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Estrogens Regulate Metabolic Substrate Concentrations in Brains of Young Adult Female Rats: A Multiple Memory Systems Approach

Ella D'Amico

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Estrogens Regulate Metabolic Substrate Concentrations in Brains of Young Adult Female Rats:
A Multiple Memory Systems Approach

A Capstone Project Submitted in Partial Fulfillment of the
Requirements of the Renée Crown University Honors Program at
Syracuse University

Ella D'Amico

Candidate for Bachelor of Science
and Renée Crown University Honors
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Honors Capstone Project in Biology

Capstone Project Advisor: _____
Donna L. Korol, Associate
Professor, Biology

Capstone Project Reader: _____
Paul Gold, Professor, Biology

Honors Director: _____
Stephen Kuusisto, Director

Abstract

As the most potent circulating form of estrogen in most young adult female mammals, estradiol has extensive effects on physiological functioning. Estradiol effects are especially notable with the drop in the hormone observed when a woman transitions into menopause, with accompanying changes not only in overall physiology but also in brain functions. Recent research reveals the complex effects estradiol has on regulating learning and memory that vary by type of cognitive task among other variables. In particular, increased estradiol levels improve performance on hippocampus-dependent tasks, but impair performance on striatum-dependent tasks through direct actions on these different memory systems. Estradiol's bidirectional cognitive effects might result from regulation of energy substrate availability and metabolism, a hypothesis tested in the present research. Prior research done in our lab revealed via microdialysis that peripheral injections of estradiol to ovariectomized female rats significantly increased glucose and lactate concentrations in the extracellular fluid of the hippocampus. The goal of the current study is to determine the effects of estradiol on basal extracellular substrate concentrations in the striatum using similar microdialysis procedures. Results showed no significant effects of low-dose peripheral estradiol injections (4.5 $\mu\text{g}/\text{kg}$) on extracellular glucose concentration, which was 2.75 ± 0.17 mM with control oil injections and 2.61 ± 0.14 mM with estradiol injections. However, rats treated with estradiol had significantly lower extracellular lactate concentrations of 0.508 ± 0.018 mM compared to 0.617 ± 0.029 mM in oil-treated controls. Further, assays on serum showed no change in peripheral concentrations of glucose and lactate with estradiol, suggesting changes are specific to the striatum and not simply a result of altered systemic availability. These findings reveal the role of bioenergetics underlying estradiol's regulation of memory systems and point to potential neural processes that become dysregulated with female aging and reproductive senescence.

Executive Summary

Hormones fluctuate throughout an organism's lifecycle, causing changes in both reproductive function and physiological functioning. Fluctuations in gonadal hormones are characteristic of the reproductive cycles of many female organisms. For many animals, aging in females is accompanied by reproductive senescence whereby reproductive cycles cease, such as menopause in humans and other primates. There are drastic changes in hormone levels with the onset of menopause. For example, 17β -estradiol, the primary circulating form of estrogen in young adult females, drops significantly. Unfortunately, there are a number of known consequences of menopause that may be related to these hormonal changes, such as hot flashes, anxiety, depression, irritability, night sweats, weight gain, difficulty concentrating, decreased libido, fatigue, and mood swings. The functional change of particular interest to this study is altered learning and memory with different levels of estradiol. Research suggests that performance on certain recall tasks is worse in women following surgical menopause compared to controls, impairments that are rescued with estrogen treatments (Sherwin, 1992; 1997). Because these changes occur specifically in otherwise healthy women who have had a surgery to remove their ovaries, and because replacing lost circulating estrogens reverses these changes, it can be concluded that estrogen levels, in addition to any age-related deficits that may occur in menopausal women, play an important role in learning and memory.

Unfortunately, while hormone replacement therapy, which attempts to restore circulating hormones such as estradiol, is able to rescue some of the memory changes that occur with menopause, hormone therapy can also have many adverse side effects, such as increased risk of stroke and breast cancer, that outweigh these potential benefits (Pisani et al., 2012; Writing Group, 2002; Craig, Maki, & Murphy, 2005). By better understanding the mechanisms of

estradiol's affects on the brain, safer therapies can be developed to reverse the negative effects of menopause.

An interesting finding from the experiments with menopausal women is that the cognitive enhancements found with estrogen treatments were not seen for all types of tasks; only select forms of learning and memory showed improvements while others remained unaffected (Sherwin, 1992; 1997). Research using female rats also suggests that the direction of estrogen regulation of cognition depends heavily on the type of cognitive task to be solved. There are consistent results of a shift from hippocampus-based to striatal-based learning that accompanies a shift to low circulating estrogens, creating a valid model for menopause (Korol & Kolo, 2002; Korol, 2004). It is thus likely that with menopause and the loss of hormones would come both declines and improvements in learning and memory depending on whether the hippocampus or striatum, respectively, is critical for the task at hand.

As shown through lesion studies, certain types of learning and memory occur in two important brain regions, the hippocampus and striatum. This research asserts that when destroyed or metabolically impaired, such as through local anesthetics or inhibitors, memory that is controlled by those regions is also impaired (Morris et al., 1990; Jarrard, 1995; Brasted et al., 1997; Doyon et al., 1997). The hippocampus is important for memory that relies on spatial and contextual integration, while the striatum is necessary for implicit memory and memory dependent on egocentric space (Morris, Schenk, & Jarrard 1990; Jarrard, 1995; Brasted et al., 1997; Doyon et al., 1997). Because these two brain regions are critical for learning and memory, they have become the focus of studies determining the effects of estrogens.

There are several candidate mechanisms that may underlie estrogenic regulation of cognition, including metabolic regulation. Glucose and lactate are two metabolic substrates

critical for good learning and memory, as demonstrated through a number of studies. These molecules act as fuel for the brain. Engagement in a task is reflected in changes in glucose and lactate (McNay & Gold, 1999; Newman et al., 2011). A large reduction of glucose concentrations in the brain is observed while performing memory tasks (McNay & Gold, 1999; Newman et al., 2011). Simultaneously, lactate levels may rise in attempt to compensate for glucose depletion (Newman et al., 2011). Injections of these substrates either peripherally or directly into the brain improves memory scores and can also reverse age-related deficits related to inefficient metabolism in hippocampus-sensitive learning and memory (Ragozzino et al., 1998; McNay, 2000; Gold, 2005; Newman et al., 2011). Collectively, prior research suggests a likely mechanism for estrogen up- and down-regulation of cognition is via manipulation of metabolic resources.

The levels of glucose and lactate in the hippocampus and striatum are readily increased by engagement in certain mental activities (Korol & Gold, 2014). Interestingly, levels of these metabolites rise more in the brain region selectively engaged by specific learning tasks (Korol & Gold, 2014). Thus, the bidirectional effects of estrogens on hippocampus-based and striatum-based learning is likely through regulation of available bioenergetics reserves. Determining the concentration of these substrates in the hippocampus and striatum of rats with and without estradiol treatments while they are at rest will reveal how well prepared the brain is to perform during cognitively demanding tasks. We previously found that in female ovariectomized rats, estradiol treatments improve hippocampus-sensitive learning and increase basal lactate and glucose in the hippocampus (Korol & Kolo, 2002; Korol, 2004; Wang et al., 2015). The current study tests the effects of estradiol on metabolic substrate concentrations in the striatum. Given

that estrogens impair striatum-sensitive learning, it is hypothesized that glucose and lactate levels in the striatum will decrease with estradiol treatment.

As my senior thesis, I was involved in all aspects of the present study, including research design, surgeries, animal care, microdialysis, tissue preparation, chemistry, data analysis and interpretation, and writing. This study used 85-90-day-old virgin female Sprague-Dawley rats. Rats received an ovariectomy surgery to remove their ovaries, allowing for experimental control of circulating estrogens. After one week of recovery, rats received a cannula surgery where a probe was implanted in either their left or right striatum. Following another week of recovery, rats were handled every day to accustom them to humans, and received a peripheral injection of estradiol or oil, a control, 48 and 24 hours prior to testing to mimic the natural estrous cycle. During testing, rats were placed in a holding container and a microdialysis probe was inserted into the striatum and perfused with aCSF while collecting effluent samples of striatal extracellular fluid. Because glucose and lactate can diffuse freely across the probe membrane, we were able to determine striatal concentrations of these substrates by measuring their levels in collection of artificial cerebral spinal fluid that was taken after it passed through the probe.

Results showed that estradiol treatments have no significant effect on glucose concentrations, but did significantly lower lactate levels in the striatum. While there is no change in glucose, the decrease in lactate suggests there is less of a fuel resource during cognitive demand. When glucose is depleted, less lactate is readily available to compensate for this dip. This is congruent with prior research suggesting striatal-based learning decreases with estradiol treatments (Korol & Kolo, 2002; Korol, 2004).

Because this test looks specifically at concentrations of substrates in the extracellular fluid, further research must be done to determine why these changes are occurring, such as

testing effects of blocking specific transporters that carry the substrates in and out of astrocytes and neurons. It should also be determined how these changes in baseline conditions affect metabolism during cognitive demand by measuring substrate concentrations during hippocampal- and striatal-specific tasks.

The results of the current study reveal the profound effects of estradiol on metabolic functioning. Further, these changes are specific to the striatum. While substrate levels are depleted in the striatum, they are augmented in the hippocampus, suggesting that estradiol acts differently in different brain regions. This research is only one factor in determining how estradiol causes a shift in learning strategies. Regardless, it brings us one step closer to fully understanding estradiol's effects, and one step closer to developing effective supplements to reverse the negative consequences without counteracting the positive changes following the loss of circulating estrogens after menopause.

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Donna Korol, Associate Professor, Biology

Paul Gold, Professor, Biology

Wei Wang, Graduate Student, Biology

Center on Aging Studies at Syracuse University

Department of Biology, Syracuse University

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Introduction

Estrogen levels fluctuate constantly throughout a woman's life, causing known changes in behavior and reproductive function that are the most radical with a woman's transition into menopause. 17β -estradiol, the primary circulating form of estrogen in young adult females, decreases the most prominently compared to other estrogens during menopause and is highly related to the adverse side effects of menopause (Kuhl, 2005; Rannevik et al., 1995). Estrogens also play an important role in cognitive function. The effects of estrogens on cognition are not consistent; estrogens can both enhance and impair learning and memory depending on task attributes and the brain regions engaged by those attributes. Research suggests that performance on certain recall tasks is worse in women following surgical menopause compared to controls, impairments that are rescued with estrogen treatments (Sherwin, 1992; 1997). Because these changes occur specifically in otherwise healthy women who have received oophorectomies, a surgery to remove their ovaries, and these changes can be reversed by replacing lost circulating estrogens, it is clear that estrogen levels affect memory in addition to deficits more generally associated with aging in menopausal women.

The hippocampus and striatum are two brain regions important to learning and memory. Studies using lesions have shown that when these regions are destroyed or metabolically impaired, such as through local anesthetics or inhibitors, certain types of memory are impaired (Morris et al., 1990; Jarrard, 1995; Brasted et al., 1997; Doyon et al., 1997). Hippocampal disruption compromises memory that relies on spatial and contextual integration, while striatal lesions impair implicit memory and memory dependent on egocentric space (Morris, Schenk, & Jarrard 1990; Jarrard, 1995; Brasted et al., 1997; Doyon et al., 1997). Due to their importance for

learning and memory, they have become the focus of studies determining the effects of estrogens.

As mentioned earlier, estrogen levels cause a shift between memory systems. Rats with high levels of estrogens do better on hippocampal-dependent spatial memory tasks but worse on striatal-dependent memory tasks that use egocentric body-turn rules compared to rats with low level of estrogens (Korol & Kolo, 2002; Korol, 2004). Parallel to studies done on young rats, estradiol treatment to aging rats that have been ovariectomized to mimic the depletion of ovarian hormones in women improves hippocampal-based learning and impairs striatal-based learning under certain conditions (Korol et al., 2007; Wang et al., 2009; Korol & Pisani, 2015). For post-menopausal women and women who have received oophorectomy surgeries, hormone replacement therapy (HRT) is a method to replace lost circulating hormones by taking a supplement containing conjugated equine estrogens and synthetic progestin. Estrogen therapies can be used to ameliorate the clinical side effects of menopause, and may also act to restore the role estrogens play in learning and memory. While relieving some of the side effects of menopause and restoring learning and memory, findings from the Women's Health Initiative (WHI) showed that HRT leads to complications related to both the physiological and cognitive functions of hormones that may outweigh its potential benefits (Writing Group, 2002; Pisani et al., 2012). Further, hormone therapies are related to a variety of health consequences, such as increased risk of stroke and breast cancer (Writing Group, 2002; Craig, Maki, & Murphy, 2005). It is unclear, however, how generalizable results from the Women's Health Initiative are due to inconsistencies between individuals and treatments (Sherwin, 2009). By understanding the cellular mechanisms of estradiol on learning and memory, safer therapies can be developed to mimic the positive effects of estradiol and regulate its negative effects.

There are several candidate mechanisms that may underlie estrogenic regulation of cognition including gene activation, pathway signaling and metabolic regulation. Estrogens likely modulate different memory systems, as suggested by the estrogen-mediated shift from striatal- to hippocampal-based learning strategies. Metabolism is an attractive possibility because it is easily regulated by the supply, storage and consumption of energy in different brain regions. This dynamic quality makes metabolism a feasible mechanism for the differential participation of the hippocampus and striatum in cognition under different estrogenic conditions. Because glucose is a major fuel source used by neurons and astrocytes, determining brain levels of glucose will likely give key insights into these dynamics. The important role of glucose in memory processing is reflected by the improved performance on memory tasks that is observed in males with both peripheral and central injections of glucose (Ragozzino, Pal, Unick, Stefani, & Gold 1998; McNay, 2000). Glucose availability itself limits memory processing (McNay, 2000). Decreases in extracellular glucose concentrations from baseline are observed in the hippocampus while rats perform cognitive tasks such as a hippocampus-sensitive working memory task called spontaneous alternation (McNay & Gold, 1999; Newman, Korol, & Gold, 2011). The higher the cognitive demand, the greater the dip in glucose (McNay, 2000). Further, replacing glucose before testing improves learning and memory and reverses deficits due to age and cognition-blocking drugs (Gold, 2005; Newman et al., 2011). Similar results have been observed in the striatum, such that when driven by a water reward on a response-learning, striatal-sensitive task, extracellular glucose is initially depleted (Korol & Gold, 2014). The availability and usage of glucose clearly reflect the capacity for mental functioning during cognitive demand.

While the brain relies on glucose for learning and memory tasks, it is depleted in

extracellular fluid in the hippocampus during testing on a spatial memory task (McNay & Gold, 1999; McNay & Gold, 2002; Newman et al, 2011). During times of high metabolic demand when glucose depletion is seen, energy metabolism may be maintained by a simultaneous rise of extracellular lactate to be used as fuel by neurons (Newman et al., 2011). Such upsurges of lactate are observed in both the hippocampus and striatum during tasks sensitive to each brain region (Newman et al., 2011; Korol & Gold, 2014). Glycogen stored in astrocytes is broken down to lactate via glycogenolysis and other enzyme metabolism and released into the extracellular fluid to be used by neurons (Newman et al., 2011). Lactate's central role in regulating memory is reflected by previous studies on the effects of altered lactate metabolism on memory task performance. Intrahippocampal administrations of lactate increases performance on spontaneous alternation tasks, while blocked glycogenolysis and blocked lactate transport into neurons both impair performance (Newman et al., 2011). Because glucose and lactate are so critical for normal mental functioning, and because their concentrations seem to change in relation to each other, a change in basal concentrations of either substrate would force downstream bioenergetics to adjust.

Altered glucose and lactate availability and usage provide a promising mechanism to explain estradiol's effects on cognition. In a mouse model of Alzheimer's disease, ovarian hormone loss stimulates a transition from glucose metabolism to the metabolism of alternative fuels including lactate and ketone bodies in the hippocampus (Ding et al., 2013). Other research has shown that estradiol acts by increasing the amount of glucose transport through the blood brain barrier for use during metabolic demand and impairing the activity of enzymes important to glucose metabolism, such as hexokinase (Bishop & Simpkins, 1995; Ding et al., 2013). Our preliminary data showed that ovariectomized rats with estradiol treatment have higher

extracellular glucose and lactate concentrations in the hippocampus compared to oil-treated rats (Wang et al., 2015). While these data are important in highlighting a shift in bioenergetics with circulating estradiol, findings were from hippocampus only or large, anatomically nonspecific, brain samples. Other research has shown increased glucose uptake in the striatum with high-dose estradiol treatment over the course of 24 hours (Bishop & Simpkins, 1995). The estradiol treatment paradigms used in these studies were different from those in which we find shifts in learning and memory. Moreover, the spatial resolution of the results was low, making it difficult to determine whether estradiol regulates basal levels available to neurons and astrocytes in the extracellular fluid. The lack of knowledge about estrogen regulation of the striatum is hindering a greater understanding of estradiol's effects on all memory systems, including those that may be insensitive or may benefit from hormone loss.

To establish how estradiol regulates learning and memory through bioenergetics, this research determined how estradiol changes the physiological conditions of brain regions by altering basal levels of glucose and lactate. The current study used *in vivo* measurements in rats with and without estradiol treatments to determine baseline extracellular concentrations of the energy substrates glucose and lactate in the striatum. Because high levels of estradiol impair performance on striatal-based memory tasks, lower concentrations of metabolic substrates in the striatum with estradiol treatment were expected. This finding would support the hypothesis that estrogens modulate cognition through regulation of metabolic status of specific brain regions.

Methods

Subjects

85-90-day-old virgin female Sprague–Dawley rats were purchased from Harlan Sprague–Dawley Breeders (Oregon, WI). The rats were housed individually and maintained on a 12hr:12hr LD cycle with *ad libitum* access to food and water. Handling began nine days prior to testing to ensure all subjects were comfortable with humans before injections and testing were performed. All animal procedures adhered to the National Research Council's *Guide for the Care and Use of Laboratory Animals* and were approved by the Syracuse University Animal Care and Use Committee, accredited by the Association for Assessment and Accreditation of Animal Care.

Surgeries

Three weeks prior to microdialysis procedures, all rats were ovariectomized bilaterally under isoflurane anesthesia to remove gonadal supply of estrogens, thereby allowing experimental control of circulating estrogens. Rats were first placed in an induction chamber for 8 minutes with 500 mg/ml (5%) isoflurane anesthesia. The abdomen was quickly shaved before rats were then placed on a sterile surgery table with a nose cone with a combination of 400 mg/ml (1.5-5%) isoflurane anesthesia and oxygen to maintain a rate of approximately one breath per second. Eye ointment, Flunixin (5 mg/kg, s.c.) and penicillin (100,000 unit/kg i.m.) were administered pre-operatively. Once sterilized with 70% ethanol and betadine, ~1.5 cm long incisions were carefully made through the skin and fascia and blunt dissection of the muscle layers were made ~75 mm from the posterior point of ribs. The fat pad and ovaries were located, pulled through the muscle layer, and clamped with a hemostat around the distal point of the

oviduct. The oviduct was tied off and ovaries excised before guiding the remaining fat pad and oviduct back into the muscle layer. Muscle was closed with 2 sutures, skin pushed back together, glued in the middle of the incision, and closed with 2-3 wound clips. Finally, the incision site was cleaned with 70% ethanol and bacitracin was applied. The same protocol was followed for the second side. Ten mL of 0.9% saline was administered (s.c.) in the dorsal neck / back region. Rats then received a postoperative analgesic, Children's Motrin®, in their water bottles (2.35 ml / 500 ml water) until the next day and were closely monitored for a week.

One week after ovariectomy, rats underwent unilateral guide cannula implantation in the striatum for microdialysis. Rats were first placed in an induction chamber for 5 minutes with 500 mg/ml (5%) isoflurane anesthesia, the head region was shaved, and rat was placed back in the induction chamber for another 2 minutes. Rats were then placed on in a stereotaxic device with a combination of 400 mg/ml (1.5-5%) isoflurane anesthesia and oxygen to maintain a rate of approximately one breath per second. Eye ointment, Flunixin (5 mg/kg, s.c.) and penicillin (100,000 unit/kg i.m.) were administered pre-operatively. The scalp was cleaned with 70% ethanol and betadine, an incision was made, and skin and fascia cleared to expose the skull. A sterilized CMA 12 microdialysis probe (14 mm shaft length, 0.5 mm membrane diameter) was implanted under stereotaxic control at coordinates +0.2 anterior/posterior, ± 2.0 medial/lateral, -4.0 dorsal/ventral from bregma through a hole in the skull made using a drill bit. The cannula was cemented in place with dental cement, using 4 stainless steel jewelers' screws as anchors. One suture was placed anterior and posteriorly to the incision site to promote healing. The scalp was cleaned and treated with bacitracin. Saline (0.9%) was administered subcutaneously in the dorsal neck / back region. Animals then received Children's Motrin in their water bottles (2.35

ml / 500 ml water) until the next day and were closely monitored for a week. A timeline of the general protocol is shown in Figure 1.

Treatments

We used a relatively low dose of estradiol treatment shown previously to alter hippocampal glucose levels. Treatments of 4.5 $\mu\text{g}/\text{kg}$ of 17 β -estradiol benzoate (EB) or sesame oil vehicle were injected subcutaneously in the nape of the neck 48 and 24 hours prior to microdialysis.

Microdialysis for glucose

To ensure optimal brain conditions on the day of sample collection, rats were primed for microdialysis 24 hours before testing by placing a microdialysis probe into guide cannula for 5 minutes. The day of microdialysis, rats were placed in a holding container from 10:00 to 12:00 am (~2-4 hrs after lights on) to allow acclimation to the collection room. At 12:00, a microdialysis probe (CMA12 probe, 20kDa cutoff) was inserted into guide cannula unilaterally in the striatum of one hemisphere for measurement of extracellular fluid (ECF) glucose values. Artificial cerebral spinal fluid (aCSF) with glucose concentration of a known concentration (0.5, 1.0, 1.5, 2.0, 2.5, or 3.0 mM) was perfused through the probes. aCSF solution contained 127.65 mM NaCl, 4.02 mM KCl, 0.17 mM CaCl₂, 0.93 mM MgCl₂, 2.04 mM Na₂HPO₄, and 0.75 mM NaH₂PO₄. Unused “waste samples” were collected for the first 40 minutes after which, 20 μL of perfusate samples were collected. Samples were collected for 160 minutes at a rate of 1 $\mu\text{L}/\text{min}$ and frozen immediately for later fluorometric enzyme assays to measure glucose concentration.

Glucose assay

Glucose concentrations of ECF and blood samples were measured using a glucose assay protocol adapted from Lowry and Passoneau (1972). Samples were placed in a 96-well plate assay system, and compared to a glucose standard with a plate reader. Briefly, samples were mixed with hexokinase and converted to glucose-6-phosphate, which was then oxidized to 6-phospho-gluconate in the presence of nicotinamide adenine dinucleotide phosphate (NADP⁺) and glucose-6-phosphate dehydrogenase (G6PDH). As NADP⁺ is reduced to NADPH, the resulting increase in absorbance at 340nm is proportional to the original glucose concentration.

Lactate assay

Lactate concentrations of ECF samples were measured using a lactate assay protocol adapted from Schon (1965). Samples were placed in a 96-well plate assay system and compared to a lactate standard with a plate reader. As lactate in samples is converted to pyruvate by lactate dehydrogenase, NAD⁺ is reduced to NADH. The resulting absorbance at 340nm is proportional to the original lactate concentration.

Zero net flux

Concentrations of 0.5, 1.0, 1.5, 2.5 and 3.0 mM glucose were perfused into the striatum ([glucose]_{in}) in separate groups of rats. Glucose can freely diffuse across the probe membrane. Consequently, infused concentrations of glucose reach an equilibrium with glucose in extracellular fluid surrounding the probe, and which is collected as the efflux sample ([glucose]_{out}). When the concentration of glucose in the efflux is equal to the concentration in the influx, this is called the point of zero net flux (ZNF). This point is used as the estimate of the

concentration of glucose is in the extracellular fluid of the striatum.

[Glucose]_{out} vs. [glucose]_{in} concentrations were measured for each [glucose]_{in} concentration. Values for [glucose]_{out} – [glucose]_{in} were plotted against the known concentration of [glucose]_{in} to generate a regression line. Because ZNF is when [glucose]_{out} equals [glucose]_{in}, it is where the line crosses the x-axis (i.e. at y = 0).

Histology

Following microdialysis testing, rats were overdosed with sodium-pentobarbital. Brains were collected, stored overnight in 4% paraformaldehyde, and then moved to a 20% glycerol solution for 48 hours. They were frozen and sectioned at 40µm on a cryostat (Leica CM1850, Leica Microsystems Inc., Germany). The sections were stained with cresyl violet to better visualize cannula placement and potential blood clotting. Rats with missed placements may comprise a valuable group to show anatomical specificity of drug effects. If sections showed excessive bleeding or misplacement of the probe in a region other than the striatum, data from the rats were blindly excluded.

Uterine horn samples were collected and measured for weight and length. In the absence of circulating hormones, the uterine horn shrinks. Thus, low unitary weights are indicative of low estradiol and successful ovariectomies.

Vaginal smears were taken for 7 days prior to training. The samples were preserved with ethanol and stained with toluidine blue for staging. Staging of the slides will confirm that rats receiving estradiol treatments successfully recovered their natural estrous cycle. Rats that received oil injections and successful ovariectomies should not be cycling and will thus have diestrus cells.

Statistical analysis

Uterine horn weights and glucose and lactate concentrations in the ECF were each averaged for each treatment group. Data were then analyzed using Student's t-tests for differences between groups. Statistical significance was set at $p < 0.05$.

Results

Histology validates treatments

Brain sections confirmed correct cannula placement in the dorsal striatum (Figure 2). Five rats were excluded due to either misplaced cannulae or excessive bleeding.

Uterine horn weights of females who were given oil treatments were significantly lower than weights from females that received EB treatments, with values of 23.45 mg/cm for oil-treated rats and 60.98 mg/cm for EB-treated rats ($p < 0.05$); Figure 3). The results confirm the success of the ovariectomies in removing circulating hormones and the injections recovering lost estradiol.

Cells collected via vaginal smears also confirmed the success of the ovariectomy and estradiol injections. Females that received the estradiol injections had vaginal cytology with nucleated epithelial cells, confirming the injection restored the natural estrous cycle. Females that received the oil injections were not cycling and had only diestrous cells, typical of ovariectomy status.

Brain metabolites

The concentration of glucose in striatal extracellular fluid for females was determined by finding the point of ZNF for each treatment. ZNF was 2.75 ± 0.17 mM in oil-treated females and

2.61±0.14 in EB-treated females (Figure 4AB). The concentrations were not significantly different ($t(11)= 0.67$; $p > 0.5$) across treatments, indicating circulating estradiol had no effect on extracellular glucose levels (Figure 4C).

In contrast, extracellular lactate levels in the striatum were substantially lower in rats receiving EB treatments compared to those receiving oil (compare 0.508±0.018 mM and 0.617±0.029 mM, Figure 5). Comparisons with t-tests demonstrate that differences between groups were statistically different ($t(11)= 3.25$; $p < 0.01$); thus, estradiol reduced lactate concentrations in the extracellular fluid.

Peripheral metabolites

There was no significant difference in circulating blood glucose and lactate concentrations between females that received oil and estradiol treatments (Figure 6). Blood glucose concentrations were 6.70±0.41 mM for oil and 7.39±0.64 mM for EB treated rats ($p > 0.05$). Blood lactate concentrations were 4.04±0.45 mM for oil and 4.22±0.63 for EB treated rats.

Discussion

Altered Substrate Availability with Estradiol

The present study examined the effects of estradiol on basal levels of energy substrates in the striatum. Estrogens regulate brain bioenergetics with neuroanatomical specificity, which may ultimately facilitate the shift in learning strategies seen in previous studies. Our results show that estradiol decreases the extracellular lactate concentration while having no significant effect on glucose concentrations in the striatum. Moreover, estradiol had no effect on peripheral glucose or

lactate levels.

These changes can more easily be explained when looking more broadly at the metabolic pathways between astrocytes and neurons important to fueling striatal functions (Figure 7). As Figure 7 demonstrates, glucose can enter the neuron directly from the blood capillary or enter astrocytes to be stored for later use as glycogen. If needed for energy, glycogen can be broken down and shuttled to the neuron as lactate (Itoh, 2003). This pathway is commonly referred to as the astrocyte-neuron lactate shuttle and allows the astrocytes to regulate memory formation and support neuronal function during glucose deprivation. The neuron metabolizes both glucose and lactate for energy that can be used for learning and memory processing (Newman et al., 2011). Changes in extracellular concentrations of these substrates with hormone treatment suggest that estradiol produces a change in the transport, storage, or metabolism of these molecules.

As a key substrate used by neurons during times of cognitive demand, lactate concentrations reflect the potential metabolic ability to fuel learning strategies dependent on the brain region (Newman et al., 2011). Based on the metabolic model in Figure 7, reduced lactate concentrations in the extracellular fluid may be a result of slower glycolytic rates and lactate release by astrocytes, increased consumption of available lactate by the neurons, or a combination of the two events. Overall, decreased extracellular lactate in ovariectomized females treated with estradiol leaves a smaller reservoir to fuel striatal-dependent functions.

We did not observe an increase in basal extracellular glucose levels in the striatum with estradiol treatment. These results suggest that there is either no change in glucose metabolism or any changes caused by estradiol create no net change in extracellular glucose concentrations. For example, an increase or decrease in the consumption of glucose by astrocytes or neurons could be offset by an equal change in the amount of glucose being transported to the striatum via the

capillaries creating no overall flux of glucose in the ECF. However, high ongoing metabolic demand typically produces high concentrations of metabolic extracellular substrates (Magistretti and Pellerin 1996, 1999); such increases are observed in the striatum during cognitive testing on tasks thought to rely on striatum function (Korol & Gold, 2014). Although glucose and lactate were not measured during behavioral testing in our study, it is possible that higher neuronal demand would produce higher extracellular levels even when at rest. Thus, because there is no flux in the concentration of extracellular glucose, it is more likely that estradiol fails to alter glucose metabolism and provision in the striatum, a possibility we plan to test more directly. The lack of prior research done on estradiol's effect on striatal bioenergetics highlights the importance of the current findings and the need for future research to make more definitive conclusions on the mechanisms of estradiol-related changes.

While the mechanisms by which estradiol acts in the striatum to regulate substrate concentrations are unclear, our results suggest that estradiol effects are site-specific because circulating estradiol has no overall effect on peripheral glucose or lactate availability. In other words, the decrease in striatal lactate is not driven by a change in the periphery. While there may be no change in peripheral or striatal glucose, there is a significant increase in hippocampal glucose with estradiol treatment, further highlighting the site-specificity of these changes (refer to Figure 8; Wang et al., 2015).

The use of microdialysis with ZNF methods allows us to detect small changes in metabolites in the extracellular fluid under different experimental conditions. Thus, we believe our lack of striatal differences in glucose concentrations reflect real values and not statistical flukes or confounds due to methodological limitations. The flow rate of the microdialysis fluid impacts the time molecules have to diffuse out of the microdialysis fluid into the brain and visa

versa. Slower flow rates facilitate time for diffusion to occur. However, slower flow rates are also associated with smaller sample sizes, making it hard to measure concentrations of target molecules in the collected fluid. This issue is exacerbated by the fact that each sample was split into three smaller collection sizes in the assay to account for pipetting inconsistencies. If time for each sample collection is increased to increase collection volume at a slow flow rate, total testing time becomes unmanageable for a single experimenter in a single day. Similar microdialysis done on the hippocampus successfully used a flow rate of 1 $\mu\text{L}/\text{min}$ was used to obtain 20 μL of perfusate every 20 min, which used as a guide for the current study (Wang, 2015).

Shift in Bioenergetics Reflects Shift in Learning Strategies with Estradiol

The shift in basal metabolite concentrations in the striatum with circulating estrogens is remarkably consistent with the simultaneous shift in learning strategies from striatal-based to hippocampal-based learning (Korol, 2002, 2004, 2015). Findings from this research and preliminary data from our lab suggest that with peripheral estradiol injections, extracellular glucose and lactate concentrations both increase in the hippocampus (Figure 8; Wang et al., 2015) while glucose was unchanged and lactate decreased in the striatum in the present experiment. The data highlight how estradiol may prime the hippocampus for information processing while limiting the striatum by increasing the availability of metabolic substrates. Such results are consistent with observed improve performance on place leaning, a hippocampus-dependent task, but impaired performance on response leaning, a striatum-dependent task, with increased estradiol (Korol & Pisani, 2015). Even if the heightened hippocampus glucose concentration is completely depleted by high metabolic demand, the surplus of lactate in the hippocampus can extend support for cognitive function. In contrast, lower lactate levels in the

striatum will easily be depleted and unable to compensate for glucose consumption, forcing the striatum to metabolize stored glycogen. Further research must be done to determine glycogen concentrations and how metabolic substrates are used during learning tasks in order to make definitive conclusions on how estradiol regulates learning strategies through brain bioenergetics.

Future Directions

The current study gives new insights into the regulation of basal metabolites in the striatum by estradiol, complementing similar research being done concurrently on the hippocampus. To better understand estradiol's role in regulating brain bioenergetics and the effects it has on learning and memory, future research should be focused on determining the source of these changes and how they affect glucose and lactate usage during times of metabolic demand. To determine whether lactate changes are due to decreased release by astrocytes or increased consumption of neurons, monocarboxylate transporters (MCT) can be blocked preferentially. MCT-1 and MCT-4 transport lactate across astrocyte membranes while MCT-2 transports lactate across neuronal membranes (Newman et al., 2011). By using an inhibitor such as 4-CIN, which preferentially blocks MCT-2, in female rats treated with estradiol, a reduction in striatal lactate similar to that observed in the present study would suggest estradiol does not affect uptake of lactate by the neurons. Conversely, a recovery of lactate in estradiol-treated rats that receive 4-CIN would suggest that estradiol increases uptake of lactate by neurons. In this way, use of inhibitors provides a method to define the source of baseline changes in lactate with estradiol.

In addition to using inhibitors to explain changes in extracellular substrate concentrations, glycogen levels could also be measured. As the sole energy reservoir in the brain, glycogen levels signify lactate production during learning and memory (Newman et al., 2011; Suh et al., 2007). Especially under hypoglycemic conditions, such as those seen during learning and memory, glycogen metabolism is needed to support metabolic demand (Suh et al., 2007). The determination of glycogen depletion during memory testing would indicate neuronal dependence on lactate.

Finding the source of estradiol-driven changes during high metabolic demand, such as during learning and memory is equally important as determining how these changes affect bioenergetics. Fluctuations in glucose and lactate concentrations during hippocampal- and striatal-sensitive tasks can be measured using microdialysis or biosensor techniques. Extensive research has been done on the effects of estradiol on place and response learning, which are hippocampal and striatal dependent respectively (for review see Korol & Pisani, 2015). Consequently, these tasks may be the best method for determining changes in extracellular glucose and lactate during times of metabolic demand. Spontaneous alternation, a working memory task that is thought to be hippocampus dependent but shown, by work in our lab, to produce glucose and lactate responses in the striatum, could also be utilized. Compared to place and response learning, the task is simpler to run making it easier to collect consistent data. Regardless of what method is used, determining the changes in substrate concentrations in both brain regions during testing would shed more light on estradiol-driven changes in metabolism during high metabolic demand.

Conclusion

The results described here indicate that estradiol plays a role in regulating basal levels of energy substrates in the striatum. While there is no significant change in extracellular glucose levels, extracellular lactate levels decrease in ovariectomized female rats treated with low-dose estradiol. In conjunction with research showing augmented extracellular glucose and lactate concentrations with circulating estradiol, the present results suggest estradiol acts differently in different brain regions. As the foundation of learning and memory, regulation of metabolism provides a powerful and complex mechanism for estradiol to regulate physiological functioning. Using a multiple-memory system approach, the current study is one piece of the puzzle that describes how changes in basal concentrations of metabolic substrates caused by levels of circulating hormones prime the brain for different types of learning. These profound effects of estradiol on psychological functioning can be applied to better understand the effects of decreased circulating estrogens with menopause or oophorectomy. Ultimately, a better understanding of estradiol's effects brings us closer to developing effective supplements to reverse the negative consequences of the loss of circulating estrogens after menopause.

Figures and Figure Legends

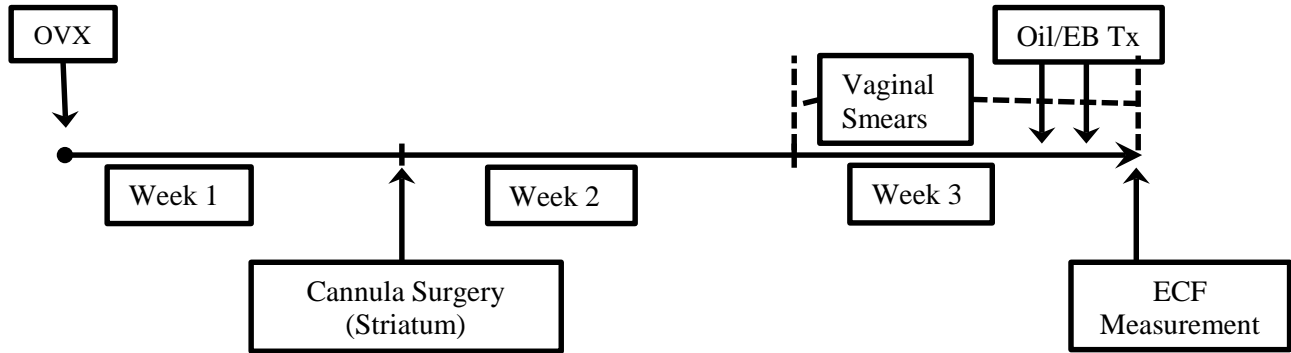


Fig 1. Experimental Timeline. Animals were ovariectomized, given one week to recover, and received a unilateral cannula surgery on day 8. On day 15, vaginal smears were taken daily until testing day. Treatments were given 48 and 24 hours before testing day.

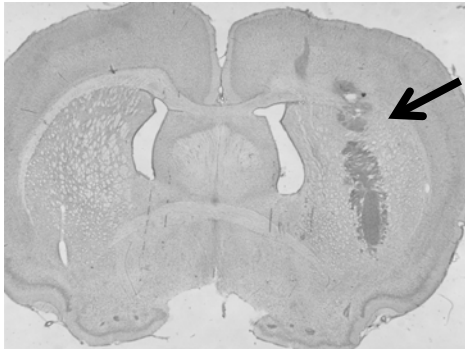


Fig 2. Proper cannula placements. Images taken from brain sectionings confirm proper placement of cannulae in the dorsal striatum.

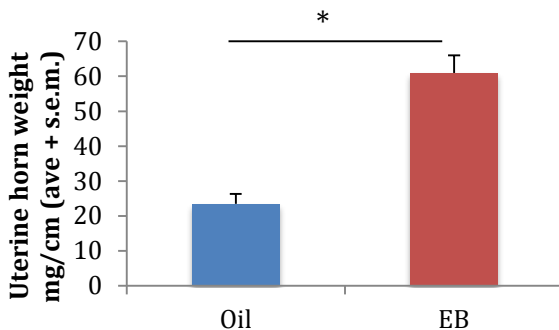


Fig. 3 Uterine horn weights. Significantly different uterine horn weights between treatments validates EB dosing. * $p < 0.5$

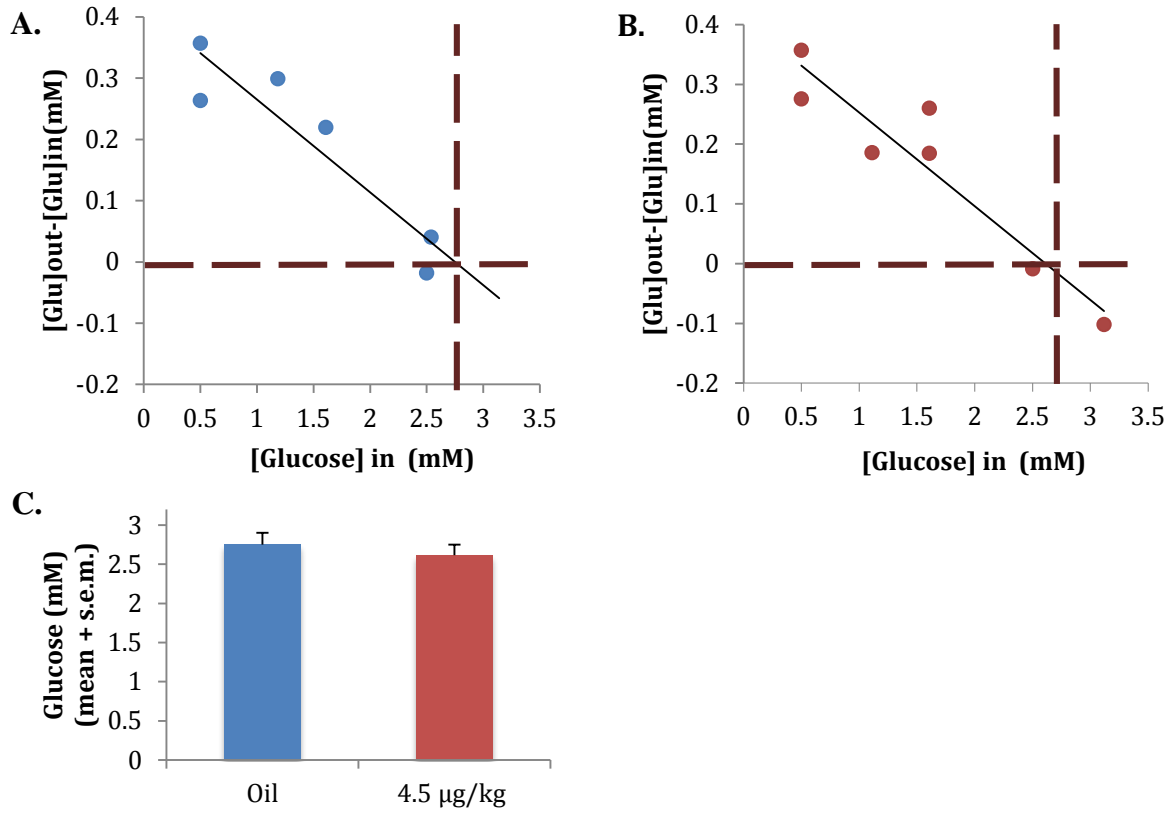


Fig 4. Striatal glucose concentration. **A.** Microdialysis results of rats receiving oil-treatments (n=6). **B.** Microdialysis results of rats receiving EB treatments (n=7). **C.** Striatal glucose concentrations are not statistically different.

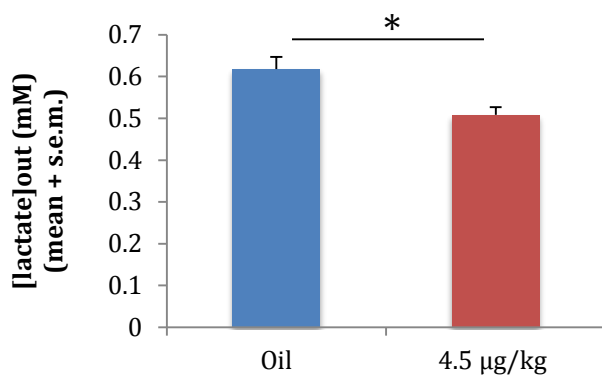


Fig 5. Striatal lactate concentration in oil- and EB-treated rats. Microdialysis samples from rats receiving the same treatment (oil or estradiol) were combined for lactate assays. Oil (n= 6) and EB (n= 6) treated rats had significantly different lactate concentrations. * p < 0.05

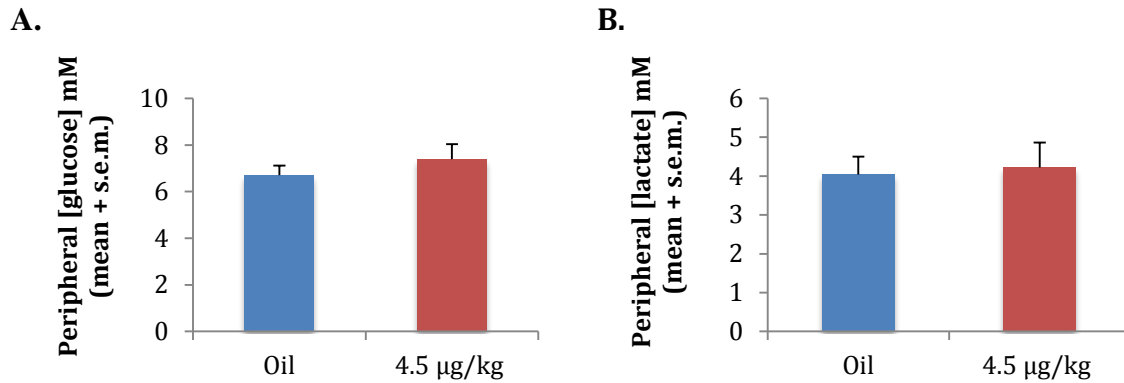


Figure 6. Circulating metabolite concentrations in oil- and EB-treated rats. **A.** Results show no significant difference in circulating glucose concentrations between oil-treated (n=8) and EB-treated (n=7) rats. **B.** Results show no significant difference in circulating lactate concentrations between oil-treated (n=9) and EB-treated (n=7) rats.

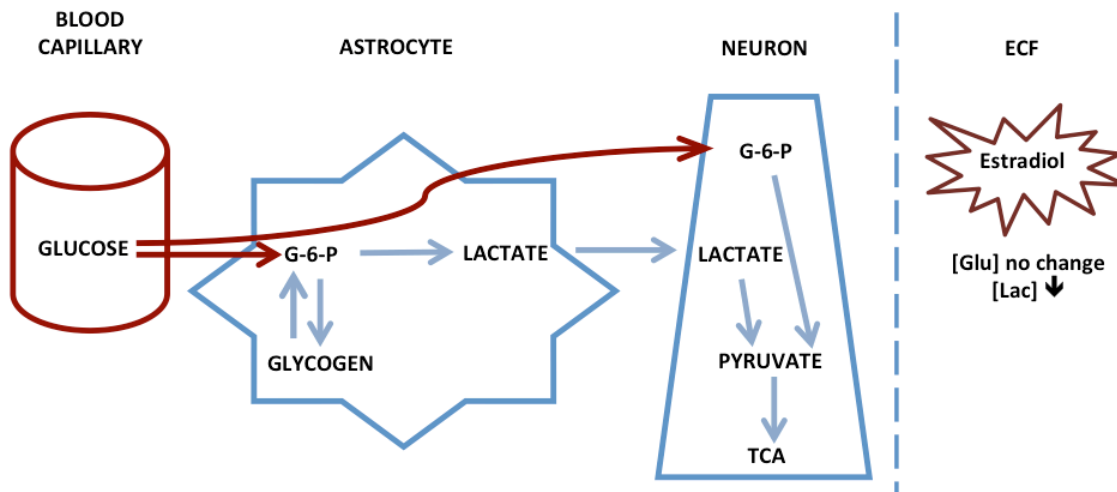


Figure 7. Model of estrogen regulation of basal bioenergetics. Estradiol increased striatal glucose and decreased striatal lactate. The changes in basal substrate concentrations affect bioenergetics and the metabolic relationships between astrocytes and neurons. Adapted from Newman et al., 2011.

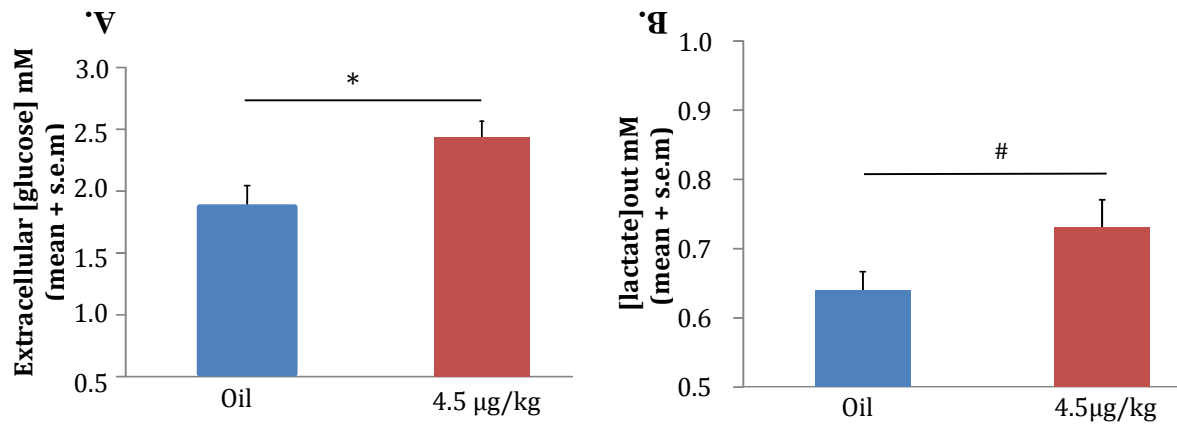


Figure 8. Hippocampal metabolite concentrations in oil- and EB-treated rats. Previous research done in the lab by Wang (2015) showed **A**, significantly different extracellular glucose concentrations and **B**, significantly different lactate (out) concentrations in the hippocampus in rats receiving oil and low-dose estradiol treatments. * $p < 0.05$ # $p = 0.07$

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