The Effects of Reproduction and Estradiol on Olfactory Function in Female Alzheimer's Model Mice

By
Megan E. Gooding
April, 2016



Allegheny College
Department of Biology

The Effects of Reproduction and Estradiol on Olfactory Function in Female Alzheimer's Model Mice

By
Megan E. Gooding
April, 2016

A Senior Comprehensive Project in Partial Fulfillment of the Requirements for a Bachelor of Science Degree from Allegheny College

I hereby recognize and pledge to fulfill my responsibilities as defined in the Honor Code and to maintain the integrity of both myself and the College community as a whole.

_____
Pledge

Approval by the Senior Thesis Committee:

_____
Dr. Mumme

_____
Dr. Venesky

Abstract

Alzheimer's disease (AD) is a neurocognitive disorder that occurs in growing numbers in the later stages of human lives and causes a loss in cognitive function. In AD model mice, research has shown that one of the earliest signs of dementia and cognitive deficits is through the olfactory system. Olfaction tests, such as the "Hidden Cookie Test," which times mice on their ability to find a cookie based on their sense of smell, can be used to observe the progression of AD and its symptoms in mice. Research and surveys with a focus on human models have shown that females are at a greater risk for Alzheimer's disease than males. Although the reasoning behind the sex differences is still unclear, one of the main factors in question is variance in hormone levels based on female reproductive history. Hormone replacement therapy is a potential prevention method that can decrease the risk for AD. I tested the effects of female reproductive history and estradiol supplementation on olfactory function of female AD model mice. Eight female AD model mice were repeatedly tested for variance in olfactory function over an eight-week time period among three treatment/control groups: post- reproductive females, non- reproductive females given placebo Nutella, and mice given 17ß-estradiol-saturated Nutella. As the disease progresses with time, mice with a reproductive history were expected to experience a more rapid loss of olfactory function and performance on the "Hidden Cookie Test," whereas mice given 17ß-estradiol were expected to have a slower progression of olfactory decline. The results of experiment showed no significant treatment effects on performance of the HRT. However, each group of mice statistically improved in the amount of time to find the cookie throughout the eight weeks of testing. Additional testing is needed with older mice in attempt to show olfactory deficits.

Introduction

Alzheimer's disease (AD) is a disease that slowly diminishes brain function and cognitive abilities until death. Currently, AD affects over five million American citizens, and is one of the top ten leading causes of death in the United States (Alzheimer's Association 2008). According to the Alzheimer's Association, "Every 71 seconds someone in America develops Alzheimer's disease; by 2050 it is expected to occur every 33 seconds" (Alzheimer's Association 2008). Although there is a high incidence of AD, there is still no cure or prevention. Some techniques have been used to inhibit the early stages of the disease, but those treatments still do not prevent the later stages from occurring. However, if diagnosis of AD can occur before cognitive impairment, and before post-mortem autopsy, the progression of the disease may develop on a slower trajectory. One of the first deficits to occur from AD is a loss of olfactory function, which is a potential biomarker for the disease.

Alzheimer's disease, like many others, is caused by a gene by environment interaction. Genetically speaking, humans with the apolipoprotein E ($APOE^{-/-}$) gene have an increased risk of developing Alzheimer's disease (Zandi et al 2002). To induce AD in mice, there are mutations, such as BACE1 mutant, which can cause plaque formation. Environmentally speaking, many factors, such as hormone differences due to reproductive histories, can also play a role in the onset of Alzheimer's disease. Thus, AD is a mix of genetic and environmental risk factors, making diagnosis and treatment options both difficult to assess.

Through initial testing on mice and other species, assessments can be used to

determine not only if the AD is early-onset (the more aggressive form) or late-onset, but also what treatments may be more effective for the individual. Although several clinical studies have shown that patients affected with AD have a lower ability to detect and identify odors, there is currently little research that identifies the cause of early deterioration of the olfactory lobe in the brain. Wesson et al. (2010) compared wild-type (WT) mice to AD model mice across varying age groups to assess olfactory deficits using an odor cross-habituation test (Wesson et al. 2010). Various odors were delivered via concentrated cotton applicators for 20 seconds each, separated by 30 seconds in between trials. The mice were analyzed according to the amount of time they spent sniffing the scent. In addition to the cross-habituation test, tissue collections monitored individual brain areas to quantify plaque deposition (Wesson et al. 2010). The results from this study indicate that the AD model mice demonstrated a weakness in odor discrimination and an increase in amyloid-β deposits in the olfactory lobes (Wesson et al. 2010). The earliest detected olfactory deficit emerged at three months of age and elevated deposits in the brain were spotted at seven months of age. Amyloid-β deposits began to occur in the hippocampus and neocortex around ten months of age (Wesson et al. 2010). Hence, there was a seven-month gap between when deficits in olfactory function began and deposits occurred in the hippocampus, indicating a potential biomarker for AD.

In addition to research about early loss of olfactory function and plaque deposits, research conducted by Turana et al. (2014) was focused on determining diagnostic values of the olfactory test. Amnesic Mild Cognitive Impairment (aMCI) is an early stage of dementia that usually progresses to Alzheimer's Disease. By identifying olfactory biomarkers of AD during the aMCI stage, predictions can be made about the disease

outcome. Turana et al. (2014) performed clinical research on 109 human subjects over the age of sixty. After baseline data were collected, participants were given five seconds to smell and identify ten distinct odors to test for a loss in olfactory function. In addition to olfactory testing, the authors examined changes in pupillary response, Apolipoprotein E (APOE), plasma levels, and changes across a variety of cognitive domains, such as word and sentence memory (Turana et al. 2014). A score of less than seven correct out of ten odor identifications would indicate a diagnosis of aMCI. Results indicated that testing an olfactory deficit in combination with a pupillary hypersensitivity test yielded an 87% positive predictive value. Turana et al. (2014) recommend combining biological markers, such as APOE and plasma levels, with clinical markers, such as olfactory tests, to attain the highest accuracy (Turana et al. 2014). Although this study collected baseline data, a loss in odor identification abilities tends to occur with aging. The results from the research conducted by Turana et al. (2014) were significant, but the data may have had a natural trend toward decreasing olfactory awareness with an increase in time. Another potential problem with this study is that subjects may have been able to smell the odor, but not identify the scent of the odor. This error implies difficulties in cognitive abilities rather than olfactory deficits.

Based on the work of Jocelyn Gruskiewicz in her Allegheny College Senior Comprehensive Project in 2013, Alzheimer's model (APOE $^{-/-}$) mice develop olfactory deficits at approximately two months of age, while typical cognitive deficits have been found to occur around four months of age (Gruskiewicz 2013). Olfactory testing was done using a "Hidden Cookie Test," which places mice in an arena and records the time it takes for them to find the cookie under blind placement conditions. By observing APOE $^{-/-}$

mice from weaning to four months of age, there were distinct differences between the wild type mice and the APOE $^{-/-}$ mice, specifically from 32 days and onward. Data for six wild type mice and four APOE $^{-/-}$ mice showed that completion differences between mouse types were significantly different with a p-value of 0.002 (Gruskiewicz 2013).

Research has also shown that late-onset AD is more common in females than in males. Hormone Replacement Therapy (HRT) is a controversial treatment in which oral supplements of estrogen are given to women after menopause. Despite the fact that HRT has worked for some, there is significant variance in its effectiveness (Reimen and Caselli 1999). Although there may be significant cognitive distinctions between men and women affected by Alzheimer's disease, successful cognitive therapy is difficult to achieve (Musicco 2009). Zandi et al. (2002) tested the relationship between Hormone Replacement Therapy (HRT) and the risk of AD in elderly women. To look at the incidence rates of dementia, 1457 men and 1889 women averaging between the ages 70-75 were assessed in 1995-1997 and then participated in a follow-up in 1998-2000. Out of the participants, 2.6% of men and 4.7% of women developed AD after the initial assessment. Twenty-six women who used HRT were later diagnosed with AD compared to 58 women who did not use HRT and were later diagnosed (Zandi et al. 2002). Although the effects of HRT were significant, there may be variance among HRT use because treatments and dosages may have been variable throughout the three-year time period. Other factors, such as the allelic frequency of the APOE genotypes (dependent upon the client's familial history), intellect, and overall health, may have also played a role in the statistical analysis. While HRT may be effective for some women, it is not a cure and it should not be used to prevent AD (Zandi et al. 2002).

Although women have a higher risk of late-onset AD, there is a low incidence of women with Alzheimer's Disease in their 50's, which is the typical age for the ending of menopause (Henderson 2006). At the end of menopause, there is a decrease in estrogen production. When women encounter their later stages of life, estrogen therapy is typically successful because it tends to decrease stress, increase cerebral blood flow, and promote the growth of nerve processes (Henderson 2006). However, the oral intake of estrogen can also have serious negative side effects, such as an increased incidence of ischemic stroke and hypertension. Since estrogen is normally released into the body on a cycle, taking daily hormone therapy medications may reduce the effectiveness of the estrogen treatments. The success of hormone therapy not only depends on the reproductive history of the woman, but also genetic differences, along with the stages of aMCI. Hormone therapy may not be useful for people with severe symptoms of AD, but may be useful for women who are ending menopause and have genetic history of AD (Henderson 2006).

Ptok et al. (2002) examined the effect of varying female reproductive histories on the risk of Alzheimer's disease. They studied 106 women who had symptoms of AD and compared them to 189 females who served as controls by lacking demential symptoms. The women in the study were compared based on the number of successful births. They found that fertility was more frequent in female patients with AD symptoms than control females with a p-value of 0.03 (Ptok et al. 2002). The researchers also examined the effects of reproductive history on males but found no predictive effect for the diagnosis of AD.

With this information, further research should be done to test the effectiveness or estrogen replacement in the prevention of AD to help reduce the risk for women with a

reproductive history (Ptok et al. 2002). The objective of my research was to examine the effects of reproduction and hormone therapy on olfactory function in Alzheimer's model mice. Since there has been significant research indicating that olfactory sensitivity is lost in AD model mice prior to the loss of cognitive abilities and that females are at a higher risk for AD, it is important to assess what increases female risk factors through olfactory testing. I predict that mice treated with HRT will take less time than the control mice to find the cookie. I also predict that mice with a reproductive history will take more time to find the cookie than the control or HRT mice.

Methods

The Alzheimer's model mice used were bred with a BACE1 mutation to induce Alzheimer's disease via plaque formation. Mice were initially obtained as a breeding pair from Jackson Labs but were then bred in the Allegheny College Biology department animal colony. Eight female AD model mice were used for testing. I randomly assigned two mice for estradiol therapy, two mice for placebo/control, and the rest of the mice for mating with male AD model mice. Random assignment was used to increase validity of the results. Each mouse (other than the mating mice who were housed with males) was housed in an individual cage in order to control Nutella estradiol and placebo dispersals. All mice were held in the Allegheny College Mouse lab under stable and quiet conditions.

The four BACE1 mice assigned to the Reproductive History (RH) treatment were given the opportunity to reproduce with male AD model mice. After birth, the offspring were taken away from their AD model mother and cross-fostered to a wild type

C57BL/6J mouse for growth, maintenance and development. The two virgin female BACE1 mice assigned to the Placebo Nutella (PN) treatment group were fed the placebo Nutella mixture every 3-4 days. The remaining two BACE1 mice assigned to the Estradiol Nutella (ED) treatment were given 17ß-estradiol via per oral administration by dissolving the hormone in sesame oil and placing it in 60mg of Nutella every 3-4 days (Ingberg et al. 2011). For this treatment, I dissolved 28µg 17β-estradiol with 5µL sesame oil and mix that with 1g of Nutella for each mouse. This was given to the Estradiol treatment group every two days. The two mice tested for HRT were kept in separate cages for the duration of the experiment to prevent cross contamination or unequal digestion of 17ß-estradiol (see Table 1).

Testing of olfactory sensitivity for all mice began at weaning (three weeks of age) and continued for eight weeks, with the Hidden Cookie Test adapted from Wersinger and Rissman (2000) being administered once weekly. The four mice in the RH treatment did not begin testing until after giving birth to the offspring to increase the chances of successful breeding by avoiding disruptions of testing. These mice began testing three to four weeks after the placebo and HRT treatment mice. The mice were not given food 18-24 hours prior to testing to increase incentive during olfactory testing.

In the Hidden Cookie Test, mice were placed in an arena (ten-gallon Plexiglas aquarium) and timed to see how long it took them to find a cookie that is out of visual sight. The ten-gallon Plexiglas aquarium that was used as an arena was covered with approximately one inch of mouse bedding to properly hide the cookie and the sides of the glass tank were lined with paper to prevent outside distractions. Based on the results of Gruskiewicz (2013), it was essential to randomly relocate the hidden cookie after each

sequential test with an individual mouse to eliminate the possibility of bias through location memory The timer started when the mouse was released from a small holding container into the arena to locate the cookie. The holding container prevented bias resulting from dropping the mice in a specific area that may have hinted to one location over another (Gruskiewicz 2013). Times were recorded based on number of seconds to the first smell of the cookie, and then the number of seconds until the mouse took the first bite. A smell time was taken if the mouse stood over the cookie for at lease one second appearing to be moving the nose and then walked away. The maximum time for testing was ten minutes.

Table 1. Graphic representation of the mice used in the testing, what group they participated in, and the number of weeks each mouse was tested.

Mice Identification	Treatment	Number of Weeks Tested
A	Estradiol Nutella	8
B	Estradiol Nutella	8
C	Placebo Nutella	8
D	Placebo Nutella	8
E	Reproductive History	5
F	Reproductive History	5
G	Reproductive History	5
H	Reproductive History	3

Data was analyzed with Stats package JMP Pro 12.0. Seconds to the first smell and first bite were long transformed to help normalize the data. Data was analyzed with a general linear mixed model in which treatment group and week of testing were used as fixed effects. Mouse ID was used as a random effect.

Results

Times of first smell and first bite for estradiol mice and placebo mice were relatively similar (Figure 1). Hidden cookie detection and bite times for the Reproductive History (RH) group were slightly higher than those for the estradiol and placebo groups (Figure 1) but the effect of treatment was not statistically significant for either first smell or first bite (Table 2, Table 3). Surprisingly, mouse performance in the Hidden Cookie Test improved over the course of the experiment in all three treatment groups (Figure 2), and the effect of test week was significant for time to first bite (Table 3).

Table 2. Fixed Effect Tests (log of time to first smell). Table shows the number of parameters, degrees of freedom, and degrees of freedom in the denominator, F ratio and the statistical significance values for time to first smell.

Source	Nparm	DF	DFDen	F Ratio	Prob > F
Week Number	1	1	37.24	0.8498	0.3626
Treatment	2	2	2.529	0.9317	0.4975
Week Number*Treatment	2	2	36.99	0.0147	0.9854

Table 3. Fixed Effect Tests (log of time to first bite). Table shows the number of parameters, degrees of freedom, and degrees of freedom in the denominator, F ratio and the statistical significance values for time to first bite (*).

Source	Nparm	DF	DFDen	F Ratio	Prob > F
Week Number	1	1	39	6.8225	0.0127*
Treatment	2	2	5.202	0.0859	0.9189
Week Number*Treatment	2	2	38.8	0.6566	0.5243

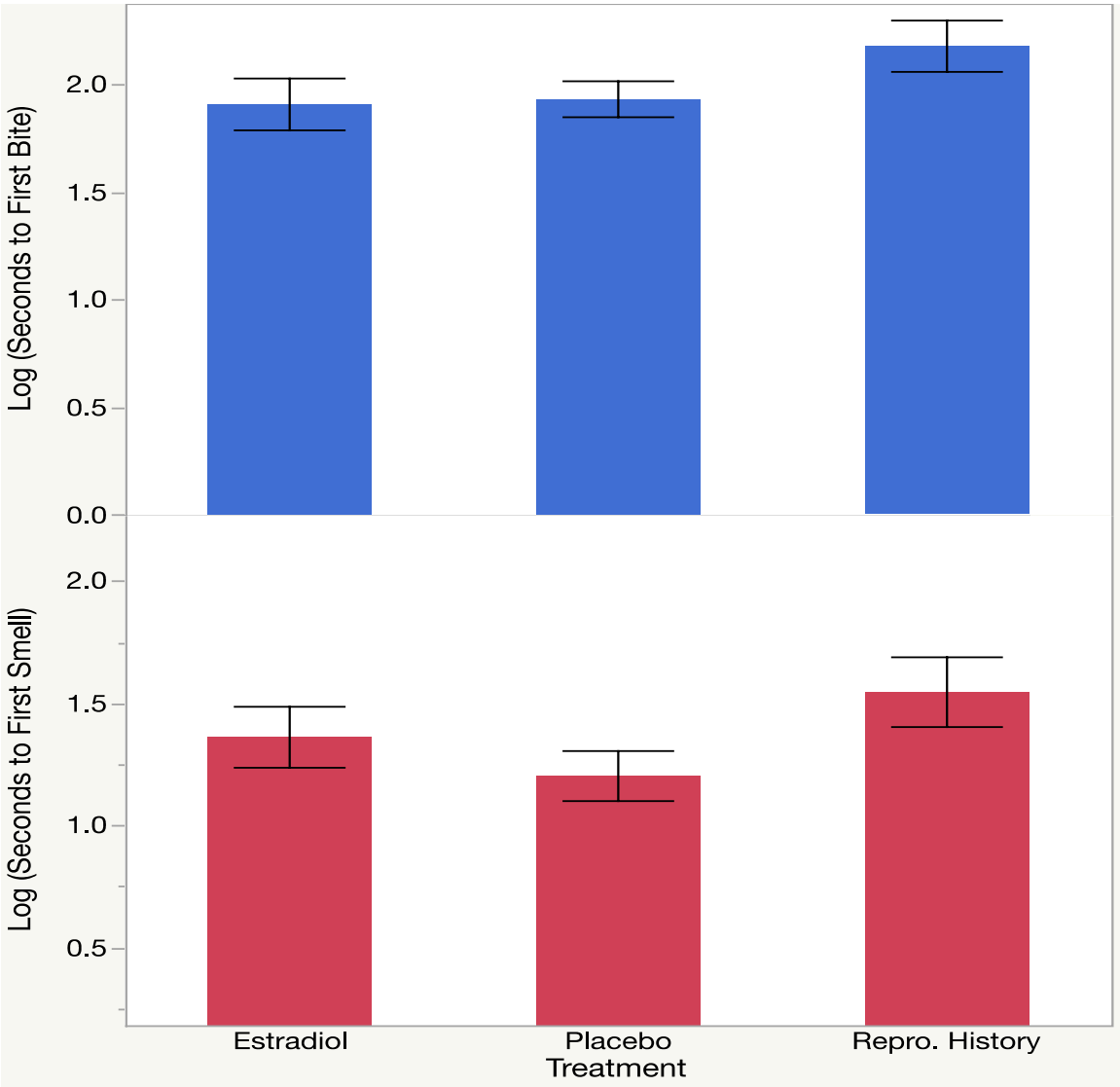


Figure 1. Bar graph showing differences between treatment and placebo groups based on the log (in seconds) of time to find cookie by both time to first bite and time to first smell. Error bars show the standard error of the mean.

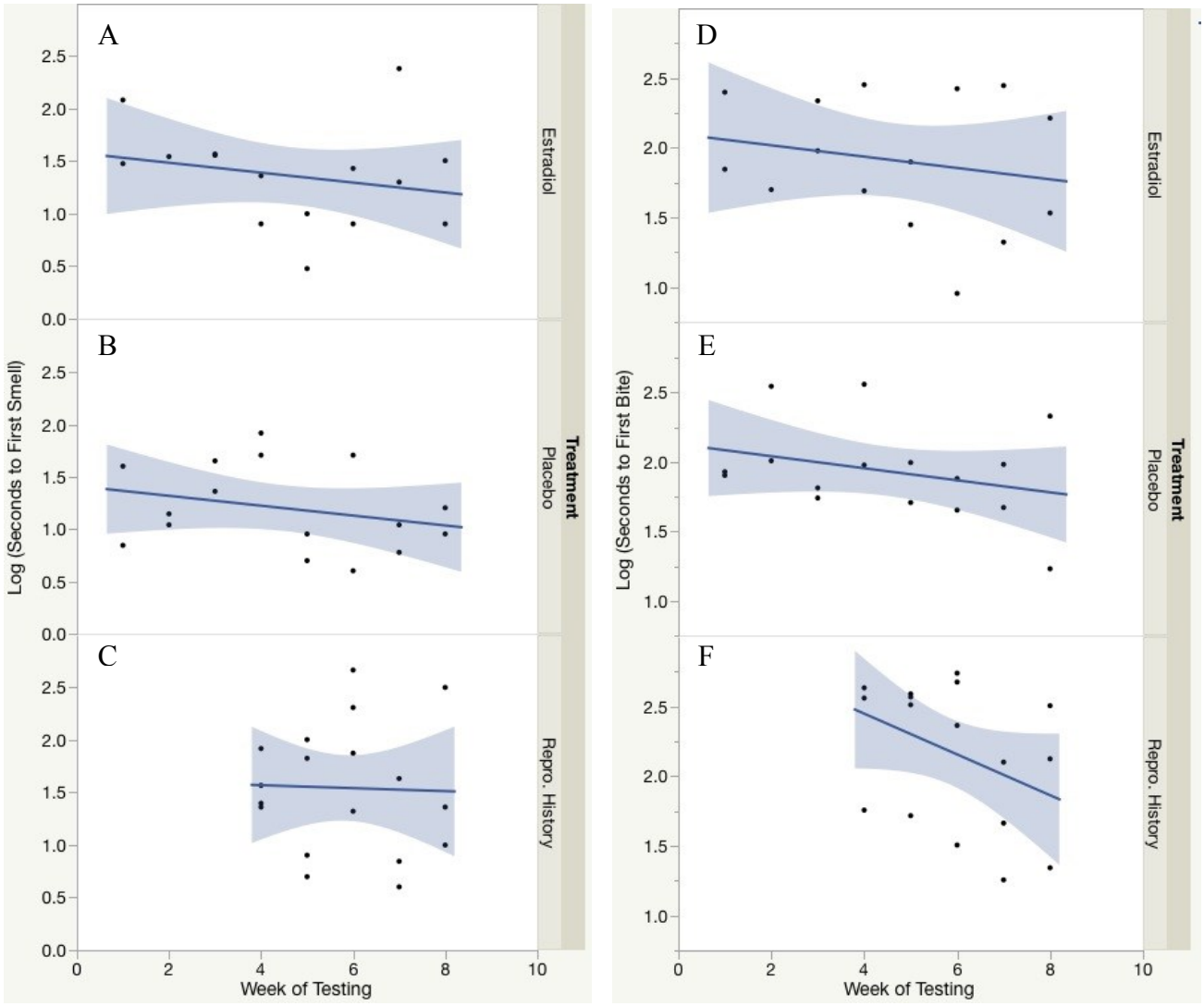


Figure 2. These graphs represent a side-by-side comparison of the difference between log (in seconds) to first smell and first bite across each treatment group through the eight-week period of testing. Graphs A, B, and C represent the time (log in seconds) of time to first smell in estradiol (A), placebo (B), and reproductive history groups (C). Graphs D, E, and F represent the time (log in seconds) of time to first bite in estradiol (D), placebo (E), and reproductive history groups (F).

Discussion

I hypothesized (1) that female mice with a reproductive history would have a greater olfactory loss than non-reproductive virgin females that were isolated from males, and (2) that mice given HRT via 17ß-estradiol Nutella would show fewer olfactory deficits than mice given placebo Nutella. My results did not support either hypothesis: comparison of the two treatments and control showed no significant treatment effects (Figure 1, Table 2). Furthermore, I surprisingly found no evidence of olfactory decline over time; mice in all three treatment groups showed improved performance on the hidden cookie test over the course of the experiment (Figure 2).

Although the main focus of my research did not yield statistically significant results, essential insight can still be gained through the testing period and behavior of the mice. It is possible that treatment effects were insignificant due to a low number of participating mice, an uneven number of mice in each treatment or placebo category, a limited number of testing days, and the timing at which the testing began. Although the procedures followed those proven effective in other studies (Wersinger and Rissman 2000) and through a previous Allegheny College Senior Project (Gruskiewicz 2013) several possible differences may have contributed to my inconclusive results.

Due to the diverse testing criteria and limited number of AD model mice available, only eight female mice were used. This is lower than many other studies, especially considering how some treatments groups comprised only two mice. Another limitation of my study is that one of the four mice assigned to the Reproductive History (RH) group failed to reproduce pups, and could not be used in the study. Due to the inability to

control reproductive success and the difficulty of breeding Alzheimer's model mice, there was an unequal distribution of mice through each treatment and placebo group (Table 1). Also, because the study had to be continued for a consecutive period of eight weeks, and the age of the female mice was important, the reproductive history mice were not able to join the study until weeks after the control and estradiol mice. Because there was a significant effect of what may be interpreted as a learned behavior in finding the cookie (Figure 2, Table 2), those RH mice had fewer weeks to adjust to the testing conditions, and the results for them may not be directly comparable to the other treatments.

The mice began testing immediately after weaning, which was a successful procedure used by Gruskiewicz (2013). However, my results suggest that olfactory deficits may not become apparent in AD model mice until later. Thus, it is possible that my study would have produced more conclusive results if the testing period began a few weeks post- weaning and continued for a longer period of time. It is also important to note that the AD model mice used by Gruskiewizc (2013) were a slightly different mutant strand, APOE $^{-/-}$, then the mutation used in the mice in my study, BACE1. This would also have allowed for the post-reproductive mice to join the study at the same time as the other treatment and placebo groups. In addition, had the study continued longer than the eight- week period, the effects of AD may have been clearer, and the results may have indicated a stronger loss of olfactory function.

To support these suggestions, I compared my results to those of similar published research studies. Wesson et al. (2010) studied the correlation between the presence and size of Amyloid- β plaques and the loss of olfaction. They found that the size of plaques did correlate with olfaction, but that the significance in this loss in olfactory function

does not occur until approximately six months of age (Wesson et al. 2010, Figure 3). The time of my experimentation was under the 3-6 month category in Figure 3 and the difference between AD model and wild type mice is almost non-existent (Figure 3). I chose to test the mice in my study directly after weaning because Allegheny College carries a different strains of AD model mice than what was used by Wesson et al. (2010), and because Gruskiewicz (2013) found significant differences in olfaction between wild type and AD model mice with a similar mutant, I decided to follow her procedure.

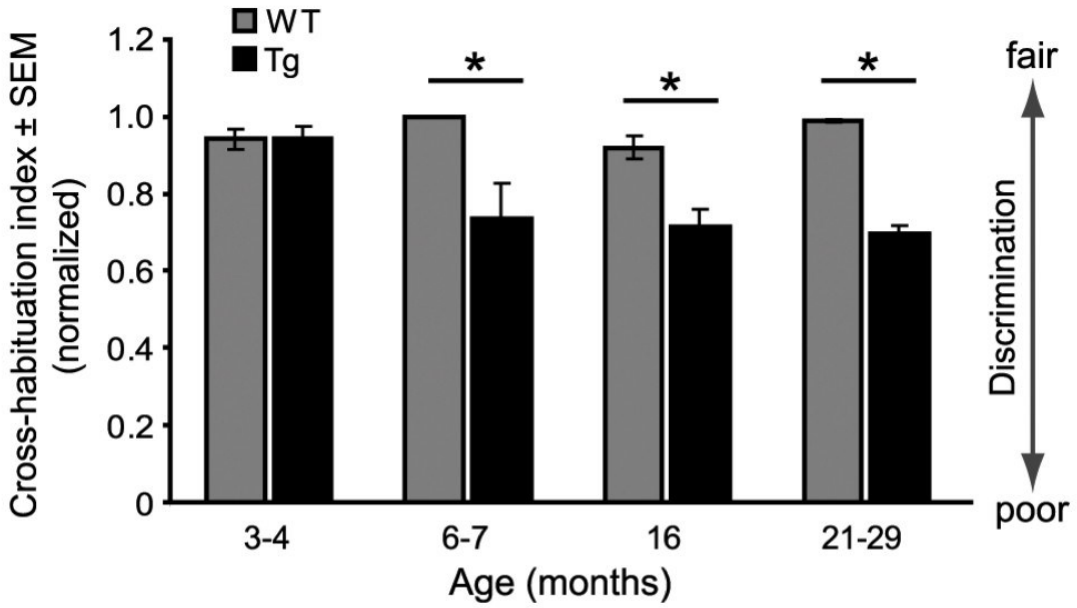


Figure 3. Bar graph from the results section of Wesson et al. 2010. Graph indicates the age of significant difference (shown by *) in olfactory and memory function through a cross-habituation test in both AD and wild type mice.

Another possible limitation of my study was the repetition of the experiment and

the possibility of learned improvement on the Hidden Cookie Test in mice through time. Roddick et al. (2013) tested the olfactory and memory capabilities of AD model mice, through reversal learning, odor discrimination, and executive function experiments. They found there were no consistent findings in a comparison of male and female of wild type and AD model mice (Roddick et al. 2013). This study is used as an example to show that even studies with multiple repetitions, many tests, and a plethora of mice can lead to inconclusive results when testing olfactory function (Roddick et al. 2013).

Saiz- Sanchez et al. (2016) examined the expression of various interneuron expressions to determine if the expression is reduced by a reduction in olfactory function in AD mice. Their results were also inclusive, but researchers believe that further studying the histopathology correlation between expression and interneurons would help identify the accuracy of olfactory tests for early diagnosis of AD (Saiz- Sanchez et al. 2016).

Although olfactory testing is difficult to attain significant results with mice, and no significant results were found comparing the control and post-reproductive model mice, HRT has been used to show significant results. Galea et al. (2001) compared pregnant wild type mice to pregnant wild type mice with estradiol treatment injections and found estradiol helped replace the high levels of pregnancy-associated hormones that were lost giving birth and there was a reduction in depressive-like symptoms (Galea et al. 2001). Thus, the use of estradiol as a HRT is effective in treating or preventing post-partum depression (Galea et al. 2001). Because other experiments have found that post-reproductive animals (and humans) are at greater risk of developing Alzheimer's disease, and HRT has been found to reduce post-partum hormonal affects, I predicted that HRT

on post-reproductive AD model mice would be effective at delaying the development of olfactory deficits (Zandi et al. 2002, Galea et al. 2001). However, Mulard et al. (2000) tested 120 post-reproductive and post-hysterectomy women with mild-moderate AD and found that one year of estrogen treatments did not slow the progression of AD or improve cognition (Mulard et al. 2000). As many studies have been done surrounding the effects of HRT, the changes in hormone levels post-partum, the decrease in olfactory function, and their correlation to AD, most results are still inconclusive as to the effectiveness of HRT. Although my research did not yield significant results, it does confirm, as do many other related studies, that more experimentation is needed in this area using both animal and human subjects.

Acknowledgements

I would like to thank my first and second advisors, Dr. Mumme and Dr. Venesky, for their support and guidance and keeping my attitude as peppy as possible throughout this process. I would also like to thank Vocelli's Pizzeria for their wonderful pies each week.

Literature Cited

Alzheimer's Association (2008). 2008 Alzheimer's Disease Facts and Figures. *Alzheimer's & Dementia* 4(2008): 110-133.

Galea, L.A.M., Wide, J. K., Barr, A. M. (2000). Estradiol Alleviates Depressive- like Symptoms in a Novel Animal Model of Post-patrum Depression. *Elsevier* 122: 1-9.

Gruskiewicz, J. (2013). Impaired Olfactory Function Related to Age and Alzheimer's Disease in Mice. Allegheny College Department of Biology.

Henderson, V.W. (2006). Estrogen- Containing Hormone Therapy and Alzheimer's Disease Risk: Understanding Discrepant Inferences from Observational and Experimental Research. *Neuroscience* 138: 1031-1039.

Ingberg, E., Theodorsson, A., Strom J.O. (2011). Methods for Long-term 17ß-estradiol Administration to Mice. *General and Comparative Endocrinology* 175(2012): 188-193.

Mulnard, R.A., Cotman, C.W., Kawas, C., VanDyck, C.H., Sano, M., Doody, R., Koss, E., Pfeiffer, E., Jin, S., Garnst, A., Grundman, M., Thomas, R., Thal, L.J. (2000). Estrogen Replacement Therapy for Treatment of Mild to Moderate Alzheimer Disease. *The Journal of the American Medical Assocation* 283: 8.

Musicco, M. (2009). Gender Differences in the Occurrence of Alzheimer's Disease. *Functional Neurology* 24(2):89-92.

Ptok, U., Barkpw, K., Heun, R. (2002). Fertility and Number of Children in Patients with

Alzheimer's Disease. *Archives of Women's Mental Health* 5: 83-86.

Reiman, E.M. and Caselli, R.J. (1999). Alzheimer's Disease. *Maturitas* 31: 185-200.

Roddick, K., Schellinck, H., Slotnick, B., Brown, R. (2013). Olfactory learning and memory in the 5xFAD mouse model of Alzheimer's disease. *Journal of the Alzheimer's Association* 9:4.

Saiz- Sanchez, D., Flores- Cuardado, A., Ubeda- Banon I., Rosa- Prieto, C., Martinez-Marcos, A. (2016). Interneurons in the human olfactory system in Alzheimer's disease. *Science Direct* 276: 13-21.

Turana, Y., Ranakusuma, T.A.S., Purba, J.S., Amir, N., Ahmad, S.A., Machfoed, Moh.H., Handayani, Y.S., Asmarinah, Waspadji, S. (2014). Enhancing Diagnostic Accuracy of aMCI in the Elderly; Combination of Olfactory Test, Pupillary Response Test, BDNF Plasma Level, and APOE Genotype. *International Journal of Alzheimer's Disease* (2014): 1-9.

Wersinger, S.R. and Rissman E.F. (2000). Oestrogen Receptor Alpha is Essential for Female- Directed Chemo-Investigatory Behavior but is not Required for the Pheromone-Induced Luteinizing Hormone Surge in Male Mice. *Journal of Neuroendocrinology* (12): 103-110.

Wesson, D.W., Levy, E., Nixon, R.A., Wilson, D.A. (2010). Olfactory Dysfunction Correlates with Amyloid- β Burden in an Alzheimer's Disease Mouse Model. *The Journal of Neuroscience* 30(2): 505-514.

Zandi, P.P., Carlson, M.C., Plassman, B.L., Welsh-Bohmer, K.A., Mayer, L.S., Steffens,

D.C., Breitner, J.C.S. (2002). Hormone Replacement Therapy and Incidence of Alzheimer's Disease in Older Women: The Cache County Study. *American Medical Association* 288 (17): 2123-2129.