Drew University

College of Liberal Arts

EFFECTS OF DCP-LA ON LEARNING AND MEMORY IN AN *IN VIVO* ALZHEIMER'S DISEASE MODEL WITH OVARIECTOMIZED RATS

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Pearl A. Sutter

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Abstract

Alzheimer's disease (AD) currently impacts an estimated 5.5 million Americans and is the sixth leading cause of death in the United States. By 2050, the number of Americans living with AD is predicted to rise to approximately 13.8 million. Women over 65 are the most susceptible to AD for reasons unknown. Due to the lack of successful therapies for AD, the United States could be bordering on an AD epidemic.

Several promising studies have shown the compound 8-[2-(2-pentyl-cyclopropylmethyl)-cyclopropyl]-octanoic acid (DCP-LA), a PKC-ε activator, to reverse the effects of AD in male *in vivo* models. These studies are limited in their focus on male transgenic *in vivo* models, so this study explored a pharmacological AD model as well as its impact on females.

The Ferrous- Amyloid- Buthionine (FAB) model was used to induce AD pathologies. This model is established by pumping ferrous sulfate, amyloid beta fragments, and buthionine sulfoximine into the lateral ventricle of rats over a four-week period. The FAB model mirrors both behavioral and molecular pathologies of AD and models the role of oxidative stress in AD. Some rats also received ovariectomies (OVX) to model post-menopause conditions, since the female population over 65 is the most susceptible to AD.

Morris Water Maze (MWM) was used to evaluate spatial learning and memory. This is a commonly used test of spatial learning and memory. Intraperitoneal injections of DCP-LA were administered 24 hours prior to the first day of testing. Immunohistochemistry for NeuN and synaptophysin is currently being conducted to determine neuronal and synaptic density differences between treatment groups.

FAB treated animals showed a significant decrease in learning and memory as compared to the controls. OVX rats only showed memory deficits as compared to the controls. The learning and memory performance by FAB animals that received DCP-LA injections, returned to the level of the control. However, the injection of DCP-LA did not restore OVX animals' memory performances. The two main conclusions that can be drawn from these results are that DCP-LA appears to reverse the negative effects of the FAB model, and OVX might be creating memory deficits through pathways unrelated to PKC-ε.

This study shows that the loss of sex hormones in females could majorly impact female neurological health. These results also have exciting prospect for DCP-LA. This compound has now shown to reverse the negative effects of the FAB model in both male and female rats. To date it has shown a lot of promise, but there are many follow up studies to conduct before it can reach human trials.

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Introduction

Alzheimer's Disease: Higher Prevalence in the Female Population

Dementia impacts millions of individuals and families each year with Alzheimer's disease (AD) being the most common form of dementia, representing between 60%-80% of all dementia cases (Alzheimer's Association 2018). According to Alzheimer's Disease International's (ADI) "World Alzheimer's Report 2016," which reported meta analyses of dementia diagnoses from 44 countries, approximately 50 million individuals around the world currently live with dementia; this number is predicted to skyrocket over the next several decades, resulting in an increase to 75 million in 2030, and 131.5 million in 2050. These estimates have dramatically increased since previous estimates made by the ADI in 2010, which estimated 35.6 million cases of dementia in 2010, 65.7 million cases in 2030 and 115.4 million cases in 2050 (Wimo and Prince 2010). As dementia estimates increase, AD also is becoming one of most common causes of death in the world. The World Health Organization published a report ranking AD as the 7th top cause of death globally in 2015, which is an alarming difference compared to its ranking as the 50th top cause of death in 2010 (World Health Organization 2017; Lozano et al. 2010). The Alzheimer's Association 2015 yearly report states that this ranking is considered low however, because AD is not always listed as the primary cause of death on death certificates. Instead it is estimated to be as higher as the third leading cause of death in the world (2015 Alzheimer's)**.**

There are several populations with higher risks of developing AD. One group disproportionately more impacted is females. In a study conducted by Herbert et al. in 2013, researchers found that about 66% of all AD cases are seen in females. Interestingly,

however, many studies have shown that the incidence of AD in males and females is equal until about age 80, after which females have a much higher incidence of AD than males (Fratiglioni et al. 1997; Ott et al. 1998; Gao et al. 1998; Letenneur et al. 1999; Andersen et al. 1999; Ruitenberg et al. 2001; Miech et al. 2002; Roberts et al. 2014). A review by Baum 2005 suggests that women live longer and so the increased prevalence of AD in females is actually a misunderstanding of the population data. Other studies suggest that environmental and cultural differences, sex-linked genetic factors, and hormonal differences all increase a female's susceptibility of AD.

One contributing factor leading to women's higher susceptibility to AD as compared to males could be due to their differences in amount of exercise. A meta-analysis conducted by Norton et al. in 2014 found that physical inactivity was the environmental factor most highly correlated with the development of AD. Considering that women tend to exercise less than men due to societal constraints, this could explain why women are more likely to develop AD (Nomaguchi and Bianchi 2004). Nomaguchi and Bianchi 2004 also found that women are limited more by marriage and parental responsibilities than males, and these social limitations negatively impact their exercise time.

The analysis conducted by Norton et al. also showed that low educational attainment was a second environmental risk factor associated with developing AD (2014). The potential linkage between low education levels and higher incidence and susceptibility to AD has been supported in many studies conducted around the world (Zhang et al. 1990; Fratiglioni et al. 1991; Katzman 1993; Mortimer and Graves 1993; Bickel et al. 1994; Stern et al. 1994; Bonaiuto et al. 1995; Karp et al. 2004; Roe et al.

2007). It is possible that low educational attainment was present for females who currently are living with AD due to the culture in which they were raised. Since the females diagnosed with AD in these studies grew up during the 1920s-1940s, a time when educating females was emphasized less, many of them received lower levels of education (Vemuri et al. 2012). In the modern education system, at least in many cultures, females are now attaining similar levels of education as men and so it is possible the incidences of AD between sexes will equalize in future decades (Ryan and Seibens 2012).

In addition to environmental factors, several genetic risk factors might offer an explanation for higher susceptibility rates of AD in women. Apolipoprotien E (APOE) ε4 is currently the highest risk factor gene for sporadic AD, the most common form of AD (Corder et al. 1993; Slooter et al. 1998; Cosentino et al. 2008). Several studies have linked one copy of APOE ε4 with a 2-3 fold increase in the probability of developing AD, and two copies of APOE ε4 with a 12 fold higher risk of developing AD (Corder et al. 1993; Saunders et al. 1993; Roses 1996; Michaelson 2014). The dramatic increase in AD susceptibility associated with APOE ε4 is alarming because at least 30% of all Americans carry at least one copy of APOE ε4 (Roses 1996). This allele has been linked with impaired cognitive function, synaptic remodeling, and reduced neuronal survival; all of which are major deficits seen in AD brains (Arendt et al. 1997; Ji et al 2013; Teter 2014).

The presence of the APOE ε4 allele also appears to lead to greater neurological troubles in females. In a couple meta- analyses conducted by Farrer et al. 1997 and Altmann et al. 2004, it was found that women with one copy of APOE ε4 have a four fold increase in risk of developing AD compared to individuals with the common APOE allele, APOE ε3. These analyses also found that one copy of APOE ε4 in males did not increase

risk of developing AD at all, and only that males who were homozygous for APOE ε4 showed an increased risk (Farrer et al. 1997; Atlman et al. 2014). Not only does the presence of APOE ε4 increase risk of developing AD, but APOE ε4 also has more severe effects in females than males with regards to memory performance, cortical thickness, hippocampal pathology, and amyloid beta plaque accumulation (Fleisher et al. 2005; Liu et al. 2005; Damoiseaux et al. 2014; Kunzler et al. 2014). It is important to recognize that APOE is not sex linked, so males and females have the same probability of possessing the APOE ε4 allele, but its presence results in more extreme impacts in females (Fallin et al. 2001). APOE ε4 is also not present in every AD patient, so that this gene cannot be considered the only cause for AD development (Ward et al. 2012).

Hormonal differences have also been implicated in the higher susceptibility to AD in women. Women undergo menopause usually in their early 50s, which is followed by a rapid loss of two major sex hormones, 17-β estradiol and progesterone (Nugent et al. 2012). Because 17-β estradiol has been shown to have neuroprotective effects in both *in vitro* and *in vivo* models, researchers suspect that the loss of 17-β estradiol might result in less neuroprotection for the brain, resulting in an increased risk of developing AD (Kwakowsky et al. 2016). This decrease in hormone levels results in females having lower levels of estrogen than males, which supports the hypothesis that females are lacking the neuroprotective features of estrogen that males still have through their conversion of their testosterone to estradiol (Janicki and Schupf 2010).

Although there are many risk factors that have been linked with increased susceptibility in the development of AD in females, it is important to consider that the higher prevalence of AD in females might be due to a combination of these risk factors.

Individuals should focus on eliminating the risk factors that can be removed, such as low educational attainment and physical inactivity, while researchers should continue to focus on understanding how the APOE ε4 gene or low estrogen levels might also pose an increase risk of developing AD. This study will focus on exploring the role estrogen plays in the development of AD and potential therapies to combat this risk factor.

Estrogen

Estrogens are a group of steroid hormones that are critical for proper growth and development in both males and females. Although estrogen is present in both sexes, estrogen levels surpass male estrogen levels around puberty and remain much higher during the entirety of reproductive age from approximately 12 to 49. Estrogen levels in females then drop below the male's estrogen levels after menopause (Dalal et al. 2015). This group of hormones is necessary for the formation of pubertal and reproductive development in females as well as fetal lung formation, and bone maturation and stability (Korach 1994; Cutler 1997; Massaro and Massaro 2004; Brisken and O'Malley 2010). Estrogen has also linked with regulation of metabolism, coagulation, fat deposition, and fluid balance in the peripheral nervous system, and has exhibited neuroprotective and anti-inflammatory effects in the central nervous system (Bonnar 1987; Heine et al. 2000; Stachenfeld and Keefe 2002; Brann et al. 2007; Coyoy et al. 2016; Villa et al. 2016).

The loss of estrogen can lead to serious health problems including osteoporosis, a variety of mental illnesses, and infertility. Osteoporosis is a skeletal disease resulting in fragile bones due to decreased bone density. Estrogen aids in bone reabsorption by increasing osteoclast populations, and so low estrogen conditions are a major risk factor

for osteoporosis (Riggs 2000). For reasons currently unknown, low estrogen levels also are associated with the development of mental disorders such as obsessive-compulsive disorder, depression, and sometimes severe mood swings (Douma et al. 2005). Finally, estrogens are crucial to female reproductive function, and low levels are directly correlated with loss of fertility such as that seen in menopause. Hormone replacement therapy (HRT) has shown to restore hormone levels menopausal women to increase bone density and re-stabilize mood; however the use of HRT remains controversial because it increases the risk of developing fatal breast cancer (Cauley et al. 2005). A specific type of HRT, estrogen replacement therapy (ERT), has also been studied with hopes that the neuroprotective effects of estrogen without progesterone will show more consistent results. These studies produced controversial results similar to studies of HRT; however ERT was not found to increase the risk of developing fatal breast cancer as much as HRT (Ross et al. 2000)

In the central nervous system, effects of estrogen on learning and memory have been widely studied. Studies found that 17-β estradiol, one of the three naturally occurring forms of estrogen, plays a critical role in memory consolidation and enhancement (Davies et al. 2005; Gresack and Frick 2006; Luine 2008). Loss or removal of estrogen corresponds with lower performance on learning as well as and spatial and recognition memory tasks (Monteiro et al. 2005; Luine 2008). Administration of exogenous 17-β estradiol also enhances cognitive abilities during learning, but showed varying effects based on different memory types (Fader et al. 1998; Luine et al. 1998; Fader et al. 1999; Dohanich 2002; Luine 2008; Frick 2009). Spatial memory and working memory both showed improvement with administration of exogenous estradiol, but no

effects were observed on reference memory (Fader et al. 1998; Luine et al. 1998; Fader et al. 1999).

Numerous studies confirm the findings that the loss of 17-β estradiol leads to specific mechanisms that are dysfunctional in AD, including neuroprotection of brain areas involved with learning and memory, modulation of overactivation of immune cells in the brain, and clearance of accumulated amyloid beta (Aβ) plaques (Yamada et al. 1999; Kwakosky et al. 2016; Villa et al. 2016). One particular area of interest, however, is the ability of 17-β estradiol to activate protein kinase C-ε (PKC-ε), a protein that when activated has shown neuroprotective effects (Cordey et al. 2003; Sun and Alkon 2014). Several studies have linked PKC-ε deactivation with development of AD hallmarks (Takashima 2006; Nelson et al. 2009).

Researchers hypothesized that restoring estrogen levels in females with hormonal replacement therapy (HRT) might reactivate PKC-ε in order to prevent or reverse AD. Many studies were conducted using hormonal replacement therapy (HRT) to reduce the risk of developing AD, but the results are controversial (Mielke et al. 2014). Some studies showed that if HRT treatment was started after menopause, the risk of developing AD was reduced (Henderson et al. 2005; Morrison et al. 2006; Whitmer et al. 2011). However, a study conducted by Shumaker et al. 2004 concluded that women who received HRT after age 65 actually had an increased risk to AD as compared to the controls. Additional studies concluded that HRT does not improve cognitive function in AD patients, and so HRT should mainly be used as a preventative therapy (Mulnard et al. 2000; LeBlanc 2001; Daniel et al. 2006). In order to explain these contradicting results, Rocca et al. 2011 suggested that HRT only has an impact on the cognitive abilities of

women at certain times in relation to the onset of menopause. This study suggested that there is narrow window immediately following menopause, during which HRT could show preventative effects (Rocca et al. 2011). This finding was promising until the side effects of HRT were considered. HRT has directly been linked to increasing risk of fatal breast cancer, ovarian cancer, and cardiovascular disease if used for over 10 years (Nabulsi et al. 1993; Collaborative 1997; Lacey et al. 2002; Rossouw 2002; Chlebowski et al. 2003). Because HRT would be used as a preventive therapy for AD, HRT would be introduced without knowledge if the individual would develop AD and so the risk of breast cancer would increase regardless if the neuroprotective effects of HRT were needed. Further studies need to be conducted to understand whether HRT therapy is an option for reducing AD prevalence in females before it is pursued as a treatment option.

PKC-ε and AD

Three of the main hallmarks of AD are the accumulation of Aβ plaques, tau hyperphosphorylation, and oxidative stress, all of which stem from PKC-ε activation. Aβ plaques are accumulations of naturally produced proteins in the brain which can stick to each other and block synaptic communication (Mohktar et al. 2013). This can lead to neuronal cell death and disrupted signaling throughout the brain (Cras 1991). There are two main mechanisms explaining the modulation of Aβ plaque levels by PKC-ε. A study by Zhu et al. in 2001 concluded that PKC-ε promotes cleavage of the amyloid precursor protein (APP) by α-secretase rather than β-secretase. Two neuroprotective fragments are produced from APP after cleavage by α-secretase, whereas Aβ is the result from cleavage by β-secretase (Klatt et al. 2013; Gouras et al. 2015). By increasing activity of α-secretase,

PKC-ε would lower Aβ production, leading to reduced accumulation of Aβ plaques (Zhu et al. 2001). This study was supported by several additional studies (Akita 2001, Nelson et al. 2009, and Hongpansian et al. 2011). Eckman et al. 2001 proposed a second mechanism the same year as Zhu et al. 2001, hypothesizing that PKC-ε increases the activity of the enzyme endothelin-converting enzyme-1 (ECE-1), which degrades Aβ. Several follow-up studies have confirmed this finding (Funalot et al. 2004; Choi et al. 2006). The development of a PKC-ε activator would be useful for breaking down current Aβ plaques, while also limiting Aβ plaque production.

PKC-ε is also involved in the mechanisms underlying tau hyperphosphorylation. In healthy brain, the cytoskeletons of neurons are structurally supported and stabilized by microtubules bound to tau proteins (Hammond et al. 2008; Wang et al. 2013). However, in an AD brain, glucose synthase kinase 3β (GSK-3β) phosphorylates tau proteins, thus removing their ability to bind to and provide structural support to the microtubule (Takashima 2006). The hyperphosphorylated tau also binds to normal tau proteins and further destabilizes the microtubule (Santa-Maria et al. 2012). PKC-ε inhibits GSK-3β through both direct inhibition and also indirect inhibition by reducing the production of $A\beta_{1\text{-}42}$ protein, which is a GSK-3β activator (Isagawa et al. 2000). The reduction of Aβ protein could be due to either of the two mechanisms described above.

PKC-ε is also involved in the oxidative stress seen in AD. While some reactive oxygen species (ROS) are present in the normal brain, in AD, the levels of these potential damaging agents can become excessive (Andreyev et al. 2005). ROS, which include oxygen radicals, hydrogen peroxide, and hypochlorous acid, the oxygen radicals and hydrogen peroxide can generate hydroxyl radicals, which are highly reactive and can

result in tissue damage when present in high amounts (Leeuwenburgh and Heinecke 2001; Andreyev et al. 2005). In a study by Maher 2001, PCK-ε was found to protect neurons from oxidative stress-induced cell death. The neuroprotective effects of activated PCK-ε were supported by two follow-up studies in 2003 that found the activation of PKC-ε beneficial in two different oxidative stress-induced cell death models (Di Capua et al. 2003; Uemura et al. 2003). The study by Uemura et al. 2003 suggested that PKC-ε might be involved in suppression of glutamate receptors to reduce damage from methamphetamine-induced cell death. Di Capua et al. 2003 found that PKC-ε has protective effects on cell survival in ischemia-reprofusion injury models. In both studies, the exact mechanistic properties of PKC-ε were not determined. Instead activation of PKC-ε was linked with neuroprotection in these oxidative stress conditions.

The involvement of PKC-ε in AD makes it a promising target for AD therapies. Because PKC-ε is not activated in high levels in AD, researchers are currently exploring PKC-ε activators with hopes that the re-activation of PKC-ε will show preventive or even curative effects.

DCP-LA: A PCK-ε activator

One particular PKC-ε activator that has shown promise as an AD therapy is a linoleic acid derivative called 8-[2-(2-pentyl-cyclopropylmethyl)-cyclopropyl]-octanoic acid (DCP-LA). First synthesized in 2003 by Tanaka and Nishizaki, DCP-LA is a relatively new compound, and therefore has only received limited attention. Even though DCP-LA has not been extensively studied, the several studies that explore the function

DCP-LA in both *in vitro* and *in vivo* model systems have shown consistent and exciting results for its potential as an AD therapy.

The *in vitro* studies aided researchers in both determining DCP-LA's function as well as its potential as a successful drug compound. Two studies, one by Takana and Nishizaki 2003 and the other by Kanno et al. 2006, found that DCP-LA specifically activates PKC-ε with a greater than 7-fold potency over other PKC isoenzymes. This specificity for PKC-ε is hypothesized to be due to DCP-LA's affinity for the phosphatidylserine binding site, a binding site required for PKC-ε activation, and PKC-ε activation being calcium independent (Kanno et al. 2006; Sun and Akon 2014). Sun and Akon propose that because PKC-ε activation is calcium independent, activation of PKC-ε only requires binding to the phosphatidylserine binding site, whereas the need of calcium for other PKC isoforms limits their activation levels when DCP-LA binds to their phosphatidylserine binding sites (2014). This would explain the observed 7-fold higher activation of PKC-ε by DCP-LA as compared to other PKC isoforms.

These studies also characterize DCP-LA as an excellent drug candidate because they demonstrate DCP-LA's affinity and specificity, two critical considerations for medications. DCP-LA's high specificity and affinity for PKC-ε is important because high specificity and affinity can help eliminate unwanted side effects through non-specific binding, helping to focus the treatment (Roy and Chaguturu 2017). DCP-LA also demonstrated ability to cross the highly selective blood brain barrier, which is a major challenge in drug development for neurological disorders (Takana and Nishizaki 2003). In order for medications to have effects on brain chemistry, they need to be able to access the brain by crossing the blood brain barrier. This finding was confirmed by several *in*

vivo studies that showed neurological impacts of DCP-LA after administration of DCP-LA through intraperitoneal injections (Nagata et al. 2005; Nagata et al. 2010; Yaguchi et al. 2010; Hongpasian et al. 2011).

The *in vitro* studies are important for understanding mechanisms underlying DCP-LA's function, but the *in vivo* studies helped examine DCP-LA in a system. In *in vivo* studies conducted to date, the administration of DCP-LA restored learning and memory abilities, protected neurons from oxidative stress, and prevented synaptic loss, Aβ accumulation, and tau hyperphosphorylation (Nagata et al. 2005; Nagata et al. 2010; Yaguchi et al. 2010; Hongpasian et al. 2011; Candia 2017). The reduction of Aβ accumulation and tau hyperphosphorylation, as well as neuronal protection from oxidative stresses are direct linkages to impacts DCP-LA has on specific AD pathologies. In addition to demonstrating impacts on the molecular level, DCP-LA administration also reversed more complex behaviors such as learning and memory. The restoration of learning and memory in AD models is particularly exciting because the current AD drugs are limited to only slowing the progression of AD, not reversing the behavioral deficits (Alzheimer's 2017). These results demonstrate that DCP-LA's activation of PKC-ε is important in eliminating AD disease pathology, and further support targeting PKC-ε for AD therapies.

One major limitation of these studies is that they were only conducted in male animals. As was previously discussed, AD impacts females disproportionality more than males, and so females are an important part of the AD population to study. One hypothesis to explain increased female susceptibility to AD suggests that women's loss of 17-β estradiol after menopause leads to diminished PKC-ε activation. Because HRT

neither restored PKC-ε activity nor learning and memory deficits in a majority of previous studies, the introduction of a different PKC-ε activator, such as DCP-LA, might be needed. Both DCP-LA and 17-β estradiol activate PKC-ε, but DCP-LA has proven more successful in *in vivo* studies than 17-β estradiol. It is possible that DCP-LA will not only restore cognitive deficits caused by the AD model, but also deficits resulting from the loss of 17-β estradiol.

Experimental Design

In order to investigate DCP-LA as a potential AD therapy in females, the appropriate *in vivo* models need to be selected including models for AD and menopause. This study selected the Ferrous Amyloid Buthionine (FAB) model because it mimics late onset AD through its production of amyloid beta plaques and oxidative stresses (Lecanu et al. 2006). Late onset AD, also known as sporadic AD, is important to study as opposed to early onset, or genetically linked, AD because late onset AD makes up about 95% of all AD cases (Alzheimer's 2018). The FAB model, developed by Lecanu et al. 2006, is the only sporadic AD model that elicits two of the three hallmarks of AD as well as produces learning and memory deficits. Previous DCP-LA studies conducted by Nataga et al. 2005 and Nataga et al. 2010 attempted to model sporadic AD with intracerebroventricular infusions of amyloid beta. Although this model led to the formation of amyloid beta plaques and produced learning and memory deficits, it is limited in its ability to model more than one hallmark of AD. The FAB model, first used in combination with DCP-LA by Candia 2017, combines two of the three hallmarks creating an AD model producing more of the AD pathologies. It is important to recognize that the FAB also has its limitations because it cannot induce neurofibrillary tangles;

however it is one model of sporadic AD that currently exists (Lecanu and Papadopoulos 2013).

Another important characteristic of in females is low levels of 17-β estradiol. A dramatic drop in estrogens and progesterone occurs after menopause in older women, and this drop is thought to be a risk factor for developing AD. Ovariectomies (OVX) in rats are a common model for menopause because they demonstrate both similar drops in estrogen levels and decreased bone density as are seen in human menopause (Kalu 1991). This study aims to create a post menopause sporadic AD model through combination of OVX with FAB that can be utilized for studying AD in females. No other studies are known to have combined FAB with OVX to model AD in females. One possible limitation to using OVX to model menopause is the difference in timing of hormone reduction between the gradual process of menopause and the abrupt surgical process of an OVX. The OVX leads to similar symptoms of menopause, but a more accurate model would mimic the slow hormone decrease observed in menopause.

The combination FAB and OVX to create the post menopausal sporadic AD model will aid in determining if DCP-LA can be used as a replacement for 17-β estradiol to restore PKC-ε activation in AD. If learning and memory deficits are observed from the OVX treatment, this study will help to answer if an injection of DCP-LA will restore learning and memory deficits from OVX as well as deficits caused by the FAB model.

Major deficits observed in AD include declines in learning and memory (Alzheimer's 2018). A commonly used learning and memory test, the Morris Water Maze (MWM), can be employed to quantify spatial learning and memory performance (Morris 1984). This test measures the latency for rats to reach a platform hidden beneath the

surface of opaque water in a ~1.7 meter diameter pool over a five day learning period. The learning period is followed by a memory test measuring the rat's ability to remember where the platform was located, even after it has been removed. This test is often used when examining spatial learning and memory behavior in AD research because spatial awareness is a major part of cognitive decline observed in AD progression (Alzheimer's 2018). There are several limitation to the MWM use to measure learing and memory including an inability to alter motivation level to find the platform, and that immersion into water might cause additional stresses in the rat that might impact results (Morris 1984)

In conclusion, this study mainly strives to determine if DCP-LA can be used as a replacement for 17-β estradiol to restore learning and memory deficits caused by a post menopause sporadic AD model. Both DCP-LA and 17-β estradiol activate PKC-ε, a kinase that's activation has been linked with protecting the brain and possibly revering the deficits from AD hallmarks. However, because exogenous 17-β estradiol has not reversed learning and memory deficits in AD, this study aims to determine if DCP-LA might re-activate PKC-ε to restore the learning and memory lost.

This experiment has several hypotheses:

1. FAB treated rats *((+)FAB (-)OVX (-)DCP-LA)* will perform significantly worse on MWM learning and memory tests than controls *((-)FAB (-)OVX (-)DCP-LA)*
2. OVX rats *((-)FAB (+)OVX (-)DCP-LA)* will perform significantly worse on MWM learning and memory tests than controls *((-)FAB (-)OVX (-)DCP-LA)*

3. The FAB-OVX treatment group *((+)FAB (+)OVX (-)DCP-LA)* will perform significantly worse on learning and memory tests than controls *((-)FAB (-)OVX (-) DCP-LA)*
4. DCP-LA administration will reverse the negative effects on learning and memory in all (+)FAB treated rats.
5. DCP-LA administration will reverse negative effects on learning and memory in the (+)OVX rats

Materials and Methods

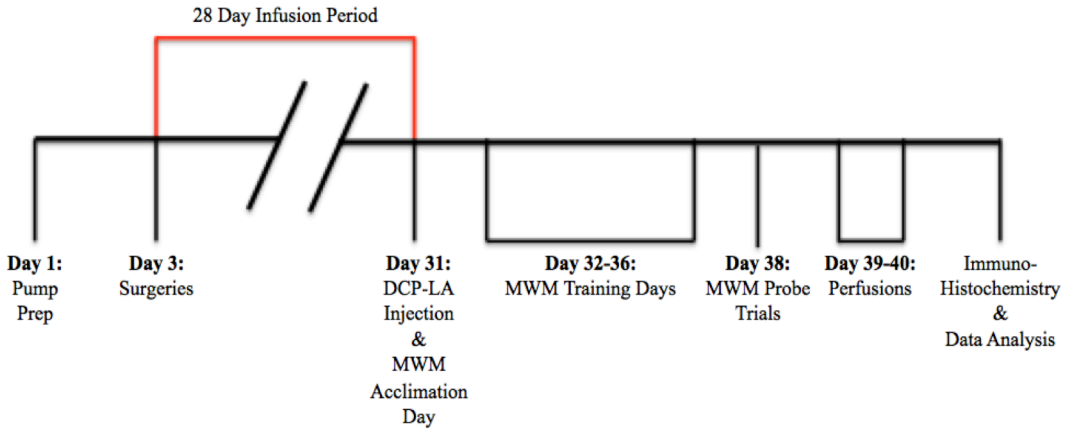

Figure 1: Experimental Timeline. All major experimental procedures are included to show the layout of this project. Day numbers are included to indicate when time sensitivity is important.

Subjects and Treatment Groups

48 female Sprague-Dawley rats weighing between 210 and 280 grams were used for this experiment. Animals were housed in pairs for the entire duration of the experiment except the first day following surgery. Immediately following surgery and the first post-surgical day, each rat was housed individually and recovery closely monitored. The rats were given access to food and water ad lib and housed under standard 12 hour light/dark conditions. All handling and experimental procedures were approved by the Drew University Institutional Animal Care and Use Committee and done in accordance with the Association for Assessment and Accreditation of Laboratory Animal Care standards.

A baseline locomotor measure of each animal's activity was taken in a locomotor chamber using infrared beam sensors to measure the horizontal activity (HA) and vertical

activity (VA) of each rat over a 10-minute period. The locomotor data was used to divide animals into counterbalanced groups. The rats were then treated +/- FAB, +/- OVX, +/- DCP-LA. There were 8 total groups: control, FAB, OVX, DCP-LA, FAB + OVX, FAB + DCP-LA, OVX + DCP-LA, FAB + OVX + DCP-LA (6 rats per group). An experimental timeline is shown in Figure 1.

Solutions

FAB (adapted from Candia 2017)

A solution containing 1.68 μM ferrous sulfate, 25.2 nM amyloid beta, and 20.16 μM of buthionine sulfoximine (FAB) was prepared in batches according to the methods described in Candia 2017, which were adapted from Lecanu et al. 2006. Briefly, 0.01 g of ferrous sulfate, 3.01 μL of 100 μM amyloid beta, and 0.09 g of buthionine sulfoximine was added to 10 ml 0.9% saline. Animals received intracerebroventricular (ICV) infusions of either FAB or 0.9% saline via osmotic mini pumps at a rate of 0.25 μL/hr for 28 days.

DCP-LA

Animals received either DCP-LA (1 mg/kg in 5% dimethyl sulfoxide (DMSO); i.p.) or 5% DMSO 24 hours prior to the first day of Morris Water Maze Training (same day as acclimation). The DCP-LA solution was a 6 μM stock made in 5% DSMO; average injection volume was 0.28 mL.

Osmotic Pump Filling

FAB was delivered via Alzet miniosmotic pumps (model 2004) and Brain Infusion Kit 2. The model 2004 mini osmotic pumps have a reservoir volume of 200 μL

and pump at a rate of 0.25 μL/hour for 28 days. Brain Infusion Kit 2 includes polycarbonate cannulae each with a 28 gauge stainless steel tube measuring 5.0 mm in length, 4 additional 0.5 mm polycarbonate spacers, and 15 cm polyvinylchloride catheter tubing with a 0.69 mm inside diameter.

Osmotic pumps were filled according to the Alzet's Mini Osmotic Pump Model 2006 manufacturer's instruction manual. Individual osmotic pump weights were recorded prior to being filled with either FAB or 0.9% saline. The provided blunt 27 gauge filling tube, attached to a 1 mL syringe, was filled with 250 μL of the desired solution and inserted into the pump opening. Once the pump was filled, the flow moderator was inserted and each new pump weight was recorded to measure the fill volume. If the fill volume was less than 90% of the desired volume, the pump was refilled.

To prepare each cannula for pump attachment, 4 spacers were added to reduce the depth of the cannula from 5 mm to 3 mm. The cannula was attached to pre-cut 6.5 mm catheter tubing. The 27 gauge filling tube filled with the desired solution was inserted into the open end of the catheter tubing to fill the entire length of tubing and cannula. The cannula tubing was attached to the pump via the flow moderator. The pumps were divided by fill solution and then placed in 0.9% sterile saline at 37 ^{o}C for 40 hours prior to implantation.

Surgical Procedures

Stereotaxic Surgery- Pump Insertion (FAB)

All surgical tools were sterilized a MicroBead Sterilizer prior to the start of each surgery. Each animal was anesthetized with ketamine (90 mg/kg) and acepromazine (10 mg/kg) i.p. and then shaved from immediately behind the eyes to the base of the neck.

The animal was secured in the stereotaxic apparatus via proper placement of the ear, tooth, and nose bars. The shaved area was cleaned with an alcohol swab and sterilized with povidone-iodine. A size 10 scalpel blade was used to make an incision from behind the rat's eyes to the base of its ears. Two bulldog artery clips were used on each side of the incision to hold back the skin and expose the skull. A suprascapular pocket was created for the pump by inserting blunt scissors and opening them to expand the space. The pump was removed from saline by mouse tooth forceps, rinsed in 70% ethanol, and inserted into the pocket. The cannula, attached to the pump by the catheter tubing, remained outside the body and was wrapped in a Kimwipe. Once the pump was secured, the fascia was scrubbed with cotton swabs to reveal the skull. Hydrogen peroxide was rubbed on the skull as an antiseptic. A thin layer of styptic powder was spread on the skull to stop any bleeding and dry the skull, then rubbed off. Flat skull was confirmed by calculating difference between the lamda's and bregma's dorsal-ventral coordinates. After bregma was located, the t-pin was moved to the desired location, above the left lateral ventricle, of 0.9 mm anterior and 1.4 mm laterally to the left (Lecanu et al. 2006). A hole was drilled in this location and cotton swabs were used to control any bleeding. The t-pin was switched out for an alligator clip to hold the cannula. After the top of the cannula was secured in the alligator clip, the cannula was positioned over the drilled hole and lowered 33% of the desired depth. Dental cement was mixed and applied on the skull directly underneath the cannula. The cannula was lowered down to the 3.0 mm desired depth and another layer of dental cement was applied to secure the cannula to the skull. A third layer of dental cement was applied after the second layer was dry. During two wait times, ovariectomies were performed on appropriate animals (see procedure below).

Once the third layer was dry, the size 10 scalpel blade used previously was heated over a Bunsen burner and used to remove the tab on the top of the cannula to allow the scalp to be fully closed over the cannula apparatus. To close, any excess catheter tubing was tucked into the suprascapular pocket, the bulldog artery clips were removed and the incision was stapled closed with wound clips. Antibiotic ointment was administered to the incision. The animal was wrapped in a towel and returned to its home cage for recovery. The animal was monitored daily for signs of distress and additional antibiotic ointment was administered as needed.

Ovariectomy (OVX)

During the wait times for the dental cement to dry, the ovariectomies or sham surgeries were performed. Rats receiving ovariectomies had previously been shaved from the bottom of their ribs to the top of their back leg on each side. The exposed skin was sterilized with povidone-iodine swabs and a 2 cm incision was made ~1.5 cm lateral to the spine that extended caudally from the bottom of the rib. Blunt scissors were inserted under the skin at the incision and opened to further expand the incision. The muscle layer was cut through to reveal the open cavity underneath. The ovary was removed from the abdominal cavity and the uterine horn was ligated. The ovary was removed and the uterine horn was replaced into the abdominal cavity. The ovary was tied off and removed. The muscle layer was sutured together with surgical silk, the skin closed with wound clips, and antibiotic ointment was administered to the incision. The procedure was repeated on the other side. After surgeries, animals were housed individually until they regained consciousness. They were then returned to pair housing and left undisturbed for 28 days.

Morris Water Maze

On day 28 following surgeries, animals began Morris Water Maze (MWM) testing. The MWM testing was done in a pool (~1.7 meters in diameter) surrounded by four curtains, each with a different pattern to provide distal visual cues during testing. The pool was divided into four quadrants using Anymaze software. A platform was placed in the center of quadrant 1. The pool was filled with water ($27^{o}C$ +/- $2^{o}C$) until it measured 1 cm above the top of the platform, so that it was shallow enough for the rats to remove themselves from the water when standing on it. The water temperature and height were maintained throughout the entire testing procedure including acclimation, training, and the probe trials. The room was indirectly lit by four lamps with 60 watt incandescent light bulbs. During the entirety of testing, Anymaze software was used to record rat behaviors.

Acclimation

Acclimation was performed the day prior to the onset of training days to allow rats to adjust to MWM conditions. Rats were placed in the darkened MWM room for an hour prior to the beginning of acclimation to adjust to the light conditions. During the acclimation period, each rat was placed in the quadrant opposite the platform (quadrant 3) and given 120 seconds to find the platform. If it did not find the platform in the allotted time, the rat was guided to the platform. Once the rat reached the platform, either independently or with help, it was left to orient itself for 30 seconds before being dried and returned to its original cage. During the acclimation period, the water remained clear so that the platform was visible to the rats under the water.

Training

One the first day of training, the water was made opaque with nontoxic black tempera paint to hide the platform's location. Each day of training consisted of four trials for each animal, where the animal's starting position in each of the four quadrants was pseudo-randomized to ensure that the rats did not have similar starting quadrant on consecutive training days. During each trial the animal was placed in the pool facing the wall and given 90 seconds to find the platform. If the animal did not find the platform in the allotted 90 seconds, it was led to the platform. Once on the platform, the rat was allowed 30 seconds to orient itself before being dried off and placed in a holding cage for 30 seconds until the start of the next trial. After the fourth trial, the rat was dried and returned to its original cage. The time it took from the rat being placed in the pool to the time it took to find the platform (latency to platform) was measured. The latency to platform for each day was calculated by averaging the four trial times.

Probe Trial

The probe trial took place 48 hours after the final training day. The conditions were identical to those used during the training days except that the platform was removed. Each rat was placed facing the wall in the opposite quadrant to the one originally containing the platform and allowed to swim freely for 120 seconds. The time taken to reach the area where the platform was during training (latency to platform-probe), the total time spent in the platform area (total time platform), and the total time spent in the quadrant previously containing the platform (total time quadrant 4) were recorded.

Data Analysis

SPSS version 23 was used to run required statistical analyses. A repeated measures ANOVA was conducted to analyze learning during the training days. To analyze memory data collected during the probe trial, a three-way ANOVAs was conducted with FAB, OVX, and DCP-LA as factors. In all cases, the α-value was set to 0.05.

Results

Weight Differences and Locomotor Activity

Animals were weighted on the day of surgery and four weeks post surgery (the day before the beginning of MWM testing). OVX animals showed significantly more weight gain over the four-week period than intact animals ($p < 0.005$; Figure 2). There were no other significant weight differences between treatment groups.

Animals received baseline locomotor testing to measure their activity levels four weeks post surgery. There were no significant differences seen in either horizontal or vertical locomotor activity between any of the treatment groups (Figures 3A, 3B).

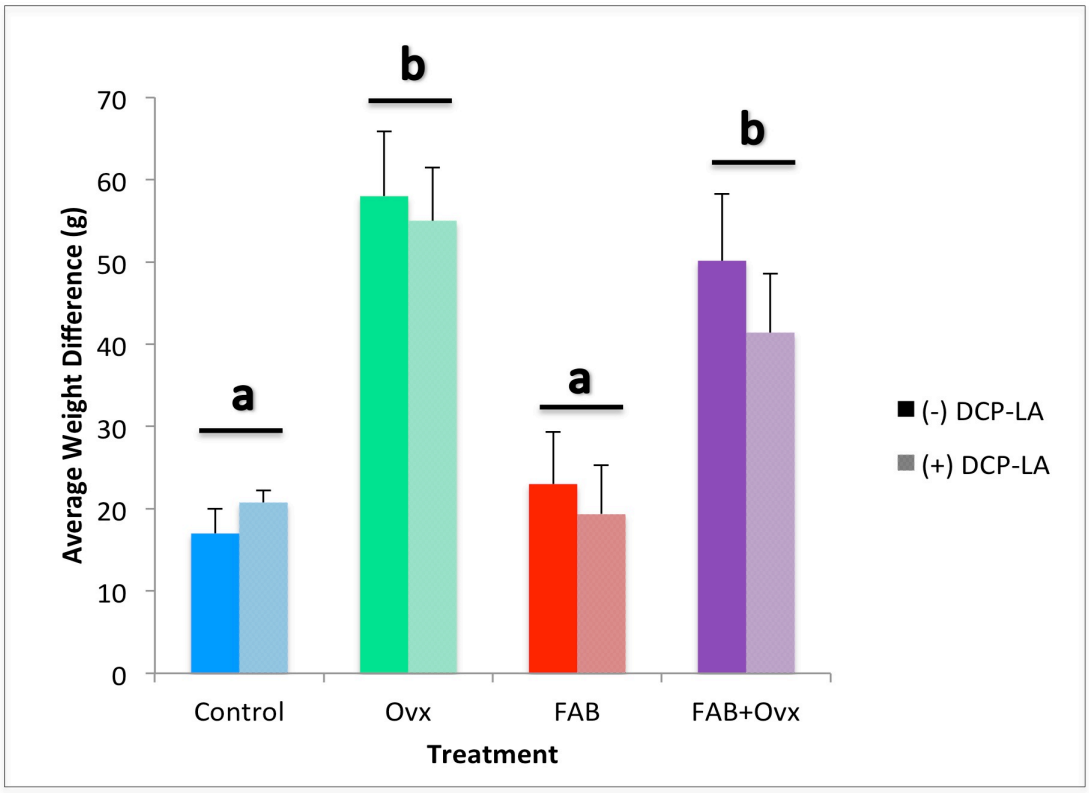

Figure 2: Weight Differences Pre-Surgery to Four Weeks Post-Surgery. The weight pre-surgery was subtracted from the weight of animals four weeks post surgery. OVX animals showed significantly higher weight gain when compared to the intact animals ($p < 0.005$). No other significant differences were observed between other treatment groups. **a** is significantly different from **b** ($p < 0.0005$). Data are all mean +/- S.E.M.

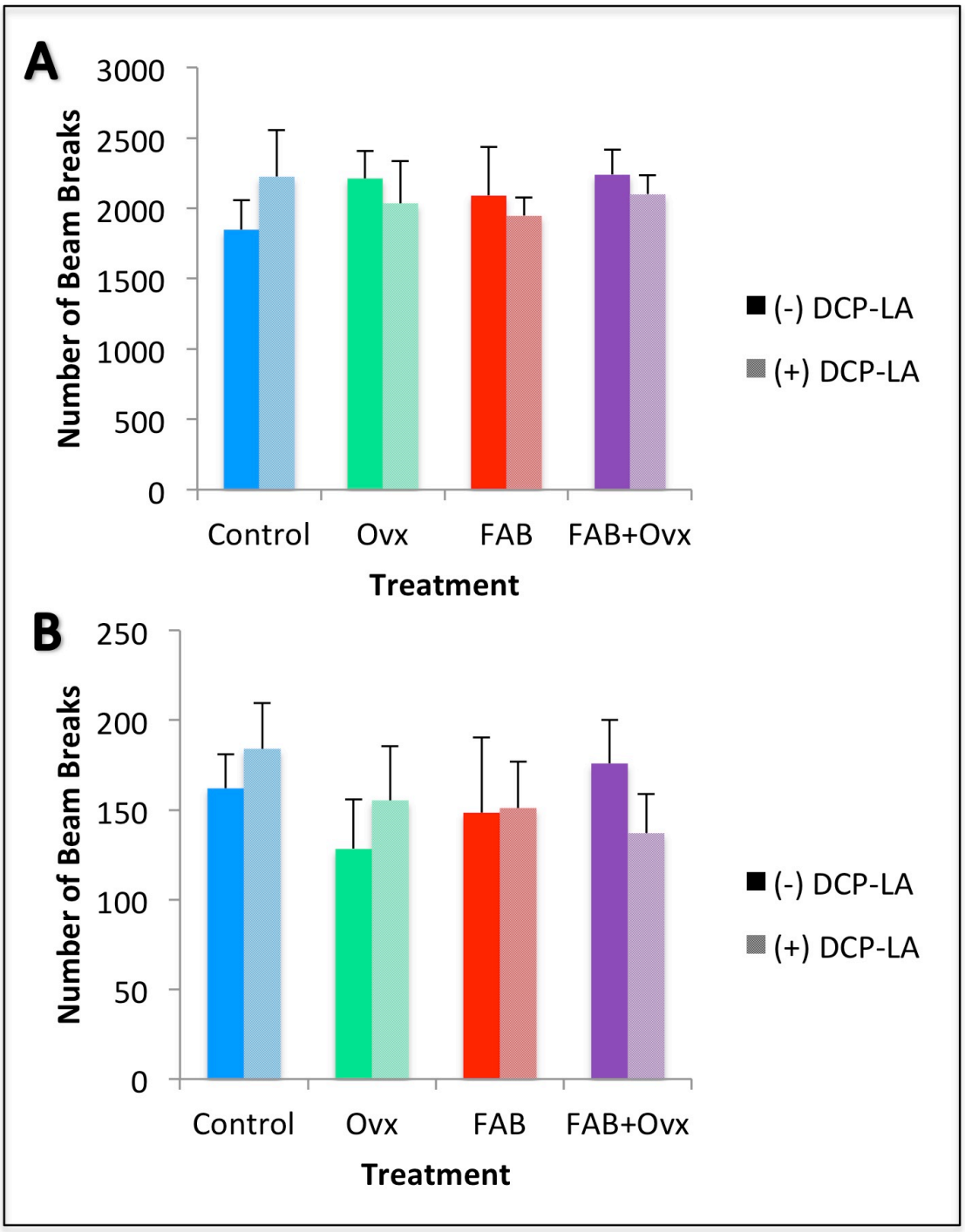

Figure 3: Locomotor Activity. A. Horizontal locomotor activity for each animal was measured four weeks post-surgery. No significant differences were observed between treatment groups. **B.** Vertical locomotor activity was measured for each animal four weeks post-surgery. There were no significant differences observed between treatment groups. Data are all mean +/- S.E.M.

Morris Water Maze

Learning Trials

Four weeks post surgery, animals underwent 5 days of training in the MWM. Each day consisted of 4 timed trials, where latency to find a hidden platform in a pool of water was measured. The four trials for each animal were averaged and then the mean and standard error of the mean was calculated for each treatment group each day. A main effect of time was seen across all treatment groups showing that as the learning trials progressed, the time to find the platform decreased ($p < 0.0005$; Figure 4A and 4B). Latency to platform for FAB and FAB + OVX animals were significantly higher on Day 5 than the control ($p < 0.0005$), DCP-LA ($p < 0.0005$), OVX ($p < 0.0005$), and OVX + DCP-LA ($p = 0.011$, $p = 0.004$) (Figure 4A). FAB and FAB + OVX treated animals also showed higher latency to platform times than the FAB + DCP-LA (both $p < 0.0005$) and the FAB + OVX + DCP-LA ($p = 0.014$, $p = 0.005$) treatment groups on Day 5 (Figure 4A). No difference was observed between the control animals and the FAB + DCP-LA treatment group on Day 5, and no difference was seen between the Control animals and the FAB + OVX + DCP-LA treatment group on Day 5.

Because no difference was observed between OVX treated animals and their respective controls, OVX and intact animals were pooled with their respective controls. Latency to platform for FAB treated animals was significantly higher than the Control on Day 5 ($p < 0.0005$; Figure 4B). FAB treated animals also had longer latency to platform times than FAB + DCP-LA treatment groups on Day 5 ($p < 0.0005$; Figure 4B). No significant differences were seen between the Control and the FAB + DCP-LA treatment group.

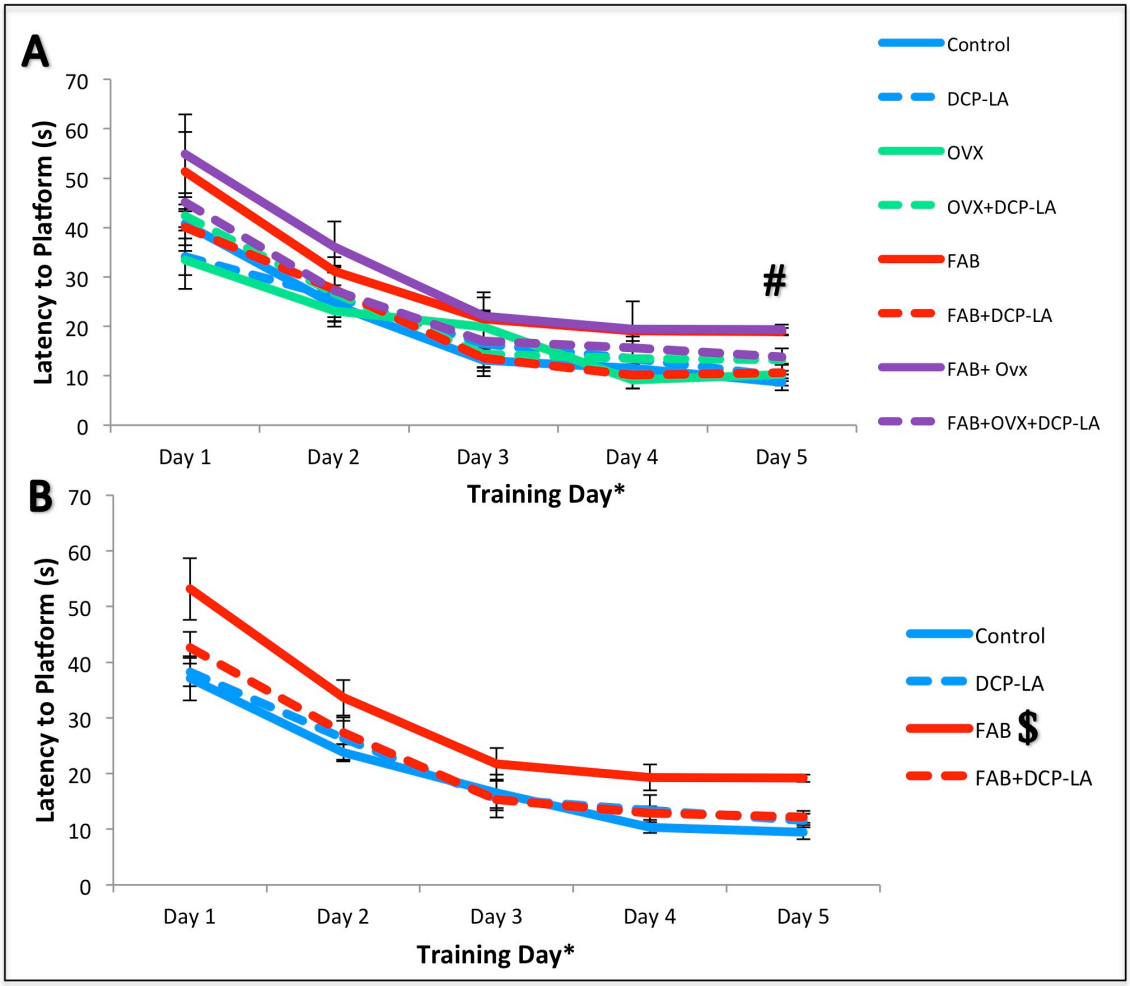

Figure 4: Latency to Reach the Hidden Platform during the MWM Training Days. Each day for 5 days, animals received 4 trials to find a hidden platform. The time from placement in the water to finding the platform was measured for each trial. The average for each treatment group on each day was calculated. * denotes a main effect of time ($p < 0.0005$). **A.** Data for each treatment group over the five day learning period. # denotes treatment is significantly higher than the control for Day 5 ($p < 0.0005$). **B.** OVX did not impact data, so data were pooled to eliminate the OVX variable. $ denotes a main effect of FAB treatment ($p < 0.0005$). Data are mean +/- S.E.M.

Probe Trial

Forty-eight hours following Day 5 of the learning portion of the MWM, the hidden platform was removed from the pool. Each animal received one 90 second trial where the latency to the position where the hidden platform previously was located was measured to test memory (platform location). FAB treated animals took significantly more time to reach the area the platform was located in during learning than the Control ($p = 0.001$), DCP-LA ($p = 0.004$), OVX ($p = 0.005$), OVX + DCP-LA ($p = 0.001$), FAB + DCP-LA ($p < 0.0005$), and FAB + OVX + DCP-LA ($p = 0.001$) (Figure 5). FAB + OVX treated animals also took significantly more time to the platform location than the Control ($p < 0.0005$), DCP-LA ($p = 0.003$), OVX ($p = 0.004$), OVX + DCP-LA ($p < 0.0005$), FAB + DCP-LA ($p < 0.0005$), and FAB + OVX + DCP-LA ($p < 0.0005$) (Figure 5). No significant differences were measured between any of the other treatment groups.

During the memory testing, total time animals spent in the quadrant where the platform was located during learning, Q4, was also measured. The mean and standard error of the mean was also calculated for each treatment group. OVX animals with and without DCP-LA spent significantly less time in Q4 compared to the Control ($p = 0.048$, $p = 0.049$; Figure 6). FAB animals also spent significantly less time in Q4 compared to the Control ($p = 0.039$; Figure 6). No significant difference was seen between the OVX and the FAB treatment groups. FAB + OVX animals spent significantly less time in Q4 than the Control ($p < 0.0005$), DCP-LA ($p < 0.0005$), OVX ($p = 0.021$), OVX + DCP-LA ($p = 0.019$), FAB ($p = 0.015$), FAB + DCP-LA ($p < 0.0005$), and FAB + OVX + DCP-LA ($p = 0.004$) (Figure 6). No significant differences were measured between any of the other treatment groups.

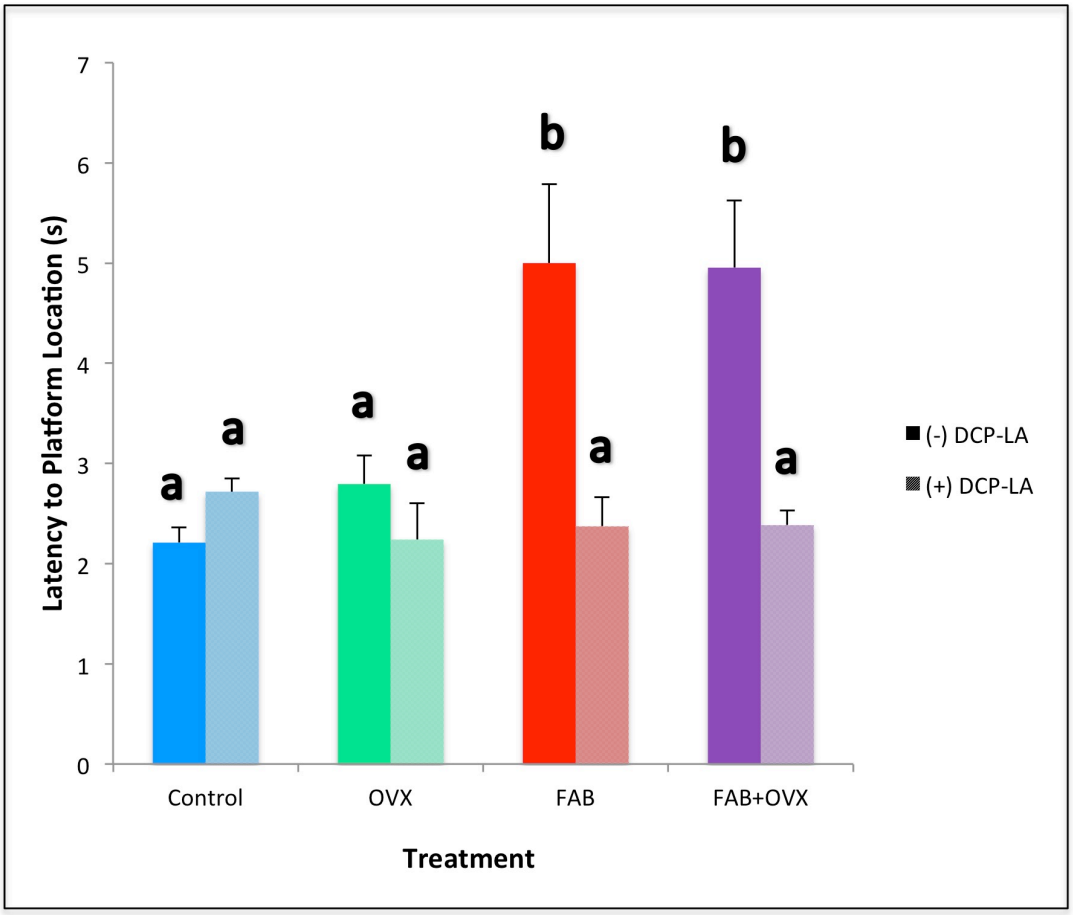

Figure 5: Latency during probe trial to location where the platform was located during learning. 48 hours following learning trials, the platform was removed from the MWM pool. Animals were placed in the pool for 90 seconds and the latency to the location where the platform was located during learning was measured. **a** significantly differs from **b** ($p < 0.001$). Data are normalized means +/- S.E.M.

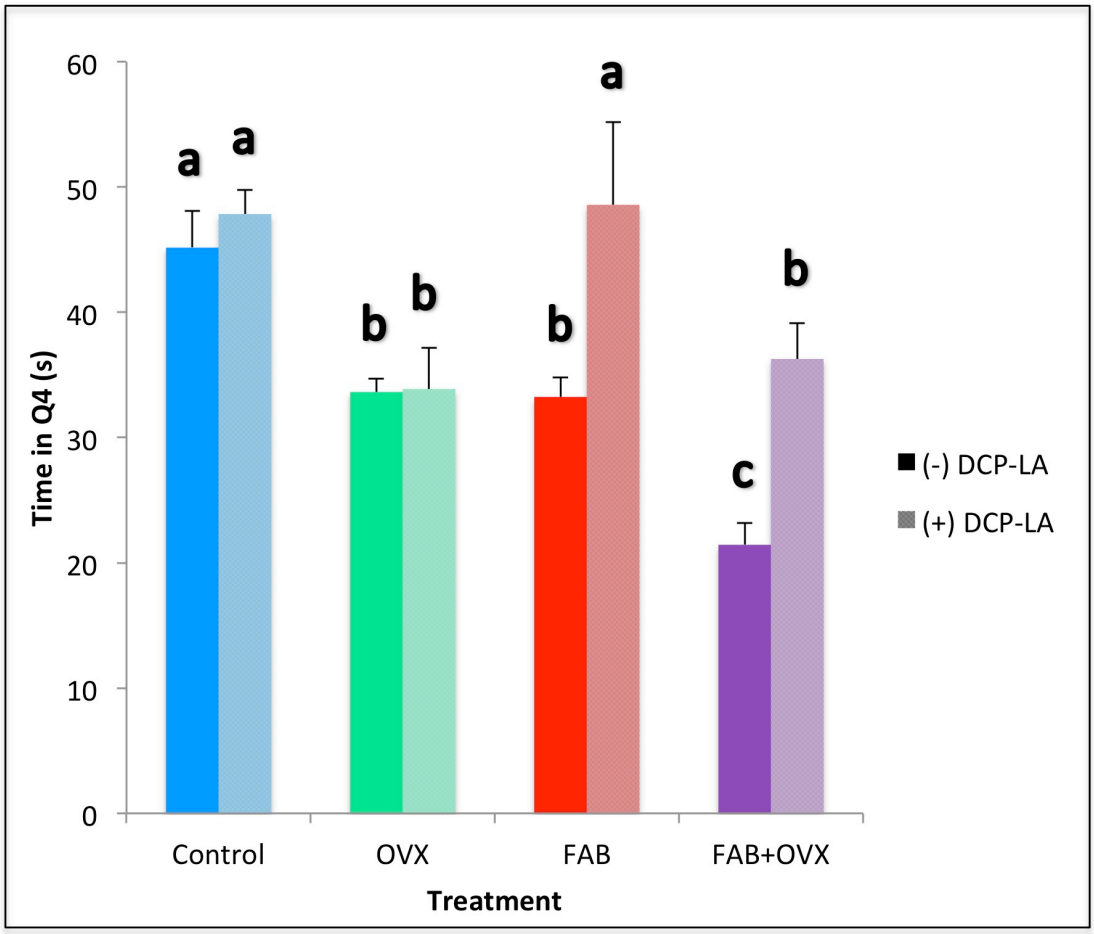

Figure 6: Total time spent during probe trial in the quadrant where the platform was located during learning. 48 hours following learning trials, the platform was removed from the MWM pool. Animals were placed in the pool for 90 seconds and the total time spent in the quadrant where the platform had been located during learning (Q4) was measured. Treatment groups were averaged and standard error of the mean was calculated. **a** significantly differs from **b** ($p < 0.05$). **b** differs from **c** ($p < 0.05$). **a** significantly differs from **c** ($p < 0.0005$). Data are mean +/- S.E.M.

Discussion

This study examined the effects of DCP-LA on learning in memory in a post-menopausal sporadic AD model, with specific aims to determine if DCP-LA can be utilized as a replacement for 17-β estradiol. The drop in 17-β estradiol levels following menopause is considered a major risk factor that explains females' higher susceptibility to AD as compared to males. However studies restoring levels of 17-β estradiol produced controversial results as to whether there is improvement or a preventive effect from restoring 17-β estradiol levels through hormone replacement therapy (HRT) (Mulnard et al. 2000; LeBlanc 2001; Henderson et al. 2005; Daniel et al. 2006; Morrison et al. 2006; Whitmer et al. 2011). Increasing 17-β estradiol levels via HRT are not without impact. HRT administration has been directly correlated with increased risks of other diseases including breast cancer, ovarian cancer and cardiovascular disease (Nabulsi et al. 1993; Collaborative 1997; Lacey et al. 2002; Rossouw 2002; Chlebowski et al. 2003).

Given that results from HRT are controversial, researchers have explored mechanisms underlying HRT, with one focus on examining the effects of PKC-ε activation. Because either PKC-ε inactivation or low activation levels possibly play a role in AD, current research examines the effects of administering PKC-ε activators as a potential AD therapy. DCP-LA, a highly specific PKC-ε activator, is focused on in this study as a compound that could restore the PKC-ε activation lost after menopause in females. Through the use of an *in vivo* postmenopausal sporadic AD model, five hypotheses were investigated and are further discussed below.

Hypothesis #1: FAB treated rats *((+)FAB (-)OVX (-)DCP-LA)* will perform significantly worse on MWM learning and memory tests than controls *((-)FAB (-)OVX (-)DCP-LA).* *(Note: Since there was no main effect of OVX on learning, the following discussion of hypothesis #1 will focus on the data that pooled OVX with respective groups as seen in Figure 4b.)*

Rats treated with FAB showed learning and memory deficits as compared to all other groups during the Morris Water Maze training (MWM) and probe trial (Figure 4-6). During the learning days, FAB animals demonstrated learning as seen by an overall decrease in latency to find the platform over the five day learning period (Figure 4b). However, the time it took for FAB animals to find the platform on certain days differed from other groups. Throughout the learning days, there were either differences or trends showing that FAB rats were taking longer to find the platform than all other treatment groups (Figure 4b). This overall effect of FAB learning performance sparked concern in the possibility of FAB impacting animal activity levels. The locomotor data was analyzed to see if the FAB treated animals had different levels of activity as compared to the other treatments, and the FAB (+/-) DCP-LA animals showed no differences in their activity levels for either horizontal or vertical movement (Figure 3a, 3b). Because there were no differences in motor activity, the learning differences in the FAB animals could be attributed to reduced cognitive function.

Although the learning performance in FAB animals was reduced as compared to the other animals, interestingly the rate of learning for FAB animals does not differ from the other groups. Overall FAB rats spent more time searching for the platform, but this

was apparent from the first day of learning and continued through the fifth day. The rate of learning itself was not impacted by the FAB treatment, only the actual average amount of time it took each day for the FAB rats to find the platform. This finding is similar to one conducted by Candia 2017 that also examined learning and memory deficits from the FAB model. Candia 2017 observed that FAB treated animals took longer to find the platform three of the five learning days, with what appears to be possible trending differences on the other two days. The consistencies with data from Candia 2017 confirm that FAB impacts the overall performance of the rat during a spatial learning task, but not necessarily its rate of learning.

The FAB model also produced deficits during the probe trial demonstrating that FAB also negatively impacts memory (Figure 5, 6). In the immediate measure of memory, the time for the rat to find the location of the recently removed platform, FAB and FAB + OVX rats (-) DCP-LA both spent more time than all other treatment groups orienting themselves before attempting to find the platform (Figure 5). Memory deficits in FAB rats (-) DCP-LA were also seen in the total time spent in quadrant 4, which is a longer memory test (Figure 6). These results were again were consistent with Candia 2017, lending support for the viability of FAB as a possible model system for producing the learning and memory deficits observed in AD.

Even though learning and memory deficits resulted from the model used in this experiment, there are several important limitations to consider concerning this model. First, the use of FAB has not produced evidence of neurofibrillary tangle formation, which is one of the main AD hallmarks. Damage from two of the other AD hallmarks, Aβ plaques and oxidative stresses, have been observed, but without all three hallmarks,

this model remains limited. There is currently no *in vivo* model that can reproduce the neuronal damages that result from all three hallmarks, and so this might be an important avenue to further explore in AD research. A second limitation to this model is that young rats were used. The conversion from rat years to human years calculates that the rats used, which were three months old, would be the equivalent of six-year-old humans (Sengupta 2013). Age should be a major consideration when examining AD because AD mainly impacts individuals over 65 years old. Therefore, the use of older animals might provide better insight into the effects of DCP-LA that could be specific to an older population. A third limitation is the rate of AD onset with the FAB model as compared to the timing in humans. AD onset in humans is gradual, usually taking decades before clinical symptoms are observed (Fratiglioni et al. 1991), but this experiment only infused FAB over a four-week period leading to a fast onset of AD. Several studies have limited the infusion of FAB to a four-week span, but future studies might expand the infusion period with hopes of mimicking the slow onset of AD (Lecanu et al. 2006; Candia 2017).

It is very important to consider the limitations of a model when examining the effects of a drug, because these limitations can negatively impact drug success in clinical trials. Only about 0.4% of all drugs tested in clinical trials successfully complete all trials to become available to the population (Wong et al. 2018). This staggeringly low number stems from problems with model systems due to non-parallels between humans and the animal models. In order to achieve success in clinical trials, the closer the animal model mirrors humans, the higher the likelihood of the drug succeeding through drug trials.

Candia further confirmed the FAB model system through immunohistochemistry by looking at molecular measures. His research showed that FAB treated animals had reduced cell survival and synapse health as compared to all other animals, which was consistent with the study by Lecanu et al. 2006 that first developed the FAB model. However, there are many other molecular mechanisms in these animals that immunohistochemistry could help explain such as the amount of synapse loss, immune cell response, microtubule instability and neuronal dysregulation, that have not yet been examined. This study is also attempting to further verify the FAB model through immunohistochemistry, but this work is still being conducted.

Hypothesis #2: OVX rats *((-)FAB (+)OVX (-)DCP-LA)* will perform significantly worse on MWM learning and memory tests than controls *((-)FAB (-)OVX (-)DCP-LA)*.

As opposed to the FAB animals, OVX treated animals only showed one specific memory deficit. They performed the same as the control animals during the learning trials and the immediate memory test, which differed from the deficits in both learning and memory observed in FAB animals (Figure 4 & 5). During the learning trials, OVX animals had no impact on their learning behavior (Figure 4a). This was surprising because it conflicted with several studies that examined learning capabilities in OVX animals. A study conducted by Warren and Juraska 1997 showed learning deficits in OVX rats measured by the MWM that were possibly due to the loss of 17-β estrdiol. This finding was confirmed by Monteiro et al. 2005 in a study that measured OVX induced

learning and memory deficits also in the MWM. The results from these previous studies raised concerns about why this study did not see a learning deficit in OVX rats. There was no question as to whether the ovaries were fully removed because the simplicity of the procedure leaves no room for uncertainty about if the ovary was removed. In addition, the weight gain observed in OVX rats was consistent with previous studies. The OVX rats gained about 2.5 times as much weight as the intact animals over a four week period, which was supported by studies and literature reviews conducted by Wade 1972, Tarttelin and Gorski 1973, Wade and Gray 1979, and Roesch 2006 (Figure 2). Because OVX rats gained such a large amount of weight, their activity levels were measured to ensure that the weight gain would not be a hindrance to their performance on the learning and memory tests. The OXV (+/-) DCP-LA rats had similar locomotor activity as all the other treatment groups, ruling out mobility difficulty as an explanation for any differences in MWM performance (Figure 3a and 3b).

OVX animals did show memory deficits during the probe trial, but only on the longer memory test. The memory test examining immediate memory of the platform showed OVX (+/-) DCP-LA rats taking no longer to find the platform area than the controls (Figure 5). The FAB+ OVX (-) DCP-LA rats took longer to find the platform than the OVX rats, but matched the FAB (-) DCP-LA rats, showing that the FAB, not OVX, was responsible for increasing latency to the previous location of the platform. The longer memory test measuring total time spent in Q4 did show memory deficits in OVX animals however. The OVX (+/-) DCP-LA animals spent less time in Q4 than all other animals, which is representative of the OVX rats showing less persistence and problem solving when attempting to locate the platform (Figure 6) (Morris 1984). The memory

deficits caused by OVX treatments were consistent with previous studies, therefore validating the OVX model (Monteiro et al. 2005; Luine 2008).

Hypothesis #3: The FAB-OVX treatment group *((+)FAB (+)OVX (-)DCP-LA)* will perform significantly worse on learning and memory tests than controls *((-)FAB (-)OVX (-)DCP-LA)*.

As was previously discussed, OVX did not have any impact on performance during the MWM learning days, therefore making the FAB and the FAB + OVX treatment groups equivalent (Figure 4b). OVX treatment also did not impact performance on the shorter memory test during the probe trial, and so FAB + OVX (-) DCP-LA showed similar memory deficits in rats treated only with FAB (Figure 5). However, the FAB + OVX (-) DCP-LA produced exciting results in the longer memory test. The FAB + OVX (-) DCP-LA treatment group performed worse than all other treatments because these animals spent the least amount of time in Q4 (Figure 6). The combination of the FAB and OVX treatments produced an additive effect of the memory deficits. This finding is not surprising because if both FAB and OVX produce lower memory capabilities in rats, it would be expected that they would elicit similar effects even when combined with another treatment.

It is interesting however, that no synergistic effect, or combined effect resulting in a greater deficit than either FAB or OXV individually, is observed between FAB and OVX since the loss of 17-β estradiol is thought to increase susceptibility to AD. Even

though the loss of 17-β estradiol has been specifically linked with mechanisms underlying AD pathology, it has also been linked with other pathways that are unrelated to AD (Yamada et al. 1999, Kwakosky et al. 2016; Villa et al. 2016). In addition, it is possible that the neuroprotective effects of 17-β estradiol could be acting through an entirely different mechanism than the FAB, meaning that the removal of 17- estradiol causes deficits for reasons completely different than deficits from FAB. Therefore without 17-β estradiol individuals are both more at risk for developing AD, but also less protected from the disease. Unfortunately, there are no known studies to compare this result to see if a synergistic effect can also be observed.

The additive effect also might also lend some insight into why females have higher susceptibility to AD than males due to the possible misdiagnosis of AD. Because AD diagnoses are guesswork due to the inability to confirm a case of AD until after death, it is possible to misdiagnose an individual presenting with memory loss as an AD patient, even if the memory loss is due to another disease (Alzheimer's 2018). This high error rate of diagnosing AD could lead to the attribution of certain cognitive symptoms to AD instead of the true cause, especially if the other disease presents in a similar manner. For example, this study shows that the loss of 17-β estradiol presents the same level of memory loss as the FAB model (Figure 6). If a physician is trying to diagnose a post-menopausal patient showing symptoms of memory loss, it would be very difficult to say with certainty whether the individual's memory loss is due to the reduction in 17-β estradiol or AD. It might be possible that females are presenting with cognitive deficits due to menopause, but these symptoms are being mistaken for AD. This would lead to

higher rates of AD diagnosis in females that translates into higher susceptibility measures of AD in females, even though these deficits might have another cause.

Hypothesis #4: DCP-LA administration will reverse the negative effects on learning and memory in all (+) FAB treated rats.

Administration of DCP-LA to FAB treated rats reversed the learning deficits induced by the FAB treatment. During the MWM learning days, the injection of DCP-LA restored cognitive function in FAB rats back to the performance of the control rats (Figure 4a, 4b). These data are supported by Candia 2017 with a study that produced similar results. Candia 2017 also saw that DCP-LA can reverse the cognitive deficits caused by the FAB model. This is due to the ability of DCP-LA to activate PKC-ε, which when activated, can reduce amyloid beta plaques and tau hyperphosphorylation (Nagata et al. 2005; Nagata et al. 2010; Candia 2017). The reduction of both of these AD hallmarks is thought to be essential for producing the restorative effects. It is not known if PKC-ε is deactivated in AD cases, so DCP-LA could either be reactivating a deactivated PKC-ε or it could just be boosting normal PKC-ε activity to promote accumulation of membrane protein, PSD-95, to promote synaptogenesis (Sen et al. 2016).

DCP-LA exhibited similar effects on restoring memory as it did on restoring learning abilities. In the memory test measuring immediate memory, an injection of DCP-LA returned FAB rat memory performance back to control levels (Figure 5). In the memory test examining problem-solving abilities, DCP-LA again reversed any deficits

caused by FAB resulting in memory performance equal to the control rats (Figure 6). It is important to notice however, that in the FAB + OVX group, DCP-LA did not restore full cognitive abilities to the rats. This finding implicates the involvement of OVX and so will be further discussed under the next hypothesis.

There are two main hypotheses as to how DCP-LA's activation of PCK-ε could be improving performance in these rats. One hypothesis is that the increased PKC-ε activity is decreasing the pathological effects of FAB. Decreased effects of FAB theoretically would show improved cognitive abilities in rats. However because only one administration of DCP-LA was given to each animal, is would be unlikely that just one injection could completely reverse the FAB pathology. A second hypothesis suggests that increased PKC-ε activity increases synaptic plasticity, which allows animals to perform better on the learning and memory tests. Increased synaptic plasticity has been linked with increased learning and memory retention, and so the single injection of DCP-LA right before MWM might improve the animal's performance (Fritsch et al. 2010). This possibility is more likely because just one injection is more likely to improve leaning immediately following the drug administration than reverse the accumulation of damage and plaques that have been building for several weeks.

Hypothesis #5: DCP-LA administration will reverse negative effects on learning and memory in the (+) OVX rats.

DCP-LA successfully reversed the learning and memory deficits produced by the FAB model, but it was not so successful with reversing cognitive deficits caused by loss of 17-β estradiol in the OVX rats. The OVX itself did not produce any learning problems, but during the memory test examining the animal's problem solving abilities, DCP-LA showed no impact on OVX rat performance (Figure 6). Similarly in FAB + OVX rats, DCP-LA only appeared to reverse the negative effects produced by FAB, and not by OVX (Figure 6).

It was surprising that DCP-LA could not reverse the memory deficits from the loss of 17-β estradiol because both DCP-LA and 17-β estradiol activate PKC-ε. One hypothesis to explain why DCP-LA reverses the memory impairments from the FAB but not 17-β estradiol is that DCP-LA and 17-β estradiol activate PKC-ε through different pathways. DCP-LA binds directly to PKC-ε with high selectivity and affinity (Takana and Nishizaki 2003; Kanno et al. 2006; Sun and Akon 2014). 17-β estradiol however binds to estrogen receptor, ESR1, leading to an indirect activation of PKC-ε (Sovershaev et al. 2006). This difference in mechanism of activation might be the key player in the modulation of PKC-ε activity.

Another hypothesis could be that there is a difference in PKC-ε activation in the FAB and OVX conditions. Because the level of PKC-ε activation is unknown in AD, it might already be high enough that just a boost in activity from DCP-LA binding is enough to produce the neuroprotective effects observed. This would differ from activated PKC-ε levels in OVX animals, because PKC-ε is known to be significantly reduced. It is possible that the DCP-LA is increasing PKC-ε activity in OVX animals, but not enough to elicit the same effects seen in the FAB animals. In order to determine if DCP-LA is

having a possible effect on restoring PKC-ε activation in OVX animals, additional analyses will need to be conducted such as increasing DCP-LA dosage to see if any of the neurological deficits in OXV are reversed.

A third possibility could be that the deficits caused by OVX have absolutely nothing to do with PKC-ε. The main hormone removed by an OXV, 17-β estradiol, can activate many different subtypes of estrogen receptors (ER) in the brain, many of which lead to different signaling cascades that have been associated with learning and memory (Korol and Pisani 2015). However it is unknown which estrogen receptor is directly responsible for activation PKC-ε. 17-β estradiol binding to estrogen receptors have been linked with the activation of several pathways including initiation of transcriptional changes and the signaling of the cyclic AMP second messenger pathway. Because 17-β estradiol can bind to many estrogen receptors to activate numerous pathways, the loss of 17-β estradiol caused by an OVX might be causing memory deficits through a pathway unrelated to PKC-ε. If this is the case, the loss of 17-β estradiol might specifically impact memory related problem solving abilities through additional pathways. The involvement of additional pathways in the memory related problem solving abilities would make sense because it is a more complex process than immediate memory recall, and this hypothesis would support the results seen in this study. OVX rats performed at the level of the control on all tests except for the memory test that examines problem-solving abilities. Because there was an additive effect of deficits from OXV and FAB and no synergistic relationship, the hypothesis that proposes that 17-β estradiol elicits its memory deficits through a pathway totally unrelated to PKC-ε is very possible.

Conclusion

In conclusion, this study determined that DCP-LA could not fully restore learning and memory impairments in a post-menopausal sporadic AD model. DCP-LA completely reversed the deficits caused by the FAB model, but had no impact on the OXV population's cognitive abilities. In the postmenopausal model that combined FAB and OXV, the reduced memory abilities due to the loss of 17-β estradiol were still observed. These deficits caused by the decrease in 17-β estradiol should be a major area of study in future AD experiments because there are no known therapies to prevent or slow the cognitive decline associated with this hormonal absence. Even though DCP-LA did not appear to have any impact on OVX animals in this study, this result does not mean that DCP-LA has no impact at all. This study can be modified to increase administration of DCP-LA to see if more long term dosages of DCP-LA might have an effect on OVX animals. Other PKC-ε activators can be introduced to this post menopause sporadic AD model to see if there are any that might elicit effects. It is also possible that these experiments will reveal that this research is not even on the right track, but instead a whole different approach to AD should be pursed. All these experiments will lead to answers that will slowly help researchers answer the main question behind this study: why are women more susceptible to AD?

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