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By

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EFFECTS OF 17β-ESTRADIOL ON LEARNING AND MEMORY AND STRATEGY USE DURING A HOLE BOARD SPATIAL NAVIGATION TASK IN AGED MALE RATS

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Abstract

Treatment with 17β- estradiol has been shown to ameliorate age related deficits in spatial learning and memory as well as to promote the use of an allocentric strategy during spatial navigation. While the majority of research using 17β- estradiol has been done using females rodents, there have been studies showing an improvement in spatial learning and memory and promoted use of allocentric strategies in spatial navigation in males as well. In males testosterone (T) is metabolized into estradiol by aromatase. As circulating levels of T decline with aging there is less available T to be metabolized into estradiol. The current study sought to determine whether treatment of aged male rats with17β- estradiol improves spatial learning and memory in a hole-board task and promotes the use of an allocentric strategy (use of extramaze cues). Control and 17β- estradiol treated groups showed decreased latencies to find all baited holes and a significant decrease in visits and revisits to unbaited holes over days. Inconsistent with prior research there was no group difference in performance, and probe tests with extramaze cues removed indicated that an allocentric strategy was not used by either group. It is possible that the lack of group differences was due to estradiol levels that were too low or too high. These results also suggest that rats tested in a hole-board task may prefer other search strategies over an allocentric strategy. Any study using the hole-board task to investigate allocentric search performance must include probe tests to confirm that the rats are actually using the provided extramaze cues to guide their search behavior.

Introduction

The Aging Brain

As mammals age, memory begins to decline and the ability to learn new tasks becomes more difficult. As the brain ages there are changes in anatomy and neurochemistry; for example, brain weight can decline by about 10%. There is also nerve cell loss present in both the brain stem nuclei and the hippocampus. The hippocampus can have anywhere from 15-60% loss of cells, contributing to the loss of overall loss of brain volume (Barnes, 1979; Foster, 2012; Nelson et al., 2013). The hippocampus is of particular interest since changes with age have been well correlated with observed changes in behavior, and are considered to play a major role in the retrieval of certain types of memories, such as spatial memory. There is deterioration of the cholinergic pathways, mainly in the septal complex and the nucleus basalis of the Meynert, which provide cholinergic input to the hippocampus. There is also a loss of dendritic branching and decreased synapses (Barnes, 1979), long term potentiation, which is "a candidate mechanism for storing information in the brain" (Berry, McMahan & Gallagher, 1997, 267), neurogenesis and the regulation of brain derived neurotrophic factor (Meyer & Korz, 2013) have all been observed in aged humans and rat models.

Changes in the hippocampus with age are considered to be one of the major factors in the decline in learning and memory with age (Barnes, 1979; Edinger & Frye, 2007; Meyer & Korz, 2013). Animal models have helped us to see changes in different subregions of the hippocampus such as the dentate gyrus, CA3and CA1. The dentate gyrus, which is important for spatial pattern separation shows decreased neurogenesis with aging. The CA3 region is important for rapid encoding and pattern completion in spatial tasks. CA3 shows more susceptibility to stress induced dendritic changes with age. This is thought to be from dysregulation of neurotrophic

factors that would normally modulate dendritic growth and branching. The CA1 region which is important for consolidation of memories, with aging shows various changes such as increased susceptibility to inflammation, dysregulation of CA^{2+}, and decreased synaptic plasticity (Edinger & Frye, 2007; Foster, 2012). All together these changes point to a decline in acquisition of new tasks, impaired encoding of both short term and long term memory, and impaired spatial navigation.

It is also important to remember that cognitive change and decline is a gradual process with aging. Neither animals nor humans hit a certain age and show a sharp decline in cognitive functions. The exact mechanisms that underlie or induce these neuronal changes that seem to produce the deficits are still not fully understood (Barnes, 1979; Foster, 2012). It is likely that multiple brain areas contribute to age related changes resulting in deficits; however, the main focus here is the hippocampus because of its involvement in spatial navigation tasks. More research into the exact mechanisms of natural and disease related changes needs to be done in conjunction with behavioral tests.

Estradiol in males and females

Estrogens and testosterone are present in both males and females, with males having more testosterone and females having higher estrogen levels. Both sexes have receptors for each hormone, with a high concentration of β receptors in the hippocampus and hypothalamus, this will be discussed in more detail later on. One of the reasons for this in males is because testosterone is converted into estradiol. Testosterone can be broken down into dihydrotestosterone via 5α-reductase, or to estradiol via aromatase (McCarthy, 2008). A lack of testosterone will produce a lack in estradiol as well.

Both hormones have an effect on behaviors and brain organization of both sexes later in life. If either sex is castrated during a sensitive period you will see changes in behavior in adulthood (McCarthy, 2008; Williams, Barnett, & Meck, 1990). Males need gonadal hormones early in development in order to induce male sexual behaviors. Females, on the other hand, need a lack of gonadal hormones in order to develop female sexual behaviors and to produce leutinizing hormone later in life (McCarthy, 2008). Gonadal steroids act during a perinatal sensitive period to permanently alter the neural architecture and restrict the response to exposure of particular hormones. Castrating a male rat at birth will lead to more female behaviors such as lordosis (McCarthy, 2008; Williams et al., 1990). They will also tend to perform more poorly on spatial and fear conditioning tasks. Administration of estradiol or testosterone eliminates the difference between castrated and intact animals (Edinger & Frye, 2006; McCarthy, 2008). It is interesting to note that if you administer a β-receptor antagonist any enhancing effects of estradiol or testosterone on spatial navigation tasks is lost.

Estradiol also plays a role in adults as well. As stated earlier, with a large concentration of β-receptors in the hippocampus, estradiol plays a role in spatial and non-spatial memory tasks such as the water maze, t-maze and radial arm maze.

The Hippocampus: Spatial navigation, learning, memory and estradiol

In humans and animal models, it has been shown that the hippocampus is important for various types of memory and cognitive tasks such as working memory, long term memory, decision making, and spatial navigation (Bizon, 2012; Frick, 2009). Pyramidal cells in the hippocampus have been shown to be selective for spatial location and spatial task performance is sensitive to hippocampal damage (Berry et al., 1997). Age related changes in the hippocampus

discussed earlier have been shown to produce deficits in spatial tasks, fear conditioning, and object recognition (Meyer & Korz, 2013).

The exact mechanisms of how estradiol affects learning and memory are still unclear. It is important to understand the basic neural mechanisms of estradiol on different systems to fully understand how and potentially why estradiol affects hippocampal-dependant learning and memory. Estradiol up regulates cholinergic function and down regulates GABA in the hippocampus. Because acetylcholine regulates hippocampal GABA, production of acetylcholine reduces hippocampal GABA and allows the pyramidal cells of the CA1 to be more active (Daniel & Dohanich, 2001). Extended exposure has been shown to increase NMDA receptor excitability, increase spine density and create new synaptic connections. The effects of estradiol on the medial septal cholinergic system may be why there is a change in GABA and NMDA in the hippocampus (Frick, 2009; Hart, Patton & Wooley, 2001). The cholinergic system must be intact in order for estradiol to have any of these corrective effects (Daniel& Dohanich, 2001; Gibbs & Gabor, 2003; Korol, 2004; Murphy & Segal, 1997; Yankova, Hart & Wooley, 2001). The cholinergic system seems to be very important for estradiol to be an effective treatment (Frick, 2009; Wu, Chen & Brinton, 2001).

Long term potentiation has also been shown to vary throughout the estrous cycle of rats, which is a potential mechanism for storage of memories (Berry et al., 1997). In addition, there seems to be impaired coding by place cells, neurons that are activated selectively when an animal moves through a particular location in space (Henriksen et al., 2008). This could be due to a decrease in dendritic spine density associated with low estradiol levels. The neurons encode less spatial information making it more difficult for the animal to find its way through the environment. When treated with estradiol, there is an increase in spine density of the

hippocampus increases, in both young rats that have been castrated and in aged rats. This increase in spine density has been associated with improved performance on spatial tasks. This could be because it helps improve synaptic connectivity that has been shown to be impaired in aged and estradiol deficient animals (Edinger & Frye, 2007; McClure et al., 2013; McConnel et al., 2012; Meyer & Korz, 2013).

Administration of 17β-estradiol, which has been shown to be the most potent of the estradiols, has been shown to alleviate the learning and memory deficits associated with age-related hormone decline in both humans and rats (Edinger & Frye, 2007; Meyer & Korz, 2013). Estradiol appears to have an effect primarily on hippocampal-dependent tasks. For example, estradiol induced improvements seem to be most robust in spatial tasks, especially ones that require the use of cues (Frye, Rhodes & Dudek, 2005; Fugger, Cunningham, Rissman & Foster, 1998). Other forms of memory may also be improved following estradiol treatment. For example during the t-maze task, the animal needs to remember which goal arm it visited last (a reliance on short-term memory) in order to enter the opposite arm to obtain reinforcement. Experiments utilizing the Morris water maze, T-maze, and other maze tasks (e.g., radial arm maze) have shown improvements in performance of rats treated with estradiol (Frye et al., 2005; Fugger et al., 1998; Galea et al., 2001; Hawley, Grissom, Martin & Halmos, 2013; Korol, 2004; Kritzer, McLaughlin, Smirlis & Robinson, 2001; Luine & Rodriguez, 1994; Mclure, Barha, & Galea, 2013; Sprtizer et al., 2011). The role of estrogen in animals of either sex may be important in understanding certain cognitive declines that are associated with aging.

Considering the current evidence, 17β-estradiol has been considered a potential treatment for the deficits associated with hormone decline during normal aging or possibly for age-related diseases, such as dementia and Alzheimer's disease. However; there have been mixed results as

to how effective estradiol really is in reducing deficits in learning and memory. Discrepancies in results seem to come from various sources, such as dosage, method of administration, age, and other environmental factors. Very high or very low levels of estradiol results in impaired spatial ability in many studies (Frye et al., 2005). Studies using supraphysiological doses tend to find no improvement or greater impairment on the task relative to controls. Studies that bring hormone levels to a physiological level tend to see improvement on performance in spatial memory tasks during acquisition and retention compared to controls (Frye & Edinger 2007; Frye et al., 2005). However, others have found that rats with high levels of estradiol out-perform rats with low levels on tasks that require use of cues in a spatial task (Edinger & Frye, 2007; Frye et al., 2005; Galea et al., 2001; Korol, 2004; Kritzer et al., 2001; Luine & Rodriguez, 1994). These contradictory results could be due to the task and strategy needed to efficiently perform the task, which will be discussed in detail.

Factors influencing the effects of estradiol

Effects of estradiol depend also on exposure length and the age of the subjects. This discrepancy in dosage and exposure length is something that has to be been taken into account when designing a hormone study. Results seem to be most consistent if the hormone levels are brought to a physiological level and remain there. There is a lot of data to support that midlevel doses of estradiol are associated with improvement of learning and memory tasks (Asthana et al., 2001; Hogervorst, Williams, Budge, Riedel & Jolles, 2000; Linzmayer et al., 2001).

Some of the conflicting results could also be coming from general age related changes, not necessarily from the estradiol treatment itself. Foster (2003) found a decrease in swim speed on the Morris water maze task in rats that had chronic exposure to estradiol, this could have affected the results of other studies that showed that estradiol had a negative effect on learning

and retention of the same task, rather it may have been a decrease in mobility. Tang et al. (1996) found that chronic estradiol treatment had a greater reduction of risk for Alzheimer's disease in menopausal women. Given these results, it would seem that if estradiol is given in the correct dose and length of time, it could aid in reducing age-related learning and memory decline. The regimen of estrogen treatment needs to be researched more to see what dosage and intervals of estrogen are most effective in reducing age related cognitive decline.

Estradiol treatment also seems dependent on when estradiol is administered. Daniel (1997) found that giving estradiol prior to training on a spatial memory task improved acquisition and retention for later performance on the task. In contrast to Frye (2005) found that an acute rise in estradiol during training is usually associated with impaired learning on the spatial version of the Morris water maze task. There are other findings that show that estradiol administered immediately after training in the Morris water maze improved performance on future trials (Frye et al., 2005).

Age at the time of administration also seems to be an important factor in determining dose and effectiveness. Foster (2003) examined the path length in the water maze task, at baseline and after treatment, to evaluate learning and memory in the different age and treatment groups, as well as to ensure that there were no other age related impairments in motility. Aged rats treated with a high dose of estrogen took a shorter path, but middle aged rats in the low dose group showed an increased path length (Foster et al., 2003). The longer path length could indicate a poorer memory of the location of the platform or it could indicate a poorer strategy for finding the platform. If the different age groups had significantly different latencies, but similar path length it would suggest a difference in swimming speed. In a spatial discrimination task, middle aged rats showed the best performance in the low dose condition, and aged rats showed

the best performance the high dose condition (Foster et al., 2003). The effects of estradiol seem to be both dose and age dependent.

The wide range of results could be due to the various methods used to study the same effects. Researchers use everything from young to old rats, rats that are intact or have had their reproductive organs removed, as well as different dosages and methods of administration. Under all of these different conditions, the results vary (Foster et al., 2003; Frick, 2009; Galea et al., 2001; Meyer & Korz, 2013). For example, when rats are left intact, it is difficult to know how quickly estradiol levels are changing, especially in female subjects. Rats have a rapid estrous cycle, which means their hormones fluctuate quite a bit and very rapidly (Galea et al., 2001). Naturally fluctuating hormone levels in both sexes, makes it difficult to pick apart the behavioral effects that estradiol or natural hormones are having in conjunction with estradiol administration (Frick, 2009).

Strategy

Estrogen also seems to aid in modulating memory formation and developing a strategy to efficiently navigate through a spatial task. Estradiol seems to promote the use of hippocampal-sensitive strategies, more specifically an allocentric or place strategy (Edinger & Frye, 2007; Berry et al., 1997; Meyer & Korz, 2013). When using a place strategy, the animal encodes information about the location of an object (Edinger & Frye, 2007; Korol, 2004; Meyer & Korz, 2013). When estradiol levels are too low, there seems to be a shift to striatal-mediated or other less effective strategies, such as egocentric strategies to complete the task. The egocentric strategy involves the location of an object relative to the rat's own body, rather than relative to other cues or places in the environment (Korol, 2004).. Korol (2004) describes estrogen as "the conductor" coordinating multiple strategies to be used on a task.

Because allocentric strategies seem to be affected by a lack of estradiol, the effects of estradiol treatment on the performance of spatial navigation tasks in aged animals is beginning to be investigated more in both sexes. While there are other factors that may be influencing strategy selection, estradiol replacement does seem to have an ameliorating effect on deficits in spatial memory. Rats with high levels of estradiol seem to use allocentric strategies on spatial tasks, utilizing extramaze cues, whereas rats that are hormone deprived tend to use egocentric strategies and in turn tend to perform poorly on the same spatial tasks.

Effects of 17β-estradiol on male rats

While the majority of the literature focuses on the effects of estradiol in females, there are more studies emerging examining the effects of estradiol in males. As described earlier testosterone is converted into estradiol, leading to research as to whether testosterone or estradiol is mediating learning and memory during aging (Edinger & Frye, 2004; Meyers & Korz, 2013). Physiological testosterone levels, when compared to low testosterone levels, have been associated with better performance on spatial navigation tasks, and fear conditioning, similar to the effects of estradiol. As males age, they tend to have lower levels of testosterone, and in turn performance on spatial tasks tends to decline. This decline in performance could be due to a decline in testosterone itself, or a lack of 17β-estradiol due to less testosterone available to be converted into estradiol (Bannerman et al, 1999; Aubele, Kaufmann, Montalmont & Kritzer, 2008; Moffat et al, 2003). Studies have shown that spatial memory performance is promoted during the perinatal sensitive period in male rats by administration of estradiol (Dawson, Cheung & Lau, 1975; Luine & Rodriguez, 1994; Williams & Meck, 2001).

As testosterone declines with age (less testosterone means less conversion to estradiol), performance is significantly impaired on spatial memory tasks and operant tests of spatial and

non-spatial memory as well as behavioral flexibility. Long term gonadectomy in male rats creates deficits in a spontaneous object recognition task (Aubele et al., 2008; Luine & Rodriguez, 1994). Learning deficits in androgen-deprived male rats can be reversed with estradiol or testosterone treatment (Meyer & Korz, 2013). By replacing testosterone in castrated or aged rats, inhibitory avoidance, object recognition, and contextual fear performance improved (Edinger, Lee &Frye, 2004). This improvement in performance could be attributed to testosterones conversion to estradiol (Frye et al., 2005).

To determine if the improvement in performance was due to testosterone, dihydrotestosterone (the other metabolite of testosterone), or estradiol researchers needed to test all of these hormones as well as block the effects of them. If β estradiol receptors are blocked the enhancing effects of testosterone are lost, suggesting that improved memory and cognitive functioning is due to estradiol's effect on the β receptors within the hippocampus (Edinger & Frye, 2007). In ER knockout mice (which is when mice are genetically altered so that the estradiol receptor is lacking) acquisition on spatial memory tasks was poorer compared to controls (Rissman, Heck, Leonard, Shupnik & Gustafsson, 2002). ER seems to be highly concentrated in the hippocampus and amygdala which are both important areas for learning and memory.

When male rats were given testosterone, dihydrotestosterone (DHT) and estradiol, both DHT and estradiol enhanced performance in an inhibitory avoidance task (Edinger&Frye, 2007). Luine and Rodriguez (1994) found that estradiol improved memory in gonadectomized and intact aged male rats on the radial arm maze. These effects could be due to the effect of androgens on the hippocampus, administration of testosterone increases hippocampal excitability (Smith, Jones, & Wilson, 2002). Adult male rats that were treated with estradiol, performance

on the radial arm maze was better than their female counterparts and their untreated male counterparts. In fact, the estradiol treated older males performed similarly to young male rats. (Luine & Rodriguez, 1994).

These enhancing effects of estradiol have also been observed in humans. Young men that have high estradiol levels performed better on visual memory tasks when compared to men with normal estradiol levels (Frye et al., 2005). In older men, low levels of estradiol have been associated with decreased learning and memory, just as in animal models. Many studies on male human participants have found that administering testosterone improves these deficits, but is thought to be linked to the breakdown of testosterone into estradiol (Frye et al. 2005).

It seems that the effect of estradiol in males is most prominent in rats whose hormones have begun to decline, in rats this seems to be between 20-24 months. Male rats treated with estradiol also showed faster acquisition on spatial tasks than control animals (Gibbs, 2005). With results showing that estradiol treatment in males can have enhancing effects on learning and memory associated with normal aging, it is even more critical that we understand the role of estradiol in both sexes. Finding out which strategy is being used to correct these age related deficits can give us clues as to which areas of the brain and which mechanisms are being influenced by estradiol.

Current Study

In the current study, I examined whether 17b- estradiol had a performance enhancing effect on aged male rat's rate of acquisition in a spatial hole- board task and how well they retained information regarding the location of food rewards. More specifically, I looked at which strategy the rats actually using and if they committed less errors overall. To examine the effects of estradiol I used the hole board which is an open field type maze that can have anywhere

between 4 and 16 holes. I used a board with 16 holes, in which 4 nonconsecutive holes were baited with sugar pellets. The rats must search the holes in order to obtain the food reward. The advantage of the hole-board is that it can use either intramaze or extramaze cues, as well as measuring multiple measures of memory.

I hypothesized that the aged rats treated with a physiological dose of estradiol will both have faster acquisition, as well as show better memory of which 4holes are baited. If the animals are indeed using a hippocampally mediated allocentric strategy, a probe test will show impaired performance. This task capitalizes on the animal's natural tendency to forage, having multiple exploratory options, and being able to include spatial landmarks within the apparatus. Spatial memory enables the animal to perceive, code, integrate, store, and retrieve information about the environment such as the location of objects and landmarks, as well as specific routes. Spatial learning and memory is crucial for survival in all mobile species, allowing flexible responding to threats and opportunities provided by a changing environment (Van der Staay, Gielin, Pinzon, Nordquist & Ohl, 2012). In order for an animal to forage successfully they must be sensitive to the environment and where they can find food within that environment. There are different strategies that an animal can use to orient itself within the environment. I used landmark cues on the walls of the apparatus, which were considered extramaze cues.

By using all aged male rats, it is assumed that their testosterone levels have dropped below normal physiologic levels; meaning there's less testosterone is available to convert into estradiol. I expected that rats in the control group (not receiving hormone treatment) would show overall worse performance than the experimental group (receiving hormone treatment) at both (1) learning the locations of the available sugar pellets in the hole board apparatus, which does

not vary across days and (2) performing with fewer errors within a test session (e.g., revisiting a previously baited hole).

I used the hole-board task since it evaluates the acquisition of the task (learning), the selection of food search strategies, spatial memory, and the animals' motor and motivational abilities (van der Staay et al., 2012). This task also allowed me to observe the strategy the rats used in order to find the sugar pellet rewards. Spatial navigation in complex environments, such as the hole- board, bias rats to use the extramaze cues to locate rewards more efficiently. Use of extramaze cues (in this case pictures on the apparatus walls) should ensure that the rats are actually using a hippocampal dependant strategy. But rats may choose other reliable landmarks in the room to navigate the maze that is unknown to the experimenters. To make the provided extramaze cues (pictures added to the walls of the apparatus) the only reliable landmarks, the apparatus was rotated daily and the rats were placed in a different corner of the apparatus on each trial. In addition every hole had a sugar pellet in it, but only the baited holes were accessible. Unbaited holes were made inaccessible by a wire mesh covering. This was done to avoid olfactory cues for finding the baited holes.

To test whether the animals were using the provided landmarks the landmark cues or some other strategy, the cues were removed for probe tests. Disruption of performance during the probe test indicates that the rats were relying on the provided landmarks to locate the baited holes. If performance is not disrupted then the rats were using another strategy independent of the landmark cues. I predicted that treated rats would rely more on extra-maze cues than the non-treated rats since previous studies have stated that estradiol treatment improves performance on spatial navigation tasks and promotes use of allocentric strategies.

I hypothesize that the experimental group receiving estradiol treatment will learn the spatial task faster and perform better all around at remembering where the sugar pellets are in regards to the spatial cues. More specifically, I hypothesize that rats receiving treatment will show faster acquisition of the task and once learned will show less short term memory errors, meaning that they will revisit already exploited holes less than the controls. Rats in the experimental group should also have fewer reference memory errors, meaning they will visit fewer inaccessible holes less than the control group, as well as have a smaller percentage of reference memory errors. In addition, rats in the experimental group should have fewer numbers of total errors, as well as shorter latency to find the first accessible hole, and to find all accessible food pellets than the control group. When a probe trial is introduced (when extra maze cues are removed), the performance of both groups should decline unless absence of estradiol treatment biases rats toward adopting a search strategy that does not dependent on hippocampal function (Fugger, Cunningham, Rissman & Foster, 1998; Galea et al., 2001; Gibbs, 2005; Hawley, Grissom, Martin & Halmos, 2013). Definitions and formulas for how each measure is calculated are listed in the methods section.

After 15 days of training using extramaze cues performance seemed to plateau. To see if performance could be improved further we added intramaze cues. Intramaze cues were cut outs of the black contact paper on the floor exposing the wooden floor underneath.

One of the issues that came up during the literature was the use of different dosages. Studies use anywhere from low levels of estrogen to supraphysiological dosages of estrogen. Not surprisingly, the results vary quite a bit between studies for this reason. However, it seems that supraphysiological doses of estrogen cause a decline in learning and memory in rats, whereas low levels improve acquisition for reference memory tasks (Barker et al., 2009; Daniel et al.,

1997; Foster et al., 2003; Frye et al., 2005; Fugger et al., 1998; Galea et al., 2001; Locklear & Kritzer, 2014; Luine & Rodriguez, 1994; Luine et al., 1998; McClure et al., 2013; McConnell et al., 2012; Meyer & Korz, 2013; Talboom et al., 2008). Another issue is how the different dosages are measured and administered. Some studies measured in ml when using subcutaneous injections, while others measured dosages based on the amount of space within a capsule when using slow release capsules implanted under the skin for long term exposure to estradiol. Subcutaneous injections tend to be used for short term examination of estradiol. When looking at longer term effects of estradiol silastic capsules are used since they release steady long term dosages of estradiol. (Barker & Galea, 2009; Daniel et al., 1997; Daniel & Dohanich, 2001; Edinger & Frye, 2006; Foster et al., 2003; Frye et al., 2005; Frye et al., 2008; Fugger et al., 1998; Galea et al., 2001; Kritzer et al., 2001; Lagunas et al., 2012; Lipitova & Toufexis, 2013; Locklear & Kritzer, 2014; Luine & Rodriguez, 1994; Luine et al., 1998; McConnell et al., 2012; McClure et al., 2013; Meyer & Korz, 2013; Narenji et al., 2013; Packard et al., 1996; Talboom et al., 2008; Zhang et al., 2008) Since the majority of studies used silastic capsules in the experiments, this is the method chosen for this study as well. Silastic capsules ensure circulating levels of estrogen for approximately one month, and can be replaced if needed. This method has best demonstrated long-term changes as opposed to ligand-interaction effects. The dose that was most consistent in creating physiological concentrations of estrogen in male rats was 5mm of estrogen in a 15mm capsule (Gibbs, 2005; Luine & Rodriguez, 1994; McConnell et al., 2012)

Method

Subjects

Eighteen male Sprague-Dawley rats (approximately 20 months of age) were used. Rats were housed in pairs in plastic shoebox cages, maintained on a 12:12 h light:dark cycle and were on *ad libitum* food and water prior to testing. Following surgery, and 2 days prior to the beginning of testing the rats were food deprived and kept at approximately 85% free feeding weight. These rats were previously used in behavioral studies in the BNS laboratory (Protocol #AH1301 and AH1302). These rats were subjects in an experiment on social housing and conditioned fear learning and extinction and were tested in an objection recognition task. Although the animals experienced different housing conditions during one of the previous studies, all rats were subsequently double housed for at least 12 months prior to the start of the present experiment. All procedures were approved by Seton Hall University IACUC.

Hormone Administration

The rats received hormone (or saline) treatment via silastic capsule pellets implanted subcutaneously. Rats were anesthetized with Ketamine (60 mg/kg, ip) and xylazine (8 mg/kg, ip). Pellet implants were placed under the skin of the nape of the neck through a small midline incision made between the scapulas after shaving the hair with an electric clipper. The incision was closed with a single stainless steel wound clip. Rats were monitored daily for 7 days after which the wound clip fell out naturally. The capsule was made of silastic laboratory tubing (.58in i.d., 0.77in o.d., Dow Corning) cut to 15mm long, filled with 5mm of hormone and was plugged at each end with 5mm of a wooden applicator stick (Fisher Scientific). The capsules are then sealed with elastomer (Sylgard 184 silicone elastomer base and curing agent, Dow Corning). Half of the rats (N = 9) received a single silastic capsule of Estradiol- 17b diluted in

sesame oil and half (N=9) received a silastic capsule containing the saline solution vehicle. This method of administration has shown to produce physiological concentrations of Estradiol and has been shown to last up to two months (Gibbs, 2005). The capsules were equilibrated by soaking in 0.9% saline solution for 24 h. Prior to implantation the capsules are rinsed with 70% ethanol (Gibbs, 2005). The animals were given 7 days to recover prior to beginning testing.

Apparatus

The hole- board apparatus is typically used to investigate exploratory behavior of rat as they are allowed to explore 16 equally spaced holes on an elevated platform. The holes are big enough for the rat to poke its head into but there is not sufficient room for the rat to pass through the holes. The hole- board can also be used as a spatial learning task by providing food cups with sugar pellet rewards (BioServ, dustless precision pellets) under the holes. The rewarded hole-board procedure presents a multiple choice situation for the rat with food consistently available in only some of the holes. If the rat chooses an incorrect hole it must remember this and continue to search for the hole that contains the reward. This procedure demonstrates learning and spatial working memory by improved ability to locate the baited holes. The hole board was made of a wood board (1 m×1 m) with 16 regularly arranged holes (7.5 cm in diameter and 7 cm deep). The holes are arranged in a 4x4 matrix as shown in Figure 1. The floor board was surrounded by Plexiglas walls 50 cm in height. Three of the walls were lined with black and white pictures (a floral picture, a striped wall, and a picture of a building) that served as extramaze cues (landmarks). Each hole had a cup beneath it, each containing a sugar pellet. All but the baited holes had a wire mesh screen over the cups so that the rats could only obtain the reward from the baited holes and were not influenced by olfactory cues to locate the baited holes. The rat was placed in a different random corner of the apparatus facing the center of the apparatus on each

trial and the experimenter always stayed in the same position during testing and AnyMaze was used to record the rat's path during each trial.

Procedure

Adaptation (5 days). All rats were given 5 days exposure to the hole board apparatus. On day one and two of adaptation 16 sugar pellets were scattered throughout the apparatus floor but not in the holes; the rat was allowed to explore and eat freely for 10 minutes or until all pellets were eaten. On day 3, 1 pellet was placed outside of the hole, and 1 sugar pellet was placed in the hole. The rat was again allowed to roam freely and eat all of the pellets for 10 minutes or until all of the pellets are eaten. On the 4th day of adaptation all of the holes were baited and no sugar pellets were on the apparatus surface, again the rats were allowed to roam freely for 10 minutes or until all pellets were eaten. On the 5th day 8 random holes were baited. If after 10 minutes the rats did not find all of the pellets the rat was gently guided to the baited hole location. The maze was rotated by 90 or 180 degrees daily so that room cues were unreliable landmark cues for the baited hole. During a trial the experimenter always remained in the same spot in the room.

Extramaze Cue Training (14 days). All rats received 2 trials per day and each trial lasted 5 minutes or until all four pellets were found. Four holes were baited with accessible sugar pellets on each trial and the pellet locations were kept consistent, as shown in Figure 1.This consistent pattern of accessible baited holes was provided as an opportunity to measure reference memory and short-term memory performance (see measures section below). From here on the holes with accessible sugar pellets will be called "baited holes". If the pellets were not found after 5 minutes rats were guided to the baited hole locations and allowed to eat the sugar pellet. The rat was placed in a random corner of the apparatus facing the center on each trial. The

apparatus floor was cleaned with a 70% alcohol solution between each trial. Testing occurred for 15 days.

Intramaze Cue Training (6 Days). All procedures remained the same during this phase as in extramaze cue training. The only difference was that intramaze cues were included. The intramaze cues were cut outs of the black contact paper on the floor of the apparatus surrounding baited holes. This exposed the wood floor and produced contrast. Testing lasted for 6 days.

Probe Tests. Two probe tests were performed during extramaze cue training, between training days 9 and 10, and 13 and 14, and one during intramaze cue training between training days 5 and 6. During the probe tests for extramaze cues, the pictures on the walls of the apparatus were removed in order to determine whether the rats were relying on the extra-maze cues or had developed another strategy to locate baited holes. If the rats were relying on the extra-maze cues, their performance should decrease once the cues are removed. If the rats used another strategy (e.g., an egocentric strategy) the rats' performance should show little to no change. The probe test during intramaze cue training covered the exposed wood floor with black contact paper. If the rats were relying on the intramaze cues their performance should decline.

Behavioral Measures. The hole- board permits the analysis of several behavioral measures including exploratory and motor behavior (especially early in training) and memory-guided behavior (especially in experienced animals later in training).

Memory-guided behavior was measured by recording the different holes visited, visits and revisits to all holes, visits and revisits to baited holes, visits and revisits to unbaited holes, and latency to locate all baited holes. Rats need to use their reference (long-term) memory to remember which 4 holes are baited across trials and days. The efficiency of memory-guided behavior was assessed by calculating memory errors. Reference Memory (RM) errors were

calculated as the number of all visits and revisits to unbaited holes. Short term memory will be considered the ability to remember which holes have already been visited during a trial. General short term memory errors will be measured revisits to baited holes and unbaited holes.

The memory-guided behaviors were recorded with AnyMaze tracking system. Latency to find all four baited holes was recorded by the experimenter for each trial. Shorter latencies indicate improvement in remembering the locations of the baited holes.

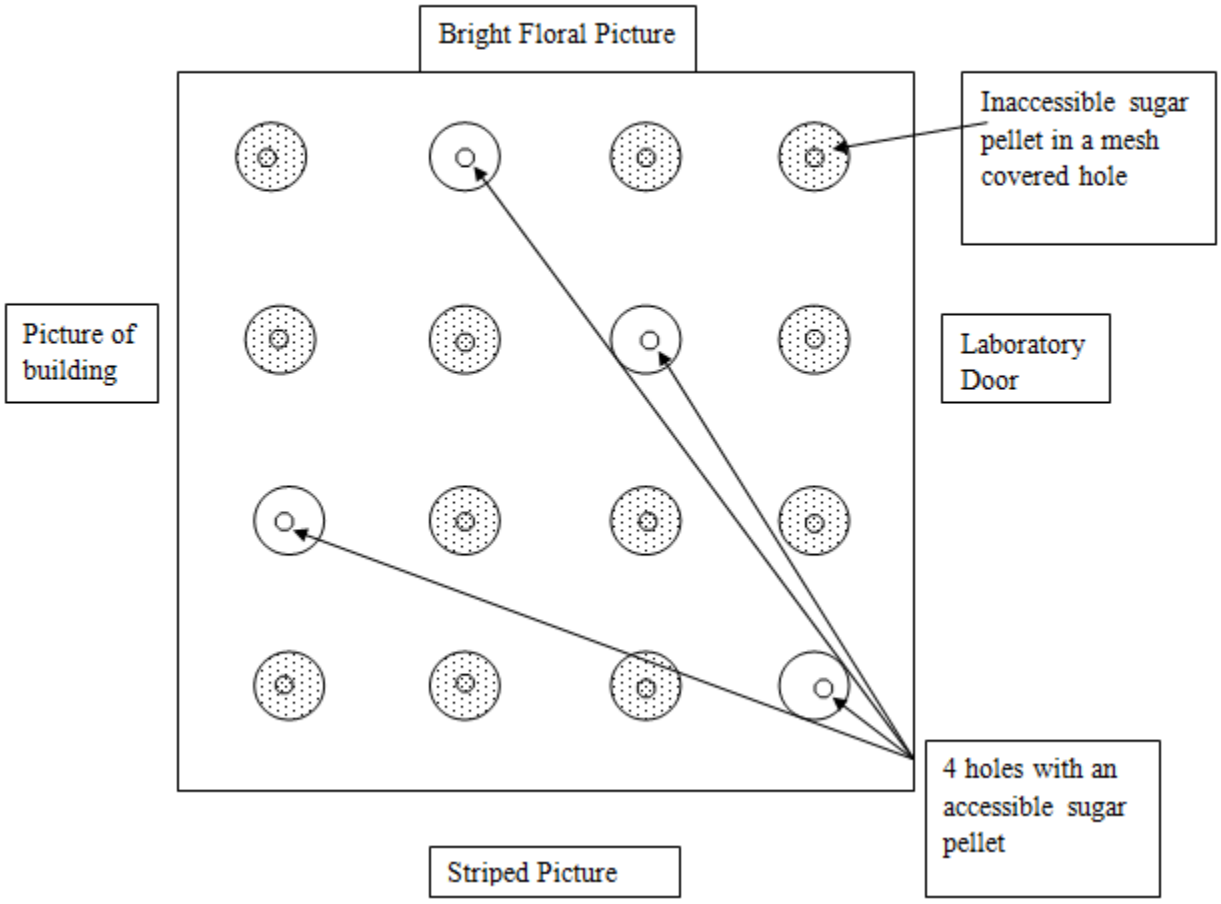

Figure 1: Hole board apparatus setup during training.

Data Analysis

The various dependent variables were analyzed with mixed designed factorial ANOVAs where Hormone Treatment (Estradiol or Vehicle) is a between groups factor and Days is the within-groups factor. For example to analyze the *revisits of baited holes* was analyzed as a Hormone Treatment (2) x Days (14[Extramaze Cues] or 6[Intramaze Cues]) mixed ANOVA. A statistically significant interaction between treatment and days would indicate differential rate of short term memory errors between the two groups. To analyze STM performance, STM errors was compared between hormone-treated rats and control. Estrogen treated males were predicted to show fewer STM errors than controls. Group differences in search strategies were also evaluated by analyzing the probe test data with ANOVAs (compared to the day prior and day after a probe test) or independent t-tests (when comparing group differences at asymptotic performance). η_ρ^2 was also looked at to assess the amount of variance explained by the current model (large$\geq$.50, medium$\geq$.30, low$\geq$.10).

Results

Extramaze Cues

There was an overall improvement in performance across days for both groups as indicated by decreasing latencies to find the food pellets in the four baited holes over training days (Figure 2). A Groups (2[Estradiol, Control)]) x Days (14) mixed factorial ANOVA revealed a statistically significant decrease in latency over days, $F(13,208)=16.81$, $p<.01$, $\eta_\rho^2=.51$, but no main effect of groups, $F(1,16)=.56$, $p>.05$, $\eta_\rho^2=.03$ and no Groups x Days interaction , $F(13,208)=.68$, $p>.05$, $\eta_\rho^2=.04$.

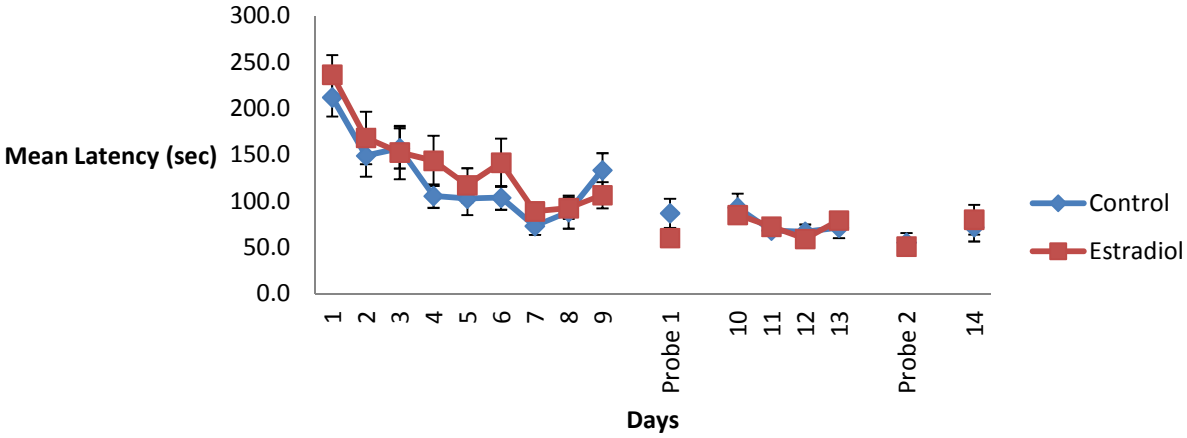

Figure2. Mean (± S.E.) latency of the Estradiol and Control groups to find all four food pellets across the 14 days of extramaze cue training, including Probe Test 1 between days 9 and 10, and Probe Test 2 between days 13 and 14

The latency data clearly shows learning in both groups. This improved performance over days may be due to increased search speed, adjustments in the number of holes visited and revisited, and /or adoption of some other behavioral strategy. To identify the cause of improved performance I analyzed the holes visited in several different ways. Figure 3 shows the total number of different holes visited during the 14 days of training. The hole-board contained 16

holes with only 4 holes baited. Thus, learning to ignore the unbaited holes would be reflected in the data as a decrease in the total number of different holes visited. However, as can be seen in Figure 3 there was no significant change in the number of holes visited across days, $F(13,208)=1.06$, $p>.05$, $\square^2=.06$. There were also no group differences, $F(1, 16) =.38$, $p>.05$, $\eta_p^2=.02$. Both groups consistently visited at least 12 of the 16 holes (75%). This suggests that neither group effectively learned to ignore any of the unbaited holes when provided only with extramaze cues.

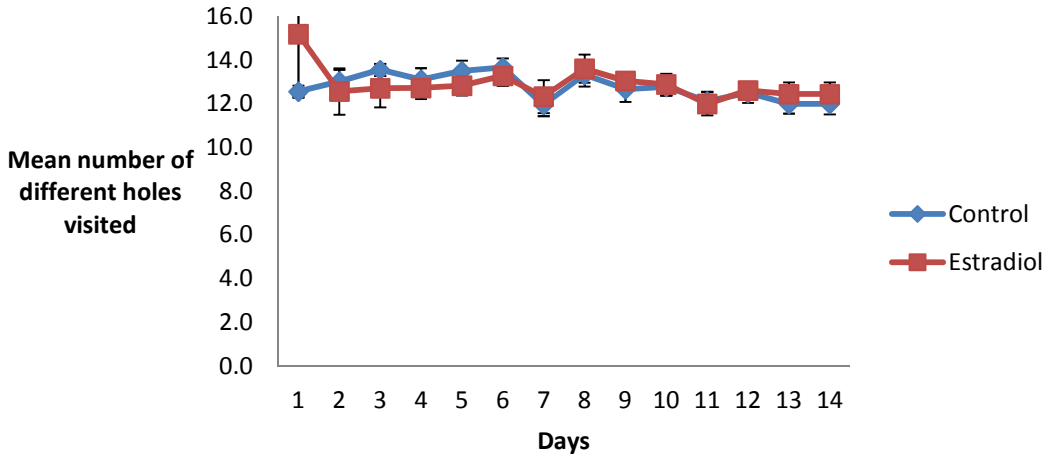

Figure 3. Mean (± S.E.) number of different holes visited, out of 16 available holes, during the 14 days of extramaze cue training.

The previous data suggests that the rats from both groups were completing the task without relying on their long-term or reference memory to identify the location of the baited holes. Thus, the decreasing latencies observed over days (see Figure 2) was not due to the gradual development of a long-term memory for the baited or unbaited holes. Another possible reason for the improvement in latencies over days is greater reliance on short-term memory so that already visited holes were less likely to be revisited. To determine if the rats' improved performance over days was due at least partly to increasing reliance on short-term memory for

already visited holes the total number of visits and revisits to baited and unbaited holes were analyzed (Figure 4). A statistically significant main effect of days indicated that both groups performed less total hole visited and revisits over days ,$F(13,208)=4.36$, $p<.01$, $\eta_\rho^2=.21$. There was no difference between the Estradiol and Control groups, $F(1, 16)=.15$, $p>.05$, $\eta_\rho^2=.01$.

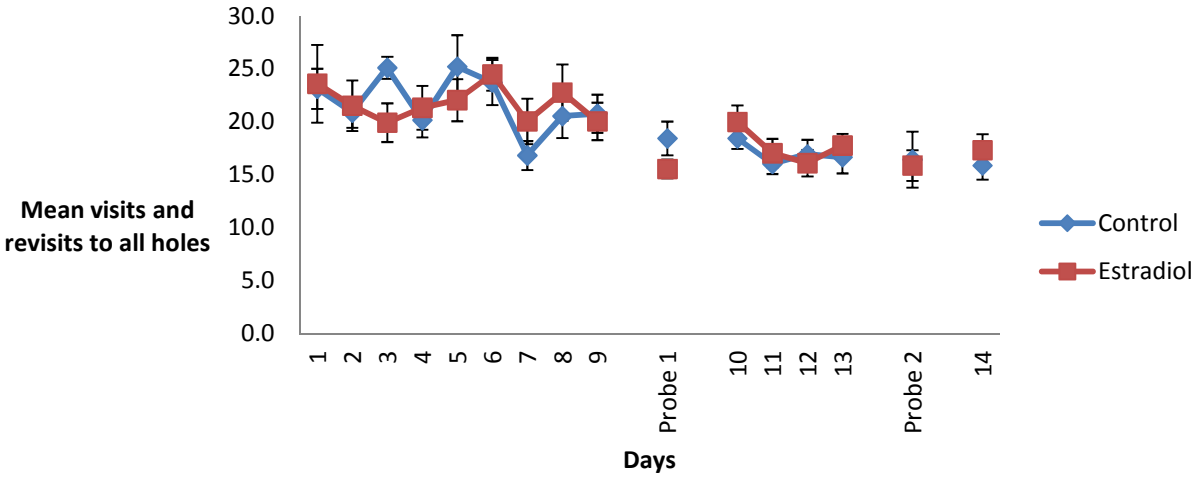

Figure 4. Mean (± S.E.) number of visits and revisits to all baited and unbaited holes over the 14 days of extramaze cue training.

Next I looked at revisits to the baited (Figure 5) and unbaited (Figure 6) holes separately. As can be seen in Figure 5 there was a statistically significant decrease over days in revisits to baited holes, $F(13,208)=3.91$, $p < .01$, $\eta_\rho^2=.20$. A similar decrease was observed in revisits to unbaited holes, $F(13,208)=5.82$, $p < .01$, $\eta_\rho^2=.27$. Interestingly, the drop in revisits appears to be greater for unbaited holes than for baited holes. No group differences were observed in number of revisits to baited holes, $F(1, 16)=.26$, $p>.05$, $\eta_\rho^2=.02$ or unbaited holes, $F(1, 16)=.53$, $p>.05$, $\eta_\rho^2=.03$. These results indicate that overall improvement in completing the task was at least partly due to increasing reliance on short-term memory for already visited baited and unbaited holes.

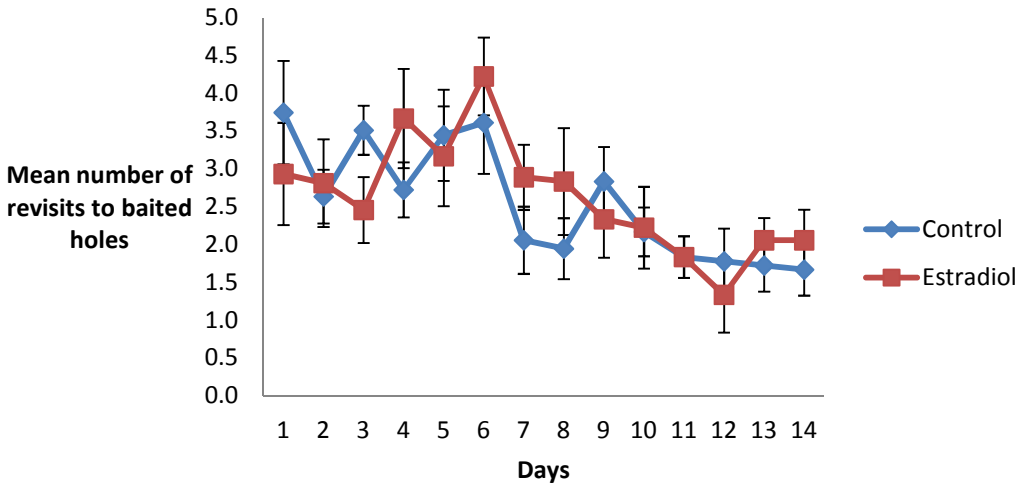

Figure 5. Mean (± S.E.) number of revisits to baited holes across the 14 days of extramaze cue training.

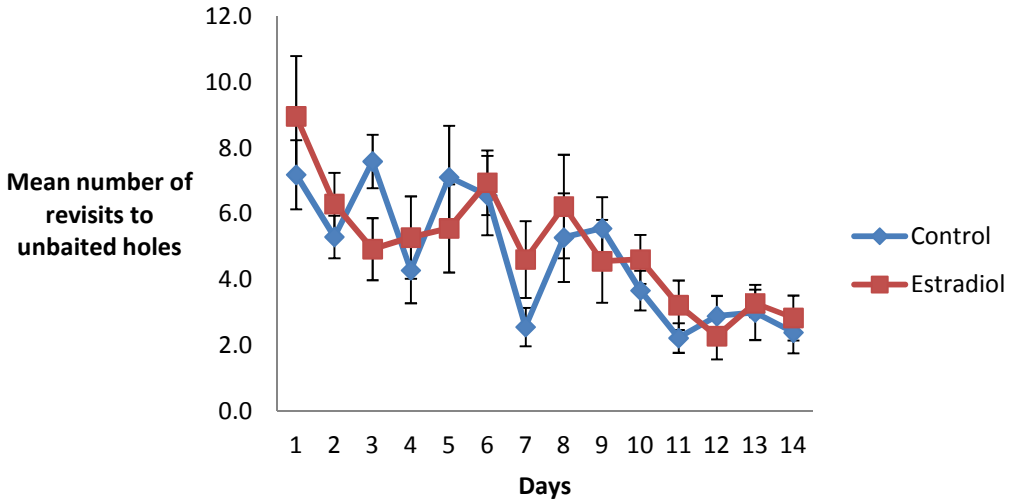

Figure 6. Mean number of revisits to never baited holes across the 14 days of extramaze cue training.

There were no group differences in any of the performance measures. This general improvement in both groups could indicate that both groups were performing equally well but using different strategies to find the baited holes. It also could be that the rats either didn't learn

to precisely locate the baited holes using the available extramaze cues, or despite learning the location of baited holes they were still motivated to explore the unbaited holes despite a lack of access to the food. If the rats were using the extramaze cues during the task, then removing the extramaze cues on a probe test should disrupt performance.

Figure 2 shows the mean latencies to complete the holeboard task during the two probe tests conducted during extramaze training. The probes took place between training days 9 and 10, and between training days 13 and 14. The probe test was a single trial with extramaze cues removed; therefore for comparison the mean latencies on the first trial of the extramaze training days before and after the probe tests are included. Increased latencies on probe tests relative to the before and after days would suggest disrupted performance, however as can be seen in Figure 6 the latencies appear to have decreased slightly on both probe tests compared to the training days that immediately preceded or followed the probe tests. These data were analyzed with a 2 (Probe [P1, P2]) x 3 (Days [Before, during, and After probe test]) x 2 (Groups [Control, Estradiol]) mixed factorial ANOVA. A main effect of probe confirmed that overall the latencies later in training (days 13 to 14) were lower than latencies earlier in training (days 9 to 10), $F(1, 16) = 7.59$, $p = .01$, $\eta_\rho^2 = .32$. This overall decrease in latency reflects continued improvement in performance with additional training. The main effect of days was also statistically significant, $F(2, 32) = 7.89$, $p = .002$, $\eta_\rho^2 = .33$. Additional pairwise comparisons indicated that latencies were significantly faster on the probe tests compared to the preceding day, $p < .001$, but failed to reach statistical significance when compared to the days that followed the probe, $p = .07$. The Probe x Days interaction was not significant $F(2, 32) = 2.39$, $p > .05$, $\eta_\rho^2 = .13$, and no significant interactions with Groups were observed.

The latency data (Fig. 2) suggest that the rats noticed the change (i.e., removal of extramaze cues) on the probe test days, but the change did not disrupt performance. To determine if the improved latencies on probe tests reflected changes in visits to the holes a similar analysis was conducted on the Mean number of visits and revisits to all holes (Figure 4). This time there was no main effect of probe, $F(1, 16) = 2.25$, $p>.05$, $\eta_\rho^2=.12$, and no main effect of days, $F(2, 32) = .94$, $p>.05$, $\eta_\rho^2=.06$. No interactions reached statistical significance.

These results from extramaze training suggest that estradiol had no effect on performance compared to the control group. In addition, it did not appear that either group was relying on the extramaze cues to find the baited holes. The slight but significant decrease in latencies on probe tests suggests that the rats noticed the change, but this decreased latency was not due to significant changes in visits or revisits to the holes. It is possible that the animals weren't using the extramaze cues provided because they weren't good enough landmark cues, or that the animals used another strategy due to the nature of the apparatus and the manipulations made to it. These ideas will be further discussed in the discussion section.

Intramaze Cues

To determine if the rats would use cues if they were better indicators of the baited holes the rats were tested with intramaze cues (black/white contrast) that surrounded the baited holes.

The latency data during the six days of training are plotted in Figure 7. A Groups 2[Estradiol, Control] x Days [6] mixed factorial ANOVA revealed only a statistically significant difference in latencies over days, $F(5, 80) =9.41$, $p<.01$, $\eta_\rho^2=.37$. However, when looking at the graph (Fig. 9) it looks like both groups latencies had plateaued between 50-60 seconds, except on days 2 and 5 which were slightly, but significantly higher, $p<.05$, and lower, $p<.001$, respectively.

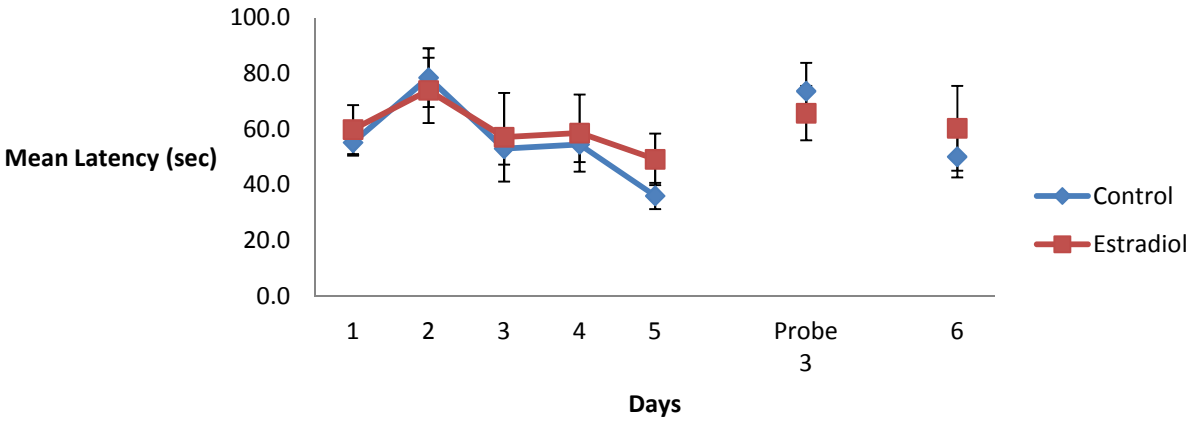

Figure 7. Mean (± S.E) latency of the Estradiol and Control groups to find all four food pellets across the 6 days of intramaze cue training.

Unlike the extramaze cue training, the latencies during intramaze cue training do not suggest any further improvement in performance. However, the amount of different holes visited (Figure 8) and total hole visits and revisits (Figure 9), both showed considerable improvement over days.

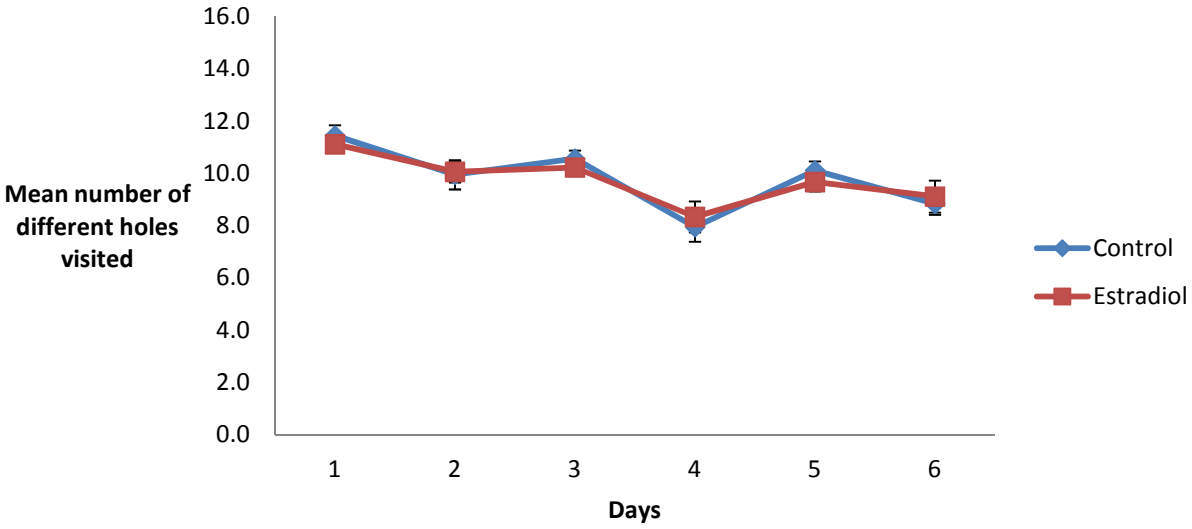

Figure 8. Mean (± S.E) number of different holes visited by the Estradiol and Control groups across the 6 days of intramaze cue training.

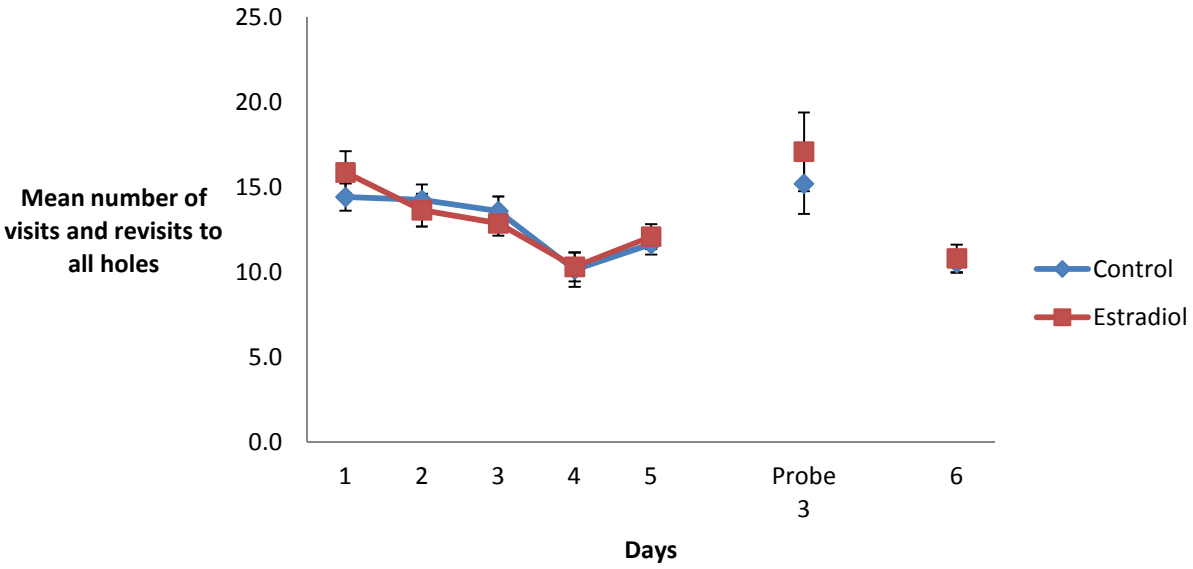

Figure 9. Mean (± S.E) number of visits and revisits to all holes by Estradiol and Control groups across the 6 days of intramaze cue training.

The improved performance over days was confirmed with 2 (Group [Estradiol, Control]) x 6 (Days) mixed factorial ANOVAs. Only the main effect of days was statistically significant for the number of different holes (Figure 8), $F(5, 75) = 12.55$, $p < .01$, $\eta_\rho^2 = .46$ and the number of visits and revisits to all (baited and unbaited) holes (Figure 9), $F(5, 80) = 10.93$, $p < .01$, $\eta_\rho^2 = .41$.

As with the extramaze cues data we next looked at revisits to the baited (Figure 10) and unbaited (Figure 11) holes separately. Despite there being an overall decrease in visits and revisits to all holes, there was no significant decrease in the amount of revisits made by either group to baited holes[Days: $F(5,80) = 1.98$, $p > .05$, $\eta_\rho^2 = .11$]. However, there was a significant decrease in the number of revisits to unbaited holes by both groups, [Days: $F(5, 80) = 6.84$, $p < .001$, $\eta_\rho^2 = .30$]. On average both groups were revisiting less than .5 unbaited holes by the end of intramaze cue training, showing improvement in correct choices.

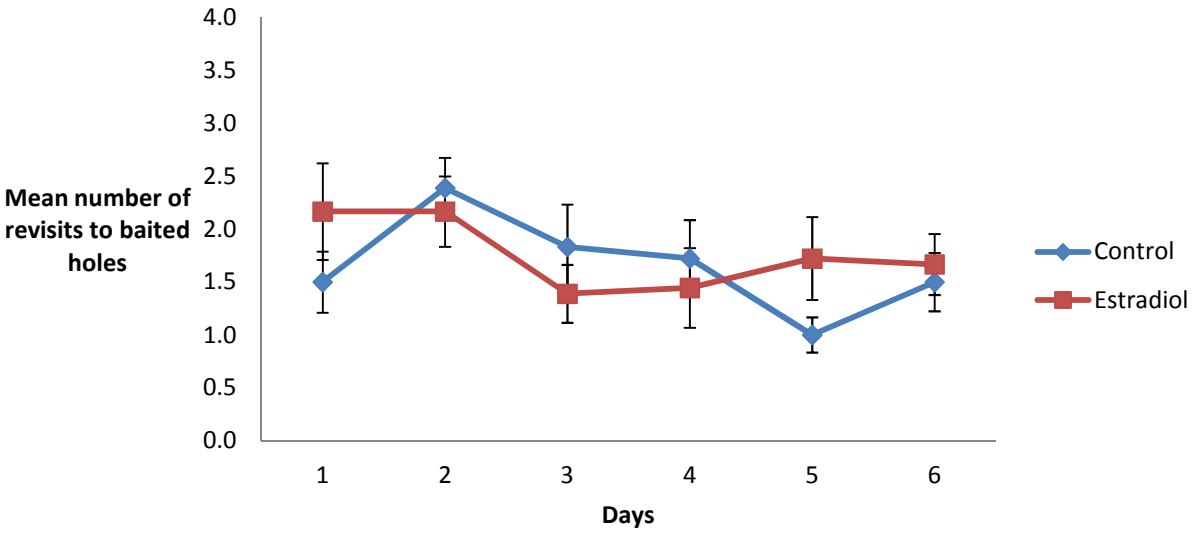

Figure 10. Mean (± S.E) number of revisits to baited holes by Estradiol and Control groups across the 6 days of intramaze cue training.

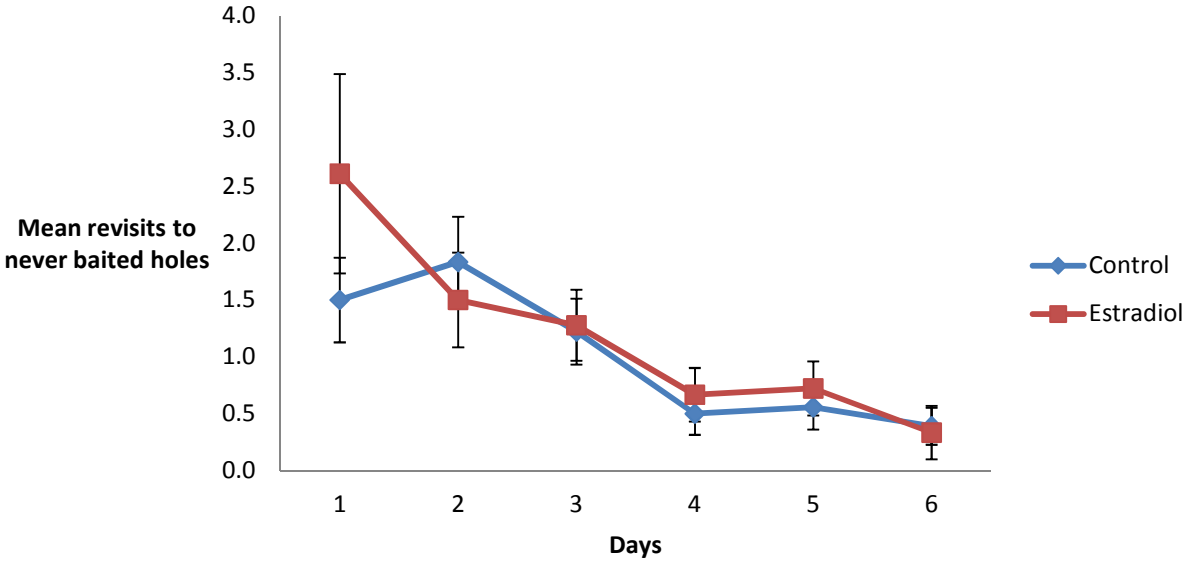

Figure 11. Mean (± S.E) revisits to never baited holes by Estradiol and Control groups across the 6 days of intramaze cue training.

If both groups were relying on the intramaze cues to locate the baited holes and to perform less visits and revisits to unbaited holes, removal of the intramaze cues should disrupt

performance. The right side of Figure 9 shows performance for probe test 3 for total visits and revisits. As with the extramaze cue probe data analysis we performed a 2 (Group [Estradiol, Control]) x 3 (Days [Before, During, and After probe test]) mixed factorial ANOVA using only the first trial from the day before and after the probe test. There was a main effect of days, $F(2, 32) = 4.87$, $p=.01$, $\eta_\rho^2 = .23$, and once again there was no main effect of group, and no group x days interaction. Additional pairwise comparisons indicated that the total number of visits and revisits to all holes was greater during the probe test day (P3) compared to the preceding day, $p<.05$, and the day following the probe test, $p<.05$. These data indicate that both groups were relying on the intramaze cues to more precisely locate the baited and unbaited holes.

To visually show how the rats' performance improved across adaptation, extramaze cue training and intramaze cue training, the AnyMaze paths for three rats have been included. These give a visual representation of how the rats' path changed with training. During adaptation all rats displayed high amounts of thigmotaxis, with training with both extramaze and intramaze cues the rats' path became much more efficient by the end of training.

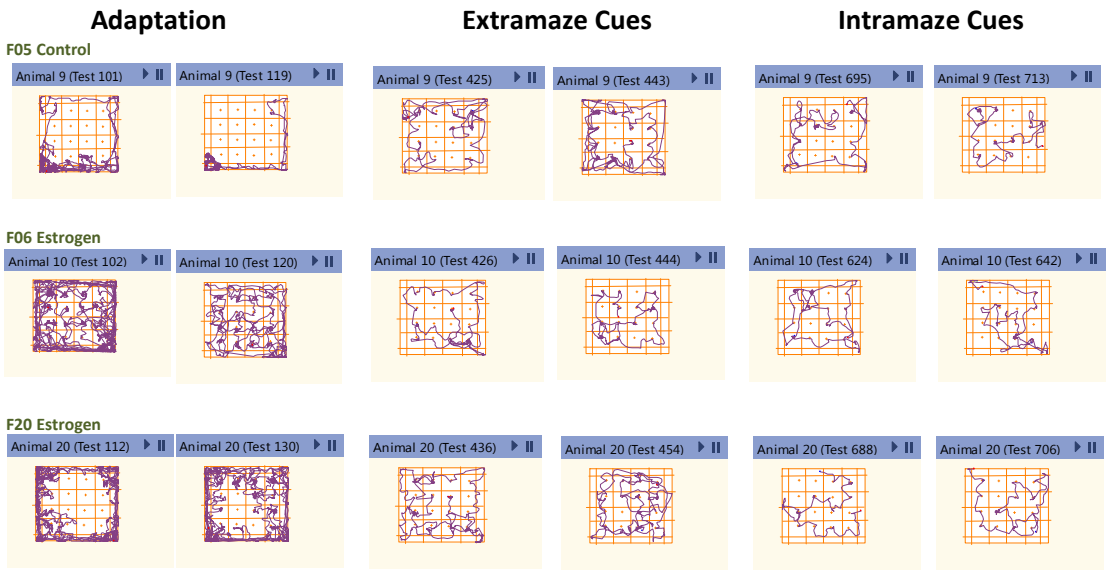

Figure 15. Representative rat paths during the start of training (adaptation) and during Extramaze and Intramaze cue training.

Discussion

The current study was unable to replicate previous results showing that estradiol improves performance on spatial navigation tasks and promotes the use of allocentric strategies in aged male rats. However, there was clear evidence learning did occur during both extramaze and intramaze cue training. Both groups had decreased latencies to find all four holes, decreased visits and revisits to all holes, and a decrease in revisits to unbaited holes. During extramaze cue training the rats' latencies and revisits declined significantly, but they were still visiting a high amount of unbaited holes (Figure 2 & Figure 6) suggesting they didn't efficiently learn to use the extramaze cues to locate the baited holes while avoiding the unbaited holes. Removing the

extramaze cues during the probe tests suggests that they noticed a difference in the apparatus, indicated by the significant decrease in latencies. However, the number of visits and revisits to baited or unbaited holes was not significant, suggesting they may have just explored less during the probe tests during extramaze cue training. When intramaze cues were added to see if performance could be improved there was a significant decline in visits and revisits to all holes (Figure 11) and revisits to unbaited holes (Figure 13). When a probe test was done during intramaze cue training the number of visits and revisits significantly increased suggesting they relied more heavily on the intramaze cues than the extramaze cues. These results indicate that when cues are useful, meaning the cues are more noticeable or relevant, the rats will use them to locate the baited holes.

The data from extramaze cue training and probe tests suggests that neither group was using an allocentric strategy to locate the four baited holes. If they had used an allocentric strategy the rats should have learned to efficiently locate the baited holes, and learn to avoid the unbaited holes, also, the removal of the extramaze cues should have disrupted performance. It's more likely that the rats developed another strategy that they found to be efficient to locate the baited holes. One of the reasons possible for the current findings is that the extramaze cues provided were not sufficient for the animals' to use. The extramaze cues provided were 3 different black and white pictures, two of which were the same size, and one that was larger than the other two. These cues may not have been relevant or distinct enough for the rats to notice and effectively use them as landmarks. It's also possible the rats were unable to differentiate between the images, making them inefficient cues to use. It's also possible that extramaze cues are useful for locating one location like in the Morris water maze (Bergado et al., 2011; Dellu et al., 1997; Guidi et al., 2014; van der Staay & Blokland, 1996) but not when they need to locate

multiple locations, in this case the four baited holes among many incorrect choices (12 unbaited holes). Future research systematically testing different kinds of extramaze cues with various versions of the hole-board apparatus (the hole-board can have anywhere from 4-36 holes and can have multiple levels) is needed to establish which cues are sufficient during this kind of task. It was clear the rats weren't using the provided cues when each probe test was performed. Other studies may have gotten significant results that extramaze cues are being used by allowing the rats to use distal visual cues (room cues) in addition to any provided cues (Korz & Frey, 2007; Kuc et al., 2006; Makracheva-Stepochkina et al., 2008; Meyer & Korz, 2013; Post et al., 2011; Schulz & Korz, 2010; Uzakov, Frey & Korz, 2005; van der Staay, 1999; Vazquez, Vasquez & Pena de Ortiz, 2000; Weichman et al., 1994). By not ensuring that the rats are using extramaze cues (either distal or on the walls of the apparatus) or by performing a probe test, these studies do not provide convincing evidence that the animals were using extramaze cues and not another strategy (Korz & Frey, 2007; Makracheva-Stepochkina et al., 2008; Meyer & Korz, 2013; Post et al., 2011; Schulz & Korz, 2010; Uzakov et al., 2005)

In addition it's possible that because of the various manipulations made to the apparatus, neither group was able to develop an allocentric strategy. Because the maze was rotated daily and the rat was placed in a different corner of the apparatus on each trial they weren't able to develop any kind of motor pattern, which was done to rule this out as a strategy. Past results showing an improvement in spatial memory in the hole-board task using estradiol may have been influenced by fixed pattern of baited holes which allowed the animals to develop a motor pattern, which would not be an improvement in spatial memory or the use of an allocentric strategy (Hoyer et al., 2014; Korz & Frey, 2007; Makracheva-Stepochkina et al., 2008; Meyer & Korz, 2013; Post et al., 2011; Schulz & Korz, 2010; Uzakov et al., 2005). It is possible that the room

cues allowed the rats to develop and use an allocentric strategy; however the studies cited above did not perform a probe test so it's uncertain as to whether a motor pattern developed or the rats really did use an allocentric strategy to find the baited holes. In the future it would be interesting to perform all of the manipulations separately, as well as trying different provided extramaze cues to see which is the most effective in eliciting the use of an allocentric strategy by rats, especially in the hole-board apparatus.

The intramaze cue training data suggests that cues within the apparatus, or cues that the rats can approach and interact with, may be more useful to successfully navigate the hole-board apparatus. In the current study intramaze cues were cut outs of the black contact paper exposing the wood floor underneath. The decrease in revisits to unbaited holes suggests that the intramaze cues were useful for learning to efficiently avoid visiting and revisiting unbaited holes. The probe data confirms this by an increase in visits and revisits to baited and unbaited holes during the probe test. A study done by Gordon and colleagues (2012) marked the baited holes with white tape and were able to randomize the baited hole locations with each trial, again suggesting that cues the rats can approach and interact with are more effective to the rats to find baited holes (Gordon et al., 2012). However, neither group showed a significant decrease in revisits to baited holes. This could be due to more time spent investigating the difference in floor color around the baited holes, especially since all experimenters noticed that the rats spent a lot of time investigating the area around the baited holes only during intramaze cue training. This behavior is similar to animals that begin sign tracking; which is when the animal will approach the stimulus signaling a reward, in this case a sugar pellet. The animal will approach the eliciting stimulus even though they don't need to interact with it and can take away time from obtaining the reward (Burns & Domjan, 1996). In the current study the rats spent time investigating and

returning to the sign for a baited hole, despite this increased investigative behavior having no benefit to the rat. This may be why there was a significant decrease in visits and revisits to unbaited holes, but the latencies were about the same as during extramaze cue training. More time spent investigating the intramaze cues, which are acting as conditioned stimuli for the presence of a sugar pellet, which may be why there wasn't much of a change in latency despite less unbaited hole visits and revisits. The holeboard is less commonly used than the T-maze or Morris water maze for studies of spatial learning. This may suggest that the holeboard isn't the best measure of spatial memory or the use of an allocentric strategy in rats. The manipulations made to the holeboard may have made the task too difficult for the rats to perform allocentrically. They were unable to develop a fixed motor pattern or smell their way through the holeboard to find baited holes. In this case they either had to use the provided cues or develop another strategy. The current study showed that the rats had developed another efficient strategy to complete the task.

Studies suggest that rodents prefer using landmark cues to navigate their way around the various mazes (Daniel et al., 1997; Daniel & Dohanich, 2001; Foster et al., 2003; Lipatova & Toufexis, 2013; Luine et al., 1998; McClure, Barha & Galea, 2013; McConnell et al., 2012; Narenji et al., 2013; Packard, Kohlmaeir & Alexander, 1996). When an animal has learned to efficiently navigate a maze, researchers assume that the animal is using an allocentric strategy to navigate their way around the maze and this strategy is considered to involve cognitive processes. This assumption needs to be confirmed with appropriate probe tests to confirm that allocentric strategies are being used, but often probe trials are not included in the studies. When there is a deficit, researchers assume there is some kind of cognitive deficit in the animal. Many studies have shown impaired performance on spatial navigation tasks in animal models of aging

(Daniel et al., 1997; Daniel & Dohanich, 2001; Foster et al., 2003; Hoyer et al., 2014; Lipitova & Toufexis, 2013; Luine et al., 1998; McConnell et al., 2012; Meyer & Korz, 2013; Narenji et al., 2013; van der Staay et al., 1990). Humans also show impaired learning and memory in spatial navigation as they age (Bimonte-Nelson, Acosta & Talboom, 2010; Bissiere et al., 2005; Hogervorst et al., 2013; Iachini, Ruggeiro & Ruotolo, 2009; Martin, Wittert & Burns, 2007; Verdile et al., 2008; Yeap et al., 2008). If animal models are being used in clinical pre-trials for a development of potential treatment these animal studies must include probe tests to confirm that allocentric strategies are being used.

The current study's data suggests the hole-board may be more useful to assess short term memory performance. Both groups showed decreased revisits to holes with training (Figures 4, 5 & 12), but the amount of different holes visited didn't significantly decrease (Figures 2 & 9). Because both groups were still going to around the same amount of holes, it suggests that the hole-board isn't conducive to assess long term memory performance. Further research needs to be done with different manipulations of the hole-board to determine if long term memory is a useful measure to look at when using a hole-board.

With both extramaze and intramaze cues both groups performed roughly the same, this may be from the dose of estradiol used and the method of administration. The literature on how much 17-β estradiol will produce an effect is highly variable; studies have shown significant effects using dosages ranging from picograms(Daniel et al., 1997; Foster et al., 2003; Kritzer et al., 2001; Locklear & Kritzer, 2014; Luine & Rodriguez, 1994; Luine et al., 1998; McConnell et al., 2012) , to micrograms (Barker & Galea, 2009; Frye et al., 2005; Fugger et al., 1998; Galea et al., 2001; Lagunas et al., 2012; McClure et al., 2013; Meyer & Korz, 2013; Narenji et al., 2013; Packard et al., 1996; Zhang et al., 2008), all the way up to milligrams (Daniel & Dohanich, 2001;

Edinger & Frye, 2006; Frye et al., 2008; Lipitova & Toufexis, 2013; Talboom et al., 2008). These are huge differences that need to be taken in to account when designing a study. One thing that has been agreed upon is that doses that are too low or too high have no or a detrimental effect on spatial memory performance (Barker et al., 2009; Daniel et al., 1997; Foster et al., 2003; Frye et al., 2005; Fugger et al., 1998; Galea et al., 2001; Locklear & Kritzer, 2014; Luine & Rodriguez, 1994; Luine et al., 1998; McClure et al., 2013; McConnell et al., 2012; Meyer & Korz, 2013; Talboom et al., 2008). Future research should include multiple doses of estradiol to demonstrate dose-response effects. Unfortunately, including more doses means more animals are needed to conduct each study. If this is the case future research needs to figure out what is the best does for both sexes, different age groups, and whether estradiol is being used as a neuroprotective agent or as a treatment for already existing deficits. In addition estradiol can be administered via injection, silastic capsule or in food. We chose to use silastic capsules because they are supposed to provide slow and steady hormone release bringing the animal back up to physiological levels. We also used this method because injections need to be given within a certain time range each day to produce constant physiological levels and food pellets with estradiol would be difficult to ensure each rat was ingesting the desired amount . Unfortunately, we were unable to analyze estradiol blood levels in order to confirm physiological levels had been reached. It's possible that estradiol levels were too low or too high contributing to a lack of group differences. In the future blood estradiol levels should be taken to more accurately interpret the results. Further research needs to be done to determine the most effective way to administer estradiol as a therapeutic agent.

In conclusion, estradiol treated rats didn't show an improvement or significant difference compared to controls in long term or short term memory, and didn't seem to be more likely to

use an allocentric strategy to navigate the hole-board apparatus. I was unable to effectively test the hypothesis that estradiol promotes the use of an allocentric strategy in aged male rats since my results suggests that the use of an allocentric strategy doesn't seem to be an effective strategy for navigating the hole-board task. The hole-board does seem to be a good measure of short term memory when evaluating search behavior, as well as other forms of learning such as discrimination learning. Finding out what strategies and tasks estradiol is affecting is an important issue that needs to be addressed. Hormone treatment in both men and women is something that is actively used to help age associated hormonal decline. If estradiol is helping improve age related cognitive deficits we need to determine the most effect dose range, what mechanisms estradiol is actually effecting, and what chronic administration of hormones could do. All of the factors need to be taken into account and looked at before estradiol can be deemed a treatment for age related cognitive deficits.

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