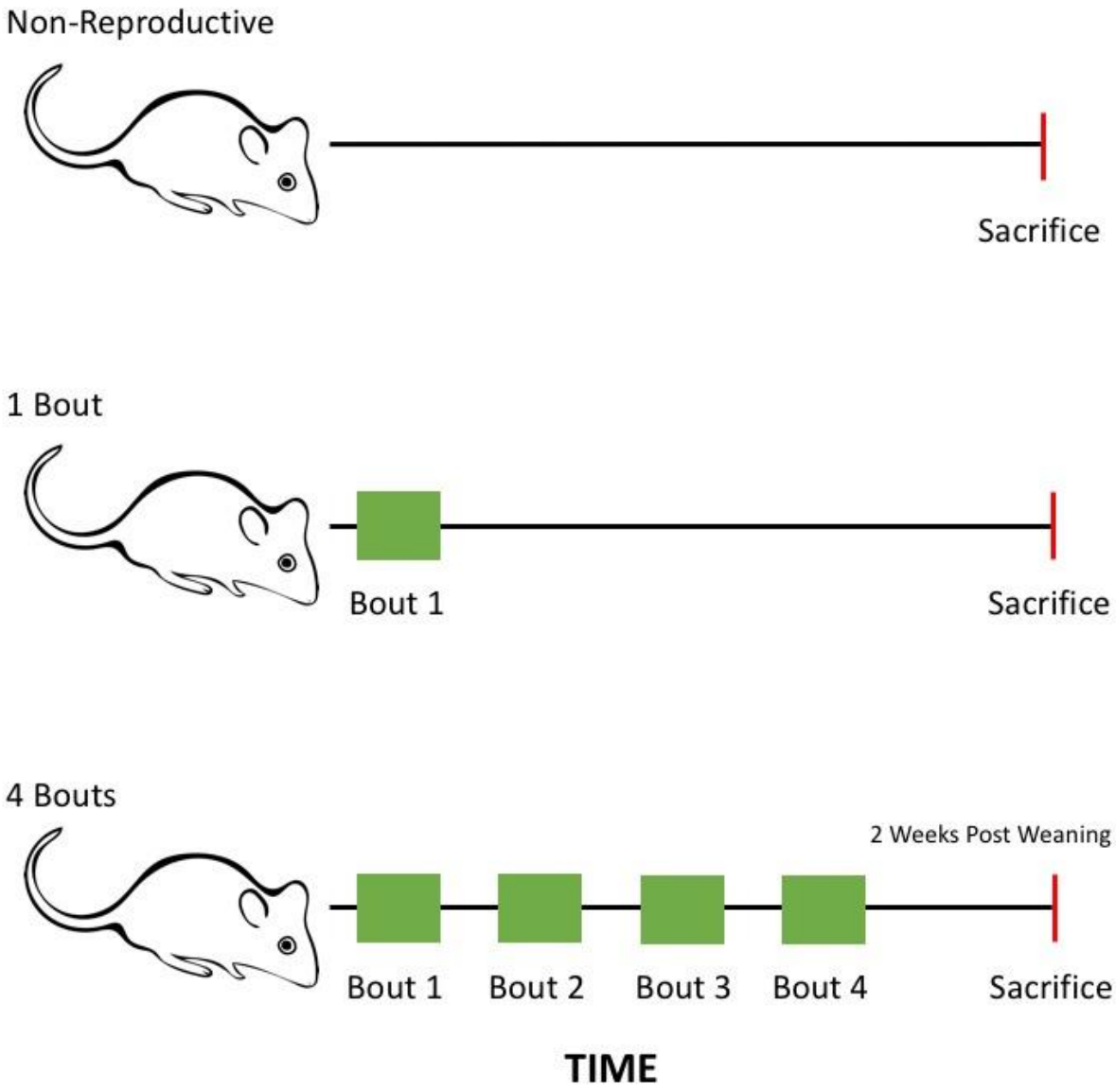

Figure 1. Experimental design. Thirty-seven age-matched female mice were randomly selected for one of three groups: non-reproductive, one-bout, and four-bouts. The reproductive females were then bred for their respective number of reproductive events. Each four-bout female had a non-reproductive control and one-bout female that was selected to be sacrificed two weeks post weaning of the four-bout female.

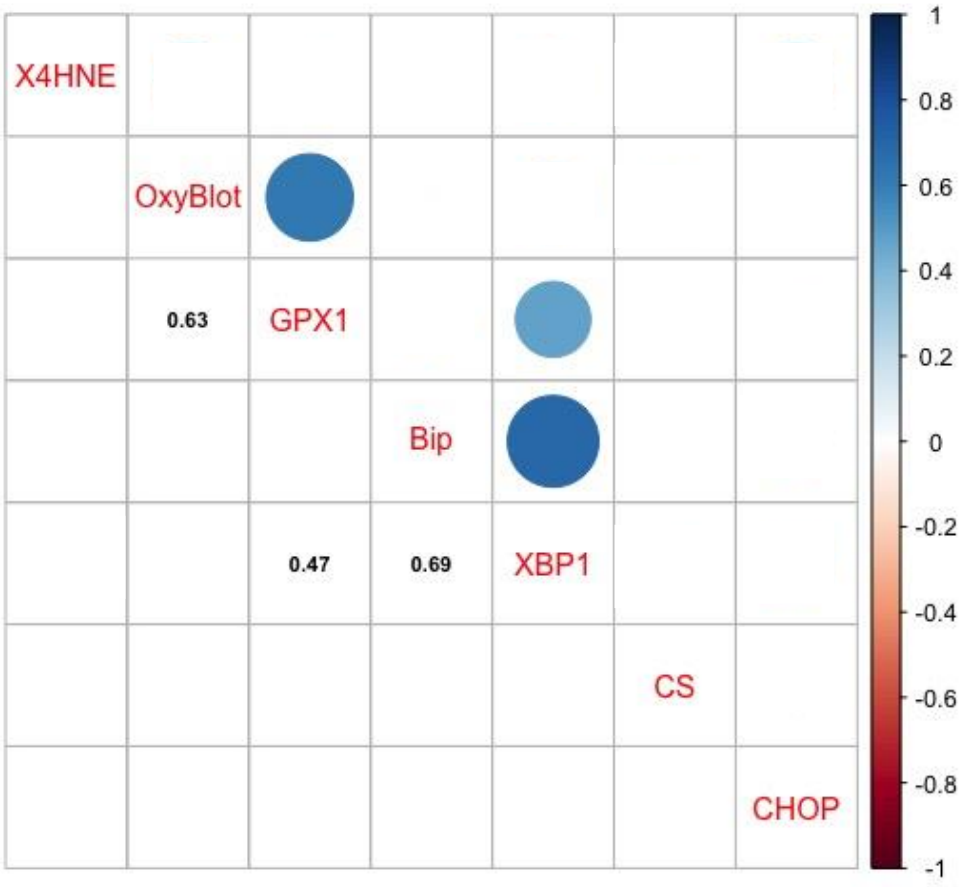

Figure 2. Shows the correlations between variables in the Cerebrum. Only correlations with a p-value of <0.005 are shown. Each variable measured is shown in red diagonally in the middle of the matrix. Above each variable are colored circles that represent the degree of correlation. The shade of the color corresponds to the chart on the far-right side of the figure. Below each variable are the R values for the correlations.

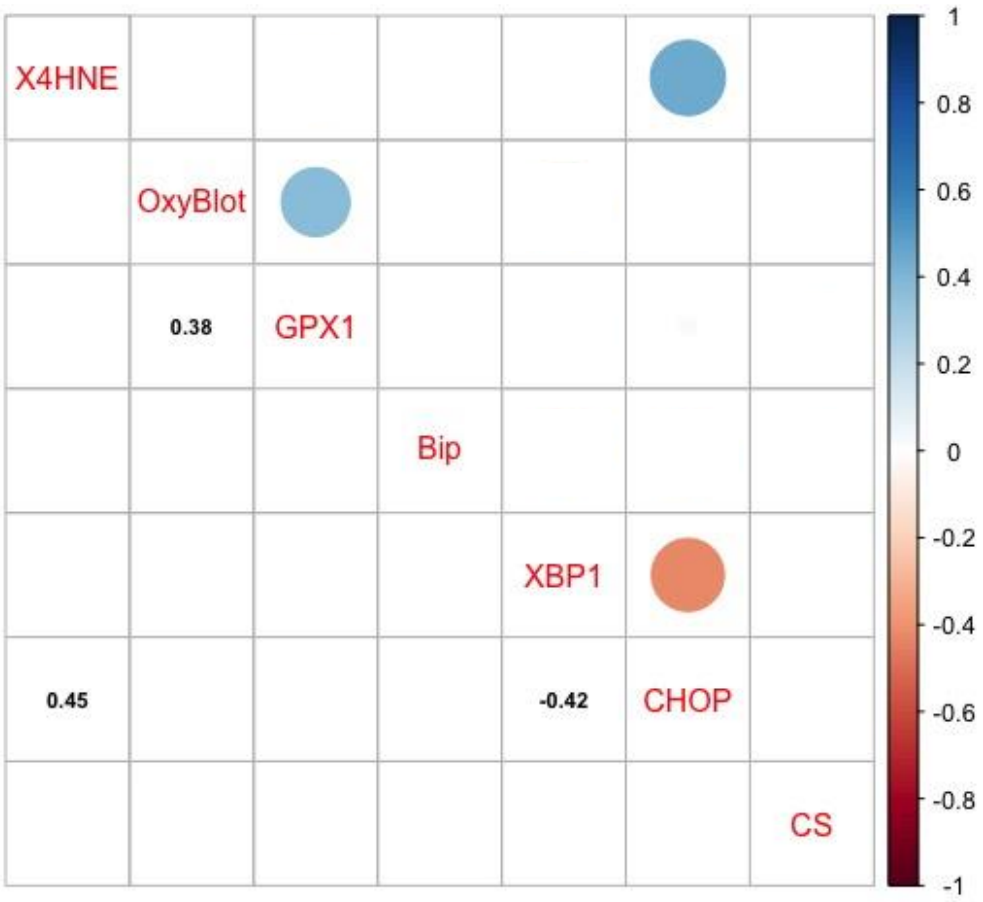

Figure 3. Shows the correlations between variables in the Brainstem. Only correlations with a p-value of <0.005 are shown. Each variable measured is shown in red diagonally in the middle of the matrix. Above each variable are colored circles that represent the degree of correlation. The shade of the color corresponds to the chart on the far-right side of the figure. Below each variable are the R values for the correlations.

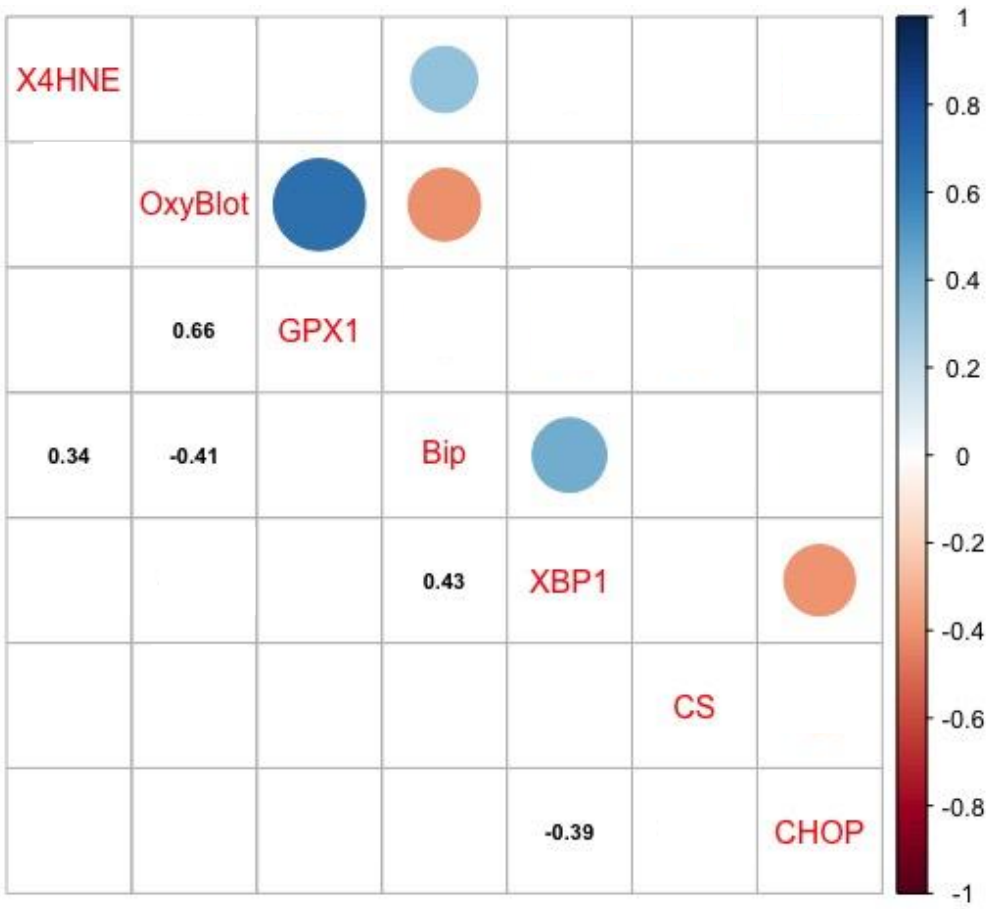

Figure 4. Shows the correlations between variables in the Cerebellum. Only correlations with a p-value of <0.005 are shown. Each variable measured is shown in red diagonally in the middle of the matrix. Above each variable are colored circles that represent the degree of correlation. The shade of the color corresponds to the chart on the far-right side of the figure. Below each variable are the R values for the correlations.

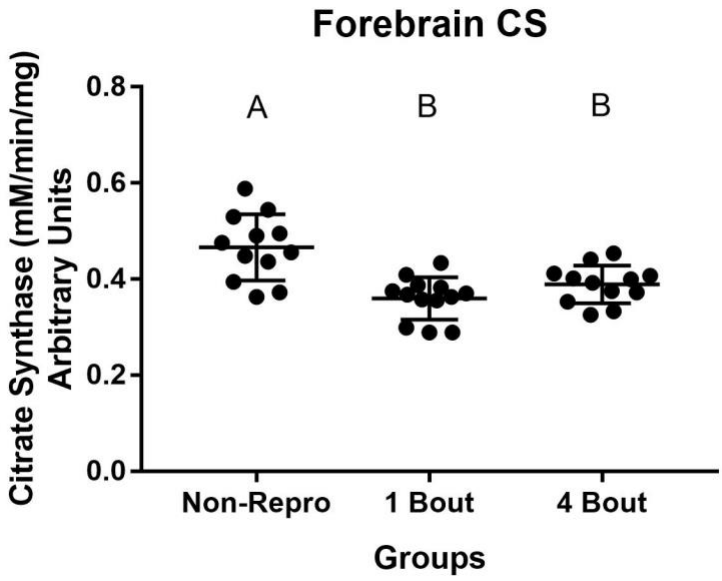

Figure 5. Effect of parity on mitochondrial density levels in the cerebrum. This graph shows the relationship between citrate synthase and parity. Letters above the data indicate a significant difference between groups.

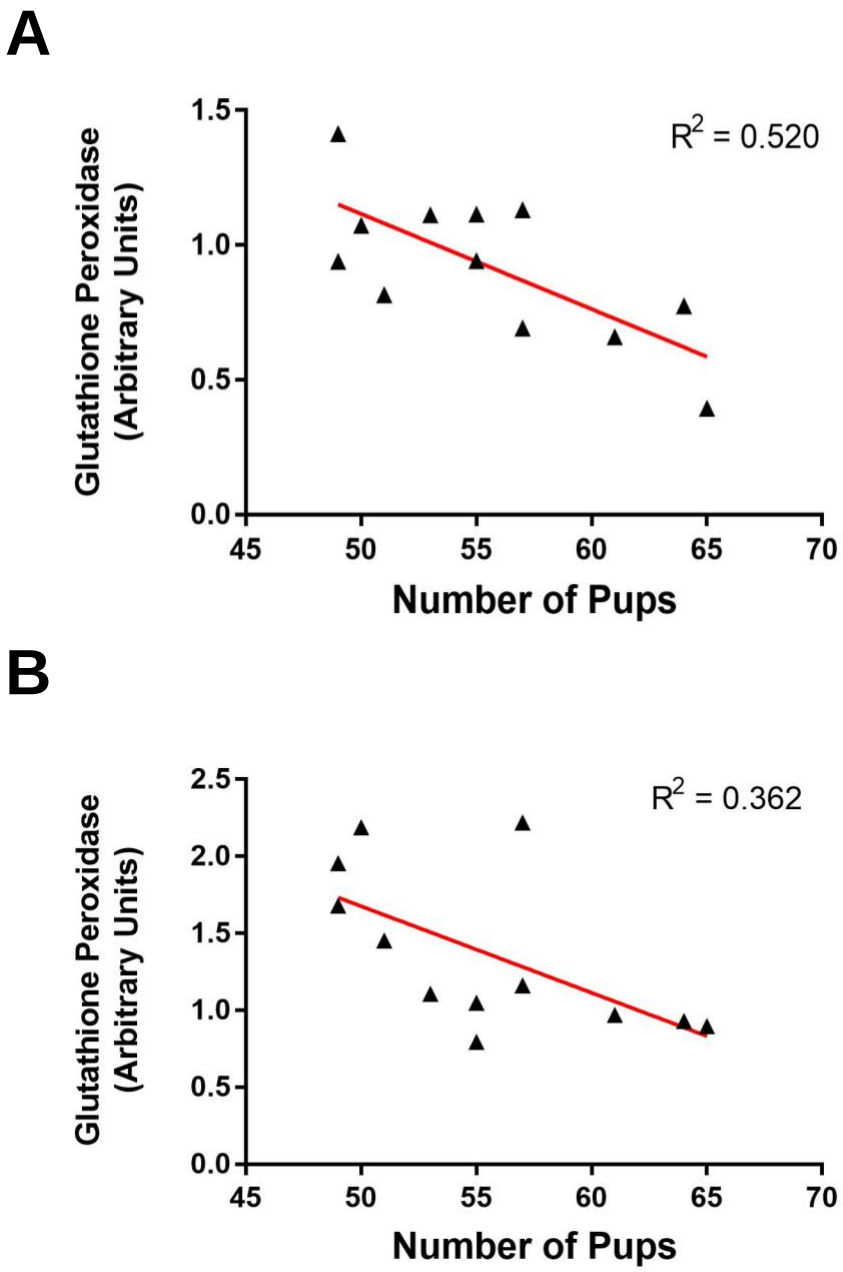

Figure 6. Effect of lifetime reproductive output on glutathione peroxidase 1 levels produced by four-bout females within the cerebrum (A) and the brainstem (B).

Table 1. Results of One-way ANOVA (p-values) comparing the relative expression levels of citrate synthase, 4-hydroxynonenal (4HNE), protein carbonyls, glutathione peroxidase 1 (GPX1), Binding Immunoglobulin Protein (BiP), X-box Binding Protein 1 (XBP1), and CCAAT/enhancer binding protein homologous protein (CHOP) in the cerebellum, cerebrum, and brainstem of non-reproductive (NR), one-bout (1B), and four-bout (4B) females. Results of linear regression models comparing all previously mentioned variables and the total lifetime number of offspring in four-bout females are also shown under the column "Regression 4B".

	NR vs 1B	**1B vs 4B**	**4B vs NR**	**Regression 4B**
Cerebrum				
Mitochondrial Density:				
Citrate synthase	**<0.001**	0.359	**0.003**	0.696
Oxidative Stress:				
4HNE adducts	0.789	0.951	0.935	0.998
Protein carbonyls	>0.999	0.849	0.894	0.172
GPX1	0.877	0.579	0.869	**0.008**
ER Stress:				
BiP	0.596	0.999	0.579	0.651
XBP1	0.879	0.997	0.848	0.590
CHOP	0.470	0.761	0.887	0.640
Brainstem				
Mitochondrial Density:				
Citrate synthase	0.052	0.453	0.462	0.458
Oxidative Stress:				
4HNE adducts	0.968	0.817	0.685	0.621
Protein carbonyls	0.845	0.741	0.976	0.303
GPX1	0.260	0.886	0.121	**0.039**
ER Stress:				
BiP	0.961	0.864	0.724	0.505
XBP1	0.616	0.962	0.469	0.264
CHOP	0.917	0.614	0.852	0.613
Cerebellum				
Mitochondrial Density:				
Citrate synthase	0.509	0.999	0.502	0.604
Oxidative Stress:				
4HNE adducts	0.837	0.999	0.859	0.677
Protein carbonyls	0.589	0.995	0.545	0.441
GPX1	0.111	0.233	0.916	0.278
ER Stress:				
BiP	0.985	0.847	0.925	0.599

XBP1	0.976	0.902	0.804	0.529
CHOP	0.780	0.667	0.981	0.130

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