Genistein as a preventative treatment for Alzheimer's disease in the female rat



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Genistein as a preventative treatment for Alzheimer's disease in the female rat

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Abstract

Alzheimer's Disease (AD) is a progressive, neurodegenerative disorder marked by the presentation of abnormal accumulations of amyloid-β plaques and tau proteins, which can result in memory loss and impaired awareness. Women are twice as likely to develop AD later in life. After menopause, there is a sharp decline in estradiol, which affects the production of proteins in the brain. This study considered the side effects of estradiol treatment, such as increased risk of breast cancer, and explored the preventative effects of the phytoestrogen genistein. Two rat models of AD were used, an insulin-deficient model from streptozotocin (STZ) administration and a model injected with misfolded amyloid-β fragments (25-35), with a treatment and non-treatment group for both models. All rats were ovariectomized and fed a soy-free diet following the procedure while the treatment rats received genistein orally for two months. The rats were then given intraventricular injections of STZ or amyloid-β plaques and allowed to heal before behavioral testing. The rats, including a control group, were tested for spatial memory in a labyrinth water maze and spontaneous alternation behavior in a y-maze. After completion of testing, all rats were perfused, and their brains collected for a silver staining protocol. The STZ group showed a greater response to treatment, which was seen in water maze performance and the different densities of neuronal death between treatment and non-treatment rats.

Introduction

Currently, 5.7 million people in the U.S. population are diagnosed with Alzheimer's disease according to the Alzheimer's Association. Yet these only account for 1 in 4 cases of Alzheimer's, due to its confusion with other types of dementia. A main behavioral characteristic of this disease is memory loss, which also occurs with other neurodegenerative dementias. A true diagnosis of Alzheimer's can only come from an analysis of the brain after death. Alzheimer's typically comes with old age and disproportionately affects more women than men, where women are 1.5 times more likely to develop the disease than men (Salkovic-Petrisic et al., 2012). This has been primarily attributed to the loss of estrogen production after menopause, which leads to a sharp decline in estrogens, such as estradiol, present in the body.

Estrogen has been found to have neuroprotective qualities (Chakrabarti et al., 2016; Tian et al., 2013). This is attributed to the estrogen receptor β that is found throughout the body and within the brain. This receptor is highly expressed in the hippocampal formation and has several functions that protect the brain from damage (Lephart et al., 2002). The hippocampus is one of the first regions of the brain to suffer damage from Alzheimer's disease and has a major role in working memory (Jarrard, 1993). Working memory is known a type short-term memory that pertains to immediate tasks. Estrogen is an allosteric effector for the beta-receptor, which is found within the cytoplasm and has an N-terminal binding domain for DNA. Once bound the receptor goes through a conformational change and the DNA is carried into the nucleus for transcription. Through this estrogen can regulate gene transcription, specifically the gene APOE which is indicated in cases of Alzheimer's disease. The β receptor also maintains levels of the amyloid precursor protein (APP) and amyloid-β peptides. When these levels get too high the receptor signals to enzymes responsible for degradation (Li et al., 2014). When the β receptor is

not functioning correctly or is not being activated, it will not keep down the levels of amyloid-β peptides, leading to plaque buildup. For treatment options, some have looked at hormone replacement therapy (HRT) with estrogen to stimulate this receptor. Carroll et al. (2007) did just this, using estrogen as a treatment method for a 3xTG-AD model in mice. In their study, they saw significant increases of Aβ plaques in estrogen-depleted subjects and that treatment with estradiol did prevent these effects. Estrogen was also used as a treatment for a streptozotocin model of Alzheimer's disease by Lannert et al. (1998), who saw increased memory functions in the treatment groups. This was assessed by a closed field activity test, for examining spontaneous locomotor activity, and a single-trial passive avoidance test using a shock floor. While several studies have found significant improvements with estrogen treatment, many others have not.

As Alzheimer's is not easily predictable, treatments methods are usually implemented after the onset of the disease. Nothing has been found that cures Alzheimer's, so all that can be done is to treat individual symptoms. More preventable measures need to be implemented for those at risk, or with a documented family history of dementia. Women commonly undergo HRT after menopause to treat the symptoms that come with it, such as bone density loss and hot flashes, which could be potentially preventing Alzheimer's. According to the Agency for Healthcare Research and Quality in 2008 about 3.5% percent of women over the age of 30 use HRT. While this may prolong cognitive functioning for older women, HRT comes with several risk factors. Prolonged use of HRT in women can lead to cardiovascular disease and increased risks for several types of cancers (Salkovic-Petrisic et al., 2012; Choi et al., 2013; Zhao et al., 2015). This should be a push for people to begin looking at agonists for the β receptor that do not come with adverse health risks.

While most of the literature does focus on estrogen itself as a treatment, some investigations have begun looking at phytoestrogens. Phytoestrogens are found in common plant foods, like soy, and have been found to selectively bind to the estrogen receptor β as agonists (Choi et al., 2013; Sirotkin & Harrath, 2014). Genistein and daidzein are two main compounds found in soy that selectively bind to the β receptor. In a study by Bagheri et al. (2010), they found that treatment with genistein significantly improved the short-term spatial memories of in rats injected with amyloid-β (1-40) fragments. They also introduced estrogen receptor β antagonists, which blocked the effects of genistein completely, indicating that is it selective to this receptor (Fig. 1). Dong et al. (2013) examined the therapeutic effects of α-zearalanol, another phytoestrogen, in ovariectomized mice and found that α-zearalanol effectively prevented the effects of estrogen deficiency in mice. α-zearalanol is also a phytoestrogen found in soy that has a high binding affinity for the β receptor. Countries whose diets contain high amounts of soy have been shown to have improved cognitive function later in life and overall increased lifespans, such as countries in eastern Asia. Soy beans and legumes have been found to have cholesterol-lowering effects (Anderson et al., 1999). These populations have a lower rate of deaths linked to cancer and oxidation-linked diseases because of the isoflavonoids, also known as phytoestrogens, found in soy which can prevent the transformation of healthy cells into premalignant ones. (McCue & Shetty, 2004). Employing a phytoestrogen, such as genistein as a

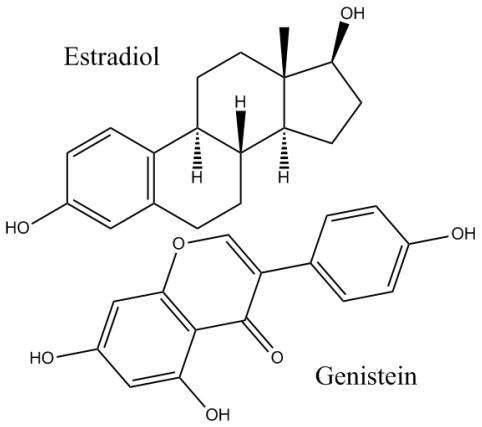

Figure 1. Molecular structure of estradiol and genistein. Sourced from Fuentes et al. (2013).

preventative treatment for Alzheimer's disease may be a more successful method than HRT with estrogen (Henry-Vitrac et al., 2010).

A model of Alzheimer's disease that has been well documented in the last two decades is a streptozotocin-induced model. Streptozotocin (STZ) is a compound that has been used for diabetes research, as it can induce type 2 diabetes in mammal models for research. People with diabetes have also been found to have a greater risk of developing Alzheimer's disease (Wang et al., 2014). Studies, such as that by Lannert & Hoyer (1998) and Chen et al. (2018), found that the insulin and metabolism disruptions caused by this compound impacted the cognitive functioning of rats. They saw a decrease in memory and learning abilities in rats that had this compound intracerebroventricularly (icv) injected. Wang et al. (2014) found that injecting STZ into the femoral vein of rats also causes neurodegeneration in the hippocampus and frontal cortex. STZ creates a model of sporadic Alzheimer's disease that can be induced with this compound and studied long-term (Salkovic-Petrisic et al., 2011). Immediately after injection, STZ causes an acute diminishment in memory functioning, which then plateaus for about 4-5 months, before an irreversible decline of cognitive functioning and a build of up plaques and tangles in the brain. Despite the presence of plaques in this model, STZ is noted in that it first causes an insulin deficiency in the brain, leading to protein misfolds and tau hyperphosphorylation (Grünblatt et al., 2007; Salkovic-Petrisic et al., 2012). For this reason, several researchers have investigated the theory of Alzheimer's disease being type-3 diabetes (Lester-Coll et al., 2006; Suzanne de la Monte, 2014). This model has also responded to estrogen treatment as shown by Lannert et al. (1998) who saw an overall increase in memory function of the treatment group during a modified hole board test, which tests the rats abilities to find food hidden in holes.

A way to showcase and treat the amyloid-β plaques that are symptoms of Alzheimer's is to create a model with injections of amyloid-β fragments. This is an effective way to develop an Alzheimer's disease model when access to a genetically predisposed lab animal is not an option. Amyloid-β fragments are typically injected into the lateral ventricles or directly into the hippocampal formation. There are several types of fragments that can be used, such as amyloid-β (1-40) (Bagheri et al., 2010) and amyloid-β (1-42) (Zussy et al., 2011). Both create the characteristic plaques and associated neurotoxicity. There is another fragment, amyloid-β (25-35), which is much shorter, yet still has the same impact (Rush et al., 1992; Kaminsky et al., 2010). Zussy et al. (2011) used this fragment, alongside the 1-42 fragment, and found that it produced denser and more widely spread plaques throughout the brain. This model does respond to estrogen treatment and has been treated with injections of estrogen receptor β plasmids, which reduced the inflammatory aspects of the disease and reduced memory impairment (Tian et al., 2013). In the study by Bagheri et al. (2011) they showed genistein as an effective treatment against Alzheimer's when using rats injected with amyloid-β (1-40). The study used a radial arm maze and a passive avoidance test using a shock to test memory and learning.

Analysis of the cognitive effects of these models can be done through behavioral testing and histology. The Morris water maze is a widely used test for assessing spatial learning and memory (Vorhees & Williams, 2006). The hippocampus has a large role in spatial memory, being shown to process and remember spatial learning (Jarrard 1993). There is a modified water maze, which uses a labyrinth design, rather than a tub of open water. This type of maze can be rearranged, to further test the learning and memory skills of subjects. A Y-maze is used to look at the working memory and the anxiety of rats, which can be impacted by cognitive impairments (Carroll et al., 2007; Rosario et al., 2006). The Y-maze measures spontaneous alternation

behavior (SAB) which is the exploration and movement between the arms of the maze. Rats will typically explore freely and alternate to less visited arms, while cognitively impaired rats may repeatedly explore the same arms, not necessarily remembering visiting them before or because they are familiar.

Histology is an important tool in examining Alzheimer's, as this disease is characterized by its pathology in the brain. There are many techniques used to identify the characteristic plaques in the brain, many of which employ immunohistochemistry. Recently, more accessible staining techniques have produced results comparable to those of immunohistochemistry. FD Neurotechnologies Inc. developed a silver stain, the NeuroSilver Kit II. This is effective at staining for the degeneration of neurons in the brain, due to their increased affinity for silver. Rebeck et al. (2010) had success using this method to identify neuronal damage due to their transfer of lentiviral amyloid-β(1-42) into the brains of rats. The stain was also effective in staining for degenerating neurons in mice infected with measles (Lehrmann et al., 2008).

My study has two main objectives. The first is to show that the phytoestrogen, genistein, is a more targeted preventative treatment for Alzheimer's than estrogen. I predict there will be significantly less cognitive impairment in rats treated with genistein as compared to those that are not. The second objective is to compare genistein treatment in two different Alzheimer's models: a protein initiated and an insulin-deficient model. My prediction is that genistein will have a more marked effect on the protein model, as the β receptor plays a close role in preventing plaque buildup. There should also be an improvement in the treated STZ model, although estrogens would work on secondary effects of insulin deficiency, such as cell death and protein build up. Several studies have looked at these two models separately and how they respond to phytoestrogens and estradiol 17β. I hope to expand on those studies with my project

and successfully compare the two models by their individual response to the preventative treatment of genistein. Both objectives were assessed through behavioral and histology methods later described.

Methods

Subjects

There were twenty-five female Sprague-Dawley rats used for this study. The rats were provided by Allegheny College's animal colony located in Carnegie Hall. The rats were split into five treatment groups with five rats each. All the rats were from the same litter or a litter born within the same week as the others, so all of the rats were roughly the same age. The rats were housed five to a cage and housed separately after each surgery for two weeks to allow for recovery. They were given access to food and water ad libitum. To avoid the variable of dietary phytoestrogens being present in food, rats were fed the Basal Diet (#5755) from LabDiet which contained no dietary soy immediately after their ovariectomies. The food was mixed with the regular rat chow for several days to ensure that the rats consumed it.

There were two treatment groups that received genistein orally daily and two non-treatment groups which did not. The genistein was dissolved in sesame oil and mixed into peanut butter at a ratio of 162 µg genistein, 30 µL canola oil, and 5.7 grams peanut butter per kilogram. This ratio was adapted from the recipe in Ström et al. (2012). This dose of genistein was described in Neese et al. (2010) as comparable to phytoestrogen levels following supplementation in humans. The genistein treatment began after the rats are ovariectomized and continued for 50-55 days depending on the group. This length of time was chosen to ensure full estrogen production decline in the nontreatment groups, which received no supplement.

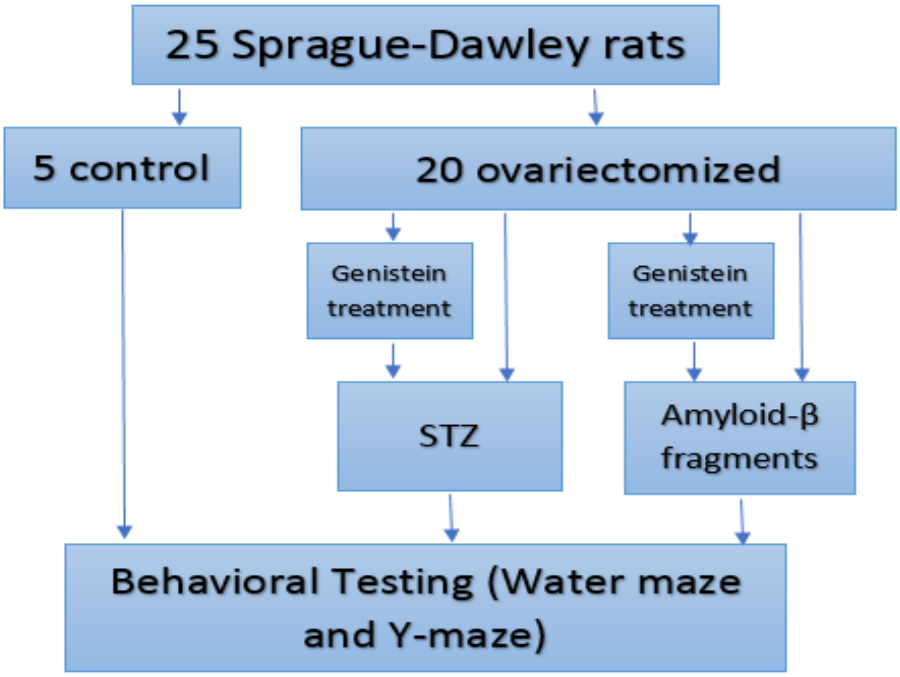

Figure 2. Flowchart of experimental protocol.

Surgical Procedures

To control the estrogen levels in the rats they all underwent ovariectomies, except for the control group (Carroll et al., 2007). These all followed the procedure listed in Ström (2012) and as well as the procedure used by Allegheny College. This involved a cauterizing method of the ovaries. The rats were anesthetized with a ketamine-xylazine mixture (0.1 mL/100 grams body weight) injected intraperitoneally. A supplemental dose (10% of the initial dose) was prepared in case of anesthetic wearing off during surgery. Consciousness was monitored by checking the paw-pinch response every 10 minutes. All rats were given two weeks of recovery after the surgery and three days of an antibiotic treatment of terramycin mixed with peanut butter to ensure no infections occurred.

The second surgery took place after 50 days of treatment with genistein (Wang et al., 2016). This was a stereotaxic surgery, which exposed the skull for an intraventricular injection of

streptozotocin (STZ) (3 mg/kg) for the insulin-deficient model or an injection of amyloid-β fragments (25-35) for the protein model. STZ was used to induce the AD model shown in Song et al. (2004) and Salkovic-Petrisic et al. (2011). The STZ was combined with 4 µL of a 0.05M citrate buffer, resulting in 2µL injected into each of the lateral ventricles. The coordinates used were +/- 2 mm lateral and 0.7 mm caudal to bregma, and 4 mm ventral to the surface of the brain, which is where the burr holes in the skill will be made. A Hamilton micro syringe was used to deliver the STZ. The protein model group received intracerebral injections of amyloid-β fragments (25-35) (0.5 nM/µl dissolved in 0.9% normal saline; pH = 8.0). Each rat received 4µL split between the two hemispheres. The coordinates used were the same as the STZ injections (Zussy et al., 2011). Injections were delivered in two doses, one to each hemisphere, for a total of 4µL of the solution. The rats were given two weeks of recovery before any behavioral testing commenced. For three days after the surgeries, they were given an antibiotic, terramycin, mixed into peanut butter to prevent infection.

After completion of the study the rats were euthanized and perfused for the collection of the brains. For this, the rats were given intraperitoneal injections of sodium pentobarbital (somnasol) (0.1 mL/kg). Once breathing ceased an incision was made below the rib cage, allowing for lateral cuts on either side of the rib cage. The rib cage was then clamped to the jaw to allow access to the heart. A flow of 4% paraformaldehyde was introduced into the left ventricle immediately after snipping the right atrium. This was allowed to flow until the liver paled and the flow from the right atrium became clear. The rat was then decapitated, and the brain carefully removed from the skull to be placed in a jar of 4% paraformaldehyde overnight.

Behavioral Testing

This experiment used two behavioral tests; a labyrinth water maze and a y-maze. The labyrinth water maze is a modified Morris water maze (Fig. 3). This allowed analysis of the spatial memory and learning of the rats (Vorhees & Williams, 2006). Both latency (time) and errors were recorded. The rats received training in the maze prior to any surgeries. This was done for two days, with three trials a day. For the protein model, the testing was done immediately after recovery from surgery. They were tested every day for one week with three trials a day. Each trial had a 120 second time limit, and if a rat did not reach the platform by then, they were guided to it and placed on it for 15 seconds. The path of the maze was changed every 3-4 days to assess the learning abilities of the rats. For the insulin-deficient model rats, the behavioral testing was done at two different time points: a few weeks after recovery from surgery and 4 months after that. This model takes longer to display Alzheimer's behavioral and physical pathologies. For both testing periods, they underwent a week of daily testing like the plaque model rats. The water was kept around 22-25 C to ensure rats were comfortable. They were dried off between trials and given at least 30 seconds of rest. After completion of three trials, the rats were put in a heating chamber before being returned to the colony.

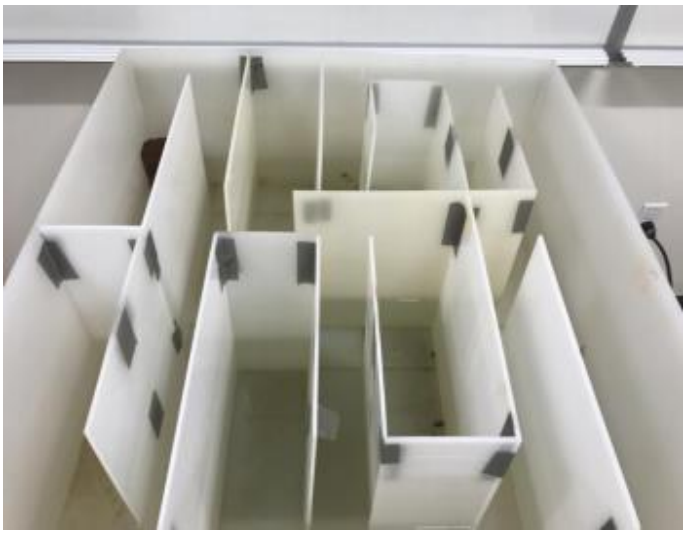

Figure 3. Modified Morris water maze arranged in a labyrinth. Located in the Carnegie basement.

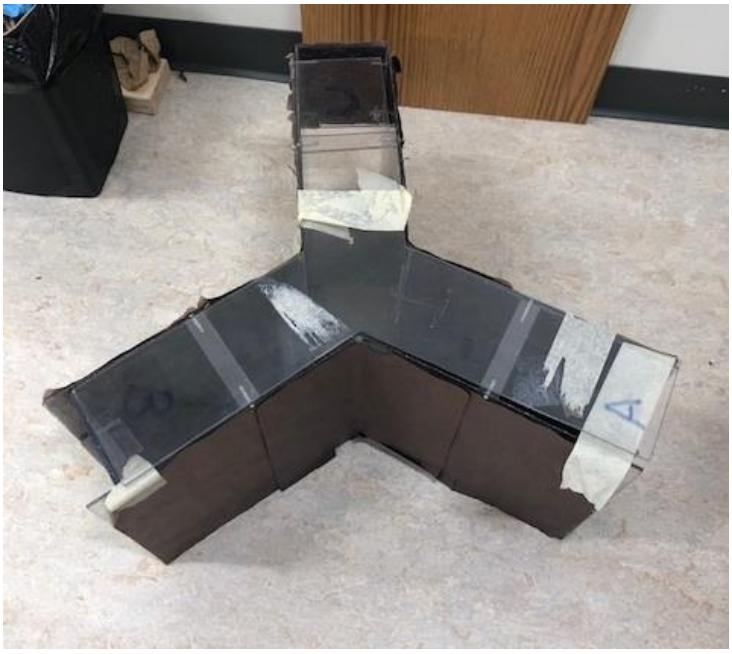

Figure 4. Y-maze used for assessing SAB. Has three arms labeled A, B, and C.

The second behavioral test used was a y-maze used to examine SAB of the rats as described by Carroll et al. (2007). The apparatus used is shown in figure 4. This measured the working memory and exploratory behavior of the rats. Each rat went through one trial after completion of all water maze testing. Each animal was placed into arm B and allowed to explore the maze for a total of five minutes. Alternations and the total number of arm choices were recorded. Spontaneous alternation, expressed as a percentage, refers to the proportion of arm choices differing from the previous two choices. Rats displaying exploratory behavior would have a higher percentage of alternation than rats who do not display that behavior. Rats that made 10 or fewer arm choices were excluded, which was not the case with this study.

Histology

The primary staining technique used was the NeuroSilver Kit II developed by FD NeuroTechnologies Inc. This kit is used for the detection of neurodegeneration, as they have found that degenerating neurons have a high binding affinity for silver. This kit can also detect amyloid-β plaques. To prepare the brains for staining, rats were perfused with 4% formaldehyde and the removed brains remained in 4% paraformaldehyde overnight at 4 C. For the following 72

hours the brains were immersed in a 0.1 M phosphate buffer containing 20% sucrose before freezing. The brains were sectioned at a 60 μm thickness and stored in 0.1 M PB containing 4% paraformaldehyde for a week. The kit contains seven solutions, A through G, which were used in a free-floating staining process, the protocol for which is provided by NeuroTechnologies Inc and shown in Appendix I. The brains were left in the final solution for 1-2 hours at 4 C before being mounted and left to dry overnight before being cover slipped. The slides were kept out of direct light once mounted on slides to reduce any effect on the staining. The sections were then examined using a Leica light microscope. The Leica application suite was used for capturing images of the hippocampal region and caudate putamen.

Data Analysis

For the labyrinth water maze, there was an independent variable of the drug treatment group and the two dependent variables: escape latency measured in seconds and the number of errors made during each trial. All data were expressed at the mean +/- SE in graphs. A two-way ANOVA with replication was used to examine the water maze data. SAB in the Y-maze was measured by the proportion of arm choices differing from the previous two choices, which was expressed as a percentage. The significance was measured with a paired t-test, assuming equal variance, for each group's performance before and after stereotaxic surgery. Tissue stained with the NeuroSilver Kit II was inspected for the density of staining of dying neurons and amyloid-β plaques. Alzheimer model rat's brains were compared with control rat's brains as a baseline.

Results

Labyrinth water maze

Data for the labyrinth water maze was recorded as latency and errors. Latency was the times in seconds it took to complete the maze, with a limit of 120 seconds, and errors were counted as wrong turns and backtracking. Significance was regarded as $p < 0.05$. The Aβ group went through a week of testing, with the data from the last three days used for statistical analysis. In Figure 5 the average trial times across those three days are shown for the two groups. The average time for the treatment group to complete the maze increased over the three trials while the time for the non-treatment group decreased over the trials. A two-way ANOVA with replication was used to see if there was a difference between the groups in regard to each trial. This gave no significance ($p=0.272$).

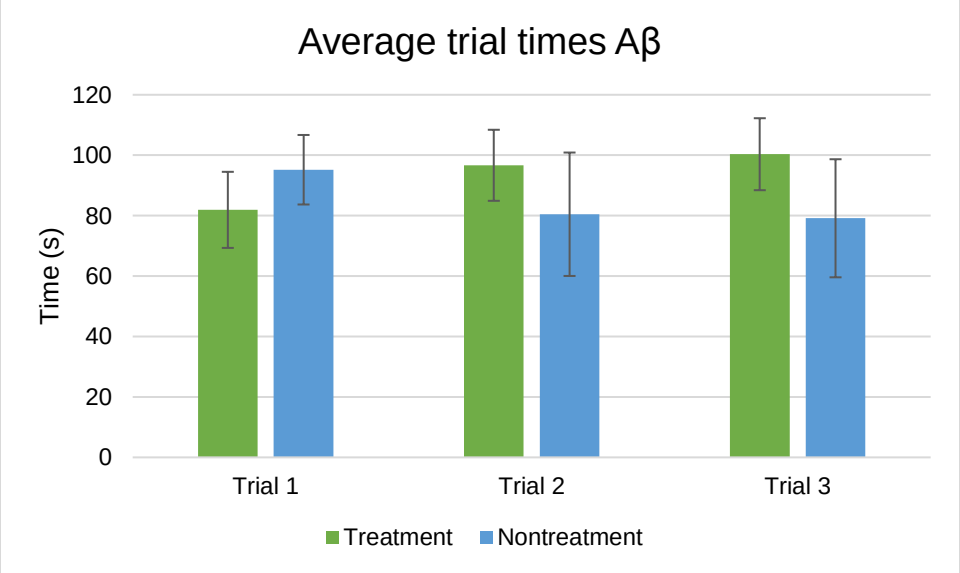

Figure 5. The average trial times for the treatment and nontreatment group of the amyloid-β plaque model rats. Standard error is represented by the error bars.

The STZ group had two testing periods, the first being three months after injection and four months after. Figure 6 shows the average time per trial for both groups at the four month mark. The times for the treatment group show a slight downward trend while the non-treatment

group shows a similar average for each trial. A two-way ANOVA with replication was run to find the interaction between groups with reference to trial number. The ANOVA showed significance between the times of the two groups for each trial (p=0.036).

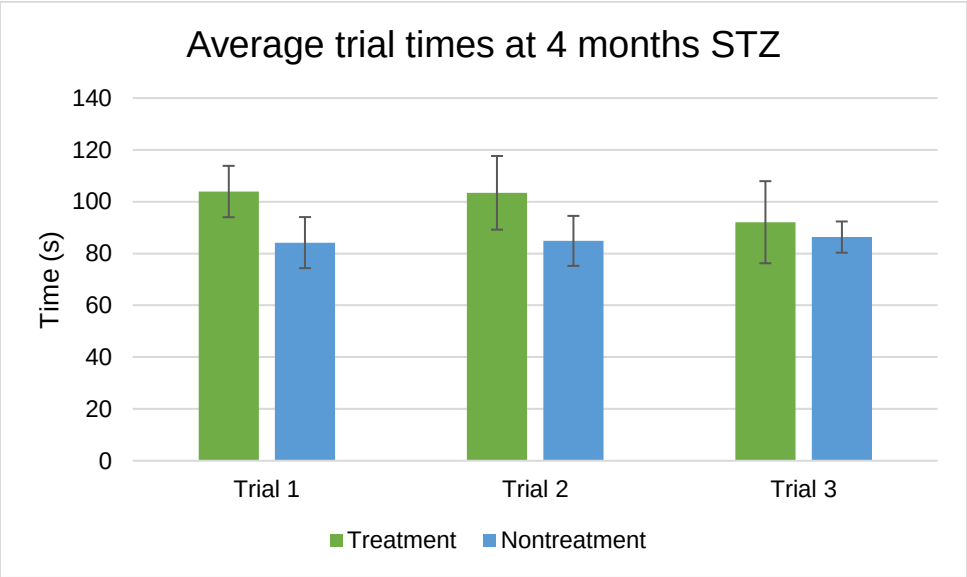

Figure 6. Average trial times for the treatment and nontreatment groups of the STZ model rats. Standard error is represented by the error bars.

Figure 7 shows the trial times for the treatment group at the three and four month marks. There are no obvious trends in the graph and a two-way ANOVA with replication showed no significant difference between the time periods (p=0.759). Figure 8 shows the trials times for the non-treatment group at the two time periods. At the three month mark there is a downward trend in time, which is not present at the four month mark. A two-way ANOVA with replication showed significance between the times for each time period (p=0.0029).

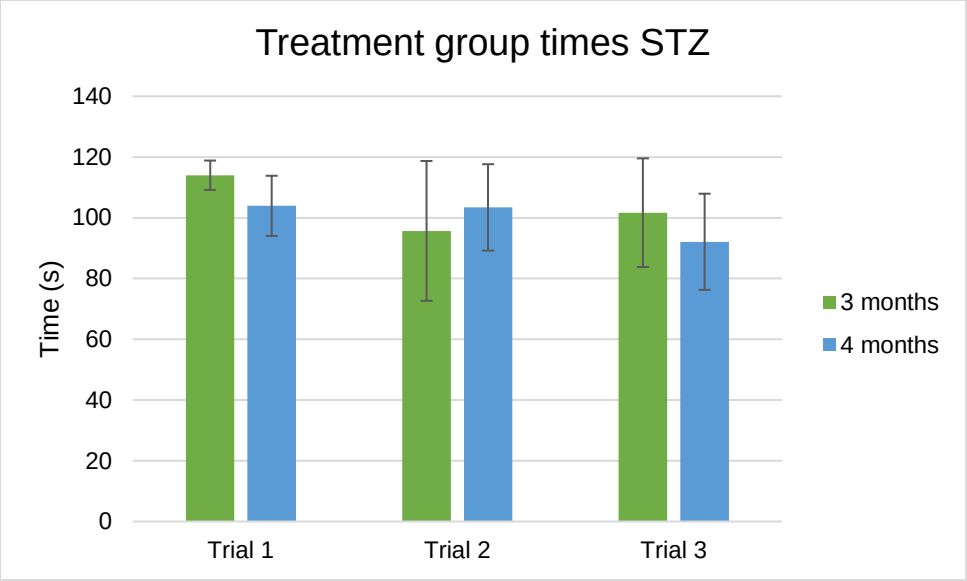

Figure 7. Comparison of average trial times of treatment group at two time periods: 3 months and 4 months. Standard error is represented by error bars.

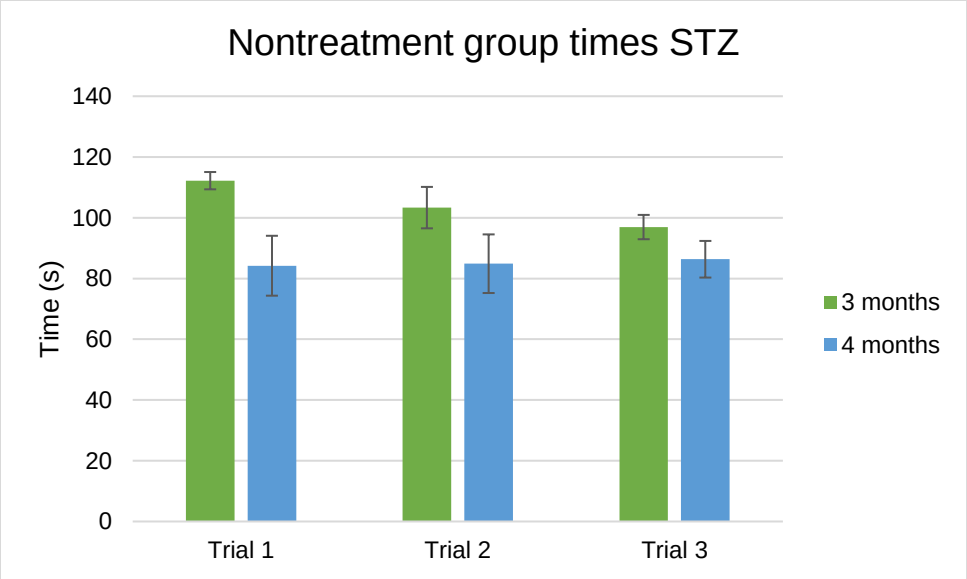

Figure 8. Comparison of average trial time for non-treatment group at two time periods: 3 months and 4 months. Standard error is represented by error bars.

Errors in the water maze were regarded as wrong turns and backtracking. In figure 9 the average errors per trial are shown for the treatment and non-treatments groups of the amyloid-β model group. The nontreatment group can be seen as having a higher initial error rate that then

matches with the treatment group. A two-way ANOVA showed no significance between the groups error rates (p=0.152).

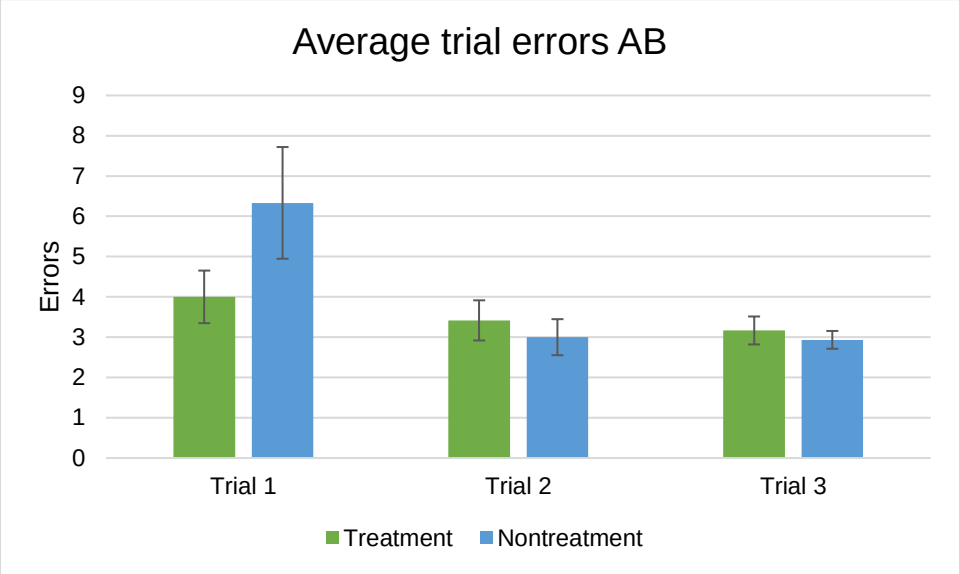

Figure 9. Average number of errors per trial for the treatment and nontreatment groups of the amyloid-β plaque model rats. Standard error is represented by the error bars.

The STZ group had two testing periods where error was also measured. Figure 10 shows the average errors per trial at the four month testing point for each group. The graph shows lower error rates for the treatment group, while both groups have a downward trend. A two-way ANOVA found a significance between the performance of each group (p=0.036).

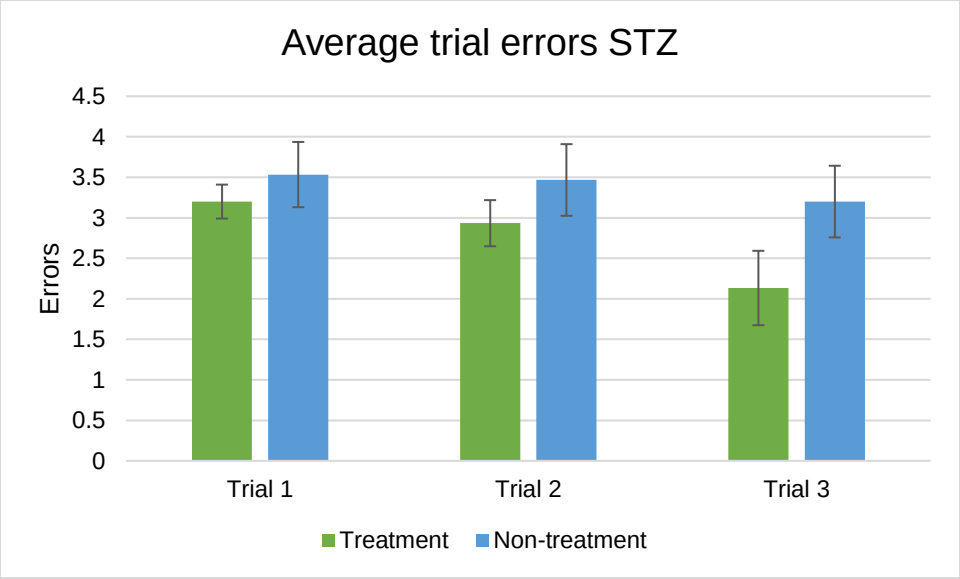

Figure 10. Average errors per trial for treatment and nontreatment groups of the STZ model rats. Standard error is represented by error bars.

Performance of treatment and non-treatment groups were looked at separately at each time period. In figure 11 there is a slight downward trend for each time points for the treatment group while in figure 12 the non-treatment group shows a downward trend only at the three month period. A two-way ANOVA was run for both groups and found no significance in errors between the time periods for the treatment group ($p=0.56$) or for the non-treatment group ($p=0.712$).

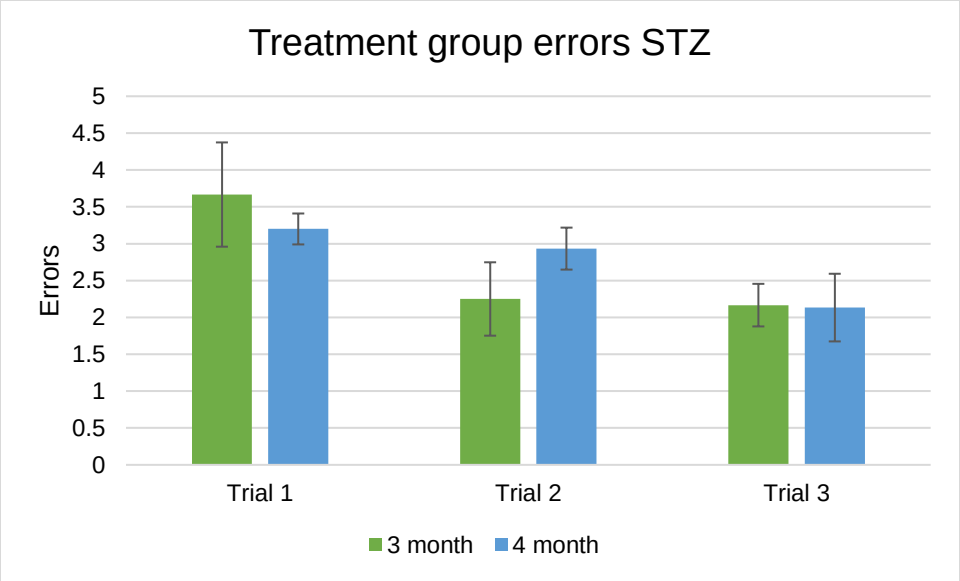

Figure 11. Average errors per trial at two different time points for the treatment group of the STZ model. Standard error is represented by error bars.

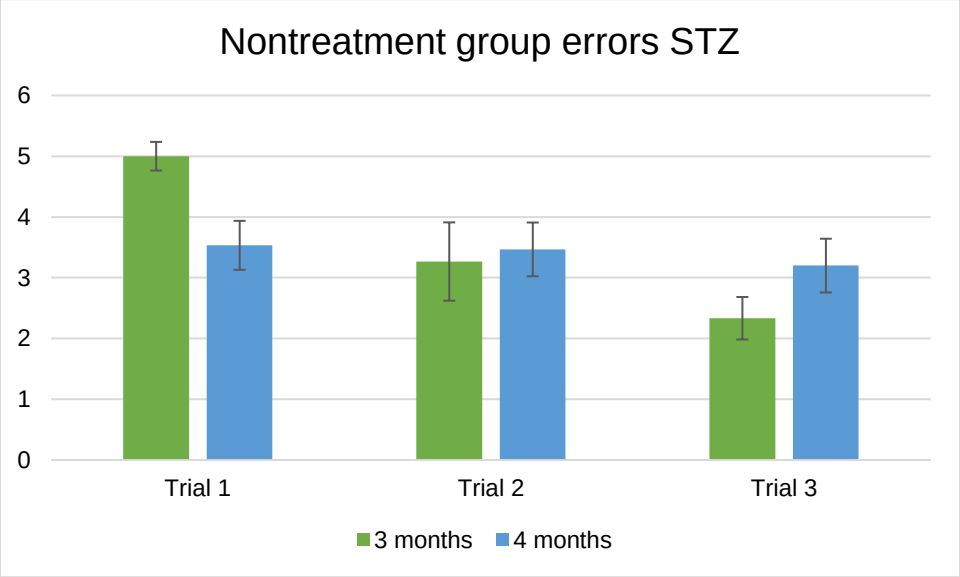

Figure 12. Average errors per trial at two different time points for the non-treatment group of the STZ model. Standard error is represented by error bars.

Spontaneous Alternation Behavior

SAB represents alternations and total number of arm choices made. Spontaneous alternation, expressed as a percentage, refers to the proportion of arm choices differing from the previous two choices. Rats displaying exploratory behavior would have a higher percentage of alternation than rats who do not display that behavior. In figure 13 the SAB for all groups are

shown. For each model group there is a decrease in activity after the induction of Alzheimer's disease. The control group is shown and has a higher SAB percent than the Alzheimer's rats after their surgeries. Only the A2 group showed a significant difference in activity when they were compared in a paired t-test (p=0.0199). The A1, S1, and S2 groups showed no significant difference in their activity (p=0.075, 0.256, 0.215).

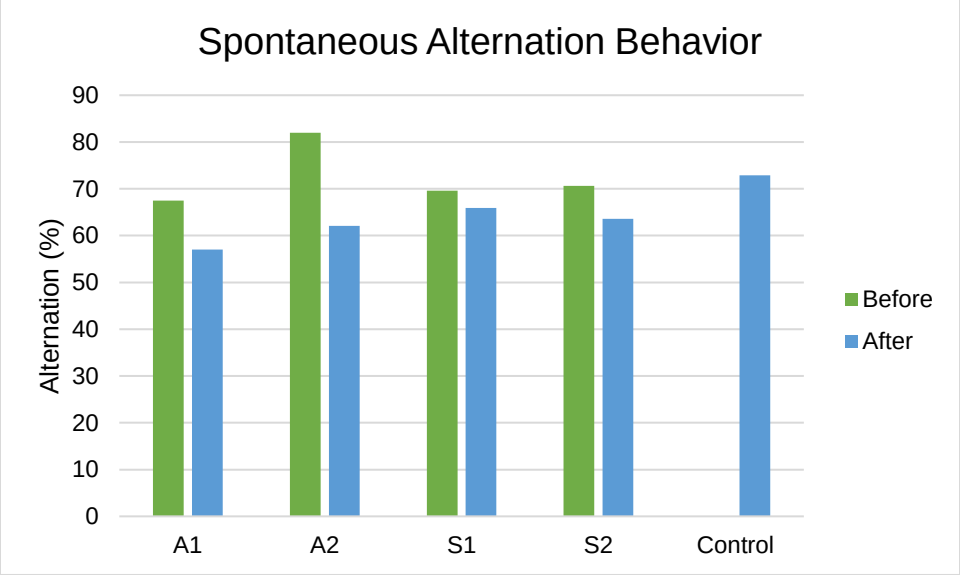

Figure 13. SAB of all groups before and after injection of amyloid-β plaques (A) or STZ (S), as well as a control group. Treatment groups are represented as 1 and non-treatment as 2. SAB is shown as a percentage that represents amount of spontaneous activity, with 100% being the most spontaneous.

Histology

To investigate the effects of the treatment on the two models the silver staining of the rat's brains was evaluated. The NeuroSilver Kit II stains for argyrophilic cells, which are typically dying neurons. Stained neurons are dark black in colour or have a speckled black appearance, while normal neurons and cells have a golden brown staining. The level of background staining varied between slices, as some took on more colour than others. In figure 14 there are captures of the hippocampal region at a 40x magnification. There is noticeable staining

in the amyloid-β group, with heavier staining seen in the brain that did not receive genistein treatment (Fig. 14A,B). The STZ group brain that received treatment is noticeably lighter with less black neurons as opposed to the non-treatment brain (Fig.14C,D). Figure 14E shows the hippocampus of a control rat which is comparable in colour to the two brains that received treatment.

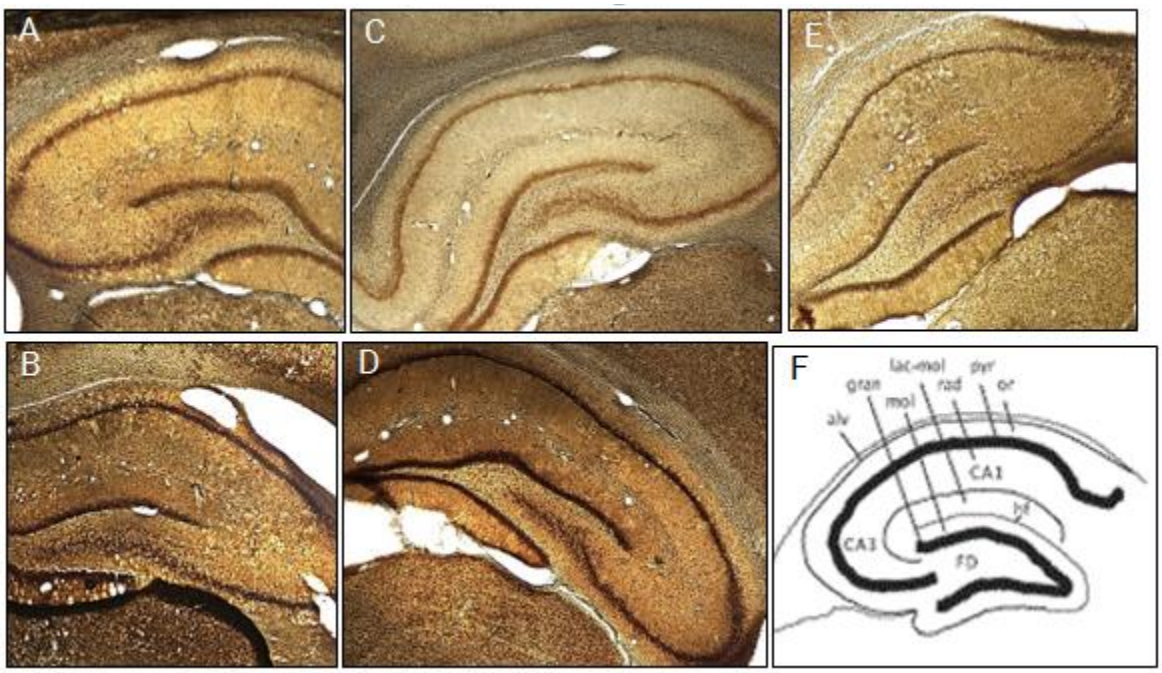

Figure 14. Pictures of the hippocampus of silver stained brains at 40x magnification. (A) and (B) show treatment and non-treatment of amyloid-β group. (C) and (D) show treatment and non-treatment of STZ group. (E) shows silver stained brain of control rat. (F) shows diagram of hippocampal regions sourced from Lehrmann et al., (2008).

Figure 15 shows the CA3 region of the hippocampus magnified to 400x. The neurons can be clearly seen at this magnification. Both of the amyloid-β group brains have darkly stained neurons, but the brain that did not receive treatment show few, if any, unstained neurons while the treatment brain has a mixed of stained and unstained (Fig. 15A,B). The STZ group brains show a great difference in presence of stained neurons. The treated brain (Fig. 15A) shows only a few stained neurons with the majority unstained, while the non-treatment brain shows heavy

staining and an obvious plaque which is indicated by the red arrow (Fig. 15D). In the control brain in figure 15E there is very light staining of neurons similar to the treated STZ brain.

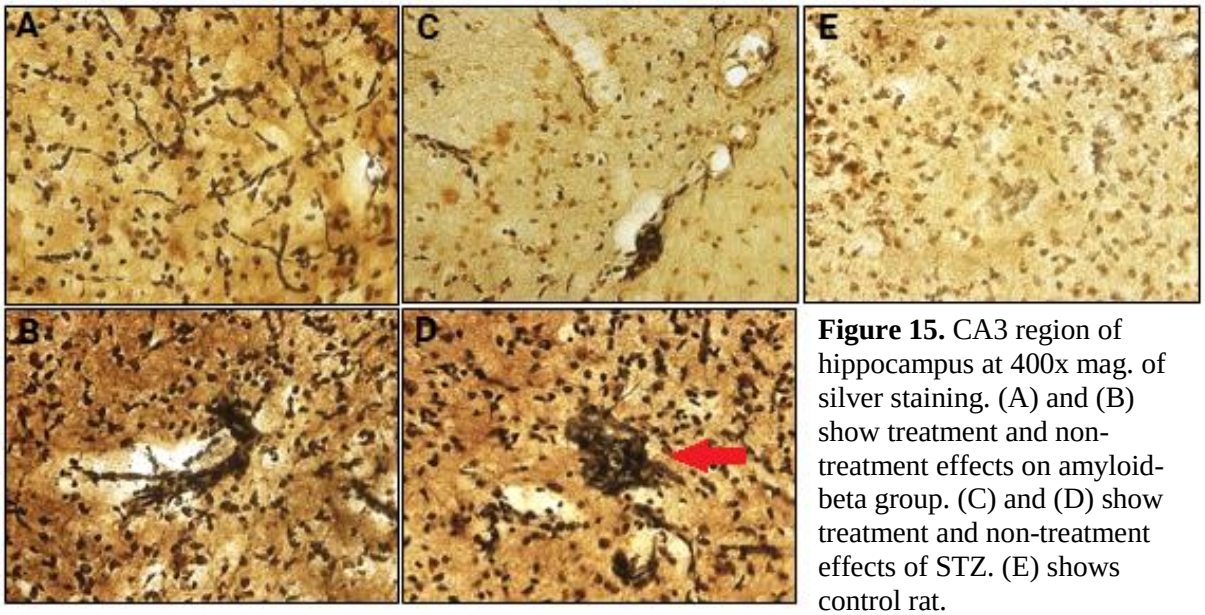

Figure 15. CA3 region of hippocampus at 400x mag. of silver staining. (A) and (B) show treatment and non-treatment effects on amyloid-beta group. (C) and (D) show treatment and non-treatment effects of STZ. (E) shows control rat.

As more than just the hippocampus is affected by AD, the thalamus of the sectioned brains was examined under microscope. In figure 16 the thalamus and surround regions and shown. In figure 16A the red arrow indicates an unstained neuron and the blue arrow indicates a stained neuron. There is a higher number of unstained neurons in this thalamus while the non-treatment amyloid-β brain shows heavier staining (Fig. 16B). The STZ group that received treatment has a comparable level of staining to the control brain (Fig. 16C,E). While the non-treatment group has a lot of silver stained neurons present in the thalamus (Fig. 16D).

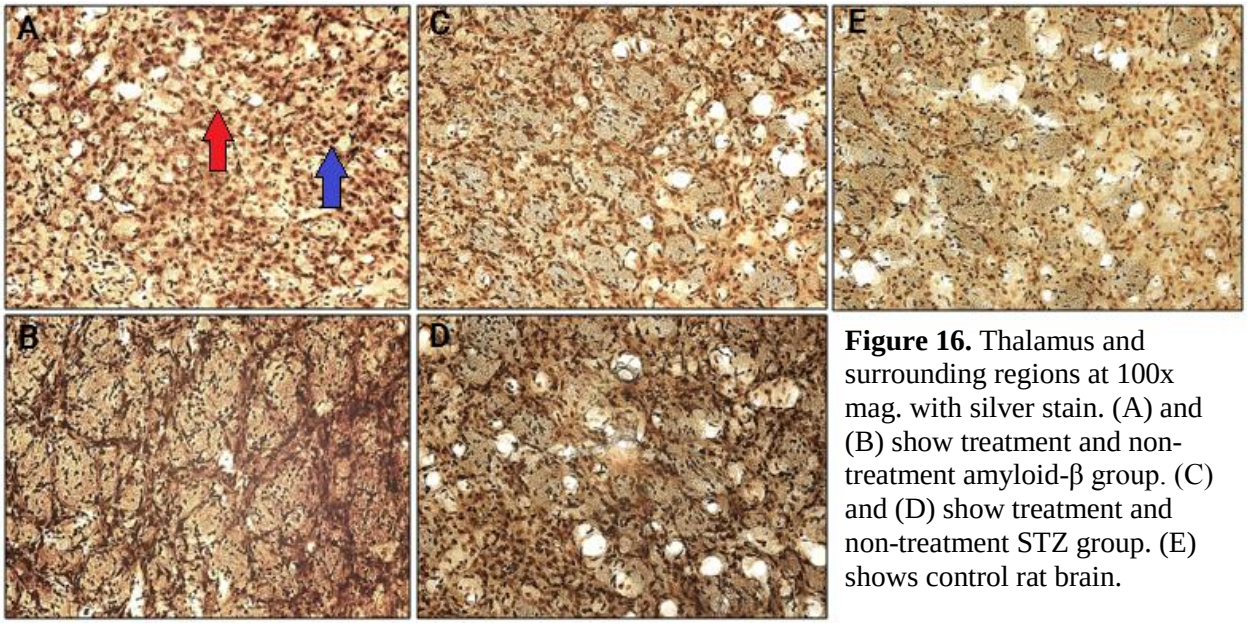

Figure 16. Thalamus and surrounding regions at 100x mag. with silver stain. (A) and (B) show treatment and non-treatment amyloid-β group. (C) and (D) show treatment and non-treatment STZ group. (E) shows control rat brain.

Discussion

This study had two main objectives which were explored through behavioral testing and histology. The first was to show that the phytoestrogen, genistein, could be a more targeted preventative treatment for Alzheimer's disease. The second was to compare the progress of the two models used and how each respond to genistein treatment.

Looking at the amyloid-β group there was no significant difference in water maze performance between the two groups for time or errors made. The non-treatment was the only group that showed a significant decline in SAB. This shows a decline in working memory which was not seen in the other groups. In the STZ group, there was a significant difference between the performances of the two groups at the four-month mark. The non-treatment group had lower maze times overall, but the treatment group showed a downward trend in their times over the three trials, showing memory retention. The treatment group overall made less errors and showed a downward trend in errors made per trial, while the non-treatment group did not show this trend. The groups were compared to themselves at two different time points as well. The treatment

group showed no significant difference between the three and four-month time points for latency or errors made. They maintained their performance, while the non-treatment group showed a significant difference in latency. Their times at the four-month period are lower, but they show no improvement over the trials (Fig. 8).

The data gathered showed some trends that support my hypotheses but not enough significance to confirm them. I hypothesized that the rats injected with amyloid-β fragments would generally do better than the rats injected with STZ, due to the link between amyloid-β plaque production and the estrogen-β receptor. In my observations, many rats from both of the non-treatment group performed much better than the treatment group rats in the water maze. This is reflected the most in the amyloid-β group, as they spent less time overall in the water maze, while the STZ group was tested more, therefore, all of the rats became better accustomed to the task. One rat from the amyloid-β treatment group was removed from the data sets due to her inability to complete the maze on all testing days. Several rats across all of the groups had difficulty in completing the maze most days. As they all received training in the maze before any surgical manipulations, I believe that this was caused by the STZ and amyloid-β fragment injections. Through observations, it appeared that several of the rats could not understand what the task was and would easily forget their way through after successful completion.

SAB in the Y-maze was only significant for one group, the amyloid-β non-treatment. They showed a significant decline in their behavior after the injection of the amyloid-β fragments. The y-maze was used to examine the working memory and exploratory behavior of the rats, which is known to be affected by AD (Carroll et al., 2007; Rosario et al., 2006). Despite there only being significant results for the one group all rats did show a decrease in alternations, even those receiving treatment, and all were less than the control group. There was no difference

in activity level seen between the time periods. In Carroll et al. (2007) they did see a significant difference between ovariectomized 3xTg-AD mice that had and had not been given supplemental estradiol, with the mice receiving estradiol displaying greater exploratory behavior.

The NeuroSilver Kit II was used to examine the pathology of the models used and how each responded to treatment. The kit is a modified silver staining protocol based on the theory that dying neurons are argyrophilic (Gallyas 1971). There was a lot of variation in general background staining between brains, which could be most likely attributed to slight variations in times the sections sat in certain solutions. Despite the darker staining on some sections, the stained neurons were still easily distinguishable. The staining was successful in that there is definite silver staining of the AD rats in reference to the control rat brain. Baizer et al. (2015) made several useful observations of this staining technique. Their study examined human and chimpanzee brains and so were able to view larger neurons at a higher magnification. They saw that the neurons had a speckled appearance from the stain and hypothesized that these were lysosomes within the cell, as these are argyrophilic when within a degenerating neuron.

The hippocampal formation is a major focus of Alzheimer's research because it plays such a large role in memories (Jarrard 1993). It is easy to ignore other regions of the brain, such as the thalamus. Aggleton et al. (2016) make a strong case for the importance of the thalamus and how it is affected by AD. The thalamus is part of the Papez circuit, a limbic system circuit, which also includes the hippocampus. According to Aggleton et al. (2016), this circuit plays a large role in episodic memory formation and recall. Staining from a paper by Braak & Braak (1991) shows obvious silver staining of neurons in the thalamic region in AD mice. The paper comments that there were primarily neurofibrillary tangles seen in the thalamus. For this study, the hippocampus was the main focus, but through examining the resulting staining there was

significant staining found throughout the limbic system. For both the thalamus and hippocampus there was an obvious difference in staining between the brains of groups that had received treatment those that had not. There was silver staining present in all brains examined, including those of the control brain, which was generally expected. By observing density of stained neurons, the biggest difference was seen in the STZ groups. This is the opposite to my hypothesis that the amyloid-β group would be more responsive to a phytoestrogen treatment. A theory for this could be that the genistein was a better protectant against a progressive model rather than direct injection of amyloid-β fragments.

A limitation of this study is that the STZ model of AD is typically observed over a longer period of time, such as in the study by Salkovic-Petrisic et al. (2011) who observed STZ model rats for nine months. Due to time restrictions, this study could only observe the STZ rats for four months. There have been other studies that have studied the model for a similar amount of time (Grunblatt et al., 2007; Lannert & Hoyer, 1998). In a review paper by Salkovic-Petrisic et al. (2012), it was emphasized that this model showed fluctuations in AD pathology, especially at the three to four-month period. For future studies, the observation period of this model should be lengthened to more accurately compare this model with the progression of AD. Another expansion of this study would be to increase the sample size of STZ model rats and euthanize at set time periods in order to compare silver staining of their brains over several months.

A future study could use a different phytoestrogen, such as daidzein (Lephart et al., 2002) or α-zearalanol (Dong et al., 2013). Each compound has a slightly different chemical makeup and different binding affinities for the estrogen-β receptor, which can impact how they function as a neuroprotective. The length of treatment with genistein was based off the study by Wang et al. (2016). This is a variable that could be easily changed to a shorter or longer period of time to

observe what amount produces the most prevention. This study did not continue genistein treatment after inducement of the AD models, because the focus was on the preventative effects rather than ongoing treatment. This may have influenced the results seen and continued administration could be more beneficial.

This study set out to explore alternative estrogens and how they could play a role in preventing AD pathology. In previous studies estrogens, such as estradiol, have been shown to reduce the development of memory loss and neurodegeneration. Although the risks of prolonged HRT with estradiol make it necessary to find alternatives. While the behavioral tests used in this study gave little significant data, the histology provides a great look into the preventative effects of genistein. Both models used showed a response to treatment. The lack of argyrophilic neurons present shows that genistein was effective in slowing down their progression of AD pathology, but possibly not enough to salvage working memory. The two models showed neural death and plaques were seen throughout the brain, indicating that both injection of STZ and amyloid-β fragments can induce AD pathology. There was little difference between the physical presentation of AD in these models when examining the staining. The only difference would be the application of these models, as STZ initiates an insulin deficiency that occurs over a long period of time and injection of fragments creates a fast, specific pathology. It is important to understand that AD is an ever-changing, plastic disease and to study it, it must be able to be replicated within an animal model. The importance of this study was to highlight that treatment for AD must be applicable to all variations of the disease, and not just one animal model.

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Appendix

I. FD NeuroSilver Kit II Protocol

a. Store brain sections in 0.1 M Phosphate Buffer (PB) containing 4% paraformaldehyde for 7 days at 4 degrees C.

b. Rinse sections in Milli-Q water 2 times for 5 minutes each.

c. Transfer to mixture containing equal volumes of solutions A and B, 2 times, 10 minutes each.

d. Place in mixture containing equal volumes of solutions A and B with a drop of solution E for every 8 mL of total volume for 10 minutes.

e. Place in mixture of solution C with one drop of solution F for every 25 mL of total volume twice for 2 minutes each.

f. Place in mixture of solution D with one drop of solution F for every 25 mL of total volume for 5 minutes.

g. Rinse in Milli-Q water 2 times, 3 minutes each.

h. Place in 1x solution G (10x solution diluted 9:1 with Milli-Q) 2 times for 5 minutes each.

i. Mount sections directly from 1x solution G and let air-dry, rinse with xylene 3 times for 3 minutes and then coverslip with permount.