

5-2018

The Effects of Intrahippocampal Estradiol Administration on Spatial Memory and Protein Expression in Rats Fed a High Fat Diet

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The effects of intrahippocampal estradiol administration on spatial memory and protein expression in rats fed a high fat diet

An Honors thesis presented to the
Department of Biological Sciences
University at Albany, State University of New York
In partial fulfillment of the requirements
for graduation with Honors in Biological Sciences
and
graduation from The Honors College

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May 2018

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Abstract

Estradiol acts throughout the body; one key target that expresses large numbers of estradiol receptors is the brain, and specifically the hippocampus. Estradiol can exhibit neuroprotective effects in the brain. However, the set of pathways through which this occurs is not well understood. *In vitro* work has shown that administration of estradiol to hippocampal neurons inactivates caspase 3, a protease involved in apoptosis and the cleavage of tau. Cleavage of tau results in the formation of neurofibrillary tangles. Together with formation of amyloid-beta plaques, this is one of the hallmarks of Alzheimer's disease. We tested the hypothesis that administration of estradiol to rats might attenuate the cognitive impairment caused by the ingestion of a high fat, diabetogenic diet whose long-term consumption causes insulin resistance and Alzheimer's disease pathologies. Administration of estradiol unexpectedly did not ameliorate the impairment seen on a spatial working memory task or a novel object recognition task. In addition to behavioral measures, post-mortem analyses of hippocampal tissue measured molecular markers associated with estradiol signaling and memory formation. Ultimately, this study aims to elucidate mechanisms involved in a possible neuroprotective role for estradiol in individuals prone to cognitive dysfunction brought about by metabolic disease.

Acknowledgements

I would like to extend a huge thank you to Dr. Ewan McNay for his guidance, support, and good humor that made my time in the lab the best three years of my life. His encouragement and sincere life advice have not only allowed me to become a better scientist, but a better person overall. Second, I would like to thank Dr. Pauline Carrico, for agreeing to act as second reader on this work, and also for offering unlimited guidance and friendship during my time at the University at Albany. I would like to extend a great thank you to Greg Fitzgerald, for without his support and advice I would not have been able to complete this project. Thank you, Greg, for the countless hours you have spent helping me conduct and analyze this experiment so that I may succeed. Finally, a huge thank you to all members of the McNay lab, and to my family and friends for the unwavering support you have always given me.

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Introduction

Estradiol is both neurotrophic and neuroprotective, working to preserve cognition during aging. It is important in maintaining normal brain function, such as consolidation of memory through membrane interactions and activation of inter-cellular signaling pathways. Estrogen binds to two receptors, alpha ($ER\alpha$) and beta ($ER\beta$), which are located throughout the brain, specifically in the hippocampus and cortex. As these two regions are crucial for acute learning and memory consolidation, and are most implicated in the development of Alzheimer's disease, deficits involving estrogen and its receptors would show an overall decline in cognitive function (Hwang et al., 2015). It has been shown that treatment with 17β -estradiol improves both spatial and nonspatial memory in young female mice, but the molecular mechanisms underlying the effects of estrogen on memory are still unclear (Frick, Pechenino, 2009). Estrogen rapidly affects cortical memory and synaptic plasticity by binding to the $ER\beta$ receptor; it is thought to influence both information processing and storage in both the cortex and hippocampus by increasing key synaptic proteins such as GluR1, synaptophysin, and PSD-95 (Logan et al., 2011; Liu et al., 2008).

In addition to increasing synaptic plasticity, estrogen has also been shown to play a role in reducing the level of amyloid-beta protein in the brain (Hwang et al., 2015). $A\beta$ toxicity is one of the indications of Alzheimer's disease. An *in vitro* hippocampal neuron cell culture study conducted by Park et al. (2007) examined the effects of adding preaggregated $A\beta$ to P2 rat hippocampal neurons in culture. The researchers found that neurons with preaggregated $A\beta$ exhibited signs of neurodegeneration, which was measured by the amount of neurite retraction seen in each individual cell. The researchers showed that in cells that were treated with estrogen twenty-four hours prior to the addition of $A\beta$ preaggregates, only $42 \pm 6\%$ of neurons showed

signs of neurodegeneration. Park et al. also tested to see whether “A β -induced programmed cell death *in vitro* was accompanied by the activation of caspase 3” (2007). Caspase 3 is one of the main proteases involved in normal brain development, and is required for typical hallmarks of apoptosis and changes in cell morphology (Porter and Janicke, 1999). The Park study utilized hippocampal cell culture neurons with preaggregated A β and counterstained these cells with an active caspase 3 antibody. A TUNEL assay was done which detected DNA fragmentation in neurons that had received apoptotic signals. The analysis showed that “pretreatment of cultured hippocampal neurons with estrogen differentially prevented caspase 3 mediated apoptosis in the presence of preaggregated A β ” (Park et al., 2007). This was determined by the researchers comparing the amount of TUNEL(+) and active caspase 3(-) neurons (neurons that had both active caspase 3 and showed DNA fragmentation) to neurons that received no treatment and neurons that had active caspase 3 but did not display significant DNA fragmentation. From these studies Park et al. concluded that estradiol was capable of reducing A β -induced apoptosis by decreasing the number of TUNEL (+) and active caspase 3 (-) hippocampal neurons.

The addition of A β to hippocampal neurons in culture might trigger a series of cellular events that lead to posttranslational changes in the microtubule associated protein tau (Park et al., 2007). The proteolytic cleavage of tau causes neurofibrillary tangles to accumulate in the brain, and is executed by caspase 3 (Park et al., 2007). Tau acts as a substrate for caspase 3; as tau is cleaved, it is itself turned into an effector for apoptosis, generating a self-propagating positive feedback loop that occurs in an Alzheimer’s disease brain (Fasulo et al., 2005). Gamblin et al. (2003) showed that active caspase 3 cleaved tau protein at residue 421 and generated a 50-kDa truncated tau which assembled more rapidly into neurofibrillary tangles than the full-length tau filaments. This data suggests that a decrease in the factors that prevent this cleavage of tau could

have some role in the detriments associated with Alzheimer's disease. Decreasing levels of estrogen, which commonly occurs with age, could be such a factor.

A growing body of evidence indicates that estrogen administration to neuronal cells can affect apoptotic processes by partially preventing the activation of caspase 3 and the generation of cleaved tau proteins *in vitro* (Park et al., 2007; Kajta et al., 2002). This process is likely to occur through the activation of caspase inhibitory factor (CIF). A study conducted by Zhang et al., (2001) indicated that activation of caspase inhibitory factor occurs within ten minutes of neuron exposure to 17β -estradiol, but not 17α -estradiol *in vitro* which likely occurs through a receptor-mediated nongenomic pathway (Zhang et al., 2001). The study showed that administration of physiological levels of 17β -estradiol to neurons in culture was able to induce a neuronal CIF that directly inhibited caspase 6 and well as caspases 3, 7, and 8, all of which are involved in apoptotic processes within the cell (Zhang et al., 2001). These effects were not seen in astrocyte cell culture. One possible explanation for this could be that astrocytes do not contain a high level of estrogen receptors compared to other neurons, which likely affects the ability of estrogen to bind and induce CIF activity in these cells (Zhang et al., 2001). This data gives probable evidence as to the importance of estrogen involvement in caspase deactivation, and further provides reasoning for the role of hormones in therapeutic treatments for cognition deficits.

Due to the vast amount of work showing that estrogen treatment of neurons *in vitro* is capable of reducing caspase 3 activation and preventing neuronal cell death, this study explored whether or not the same effects will be seen *in vivo* when estradiol is administered to an Alzheimer's disease rodent model. Administration of estradiol exhibits neuroprotective effects in the hippocampus, which allows for insight as to the specific metabolic pathways through

which this induction occurs. This study explored the hypothesis that inactivation of caspase 3 via estradiol administration allows the rats suffering from metabolic disease to regain normal cognitive function when put through two different behavioral tests.

Materials and Methods

Animals

Four groups of male, Sprague-Dawley rats (Charles River, Wilmington, MA) were purchased and received at 4 weeks of age and doubly housed in enriched conditions on a 12-hour light/dark schedule (lights on at 7:00am). Room and cage temperatures were maintained at 25 degrees Celsius. The forty rats were randomly assigned to create four groups of animals in a 2x2 diet x treatment factorial design. Rats received either standard chow diet (control) and regular water or a high fat, high fructose diet consisting of 20% fructose, 60% fat (Research Diets #D16030909) and a 25% fructose water solution. Access was given to food and water *ad libitum*. Each rat was handled daily for ten minutes per day prior to receiving microinjection cannula surgery at eleven weeks of age. After a week of recovery post-surgery, in which the rats were again handled for ten minutes per day, the forty rats were subjected to glucose tolerance testing, as well as spontaneous alternation and novel object behavioral testing before being euthanized. All procedures were approved by the University at Albany, SUNY animal care facility.

Surgical Procedures

Rats were anesthetized with 5% isoflurane and 3% oxygen administered intranasal before surgery. Throughout the duration of the surgical procedure the isoflurane was lowered to 3% and oxygen to 2%. All rats received a microinjection guide cannula into the left hippocampus with coordinates relative to bregma X= +4.6 mm lateral, Y= -5.6 mm posterior to bregma, Z= -3.3 mm ventral from the dura. The nose bar was set at 5.0mm above interaural line (McNay et al., 2010). Rats received injections of sterile saline (1mL before the procedure and 3 mL post-surgery) in order to maintain hydration. A 1:1 solution of epinephrine/Marcaine was delivered dropwise after the incision in order to stop any slight bleeding that occurred from the incision.

Immediately following removal from isoflurane, rats were placed in a warm incubator until they were recovered from anesthesia and deemed sternal. All rats received a 5mg/kg dose of rimadyl prior to the incision and a 2 mg rimadyl tablet for three days post-surgery. Rats were given one week post-surgery for recovery prior to behavioral testing during which they were handled daily for ten minutes per day.

Glucose Tolerance Testing

One week post-surgery, all rats were subjected to glucose tolerance testing to check fasting blood glucose levels in order to confirm diabetic status. Rats were fasted for six hours prior to the test, in which they were only given access to regular water. Blood was obtained by tail prick and measured using a blood glucose meter (OneTouch Ultra Mini Monitor). After fasting, animals were given an intra-peritoneal (IP) injection of a 2kg/g dose of 45% glucose solution dissolved in 0.9% saline. Blood glucose was measured at 0 minutes (baseline, prior to injection), 5 minutes, 15 minutes, 30 minutes, 60 minutes, and 120 minutes after injection.

Microinjection Procedures

Microinjections were given to maze-tested animals into the left dorsal hippocampus as previously described (McNay et al., 2010). The vehicle for the four groups was artificial extracellular fluid (aECF; McNay & Sherwin, 2004), with experimental groups receiving a solution of 1.0 µg estradiol (Sigma Aldrich #E4389) in 0.5 µl aECF as described (Packard et al., 1996). The volume of solution administered to the brain was 0.125 µl per minute for a total volume of 0.5 µl over the course of four minutes. Injection cannula remained in place for an additional six minutes after injections in order to ensure proper diffusion of the solution throughout the brain (McNay et al., 2010). Rats entered the maze ten minutes after the start of injection.

Spontaneous Alteration Testing

Spontaneous alternation is a task that is used to examine spatial working memory and acute learning (McNay et al., 2010). Rats were placed into the center of a four-arm maze, facing arm “B” each time, and allowed to explore the arms of the maze freely for twenty minutes. The rat was only counted as visiting a new arm when all four legs of the animal had crossed from the middle of the maze into the arm being explored. An alternation was counted when a rat visited all four arms of the maze within any span of five consecutive arm choices; the measurement of memory testing performance and percent alternation was conducted as described by (McNay et al., 2010). The maze was cleaned with 70% ethanol at the start of the testing day and in between each rat being tested. Each rat was returned to their home cage for thirty minutes after spontaneous alternation testing before being subjected to novel object testing.

Novel Object Recognition Testing

For novel object testing, each rat was placed into a clear plastic box marked with a grid of sixteen black squares and allowed to freely explore two objects placed in the center of the box for five minutes. The objects had the same placement and orientation in the box for each animal. This was used as a baseline measurement to ensure the rat did not prefer one side of the box over another. After five minutes, rats were removed from the box and subjected to microinjection of either aECF or estradiol solution as described previously. Rats were returned to their home cages and 24 hours later tested again with a novel object replacing one of the familiar objects. The replacement of the familiar object with a novel object was randomized for each animal. Each day of training and testing was recorded using a video camera, and the amount of time each rat spent

exploring both objects analyzed using Solomon Coder. The box and every object was cleaned with 70% ethanol at the beginning of the training day, testing day, and between each animal.

Euthanasia and Tissue Collection

After behavioral testing, rats were anesthetized by intranasal dose of isoflurane and subsequently decapitated. Brains were removed and correct probe placement confirmed. Only data from animals with proper probe placement was included in the analysis. The tissues collected included the left and right hippocampus, frontal cortex, trunk blood, plasma, and both adrenal glands. The trunk blood collected was spun down at 5000 rpm for 5 minutes, and the serum fraction collected for analysis.

Tissue Preparation

Collected tissues were immediately placed on dry ice and stored at -80 degrees Celsius for molecular analysis. The tissue preparation for molecular analysis was prepared as follows: each side of the dorsal hippocampus was dissected out and immediately processed for western blotting. Both hippocampal sections were homogenized with 196 μ l of homogenization buffer 13 (BioVision) containing protease inhibitor (1:500) (BioVision) from which 30 μ l of homogenate was removed and combined with 200 μ l of RIPA buffer containing a protease and phosphatase inhibitor cocktail (1:200) (ThermoScientific). Frontal cortex sections were homogenized with 210 μ l. The initial homogenate was processed according to manufacturer's instructions (BioVision) to isolate plasma membrane fractions from the total sample.

Molecular Testing

Western blotting will be used to analyze protein samples from collected tissues. The following proteins will be analyzed: total tau, phosphorylated tau, cleaved tau, GSK3 β , and caspase 3.

Statistical Methods

All data was analyzed using SPSS software. T-tests were performed, as well as two-way ANOVA tests to determine significance.

Results

High fat diet, but not estradiol, affects total body weight

Body weight and fat pad weight were measured at euthanasia to confirm effectiveness of the high fat diet. Figure 1 indicates that rats fed a high fat diet weighed significantly more (Fig. 1A) and had a greater visceral fat composition compared to body weight (Fig. 1B.) compared to control animals.

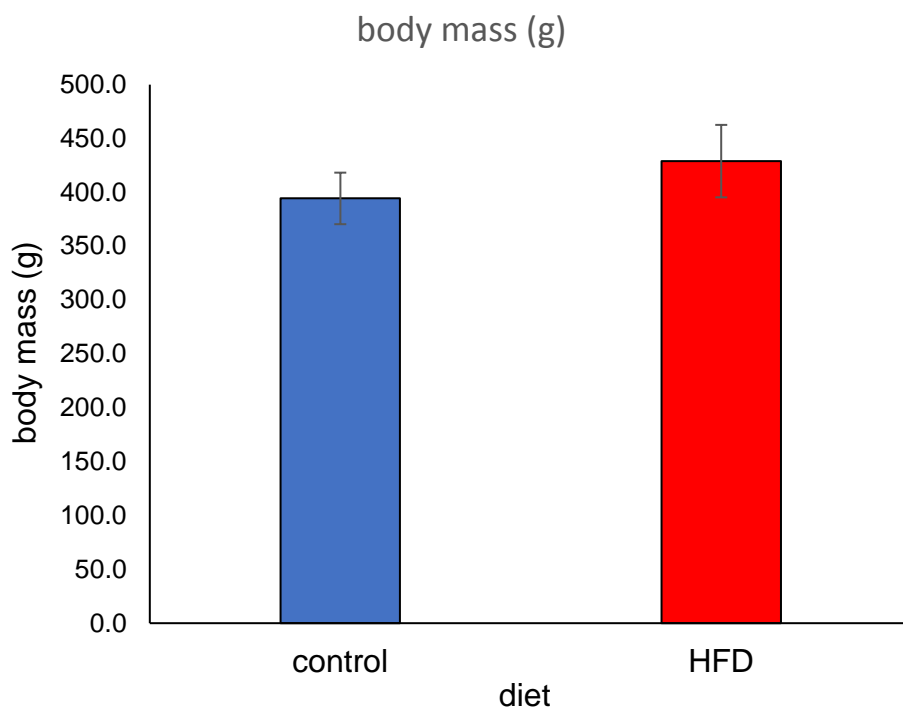


Figure 1A. Body weight at euthanasia. Rats fed a HFD weighed significantly more at euthanasia compared to control rats ($p < 0.05$).

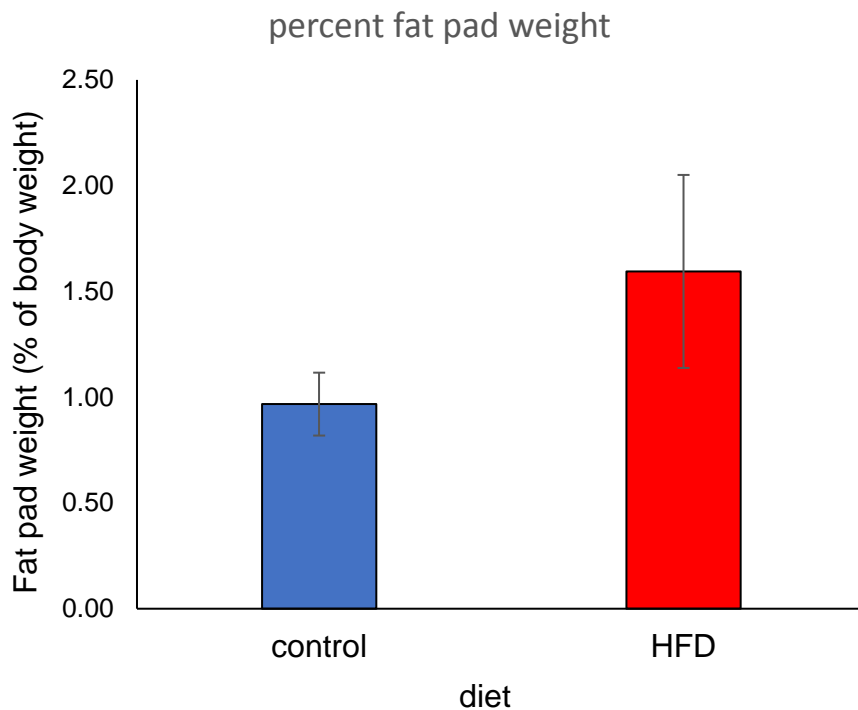


Figure 1B. Epididymal fat pad weight as a percentage of body weight is an indicator of visceral fat levels. Rats on the HFD had a significantly greater percentage of fat pad weight compared to control rats ($p < 0.05$).

High fat diet affects blood glucose levels

Administration of a high fat diet had significant effects on fasting blood glucose levels in all animals. Animals fed a high fat diet had significantly higher resting blood glucose levels, had blood glucose levels that spiked higher during the test than control animals, and took significantly longer to return to baseline blood glucose levels than control animals (Fig. 2A).

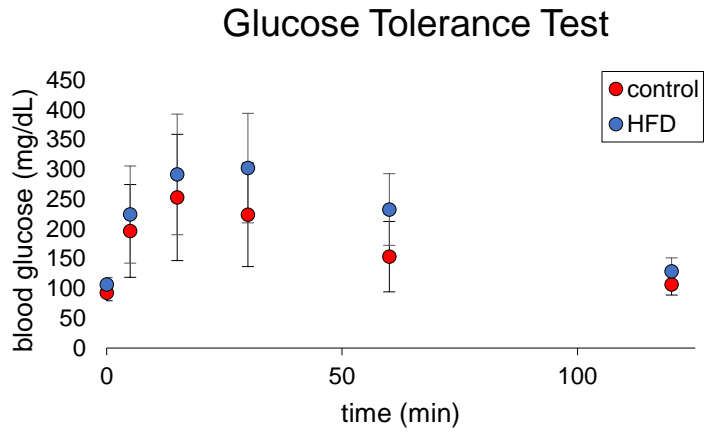


Figure 2A. Results of glucose tolerance test. $p < 0.05$.

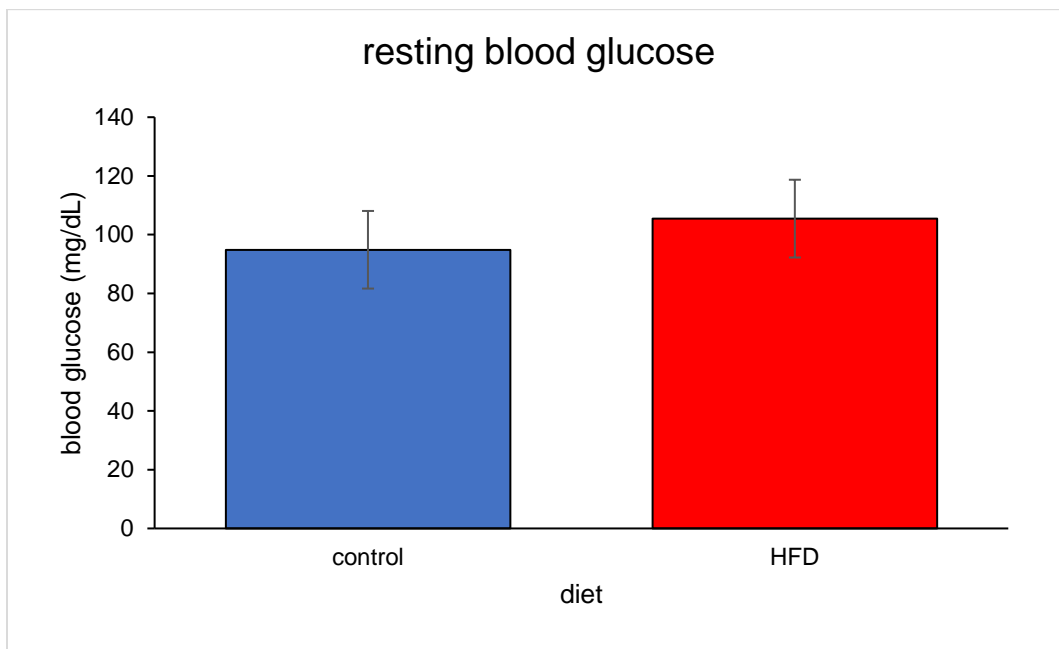


Figure 2B. Bar graph results depicting resting blood glucose levels. HFD animals had significantly higher resting blood glucose levels compared to control animals. $p < 0.05$.

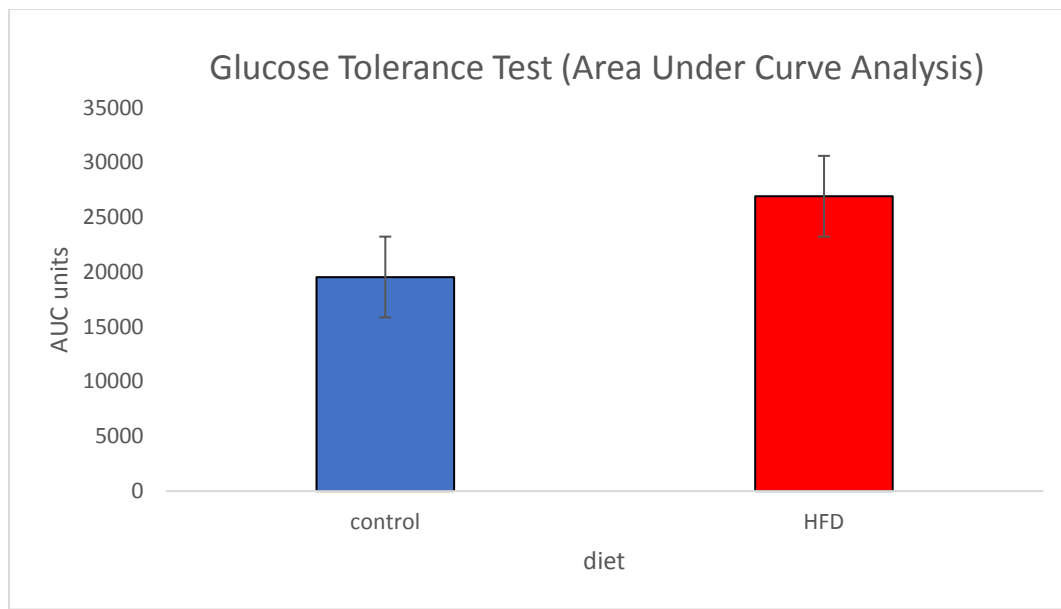


Figure 2C. Area under the curve analysis of a glucose tolerance test for control and HFD animals. HFD animals had significantly higher resting blood glucose, had higher levels of blood glucose throughout the test, and took longer to return to baseline levels compared to controls. $p < 0.05$.

Administration of estradiol into the left dorsal hippocampus did not significantly improve spatial working memory

Through trials of the spontaneous alteration task, the administration of estradiol did not improve memory functioning or cognition in the rats (Figures 3A & 3B). The groups that were administered estradiol did not perform any better than the controls did during the spontaneous alteration task. In addition, the effects of the high fat diet paired with the administration of estradiol also did not seem to have any effect on memory functioning or cognition.

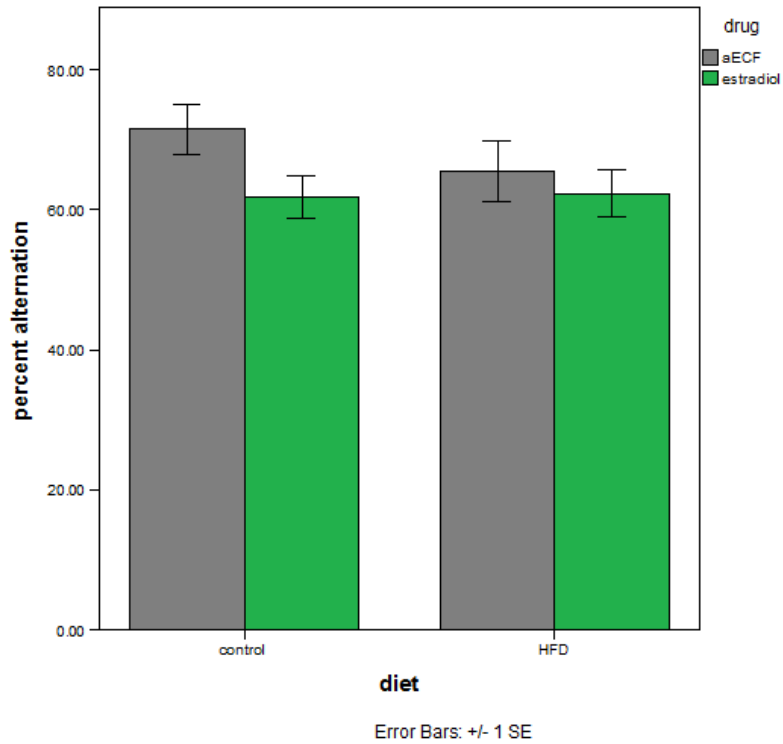


Figure 3A. Results of spontaneous alteration task. Neither diet nor estradiol administration had any significant effect on percent alteration performance. $p > 0.05$.

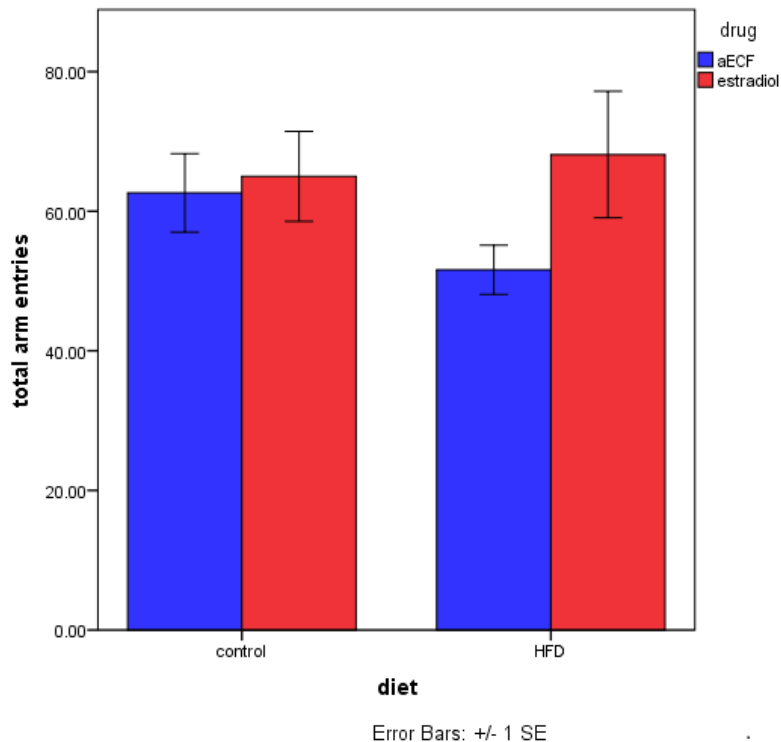


Figure 3B. Results of a spontaneous alteration task. Neither diet nor drug administration had any significant effect on total arm entries in the maze. $p > 0.05$.

Estradiol inhibits cognition seen in rats fed a control diet, and does not improve novel object recognition

The administration of estradiol to healthy rats had significant effects on recognition of the novel object. Control rats receiving estradiol did not show a significant preference for the novel object. This result was surprising, and seems to have some indication that estradiol may have inhibitory effects in the male rodent brain at the dose given. There were no significant results obtained from the high fat diet animals given vehicle or estradiol. These animals preferred the novel object over the familiar object in both treatment groups, which was expected behavior (Figure 4).

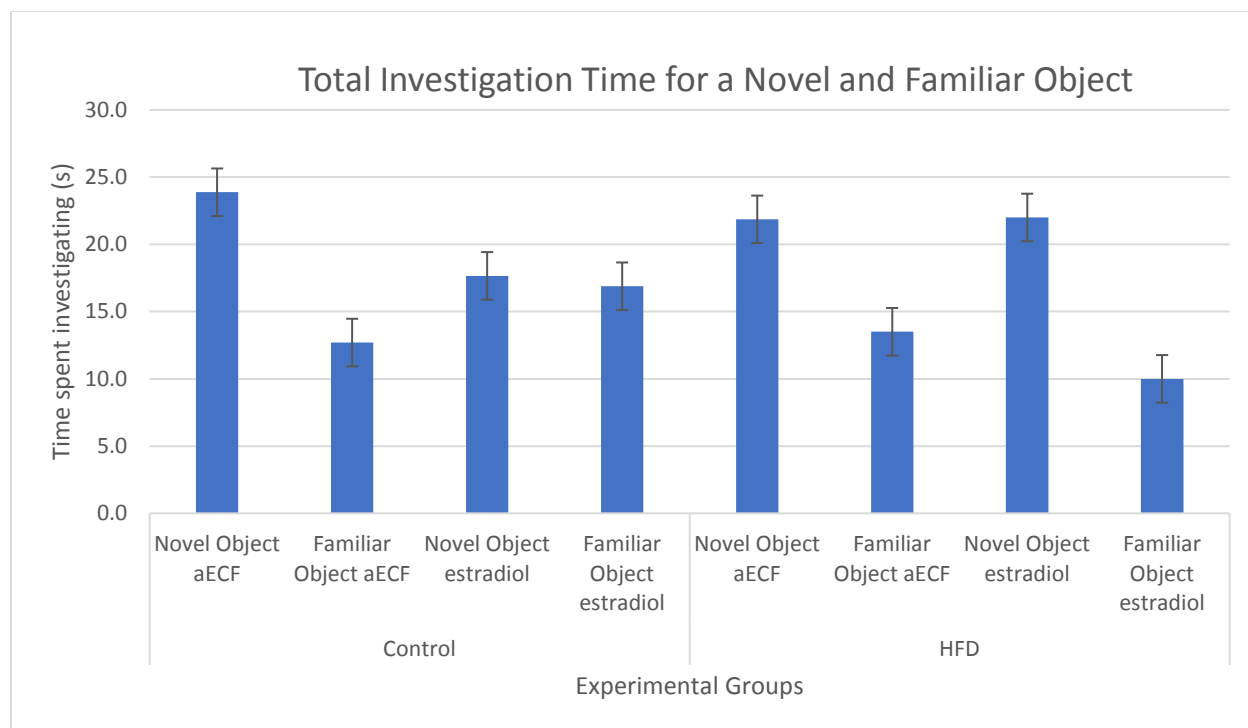


Figure 4. Results for a novel object recognition task. There were no significant differences in performance in HFD animals based on treatment. There was significant difference in control animals based on treatment, with control animals receiving estradiol performing worse than control animals receiving vehicle, indicating that estradiol had some inhibitory effects on memory. $p < 0.05$ for control animals given estradiol.

Discussion

The administration of estradiol along with a high fat diet declined to show effects on memory functioning and cognition that have been seen in previous literature. Previous studies have shown that estradiol administration increases cognitive performance during spatial working memory tasks, and that consumption of a high fat diet decreases memory functioning. With this, we expected to see a decrease in memory functioning in the animals fed the high fat diet, and expected that animals fed the high fat diet and given estradiol would perform as well as the controls during the tasks. The data obtained were not significant and gave inconclusive results.

Unexpectedly, animals that were fed the high fat diet did not perform any worse on the spontaneous alteration task than animals fed standard chow. In addition, the administration of estradiol to those animals that were on the high fat diet did not ameliorate the memory deficits that are typically seen in a diet induced obese animal. In the spontaneous alteration task, the results indicate that the control animals reached between 60-80% alteration. These results are inconclusive and do not give any insight as to the effects of estradiol administration on spatial working memory performance. Estradiol administration might work in an inverted-U dose response curve, meaning that at both low and high doses, the effects of estradiol on memory functioning are insignificant. It is possible that the dose given to the animals before the task was not large enough in order to induce the cognitive enhancements we expected to see. In addition, it is also possible that estradiol might not work in an acute manner, and perhaps only displays cognitive enhancements in the brain over a longer period of time.

Estradiol did seem to have some effect on spatial memory in control rats during the novel object recognition task. Animals fed a control diet and given an intrahippocampal injection of estradiol following the training period of the task performed significantly worse than animals

given vehicle or on the control diet ($p=0.04$). This result was unexpected, as previous research has shown that estradiol has cognitive enhancing effects and should enable animals with cognitive deficits to perform better, or at least on par with, control animals. Animals fed a standard diet and receiving a vehicle injection significantly explored the novel object more; this was expected behavior, as rats are naturally curious animals and will likely explore a novel object more than one that is familiar. Animals fed a high fat diet and given a vehicle injection did not significantly explore the novel object more than the familiar object, indicating that these animals were experiencing cognitive deficits due to consumption of the high fat diet and could therefore not form memories of which objects had been explored. It was also surprising to see that animals fed a standard diet and given an injection of estradiol did not significantly explore the novel object more than the familiar object. We expected animals in both the control/estradiol and HFD/estradiol groups to explore the novel object significantly more than all other groups, as these rats would have experienced the neuroprotective effects of estradiol if estradiol works as a cognitive enhancer. The results of the control/estradiol group seem to indicate that estradiol may be acting as a cognitive inhibitor at the dose given, and would not improve memory in a diet induced diabetic rat. These abnormal results could be explained due to the dose of estradiol given for both behavioral tasks. It is possible that the dose of estradiol given to the rats was not high enough to warrant a substantial effect on cognition, particularly in male rats. In order to gain more significant results and fully understand the role of estradiol in memory formation, differing doses of estradiol will have to be given to determine the best dose for cognitive enhancement.

A lack of behavioral effects does not mean that estradiol has no molecular effects in the brain. In order to determine whether or not estradiol affects protein expression in the male rat brain during memory formation, western blot analysis will be conducted on various brain

samples collected. Caspase 3, GSK3 β , and different variations of tau protein will be analyzed for their expression levels in order to elucidate the specific pathway through which estradiol acts in the brain. It is expected that animals fed a high fat diet will exhibit higher levels of neurofibrillary tau tangles, and that those given estradiol treatments will have lower levels of cleaved tau and inactivated caspase 3 proteins. Determining the levels of protein expression in the hippocampus of these animals will give further insight as to how exactly estradiol works in the hippocampus to enhance spatial memory and cognition.

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