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Assessing the Therapeutic Potential of the Linoleic Acid Derivative DCP-LA in a Pharmacological Rat Model of Alzheimer's Disease

A Thesis in Neuroscience

by

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Abstract

Alzheimer's disease (AD) is a neurodegenerative disorder characterized primarily by progressive memory loss. It is estimated to afflict nearly 47 million people worldwide, and its prevalence is expected to triple within 35 years. Within the last decade, 99.6% of drugs targeting this disease have failed in clinical trials, and those few that have succeeded have mild effects, at best. Recently, the serine/threonine kinase PKC-ε has become a target for drug development in AD. The kinase has been shown to play a role in critical processes related to AD pathology, including neurite outgrowth, synaptogenesis, and long-term potentiation. The linoleic acid derivative 8-[2-(2-pentylcyclopropylmethyl)-cyclopropyl]-octanoic acid (DCP-LA) specifically activates PKC-ε and has been shown to be beneficial for cognitive and molecular pathology in transgenic models of AD. However, transgenic models have many issues when it comes to the drug development process. As such, we have tested the therapeutic potential of DCP-LA in the FAB rat model, which is a pharmacological AD model that focuses on the role of oxidative stress in the molecular pathology of the disease.

The goals of this experiment were to specifically assess the effects of DCP-LA on learning, memory, neuronal health, and synaptic density. To measure learning and memory, rats were trained to complete the Morris water maze. To measure neuronal health in the hippocampus, immunostaining was performed against the neuronal marker NeuN. To measure synaptic density in the hippocampus, immunostaining was performed against the synaptic marker synaptophysin.

We report a decrease in performance for FAB rats in both the learning and memory tasks in the Morris water maze. Treatment with DCP-LA (3mg/kg) restored performance on both tasks to the level of control animals. There was significant neurodegeneration observed in the CA1, CA3, and dentate gyrus regions of FAB rats compared to all other groups. DCP-LA treatment partially restored the density of healthy neurons, but not to back control levels in the CA1 and CA3. FAB rats also exhibited significant loss of synaptic density in all three hippocampal region tested, which was recovered to control levels by DCP-LA treatment.

Together, these data suggest that DCP-LA treatment is able to reverse learning and memory deficits and improve neuronal and synaptic health in FAB rats. While this certainly suggests that DCP-LA has potential to be used in the human disease, more research is required to determine key safety data, such as systemic toxicity, optimal dosages, and treatment windows.

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Introduction

Alzheimer's Disease: Prevalence, Morbidity, and Caretaker Burden

Alzheimer's disease (AD) is a neurodegenerative disease and the single most common cause of dementia, accounting for somewhere between 60-80% of dementia cases (Barker et al., 2002; Wilson et al., 2012). It is not unreasonable to suggest that we stand on the precipice of an Alzheimer's pandemic. A 2015 report from Alzheimer's Disease International (ADI) documented a meta-analysis incorporating over 200 studies of dementia prevalence worldwide, estimating that 46.8 million people worldwide were living with dementia in 2015. This estimate is an 88% increase from the 26.6 million people living with dementia in 2006, less than a decade earlier (Brookmeyer et al. 2007). Further estimates suggest that this number will nearly triple in the next 35 years, such that 131.5 million people will have dementia by the year 2050 (Alzheimer's Disease International, 2015). In terms of incidence, a meta-analysis of 46 studies conducted worldwide suggests an estimated 9.9 million new cases of dementia arose in 2015, equating to a new case every 3 or so seconds. This is a 29% increase from a joint report by ADI and the World Health Organization, using data from just 5 years earlier (World Health Organization, 2012).

Similar population studies have been conducted in the United States alone, but the outlook is no less grim. In 2016, an estimated 5.4 million Americans have AD (Alzheimer's Association, 2016). Of these 5.4 million, 5.2 million are aged 65 and older,

while the other 200,000 have a genetic, early-onset form of AD (Alzheimer's Association, 2006). Hebert et al. (2013) estimate that, just like the global prevalence, the prevalence of AD in the U.S. will nearly triple within the next 35 years, to 14 million Americans. In that same study, the authors utilize 2010 U.S. census data to specifically estimate the prevalence of AD within the population aged 65 or older, concluding that roughly 11% of Americans aged 65 or older have AD, and that 32% of Americans aged 85 or older have AD. A similar estimate was made using data from the nationally representative Aging, Demographics, and Memory Study, suggesting that approximately 14% of Americans aged 71 or older have AD (Plassman et al., 2007).

The incidence of AD in the United States is a bit tricky to estimate, as research in that area appears to be scant. The only readily available estimate of U.S. AD incidence in the last decade comes from the Alzheimer's Association (2016). They predict that there will be 476,000 new cases of AD in 2016. This number is broken down by age group, such that 63,000 new cases are estimated for those aged 65-74, 172,000 new cases for those aged 75-84, and 241,000 new cases for those aged 85 or older. It is important to note, however, that these numbers are calculations derived from estimates made in a 2001 study by Hebert et al. and not from a recent study of AD incidence (Alzheimer's Association, 2016).

Reliable estimates of AD prevalence and incidence are especially important now because the baby boomer generation has begun to enter that 65 and older population. It is estimated that, within the next fifteen years, the population aged 65 and older will

make up 20% of the total U.S. population, an increase of 6% (Ortman et al., 2014). Within this population of those aged 65 or older, the number of individuals aged 85 and older, known as the 'oldest-old' population, is expected to increase by 8%, from 14% to 22%, by 2050 (Ortman et al., 2014). Recall that this is the population that, right now, contains the highest percentage of individuals with AD, and its rapid growth over the next 30 years becomes worrying. It has been estimated that, in 2050, roughly half of the projected 14 million individuals with AD will belong to the oldest-old population, an increase of almost 20% from what it is estimated to be in 2016 (Hebert et al., 2013).

Perhaps the most somber implication of the projected surge in AD cases comes with the realization that AD is not just amnesic, but terminal. It is currently listed as the sixth-leading cause of death in the United States (Xu et al., 2016). The CDC itself has listed AD as the primary cause of approximately 85,000 deaths in 2013, a 71% increase since 2000 (Xu et al., 2016). However, data from the Rush Memory and Aging Project and the Religious Orders Study suggest that roughly 500,000 deaths could be attributed to AD in 2010 (James et al., 2014). A major reason for this large disparity is that individuals with AD are more susceptible to certain other complications, most commonly pneumonia (Brunnstrom and Englund, 2009), that directly lead to death and undermine the role of AD in that death (Romero et al., 2014a; Romero et al., 2014b). This undermining occurs because of misinterpretation of the cause of death. The WHO defines the official cause of death as "the disease or injury which initiated the train of events leading directly to death" (WHO, 2004). While death from AD is actually caused

by comorbidities, according to the definition from the WHO, AD would be the official cause of death. However, death certificates often report conditions such as pneumonia rather than AD, and the CDC records the cause of death as such. Current estimates hold that roughly 700,000 Americans 65 or older will die with AD in 2016, with the actual number of deaths from AD being likely lower than this estimate, but higher than the officially reported number (Weuve et al., 2014). This high contribution of AD to death in the elderly is further supported by estimations that 61% of 70 year old Americans with AD will not live to see 80, compared to only 30% of those without AD (Arrighi et al., 2010).

Ultimately, the rapid projected growth of the AD pandemic affects not only the individuals who have to live with (and eventually die from) the disease, but also the caregivers and the economy at large that must support the physical, medical, and emotional needs of these individuals. Family and close friends of individuals suffering from AD are particularly impacted by the development of the disease, contributing 83% of the total help provided to seniors in the U.S. (Friedman et al., 2015). The Alzheimer's Association reports that, in 2015, 15 million Americans provided a total of 18 billion hours of unpaid care for individuals with dementia (2016). This type of care most often includes helping patients with integral daily activities, such as bathing, cooking meals, and shopping (Gaugler et al., 2002). The proportion of seniors with dementia who receive help with such tasks is high: 77% of seniors with dementia need such help, compared to 20% of seniors without dementia (Kasper et al., 2015). An estimated 40-

50% of caregivers provide this support for individuals suffering from dementia for 6 or more years (Kasper et al., 2014).

This prolonged time period of caregiving is incredibly stressful for the caregivers. In a 2014 poll conducted by the Alzheimer's Association, almost 60% of caregivers claimed to be experiencing high levels of emotional stress from caregiving, and about 50% of women with young children reported that caring for someone with dementia was more difficult than caring for children (reported in Alzheimer's Association, 2016). During the last year of life for AD patients, 59% of caregivers reported feelings of around-the-clock caregiving, and 72% of caregivers even reported feeling relieved after the death of the family member they were caring for (Schulz et al., 2004). The buildup of stress over the course of caregiving often manifests itself as deterioration of mental and/or physical health of caregivers. For example, 40% of caregivers for individuals with dementia develop depression during their time as caregivers, compared to somewhere between 5% and 17% of caregivers for individuals without dementia (Mausbach et al., 2012). Physiological signs of declining health include elevated stress hormones (von Kanel et al., 2006), impaired immune system function (Kiecolt-Glaser et al., 1991), development of hypertension (Shaw et al., 1999), and development of heart disease (Vitaliano et al., 2002). Data also suggests that the increased levels of stress may contribute to an increased mortality rate among caregivers (Schulz and Beach, 1999).

Alzheimer's Disease: Diagnosis, Symptomatology, and Epidemiology

A definitive diagnosis of AD cannot be given until after the patient has died and the disease pathology can be observed in his or her brain. However, physicians have been able to make probable diagnoses according to the criteria in the Diagnostic and Statistical Manual of Mental Disorders (DSM). These criteria include evidence of steady, progressive cognitive decline in multiple areas, such as learning and memory, language skills, or executive function, which interferes with the patient's ability to function normally in everyday life, as assessed through cognitive tests (American Psychiatric Association, 2013). Behavioral changes, such as depression, irritability, and apathy, have also been reported as symptoms manifested in AD (American Psychiatric Association, 2013). Recent research, however, has forced us to re-think the way that we approach AD diagnosis. A number of longitudinal studies have observed the development of cognitive decline and molecular pathology of AD in healthy individuals as they developed dementia, and the results suggest that cognitive decline can begin almost a full decade before the clinical manifestations of dementia would allow for a probable diagnosis (Wilson et al., 2012; Amieva et al., 2008; Johnson et al., 2009). Some of the most striking results indicate that the deposition of amyloid beta (Aβ) plaques, one of the three major hallmarks of AD molecular pathology, begins as early as 20 years before the manifestation of symptoms, slowly building up to the levels seen in the disease (Villemagne et al., 2013). This same study also suggested that atrophy of the

hippocampus, the brain region most closely associated with learning and memory, could be observed up to 5 years before dementia onset.

In 2011, the National Institute on Aging and the Alzheimer's Association responded to findings such as those presented above by proposing new diagnostic criteria for AD. The single biggest difference in these new criteria is the proposal of three stages of AD: preclinical AD, mild cognitive impairment (MCI) due to AD, and dementia due to AD. The final stage, dementia due to AD, is essentially the same as the old system for diagnosing AD, relying on the manifestation of severe and progressive cognitive deficits (particularly memory deficits) that prevent normal everyday function (McKhann et al., 2011). The middle stage, MCI due to AD, accounts for the development of cognitive deficits prior to the onset of full-blown dementia (Albert et al., 2011). This cognitive decline is defined as greater than what would be expected for the individual's age, but unlike in the dementia stage, these cognitive deficits do not interfere with everyday life for the individual and do not need to be memory-related. However, it is estimated that two-thirds of patients with MCI have memory deficits, a condition more specifically known as amnestic MCI (Vemuri et al., 2010). In addition, a major reason that MCI has been defined as a separate stage is that having MCI does not necessarily mean that the individual will progress to full-blown dementia. Studies have suggested that as many as one-third of individuals with MCI will progress to dementia within 5 years (Ward et al., 2013), but some individuals never progress past MCI, and some even

revert back to normal cognitive function (Ganguli et al., 2011). This suggests that MCI is a condition with multiple etiologies, with AD being just a subset.

The earliest stage, preclinical AD, does not have any diagnostic criteria associated with it. Rather, this stage is a theoretical response to findings that molecular pathology of AD may begin decades prior to the development of any symptoms (Sperling et al., 2011). Significant effort is being put into finding biomarkers that can be used to accurately detect the presence of AD in this preclinical phase, potentially allowing for the creation of treatments that can prevent the damage caused by the pathology in the first place. One example of this effort is using radiotracers to visualize amyloid plaques through brain scans (Pike et al., 2007; Ewers et al., 2012), although it should be noted that amyloid plaques are present in other pathologies (Masters et al., 1985), and so plaque detection alone is insufficient. Measuring the levels of amyloid beta and tau protein, the constituent of the second major hallmark of AD, in the cerebrospinal fluid of patients is another method being developed to supplement this (Ewers et al., 2012; Bertens et al., 2015).

If efforts are to be focused on early identification and prevention, then one of the most important aspects of AD research is the epidemiology of the disease. This area of research tries to identify which populations are most likely to develop the disease. For example, common population lines that are drawn when studying risk for a disease are sex and race, and this has been no different for AD. Prevalence studies have suggested that nearly two-thirds of Americans living with AD are female (Hebert et al.,

2013). The overall lifetime risk for developing AD has been reported to be higher in women than men at all ages above 65, as well (17%, 19%, 20% for women and 9%, 10%, 12% for men at ages 65, 75, and 85, respectively; Seshadri et al. 2006). In terms of race, studies have suggested that whites are the race least likely to develop AD. Older African Americans have been suggested to be about twice as likely to develop AD as elderly whites (Potter et al., 2009; Gurland et al., 1999). Elderly Hispanics carry a greater risk for AD, as well, roughly one-and-a-half times greater than the risk for elderly whites (Haan et al., 2003; Samper-Ternent et al., 2012).

A key part of the study of AD epidemiology is attempting to relate AD risk to genetic, health, and lifestyle factors. Perhaps the largest and most well known risk factor for developing AD outside of aging is inheritance of apolipoprotein E (ApoE). This protein has three different alleles (ε2, ε3, and ε4), and everyone inherits an allele from each parent. One allele in particular, ε4, has been associated with increased risk for AD. Individuals who are heterozygous for ε4 (i.e., only have one ε4 allele) have a roughly three-fold increase in AD risk, and individuals who are homozygous for ε4 (i.e., have two ε4 alleles) have a roughly ten-fold increase in AD risk (Holtzman et al., 2012; Loy et al., 2014). As with aging, however, having the ApoE ε4 allele does not guarantee the development of the disease, but an estimated 40-65% of AD patients have at least one copy of this allele (Saunders et al., 1993).

A particularly strong body of evidence links an increased risk for developing AD to many factors that influence cardiovascular health. Chief among these is the

development of type-2 diabetes, which a number of meta-analyses have implicated as a risk factor for developing AD (Gudala et al., 2013; Vagelatos and Eslick, 2013; Profenno et al.; 2010). Obesity is a factor related to both cardiovascular health and diabetes, but evidence for obesity as a risk factor in AD has not been as strong, with some studies suggesting that obesity in mid-life may be a risk factor (Beydoun et al., 2008; Loef et al., 2010; Anstey et al., 2011) and others suggesting that obesity in later life is not associated with an increased AD risk (Fitzpatrick et al., 2009; Dahl et al., 2008). A similar association was found with hypertension, suggesting that midlife hypertension may be a risk factor for dementia (Power et al., 2011; McGuinness et al., 2009) while late-onset hypertension may protect against dementia (Corrada et al., 2014).

There is also strong evidence that modification of lifestyle factors may be a viable target for reducing AD risk. Physical activity, a lifestyle factor that is associated with reduced risk for cardiovascular disease, diabetes, and obesity, has been strongly associated with a protective effect against the development of dementia (Rolland et al., 2008; Sofi et al., 2011; Ahlskog et al., 2011). The other commonly cited lifestyle factor is level of education. Numerous studies and meta-analyses have looked at the relationship between education and dementia risk, and have generally found that more years of education is associated with a reduced risk for AD (Meng et al., 2012; Sando et al., 2008; Caamano-Isorna et al., 2006).

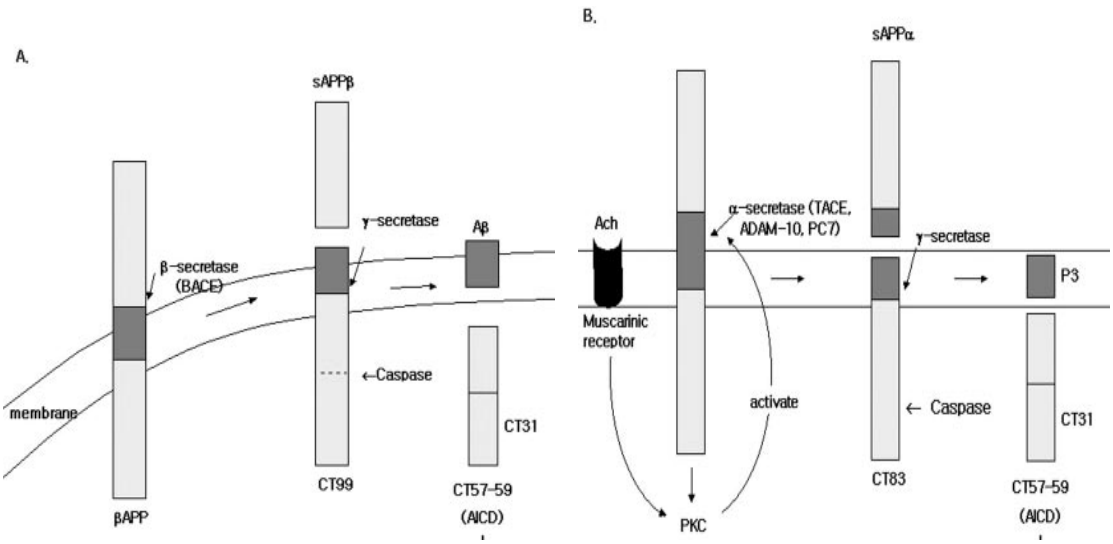
Alzheimer's Disease: Molecular Pathology

While our seemingly ever-changing understanding of AD has brought new perspectives to the clinical aspects of AD and accentuated the importance of identifying risk factors, our understanding of the molecular pathology of the disease, that is, the physical and chemical changes in the brain that underlie the observed symptoms, is still limited. We are still painfully unaware of the initial changes that begin the development of AD and which aspects of the pathology are causes or effects of which others. This reflects most strongly in the efforts to develop treatments for AD. In the decade between 2002 and 2012, 413 clinical trials were performed with drugs that were candidate AD treatments. Of these, only a single drug was approved for use by the FDA, which is a failure rate of 99.6% (Cummings et al., 2014). Even this single drug, marketed as memantine, has been shown to have at best a mild effect (Reisberg et al., 2003), often showing no effect at all during milder stages of the disease (Schneider et al., 2011). Continuing to build understanding of the molecular pathogenesis of AD is essential to overcoming this dearth of treatment options.

The molecular pathology of AD is most often described in terms of 'hallmarks' of the disease. There are three of these major hallmarks: the deposition of extracellular amyloid plaques, the formation of intracellular neurofibrillary tangles, and widespread neuronal death, or neurodegeneration (reviewed in Tatarnikova et al., 2015). The first of these, however, is possibly the most extensively studied, and these studies have lead to development of a widely (although not universally) accepted hypothesis known as the

amyloid cascade hypothesis (reviewed in Karran et al., 2011). The crux of this hypothesis is that the deposition of amyloid plaques is the central event in the development of AD pathology.

The plaques found in AD are primarily composed of Aβ peptide, which is derived from an integral membrane protein known as the Amyloid Precursor Protein (APP). This protein is processed by certain proteases, known as secretases, in one of two pathways (displayed in Model 1). The first of these pathways, known as the amyloidogenic pathway, requires the combined actions of β-secretase and γ-secretase (reviewed in Suh and Checler, 2002). Activity of β-secretase produces a soluble peptide named β-APPs and a 99-residue, membrane-bound C-terminal fragment. This fragment is then cut by γ-secretase to produce Aβ and a peptide named the amyloid intracellular domain (AICD). The activity of γ-secretase is heterogeneous, meaning that it can produce more than one form of Aβ: either a 40-residue peptide ($A\beta_{40}$) or a 42-residue peptide ($A\beta_{42}$), with $A\beta_{40}$ being much more common. The second pathway, known as the non-amyloidogenic pathway, requires the combined actions of α-secretase and γ-secretase (reviewed in Suh and Checler, 2002). In this pathway, α-secretase cleaves APP in the middle of the Aβ sequence, producing a different soluble peptide named α-APPs and an 83-residue, membrane-bound C-terminal fragment. This shorter fragment is also cut by γ-secretase and also produces the AICD, but results in a peptide named p3 rather than Aβ.



Model 1. Schematic representation of APP processing . The amyloidogenic pathway is shown on the left (A), while the non-amyloidogenic pathway is shown on the right (B). Image credit Suh and Checler (2002)

Our understanding of the processing pathways of APP is especially important because these pathways are vulnerable to mutation. There is a rare, genetic form of AD, known as early-onset AD (EOAD). Research has been able to identify exact causes for the development of EOAD, which are mutations in one or more of three genes: presenilin 1 (PS1) on chromosome 14, presenilin 2 (PS2) on chromosome 1, and APP on chromosome 21. APP, as discussed above, is the source of the Aβ peptide. PS1 and PS2 are both thought to be a part of the multiprotein complex that is γ-secretase (reviewed in Spasic and Annaert, 2008). These mutations normally have one of two major consequences: either they increase the overall amount of Aβ produced (Cai et al., 1993), or they alter the ratio of $A\beta_{40}$ to $A\beta_{42}$ (Suzuki et al., 1994), which increases the production of the more aggregation-prone $A\beta_{42}$ (Iwatsubo et al., 1994). The striking thing about these mutations is that, unlike other genetic factors such as ApoE4, they are

not risk factors. Rather, an individual with these mutations is guaranteed to develop AD, and will normally do so at a much earlier age, hence the early-onset in EOAD. In addition, patients with Down syndrome, who have an extra copy of the APP-containing chromosome 21, develop AD earlier in life (Lemere et al., 1996). This genetic evidence in particular provides the foundation for the amyloid cascade hypothesis, showing that alteration of the proteins responsible for the production of Aβ leads to the development of AD.

With the notion that increased production of Aβ could be a causal factor in the development of AD, researchers sought to identify potential candidate proteins to fulfill the role of α-secretase with the hope that these proteins might be potential therapeutic targets. This search has identified two members of the A Disintegrin And Metalloprotease (ADAM) family of proteases, ADAM10 and ADAM17. Lammich et al. (1999) observed that overexpression of ADAM10 *in vitro* resulted in increased secretion of α-APPs, which suggests an increased shift towards α-secretase-mediated processing of APP. Expression of a dominant negative form of ADAM10 drastically reduced α-APPs secretion. Buxbaum et al. (1998) showed that knockout of ADAM17 also resulted in a large decrease in α-APPs secretion. In both knockout experiments, there was still residual α-APPs secretion, suggesting that ADAM10 and ADAM17 most likely function as inducible regulators of α-secretase activity (Lammich et al., 1999; Buxbaum et al., 1998).

Perhaps the most important mechanism of AD development that we must understand is the relationship between the molecular pathology of AD and the primary

symptom: severe memory loss. Memory is often thought to be encoded by changes in synaptic strength, a phenomenon known as synaptic plasticity. As such, one of the molecular mechanisms most commonly linked with learning and memory is long-term potentiation (LTP), which is a persistent strengthening of synapses in response to repeated stimulation. In the most common model of LTP, stimulation of N-methyl D-aspartate receptors (NMDARs) leads to increased calcium influx, which activates calcium/calmodulin-dependent protein kinase II (CaMKII). CaMKII then either phosphorylates α-amino-3-hydroxy-5-methyl-4-isoxazolepropionic acid receptors (AMPARs) to increase their conductivity or leads to the insertion of more AMPARs into the membrane. It is commonly thought that nitric oxide diffuses into the presynaptic cell, where it promotes increased production of glutamate. Dendritic spines also undergo structural changes (Matsuzaki et al., 2004).

Importantly, synaptic loss, and not plaque deposition or tangle formation, is the hallmark most closely correlated with the degree of cognitive decline, leading researchers to look for ways in which Aβ may affect synapses. Perhaps expectedly, Walsh et al. (2002) showed that cerebral microinjection of naturally secreted human Aβ caused impairment of hippocampal LTP *in vivo*. When either amyloid fibrils or monomers were removed from the medium, the LTP-inhibiting effect was not diminished, leading the authors to conclude that Aβ oligomers are the synaptotoxic species. Next, Almeida et al. (2005) elucidated a potential mechanism for this inhibition of LTP. In an *in vitro* transgenic AD model, they observed fewer pre- and post-synaptic

compartments, as well as a decrease in the levels of PSD-95, a protein that is responsible for anchoring glutamate receptor subunits. Following that observation, they show a decrease in the surface expression of NMDARs. These same deficits were reproduced in wild-type neurons after addition of Aβ oligomers, solidfying the role of Aβ in causing these deficits. Snyder et al. (2005) report another interaction between Aβ and NMDARs by showing that application of Aβ induced a reduction in current through NMDARs. Rather than simply inhibiting the receptor, however, the authors show that Aβ promotes its endocytosis by activating the tyrosine phosphatase STEP, which dephosphorylates the NR2B subunit of the NMDA receptor. Similarly, Hsieh et al. (2006) report a loss of dendritic spines and increased endocytosis of AMPARs upon treatment with Aβ. Lastly, Zhao et al. (2004) have shown that application of Aβ during LTP reduces the levels of autophosphorylated CaMKII and phosphorylated GluR1, a subunit of the AMPA receptor. When taken together, all of these data show that Aβ has the potential to inhibit LTP on multiple levels by attacking the NMDARs needed for its induction, the AMPARs that ultimately result in the increased synaptic signaling, the PSD protein that anchors the receptors to the membrane, and the enzyme that leads to the increased insertion or activity of AMPARs. Importantly, a number of studies also report reductions in the number of synapses or dendritic spines (Shrestha et al. 2006; Evans et al. 2008; Lacor et al. 2007) without explicitly reporting cell death, which is a point emphasized by some older studies (Davies et al. 1987, DeKosky and Scheff, 1990) suggesting that

synaptic loss may be one of the earlier occurrences in the development of AD, as it is associated with pruning by Aβ, not the widespread neurodegeneration.

In additon to synaptic damage, an increase in the amount of $A\beta_{42}$ is thought to drive AD pathology through exerting negative effects on neuronal health. One of the primary mechanisms through which this is thought to occur is $A\beta_{42}$ exerting an effect on neuronal apoptosis. Apoptosis is a highly regulated homeostatic process that leads to cellular death (reviewed in Elmore, 2007). Apoptosis is initiated by high levels of secretion of a protein named cytochrome c, which, when released in small amounts, binds to inositol trisphosphate receptors (IP_3Rs) in the endoplasmic reticulum and causes an increase in calcium ion release. This increase in calcium leads to further cytochrome c release from the mitochondria and the initiation of apoptosis. Interestingly, Aβ injection has been shown to dysregulate calcium dynamics *in vitro*, increasing the amount of calcium moving through IP_3Rs (Ferreiro et al., 2006). Mechanistically, this disturbance is suggested to result from Aβ-mediated interruption of Na+/K+-ATPase and Ca2+-ATPase activity (Mark et al., 1995). Loss of Ca2+-ATPase function suggests that calcium ions are not reliably transported back into the endoplasmic reticulum after release, instead remaining in the cytoplasm. The other major mechanism of calcium removal from the cytoplasm is the Na+/Ca2+-exchanger, which pulls sodium in and calcium out. However, impaired function of the Na+/K+-ATPase suggests that neurons may struggle to re-establish resting ion concentrations after depolarization, which would disrupt the electrochemical gradient that the

Na+/Ca2+-exchanger relies on to perform its function. In addition to altering calcium dynamics, Aβ has been suggested to interact with some proteins that are key to the process of apoptosis. Injection of $A\beta_{42}$ into cultured neurons results in increased levels of apoptosis (Zhang et al., 2002). Using antibodies against proteins involved in the apoptotic pathway, Zhang et al. (2002) show that this increased apoptosis is mediated by an increase in the transcription of the pro-apoptotic factor Bax by p53.

Another potential driving process behind the development of AD is oxidative stress. Oxidative stress is defined as an imbalance in the production of reactive oxygen species (ROS) and the organism's ability to eliminate them. These ROS are free radicals, highly reactive molecules that possess unpaired valence electrons, and are produced in the mitochondria during aerobic metabolism. However, because of their reactivity, ROS can interact with and damage proteins and DNA in the cell, requiring their controlled production and elimination. It is currently thought the production of ROS is a primary contributor to the aging process, as damage caused by ROS production slowly builds up over time (reviewed in Finkel and Holbrook, 2000). Since aging is the primary risk factor for the development of sporadic AD, the potential contribution of ROS and oxidative stress to the development of the disease is worth investigating.

In this investigation, both the potential effects of oxidative stress on Aβ production and of Aβ production on oxidative stress should be considered. Thus far, research looking into the former relationship has pinpointed two potentially important molecules. The first of these is 4-hydroxynonenal (HNE), an aldehyde produced by lipid

peroxidation. A study by Tamagno et al. (2002) has shown that HNE results in an upregulation of BACE-1 (β-secretase) *in vitro*. They have also shown (Tamagno et al. 2005) that this upregulation is a result of the activation of stress-activated protein kinases (SAPKs), namely c-Jun N-terminal kinases and p38MAPK. These studies suggest that oxidative damage, indicated by the formation of HNE, can indirectly affect Aβ production by activating proteins that increase BACE-1 expression. Kao et al. (2004) have shown that hydrogen peroxide causes this effect, as well, and introducing siRNA against BACE-1 prevented the neurotoxicity caused by hydrogen peroxide. This again suggests that amyloidogenic processing of APP is heightened as a result of oxidative damage.

The second molecule that has been studied as a part of this relationship is Pin1, an isomerase that interconverts between the cis and trans conformations of phosphorylated Ser/Thr-Pro motifs. APP has such a motif, and, in fact, phosphorylation of Thr 668-Pro has been shown to be increased in the brains of AD patients (Lee et al. 2003). Pastorino et al. (2006) have shown that Pin1 binds to this motif and catalyses its isomerization. They also show that when Pin1 is overexpressed, Aβ secretion *in vitro* decreases. Likewise, when Pin1 was knocked out, Aβ secretion increased, but this knockout specifically increased the levels of the 42-amino acid peptide. These data suggest that Pin1 plays an important role in regulating APP processing. Interestingly, Sultana et al. (2006) have shown that Pin1 is down-regulated in the AD brain. Additionally, they show that the down-regulation of the protein is due to its oxidation,

which provides further evidence that oxidative damage ultimately results in increased production of Aβ.

Investigations into the potential effects of Aβ on oxidative stress have focused primarily on the ability of Aβ to cause mitochondrial damage and dysfunction. Crouch et al. (2005) report a high level of Aβ immunoreactivity in the mitochondrial fraction of the brains of AD mouse models. In addition, they show that Aβ functions as an inhibitor of cytochrome c oxidase (COX). COX is the terminal step in the mitochondrial electron transport chain, and loss of COX function has been associated with an increase in the production of ROS and cellular toxicity (reviewed in Srinivasan and Avadhani, 2012). The inhibition of COX by Aβ was also shown by Manczak et al. (2006). They also show, however, that AD mouse models exhibit greater levels of hydrogen peroxide production, and that this level is directly correlated with the levels of soluble Aβ.

Another set of studies have investigated the role of the mitochondrial enzyme Aβ-binding alcohol dehydrogenase (ABAD) in causing mitochondrial dysfunction in AD. The enzyme has been found to be increased in the cerebral cortex and hippocampus of AD patients and mouse models (Lustbader et al. 2004). Lustbader et al. (2004) also directly showed that Aβ can access the mitochondria to bind to this protein and that mice that overexpress ABAD exhibit increased oxidative stress. Using a decoy peptide meant to block the binding of Aβ to ABAD, Yao et al. (2011) observed an decrease in mitochondrial oxidative stress, neuronal death, and mitochondrial soluble Aβ levels in transgenic AD mouse models. These data, combined with the studies from the previous

paragraph, suggest that Aβ is capable of directly causing damage to mitochondria, ultimately leading to increased generation of ROS and neuronal death.

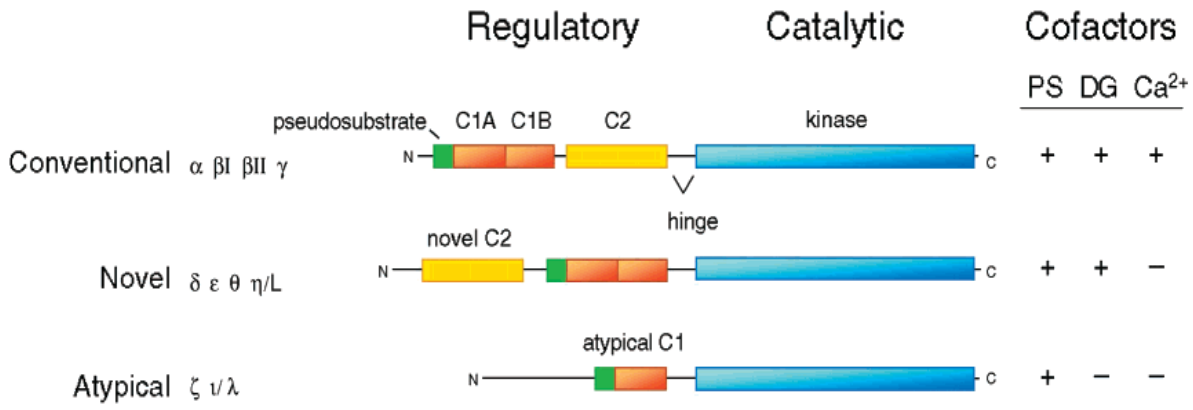
In addition to its effects on mitochondria, Aβ has been implicated in direct oxidative damage to membrane lipids. This role is believed to be centered on methionine 35 of the 42-amino acid peptide. The major evidence for this comes from a study by Yatin et al. (1999), where they used C. elegans models overexpressing human APP. When Met^{35} was converted to cysteine, there was no observed change in Aβ deposition, however, there was no longer any observable protein oxidation. Kanski et al. (2002) have proposed a model by which methionine leads to oxidative damage. The hydrophobic nature of the Aβ C-terminus allows it to insert itself into a membrane in an α-helical conformation. While in the membrane, the methionine can be oxidized to produce a methionine sulfuranyl radical, which can then take a hydrogen from a nearby lipid to produce a carbon-centered radical on that lipid capable of interacting with oxygen to form a peroxyl radical. Pogocki and Schoneich (2002) show that residues 27-36 of the Aβ peptide do adopt α-helical secondary structure, and that structure places the C-terminal peptide bond carbonyl of isoleucine 31 near Met^{35}, providing a stabilizing force for the proposed single-electron oxidation of the residue. Kanski et al. (2002) provided further support for this model by replacing isoleucine 31 with proline, which destabilizes the α-helical structure. Cells expressing this mutant exhibited less oxidative damage, reinforcing that proper secondary structure of the Aβ peptide is critical for promoting this function.

Protein Kinase C: Structure

In the last decade, many research groups have taken an interest in the potential involvement of protein kinase C (PKC) in AD and its potential as a therapeutic target. PKC is a family of serine/threonine protein kinases, meaning that their major biochemical function is to phosphorylate other proteins on serine or threonine residues. PKC exists in three major classes of isozymes: conventional (PKC-α, PKC-βI, PCK-βII, and PKC-γ), novel (PKC-δ, PKC-ε, PKC-η, and PKC-θ), and atypical (PKC-ζ and PKC-ι/λ). The structure of this family is well studied and has been extensively reviewed by Newton (1995 and 2001). All isozymes of PKC have a conserved kinase domain attached to a regulatory moiety, but the contents of this moiety vary between the classes and help to define their unique properties (see Model 2). One member of this moiety that is conserved, however, is the pseudosubstrate domain, which, as its name suggests, is able to bind to the active site of the enzyme and block it from exhibiting activity before the conditions for PKC activity are met.

There is also a semi-conserved domain called C1, which is approximately 50 amino acids long and rich in cysteine residues. In conventional and novel isozymes, this domain is repeated (called domains C1A and C1B), but atypical isozymes have only one copy. The domain is stabilized by two zinc ions coordinated to histidine and cysteine residues at either end of the domain. In the novel and conventional isozymes, these domains have been shown to contain a binding site for phorbol esters and diacylglycerol (DAG) between two β-sheets. In the atypical isozymes, however, one side of this binding

site is eliminated, meaning that the atypical isozymes cannot bind DAG and suggesting a major difference in their regulation. The novel and conventional isozymes possess a fourth domain in their regulatory moiety, called the C2 domain. These domains possess two different topologies, either following the C1 domains (in conventional isozymes) or preceding them (in novel isozymes). Regardless of this, the C2 domain is rich in β-sheets and contains a pocket formed by loops at either end of the structure. In conventional isozymes, this pocket contains several aspartate residues, which allow for coordination of 2 or 3 calcium ions. The novel isozymes lack these aspartate residues, and, consequently, do not bind calcium (Newton 1995 and 2001).



Model 2. Schematic depicting the structural and cofactor differences between the classes of PKC isozymes. Image credit Newton (2001)

There are two major factors that contribute to the overall regulation of PKC activation: phosphorylation and cofactor binding. Post-translational modification by phosphorylation is required for PKC to attain its mature conformation, and this occurs in three regions that are mostly conserved in all PKC isozymes. The first of these

phosphorylation events occurs at the activation loop on a threonine residue, and is catalyzed by PDK-1. Orr et al. (1994) found that mutation of this residue to a non-phosphorylatable residue in conventional isozymes results in aggregation of immature, unphosphorylated enzyme. At the same time, conversion of this residue to a glutamate allowed for complete maturation of the enzyme, suggesting that the presence of negative charge in the activation loop is critical to the function of phosphorylation (Orr et al., 1994; Cazaubon et al., 1994). The second phosphorylation site is a residue in a proline-rich region, known as the turn motif, which is suggested to occur by autophosphorylation (Flint et al., 1990). Researchers propose that the presence of phosphate at this motif contributes to thermal stability and phosphatase resistance in the mature enzyme (Bornancin and Parker, 1996). The final phosphorylation event is autophosphorylation at a site known as the hydrophobic motif. This is the only site that is not conserved across all isozymes, with atypical PKCs having a glutamate there to function as a phosphate mimic. The function of this phosphate is thought to be similar to that of the turn motif phosphate, increasing stability of the enzyme. However, this phosphorylation event is not essential and mature (albeit less stable) enzyme can exist without it (Bornancin and Parker, 1996). Similarly, the activation loop phosphate can be removed after activation of the mature enzyme without negatively impacting its stability or function, suggesting that the turn motif phosphate is the only one essential to activity of the fully mature enzyme (Keranen et al., 1995).

The second major mechanism of PKC regulation, cofactor binding, is where the differences between the classes of isozymes have the largest impact on regulation (see Model 2). The first of these cofactors is DAG (often replaced by phorbol esters in experiments because they are less readily metabolized and thus produce longer lasting activation). As was mentioned previously, the C1 domains of novel and conventional isozymes contain a pocket for binding these molecules. Research has suggested that the insertion of one of these cofactors causes the pocket to have a contiguous hydrophobic surface, which allows for recruitment of the enzyme into the membrane. The other major effect of this binding is increasing the affinity of the C1 domains for phosphatidylserines present in the membrane, which are the second cofactor necessary for PKC activity, and the only one that binds to all three classes of PKC isozymes. The final cofactor is calcium, which binds to aspartate-lined pockets in the C2 domain of conventional isozymes. This is thought to work in a similar manner to DAG recruitment: by coating the pocket with positive charge, the ability of the domain to interact with anionic lipids in the membrane (i.e. phosphatidylserine) is increased.

Interestingly, calcium and DAG have been shown to have a synergistic effect by independently increasing the enzyme's affinity for anionic lipids. It has been proposed that recruitment of either the C1 or C2 domain to the membrane helps to increase the odds of the other being recruited, as well. In the case of conventional isozymes, calcium binding recruits C2 to the membrane, increasing the odds of C1 finding DAG. For novel isozymes, which contain an arginine in the C2 pocket rather than aspartates, C1

recruitment may make it easier for the C2 arginine to find anionic lipids for binding (Newton, 1995). Either way, the recruitment of both of these domains is thought to be necessary for the release of the pseudosubstrate from the enzymatic active site (Johnson et al., 2000). To summarize, conventional PKC isozymes are dependent upon all 3 cofactors for activation, novel isozymes require DAG and phosphatidylserine for activation, and atypical isozymes only require phosphatidylserine for activation.

Protein Kinase C-ε: Functions and Implication in Alzheimer's Disease

Of all the isozymes of PKC, the novel PKC-ε has become a subject of many studies investigating AD pathology. PKC-ε is the most abundant isozyme of PKC in the brain, and specifically has a high expression in the hippocampus. The levels of the active enzyme have been reported to be reduced in the brains of AD patients, as have the total expression levels of the protein. In order to understand how loss of this protein may contribute to AD pathology, we must consider what specific functions this protein performs in the brain.

There is a large body of eveidence suggesting that PKC isozymes are important in neuronal differentiation and neurite growth. Using phorbol esters, Hall et al. (1988) and Roivainen et al. (1993) both showed induction of neurite outgrowth in PC12 cell lines, which are derived from the neural crest. This is supported by the finding that phorbol esters also increased signaling by nerve growth factor (NGF), which is essential in neurite outgrowth (Roivainen et al., 1995). However, these studies did not indicate which isozymes are involved in this process. Hundle et al. (1995) reported increased

mRNA for PKC-δ and PKC-ε in stimulated PC12 cells, and that PKC-ε expression was localized in the growth cones and neurites of differentiating PC12 cells. Additionally, overexpression of PKC-ε alone increased NGF-stimulated neurite outgrowth, and this was the result of increased activation of the mitogen-activated protein kinase (MAPK) signaling pathway, which is well-known to be involved in neuronal differentiation. These data suggest that PKC-ε plays an important role in the growth of neurites during neuronal differentiation, and may play a larger role in the construction of neural networks. This hypothesis is supported by expression data that showed PKC-ε as the dominant isozyme in developing chick and rat brains around E5-7 (Mangoura et al., 1993; Shirai et al., 2008).

The suggestion that PKC-ε may play a larger role in the construction of neural networks is further supported by studies of synaptogenesis. Hama et al. (2004) demonstrated that contact between neurons and astrocytes leads to activation of PKC. This contact resulted in increased excitatory synaptogensis throughout the neuron, which was blocked by PKC inhibitors. Importantly, they showed that the arachidonic acid cascade is the cause of this PKC activation. Arachidonic acid is capable of binding to PKC-ε, and this interaction is unique to this particular isozyme (Kasahara and Kikkawa, 1995). Thus, the necessity of the arachidonic acid cascade implicates this isozyme in the observed synaptogenic effect. An additional synaptogenic implication comes from a study linking PKC-ε to post-synaptic density protein 95 (PSD-95). This particular protein plays an important role in the synapse, serving as a scaffold to cluster glutamate

receptors and ensure their coupling to downstream molecules (Sheng and Hoogenraad, 2007; Bats et al., 2007). It has also been suggested to help with the stabilization of newly formed synapses (De Roo et al., 2008). Sen et al. (2016) showed that specific activation of PKC-ε increases the levels of PSD-95 as well as its localization to the membrane. Subsequently, they reported an increased number of synapses. When the reverse (i.e. knockdown of PKC-ε) was performed, they reported decreased expression of PSD-95 as well as of synaptophysin, a presynaptic protein often used as a marker for the presence of synapses. These results further suggest that PKC-ε plays an important role in the process of synaptogenesis.

Similar studies have shown that phorbol ester-mediated activation of PKC is able to mimic LTP in hippocampal neurons. Evidence for this comes from studies showing that phorbol ester treatment increased the release of glutamate by pre-synaptic neurons and resulted in excitatory post-synaptic potentials (Yamamoto et al., 1987; Malenka et al., 1986; Shapira et al., 1987), and that these potentials were blocked by PKC inhibitors (Huang et al., 1992). Evidence that PKC-ε is the isozyme responsible for this is two fold. First, Saitoh et al. (2001) showed that PKC-ε is present in synaptic terminals and that phorbol ester stimulation caused the protein to become phosphorylated and translocate to the synaptic side of the terminal. Second, the significance of this translocation is suggested by Prekeris et al. (1996), who showed that PKC-ε has a unique actin-binding site located between its two C1 domains. Actin filaments, aside from their role in cytoskeletal structure, have been implicated in vesicle

exocytosis and maintenance of a reserve pool of vesicles (Doussau and Augustine, 2000). It follows, then, that PKC-ε interacting with actin filaments and affecting actin dynamics may explain the phorbol ester-mediated increase in neurotransmitter release. Interestingly, Prekeris et al. (1996) also showed that binding of arachidonic acid along with DAG enhanced the interaction between PKC-ε and actin filaments. Arachidonic acid is produced by activation of NMDARs (Dumuis et al., 1988) and there is evidence that it is capable of acting as a retrograde signaling molecule, traveling from the post-synaptic neuron, where it is produced, to the pre-synaptic neuron, where it exerts an effect (Leu and Schmidt, 2007). This action of PKC-ε fits into the previously discussed model of LTP well, as a retrograde signaling molecule is thought to be responsible for the increased release of neurotransmitter from the pre-synaptic neuron that synergizes with the increased insertion of AMPARs into the post-synaptic neuron.

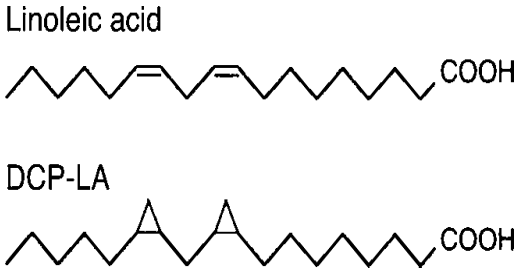
While involvement in processes such as synaptogenesis and LTP may go a long way to suggest that loss of PKC-ε is likely related to AD pathology, there is even more direct evidence available. Several studies have shown that treatment with phorbol esters resulted in a reduction of Aβ and an increase in the secretion of sAPPα, which indicates a shift in APP processing toward the non-amyloidogenic pathway (Slack et al., 1993; Jacobsen et al., 1994). Using specific inhibitors against certain PKC isozymes, Zhu et al. (2001) showed that PKC-ε is the isozyme that mediates these observed effects of phorbol esters. Furthermore, phorbol esters have been shown to lead to increased activity of ADAM17, one of the putative α-secretases (Cisse et al., 2011). This effect was

replicated by expression of a constitutively active mutant of PKC-ε and blocked by expression of a dominant negative mutant of PKC-ε, showing that PKC-ε leads to the activation of α-secretase. The mechanism by which this occurs remains unknown. However, Cisse et al. (2011) demonstrated that activation of the extracellular-regulated kinase-1/MAP-ERK kinase (ERK1/MEK) signaling cascade is not involved in this pathway. This cascade is used by ADAM17 in cleavage of other common targets (Alfa Cisse et al., 2007), which suggests a unique mechanism for ADAM17-mediated cleavage of APP that could be a viable drug target if it is identified.

DCP-LA: A Potential Therapeutic Agent for Alzheimer's Disease

A series of studies conducted by Nishizaki et al. showed that *cis*-unsaturated fatty acids, such as arachidonic acid (1999), linoleic acid (1997a), and oleic acid (1997b), could induce LTP-like synaptic potentiation, and the acids themselves, not their metabolites, were responsible for this effect. This led to the desire to produce derivatives of these acids with increased biostability in an attempt to develop cognitive-enhancing drugs for diseases such as AD. In this pursuit, Tanaka and Nishizaki (2003) synthesized 8-[2-(2-pentylcyclopropylmethyl)-cyclopropyl]-octanoic acid (DCP-LA), a linoleic acid derivative with cyclopropyl rings replacing the two double bonds (see Model 3). They demonstrated that DCP-LA reproduces this same synaptic potentiation. It was also able to pentrate the blood-brain barrier and was retained in the blood at greater than 180 times its maximal effective concentration after intraperitoneal (i.p.) injections, suggesting that the drug was efficient in an *in vivo* system. In terms of the

mechanism of function, Kanno et al. (2006) showed that DCP-LA acts as a strongly selective activator of PKC-ε, displaying greater than 7-fold specificity for this isozyme over the others. In addition, the activity of DCP-LA was not increased by the addition of dioleoyl-phosphatidylserine, suggesting that the drug works by binding to the phosphatidylserine site of PKC-ε.



Model 3. Comparison of chemical structures for linoleic acid and its derivative, DCP-LA.
Image credit Kanno et al. (2006)

The developers of the drug tested it in mouse model of AD that involved a constant 2-week infusion of Aβ into a lateral ventricle of the animals (Nagata et al., 2005). They observed a decrease in performance of these animals in the Morris water maze, which was returned to sham levels by i.p. injection of DCP-LA. Similarly, Hongpaisan et al. (2011) studied the effects of DCP-LA using the 5XFAD transgenic mouse model of AD, which contains three mutations in APP and two mutations in PS1. As in the prior study, the transgenic animals injected with DCP-LA returned to control levels of performance in the Morris water maze. However, this study also measured the deposition of amyloid plaques in the mouse models, and reported a significant

reduction in plaque deposition in the models treated with DCP-LA. Lastly, they observed an increase in the number of dendritic spines in the DCP-LA treated models, suggesting a protective effect against synaptic loss. More specifically, there was a significant increase in the quantity of mushroom spines, which are the largest, most stable, and most mature of the spine morphologies.

In order to more specifically investigate the relationship between DCP-LA and amyloid beta production, Nelson et al. (2009) applied the drug to an *in vitro* model overexpressing a mutated form of the human APP gene and PS1 gene. They show that DCP-LA was able to increase α-secretase activity in both fibroblasts and neurons, although the effect in neurons was lower than in fibroblasts. However, they also demonstrate DCP-LA-mediated activation of endothelin-converting enzyme, which is responsible for degrading Aβ, to 180% of control levels. This study raised an interesting comparison between DCP-LA and more traditional PKC activators, namely phorbol esters. Phorbol ester-mediated activation of PKC was followed by a period of down-regulation of the enzyme, while DCP-LA resulted in a consistent activation. The authors proposed that this is the result of differences in binding mechanisms, as phorbol esters bind the DAG site and DCP-LA binds the phosphatidylserine site. Whatever the reason, this finding is critical because it suggests that attempting to use non-specific PKC activators as therapies for AD may result in periods of undesirable effects when the PKC isozymes are down regulated. This has already been demonstrated by da Cruz e Silva et

al. (2009), who reported an *increase* in Aβ generation after chronic application of phorbol esters.

The ability of this drug to affect the levels of Aβ suggests that studying the effects of this drug on pathways related to Aβ activity, such as oxidative stress, may provide further evidence of therapeutic potential. Yaguchi et al. (2010) did just this, examining the ability of DCP-LA to protect against oxidative stress *in vitro*, although they used the drug sodium nitroprusside (SNP) to induce this damage rather than Aβ. SNP tretament dropped cell viability by half, and the surviving cells were smaller and had damaged axons. They showed that co-treatment with DCP-LA was able to protect the neurons from the damage caused by SNP, not only blocking the apoptosis, but also maintaining the size and structure of the cells. Mechanistically, the authors were able to attribute this result to inhibition of the pro-apoptotic enzymes caspase-3 and caspase-9. A prior study has also shown that PKC-ε is able to increase the activation of Bcl-2, which is an anti-apoptotic gene, and this was accomplished through direct association with protein kinase B (Akt), providing a possible mechanism for the observed effects of DCP-LA (Steinberg et al., 2007).

Animal Models of Alzheimer's Disease

The development of animals models of neurological disease is critical to the effort to develop therapeutics for these disorders, especially those that primarily involve cognitive deficits and cannot be completely modeled *in vitro*. These models act as an intermediate between *in vitro* studies and human trials, allowing researchers to

translate mechanistic discoveries to clinical application. Importantly, this translation requires that the animal model faithfully mimic the disease being studied. AD research has been no different, with animal models being widely used to test potential therapeutics. Recall, however, that 99.6% of drugs that were given clinical trials in the last decade failed, even though their beneficial effects in animal models of AD earned them that trial. It is possible, then, that the animal models so heavily used in AD research are not an accurate enough representation of the disease to translate reliably to human pathology.

The particular type of model primarily used in AD research is a transgenic mouse model, which contains various combinations of genetic mutations thought to promote the disease phenotype. As was discussed earlier, there is a genetic form of AD that results from mutations in either APP or PS1, and it was the characterization of these mutations that lead to the genesis of these animal models, which rely upon overexpression of different mutant form of APP and PS1 (Games et al., 1995; Hsiao et al., 1996; Holcomb et al., 1998). These models were useful for us in that they resulted in a phenotype that displayed amyloid plaque deposition and cognitive impairment in learning and memory based tasks (Games et al., 1995; Hsiao et al., 1996; Holcomb et al., 1998).

However, there are a number of flaws in these animal models that must be considered when evaluating their usefulness in the drug development process. First, these animals have been widely shown to exhibit deficits in learning and memory prior

to the deposition of plaques (Chapman et al., 1999; Hsia et al., 1999; Larson et al., 1999), but the opposite occurs in humans, with plaques being detectable decades before cognitive deficits (Villemagne et al., 2013). Second, the development of NFTs was not observed in the previously cited characterizations of these animal models. Reliable generations of NFTs has come to depend upon introduction of a third transgene coding for mutant tau protein (Oddo et al., 2003), which is not related to human AD. In addition, the use of these mutants means that both Aβ and tau protein are being overexpressed simultaneously, whereas tau pathology normally develops later in the course of human pathology (Naslund et al., 2000). These two factors together raise the question of whether or not these models reliably replicate the temporal development of human AD, which can limit the effectiveness of these models in drug development. Another challenge to these models is the fact that they require a 5-10-fold increase in APP expression (reviewed in Balducci and Forloni, 2011) when, in humans, a 50% increase in APP expression is sufficient to lead to plaque formation (Duff and Suleman, 2004). This may lead to an accelerated pathology that does not accurately reflect developments in the human brain. While accelerating the pathology may be necessary for efficient development of a model, we must find a way to account for the potential differences between the mouse and human pathologies. Yet another concern is that the traditional transgenic models exhibited little to no neurodegeneration (Irizarry et al., 1997 a, b). However, this was remedied by the creation of the previously mentioned 5XFAD model, which contains five FAD mutations and is a very rapidly and aggressively

developing model (Oakley et al., 2006). Lastly, we must consider the fact that these animals are based off of features of the genetic form of AD, which, as mentioned earlier, only accounts for about 5% of AD around the world. We do not yet understand how plaque deposition occurs in the much more common sporadic form of the disease (or even the exact role that plaques play in the disease etiology), but it is not related to mutation, which is the mechanism that these models use. As such, there may be yet unknown differences between the induced pathology in the mouse model and the sporadic pathology in humans that account for the widespread failure of drugs in humans that were successful in these model systems.

In recent years, researchers have been working to create new model systems that more closely adhere to our current understanding of sporadic AD pathology. One such model that has seen some success is the Ferrous-Amyloid-Buthionine (FAB) model, developed by Lecanu et al. (2006). This model is pharmacological, rather than transgenic, and is established by intraventricular infusion of a solution containing Aβ, ferrous sulfate, and buthionine sulfoximine. The particular defining feature of this model is that it tries to account for the role of oxidative stress in the development of AD pathology. Ferrous sulfate is known to aid in the development of ROS through a process known as the Fenton reaction, and buthionine sulfoximine inhibits the synthesis of glutathione, which is the major anti-oxidant in the brain. Lecanu et al. (2006) report a significant worsening of learning and memory in these model animals, as well as the presence of amyloid plaques, NFTs, oxidative DNA damage, and neuronal death. Thus,

the pathology expressed in these animals is consistent with our current knowledge of AD pathology. There are two major weaknesses to this model, however. The first is that its temporal development has not been characterized. While all of the major players are present, we do not have direct evidence of how the pathology develops from the FAB cocktail, and so the model cannot yet be used to test treatment strategies at different stages of severity. The second is that the pathology being modeled is very acute, occuring over only 4 weeks, compared to the decades of development that can exist behind sporadic AD pathology.

Purpose of This Experiment

A high amount of evidence exists that PKC-ε is involved in AD pathology and that its specific activator, DCP-LA, could potentially be of therapeutic benefit in AD. However, the drought of successful AD therapeutics raises the concern that our transgenic model animals do not mimic the disease as faithfully as we need them to, and the actions of DCP-LA have only been shown using these models. As such, the goal of this experiment is to assess the therapeutic potential of DCP-LA in AD using the pharmacological FAB model. This model was chosen because it is useful for studying learning and memory related tasks, as well as correlating differences in these tests with neuronal proteins. However, the fact that amyloid burden and oxidative stress are artificially induced limits the scope of studies available in this model. The FAB model was created by 4-week intracerebroventricular infusion of Aβ, ferrous sulfate, and buthionine sulfoximine. The animals were then trained in the Morris water maze task to assess their learning and

memory capabilities, with some sham and FAB animals being given DCP-LA prior to the beginning of their training. The animals were then sacrificed and their hippocampi analyzed for the presence of the marker proteins NeuN and synaptophysin, which provide a measurement of neurodegeneration and synaptic density, respectively. We hypothesized that the FAB model animals would exhibit deficits in learning and memory, as well as decreased expression of NeuN and synaptophysin, and that treatment with DCP-LA would rescue all three of these deficits.

Materials and Methods

Animals and Treatment Groups

Thirty male, 3-month old Sprague-Dawley rats weighing between 328g and 374g were used for this experiment. The animals were divided into 5 treatment groups, each containing 6 animals: control, FAB, DCP-LA, FAB + DCP-LA, and no surgery. Animals in the no surgery groups were housed in pairs, while all others were housed individually. All animals were provided with food and water ad libitum and housed under a 12hr light, 12hr dark cycle. All handling and experimental procedures were approved by the Institutional Animal Care and Use Committee of Drew University.

Solutions

All infusions were intracerebroventricular and lasted for a period of 4 weeks. Animals in the control and DCP-LA groups received infusions of 0.9% saline. Animals in

the FAB and FAB + DCP-LA groups received infusions of 1.68 µM ferrous sulfate, 25.2 nM amyloid beta, and 20.16 µM buthionine sulfoximine. FAB solution was prepared in 10 mL batches using 3.01 µL of 100 µM amyloid beta, 0.01 g ferrous sulfate, and 0.09 g buthionine sulfoximine. Animals in the DCP-LA and FAB + DCP-LA groups received an intraperitoneal injection of DCP-LA (3 mg/kg of 6 µM stock in 5% DMSO) 24 hours before the beginning of Morris Water Maze training. 4% paraformaldehyde (PFA) was prepared in 1X phosphate buffered saline (PBS).

Osmotic Pump Filling

Model 2004 osmotic mini-pumps (reservoir volume 200 µL, infusion rate 0.25 µL/hr for 4 weeks) and Brain Infusion Kit #2 (containing polyvinyl catheter tubing approximately 15 cm in length, a cannula with a 28-gauge stainless steel tube 5mm in length, and 4 spacers for adjusting the length of the cannula) were purchased from Alzet. This infusion kit is specifically designed for ICV infusions, with default length cannula tip resting 3.0 mm in the brain. Catheter tubing was cut to approximately 6.5 cm in length and a cannula was attached to one end. A small amount of the solution to be added to the pump was injected into the open end of the catheter using the provided 27-gauge filling tube attached to a 1 mL syringe and allowed to run the length of the tubing and escape from the cannula. The empty pump and its flow moderator were weighed. The flow moderator was attached to the open end of the catheter tubing. The pump was then filled with approximately 250 µL of the desired solution using the provided 27-gauge filling tube attached to a 1 mL syringe and the flow

moderator was inserted into the pump. The pump was weighed again to ensure that the full volume was injected. The pumps were allowed to prime in 0.9% saline for 40 hours at approximately 37° C and remained in saline until immediately before insertion.

Surgical Procedure

All surgical equipment was cleaned and sterilized in 70% ethanol prior to each surgery. Each animal was anesthetized with a solution of 90 mg/kg ketamine and 10 mg/kg acepromazine. The top of the animal's head and back of its neck were shaved using an electric razor. The animal was then secured into a stereotaxic frame by inserting ear bars into its ears, catching its front tooth in the tooth bar, and then lowering a nose bar. The exposed skin was sterilized by rubbing with a povidone-iodine pad. An incision was made from between the animal's eyes to the base of its neck using a size 11 scalpel blade. The skin was held back for the duration of the procedure using four artery forceps, two on each side of the animal's head. The connective tissue was removed by rubbing with cotton swabs, exposing the animal's skull. Pressure was applied with gauze pads to soak up the blood and styptic powder was applied to prevent further bleeding. Hydrogen peroxide was rubbed on the skull using a cotton swab to enhance the visibility of the skull sutures. A t-pin attached to the arm of the stereotax was placed on the location of bregma and then moved 1.4 mm laterally to the left and 0.9 mm caudally, to the location of the left lateral ventricle (Paxinos and Watson). This location was marked for drilling with a permanent marker. A subcutaneous pocket was made by holding up the skin at the back of the animal's neck with mouse tooth forceps

and inserting scissors between the skin and the muscle. A drill was then used to penetrate the skull at the previously marked location. Pressure was applied to stop the bleeding. An osmotic pump was removed from the saline and briefly sterilized by wiping with antiseptic pads. The top of the pump was grasped with hemostats and the skin at the back of the neck was pulled up with mouse tooth forceps, opening the pocket. The pump was then inserted into the pocket and allowed to sit on the animal's left flank. The arm of the stereotax was changed from a t-pin to an alligator clip and the top of the cannula was inserted into the clip. The tube of the cannula was moved over the drill hole and lowered approximately 25% of the way down. A batch of dental cement was mixed and applied thinly on the skull around the drill hole. The cannula was then lowered the rest of the way and a layer of cement was applied around the base of the cannula. The cement was allowed to dry for 10 minutes and then the alligator clip was removed from the top of the cannula. A second batch of cement was mixed and a second layer covering all but the top of the cannula was applied. After 10 minutes of drying, excess catheter tubing was tucked into the subcutaneous pocket, the hemostats holding the skin back were removed, and the incision was closed with wound clips. The animal was freed from the stereotax, wrapped in a towel to maintain body heat, and returned to its home cage to recover.

Morris Water Maze

Apparatus

The Morris water maze used for this experiment was a yellow polypropylene pool roughly 5 feet in diameter and 18 inches deep. When in use, the pool was filled with approximately 12 inches of water at room temperature (25-27°C). For the sake of the experiment, AnyMaze software was used to divide the pool into four quadrants, one of which contained a platform approximately 1 inch below the surface of the water, such that the animals could escape from the water by standing on it. There were 4 curtains of differing colors and patterns surrounding the pool to provide unique visual cues to the animals during the task.

Acclimation

The day before formal training began, animals were allowed to sit in the darkened water maze room for one hour to adjust to the room. Then, animals were placed, one at a time, in the quadrant opposite the platform and given 120 seconds to find it. If an animal did not find the platform, it was guided there. Upon reaching the platform, each animal was allowed to remain there for 30 seconds. It was then towel-dried and returned to its home cage.

Training

For training trials, the water was rendered opaque by adding black nontoxic paint in order to make the platform invisible to the animals. Training lasted for 5 days, and each animal received one training session for each of the 5 days. A training session consisted of 4 trials, and each trial started in a different quadrant, such that every animal underwent one trial in each quadrant each day. The order in which the animals

were tested and the order of the starting positions were randomized and changed for every training day. During the trials, the animal was placed in the water facing the wall of the starting quadrant. The animal was given 90 seconds to find the platform, and each trial ended when the platform was found. If the animal did not find the platform within 90 seconds, it was guided there. The animal was allowed to remain on the platform for 30 seconds, beginning from the time that the animal first reached the platform. The animal was then towel-dried and placed in a holding cage for 30 seconds before beginning the next trial. When the animal completed its fourth trial, it was returned to its home cage instead of the holding cage. During each trial, the amount of time it took the animal to reach the platform (henceforth called "latency to platform") was measured, and an average was calculated for each animal on each day.

Probe Trials

This testing was conducted 48 hours after the final training day. The platform was removed from the pool and each animal was given a single 120 second trial, starting in the quadrant opposite where the platform was located. The total amount of time spent in the quadrant where the platform was located and the total amount of time spent in a designated area around where the platform was located were recorded.

Perfusion

A perfusion pump was set up with an open perfusion tube on one side and a needle on the other. Animals were injected with a lethal dose of ketamine/acepromazine solution. After it was anesthetized, but before it was killed, the

animal's abdomen was cut open. Connective tissue below the diaphragm was cut away and incisions were made along the sides of the rib cage, up toward the animal's armpits. The rib cage was lifted to expose the heart and clamped up with hemostats. The heart was gently lifted and the dorsal aorta was clamped with hemostats to prevent perfusion solution from reaching the lower half of the body. The open end of the perfusion tube was placed in PBS and the needle end was inserted into the left ventricle of the animal's heart. The auricle of the heart was cut to monitor flow of the fluid and to relieve pressure. The flow of PBS was maintained until the fluid coming from the animal was nearly colorless. At that point, the open end of the perfusion tube was moved from PBS to 4% PFA in PBS. The flow of 4% PFA was maintained until whitening of the animal's paws and lungs were observed and its arms and neck had become rigid. The animal was then removed from the set-up and decapitated using a guillotine. The fur and skin were cleared away from the top of the head using scissors, the cannula (if present) was removed using pliers, and the skull was peeled away using rongeurs. The brain was scooped into a vial containing 4 % PFA and the animal's body and head were discarded appropriately. After overnight incubation in the 4% PFA solution, the brains were transferred to a 20% sucrose solution in PBS. They were transferred again to a PBS solution after 24 hours.

Immunohistochemistry

Sample Preparation

Each brain was removed from its vial and cut approximately a millimeter rostral from the site of cannula implantation using a surgical blade. The cerebellum and hindbrain were also removed. The remaining portion of the brain was affixed to a metal stage using superglue, the stage was placed into a vibratome, and the brain was submerged in PBS. The brain was trimmed in slices of approximately 300 micrometers until the needle track was visible. At this point, 4 slices of 40-micrometer thickness were taken and affixed to a gelatin-coated slide. The brain was trimmed again until the hippocampus was visible. At this point, 4 slices of 40-micrometer thickness were collected into a single net well on a PBS-filled 6-well plate. This process was repeated 7 more times, for 2 brains from each of the 4 treatment groups (excluding no surgery controls), with each brain having its hippocampal slices stored in a separate well.

Staining

To permeabilize the samples, the net wells were transferred from PBS to a well plate containing 0.3% Triton X-100 (TX100) and 5% normal horse serum for 30 minutes at room temperature. The net wells were then transferred to a well plate containing 1:1000 dilutions of both an anti-NeuN antibody raised in mouse and an anti-synaptophysin antibody raised in rabbit in PBS + 0.2% TX100 and 1% bovine serum albumin (BSA). The well plates were incubated with agitation for 24 hours at 4°C. The slices were rinsed by transfer to a PBS-containing well plate 3 times for 10 minutes each. The net wells were then incubated with agitation in a 1:400 dilution of goat anti-rabbit FITC-conjugated secondary antibody in PBS + 0.2% TX100 and 1% BSA for 2 hours at

room temperature. The slices were again rinsed in PBS 3 times for 10 minutes each. The net wells were then incubated with agitation in the other secondary antibody, a 1:400 dilution of sheep anti-mouse Cy3-conjugated antibody in 0.2% TX100 and 1% BSA for 2 hours at room temperature. Slices that were exposed only to primary antibody and only to secondary antibody were used as controls. The slices were washed again in PBS 3 times for 10 minutes each. The slices were mounted onto a glass slide and coverslipped using Fluoromount.

Data Collection

Using a fluorescent microscope, each slice was excited at the appropriate wavelength (blue laser [488 nm] for FITC, yellow laser [561 nm] for Cy3) to produce detectable fluorescence emission. An imaging program, Axiovert Imaging, was used to scan the slices in real-time and images were taken of the CA1, CA3, and dentate gyrus regions for both the left and right hemispheres of each slice under both emission conditions. In total, 384 images were collected (12 per slice, 4 slices per brain, 2 brains in each of 4 treatment groups). Each of the 384 images was analyzed using ImageJ. The region of interest was outlined to separate it from background staining. The mean fluorescence intensity and the percentage of the outlined area covered by fluorescence were collected from the program for each image.

Data Analysis

Morris Water Maze

Statistical analyses were performed using SPSS. A repeated measures ANOVA was used to analyze the learning data. One-way ANOVAs with Tukey's post-hoc were used to analyze each of the memory trial variables. For all analyses, α was set at p = 0.05.

Immunohistochemistry

Statistical analyses were again performed using SPSS. One-way ANOVAs (followed by Tukey's post-hoc test) with treatment group as the dependent variable were used after splitting the data file by the protein being stained for and hippocampal region. For all analyses, α was set at p = 0.05.

Some of the ANOVAs failed to meet the assumption of homogeneity of variances, and these tests were re-run as a non-parametric Welch ANOVA with a Games-Howell post-hoc test. However, there were no differences in the comparisons reported as significant using the non-parametric test, so the results of the standard one-way ANOVA are considered to be robust to the homogeneity of variances violation.

Results

Morris Water Maze

Briefly, animals were trained over a period of 5 days to swim to the location of a platform hidden under the water's surface in one quadrant of a pool divided into four quadrants. Each animal received 4 trials on each of the 5 training days, 1 in each quadrant. The latency to the platform was recorded. 48 hours after training day 5,

animals were placed back into the pool with the platform removed. The latency to former platform location and the amount of time spent in the correct quadrant were recorded.

Learning Trials

A main effect of time was found ($p < 0.0005$) such that the latency to the platform decreased as the training days passed (Figure 1). Further analysis reveals that there were differences between the some treatment groups in latency to the platform, with the FAB group having significantly higher latencies than the DCP-LA ($p = 0.006$), FAB+DCP-LA ($p = 0.01$), and No Surgery ($p = 0.01$) groups (Figure 1). There were no other significant differences detected, however.

Memory Trials

During the probe trial, FAB animals were found to have a significantly higher latency to the previous platform location compared to the Control ($p = 0.019$), DCP-LA ($p = 0.034$), FAB+DCP-LA ($p = 0.045$), and No Surgery ($p = 0.011$) groups (Figure 2). No other significant differences were detected. Similarly, FAB animals were found to have spent less time in the quadrant where the platform was located than the Control ($p = 0.025$), DCP-LA ($p = 0.001$), FAB+DCP-LA ($p = 0.001$), and No Surgery ($p < 0.0005$) groups (Figure 3). No other significant differences were detected.

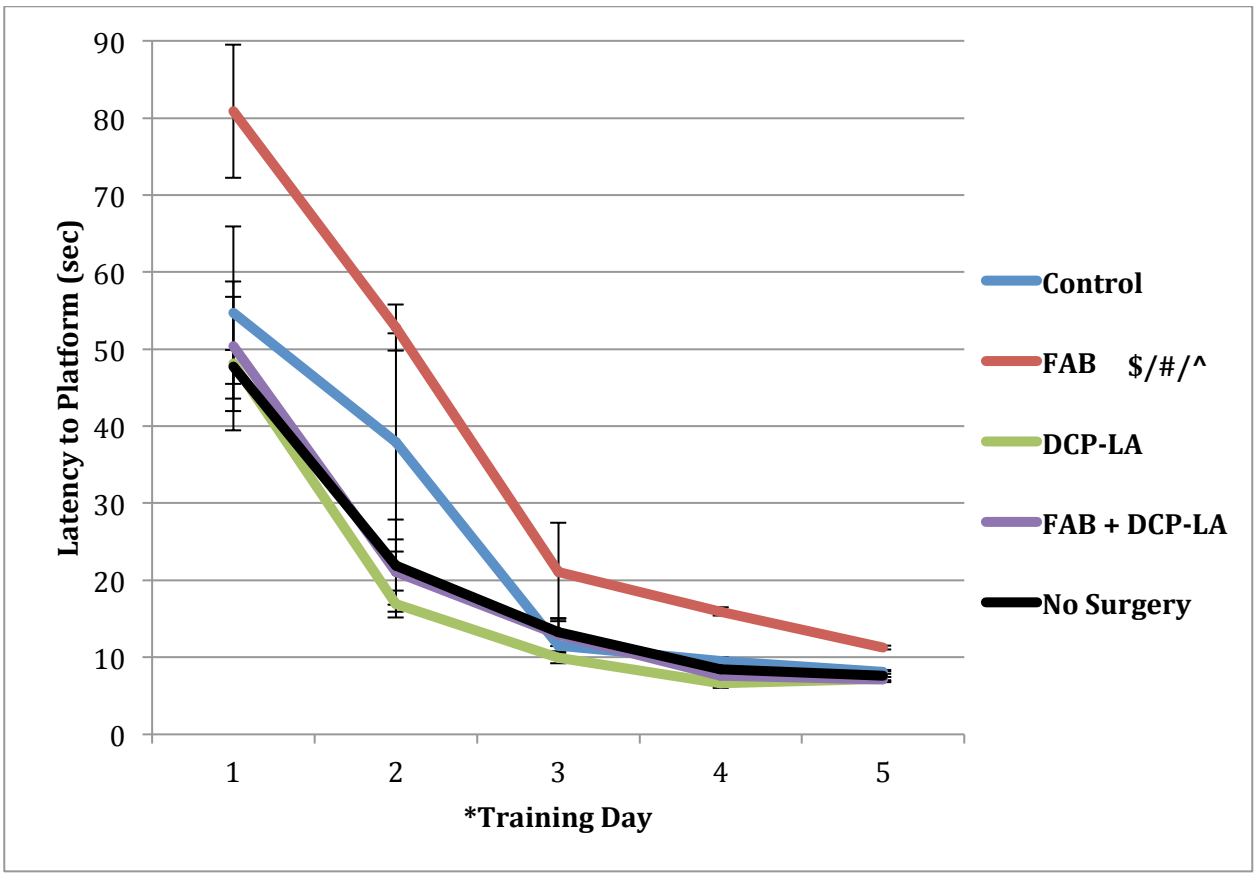


Figure 1. Latency to reach a hidden platform over 5 days of learning trials. Animals were given 4 trials each day for 5 straight days to find a platform, and the time it took for each animal to find the platform was recorded. The average for each treatment group on each day was calculated. * denotes a main effect of time ($p < 0.0005$). $ denotes a significant difference from the DCP-LA group ($p = 0.006$). # denotes a significant difference from the FAB+DCP-LA group ($p = 0.01$). ^ denotes a significant difference from the No Surgery group ($p = 0.01$). Data are mean +/- S.E.M.

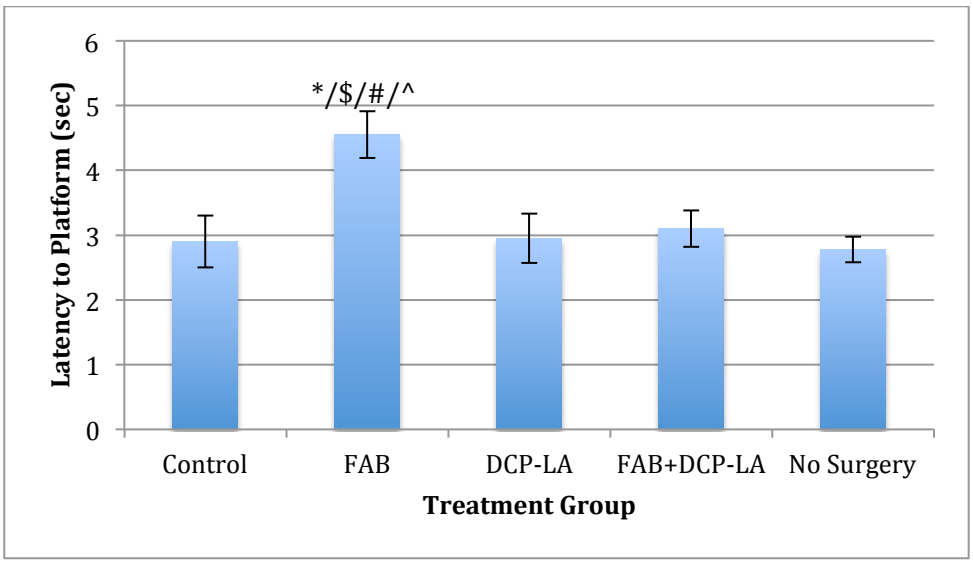


Figure 2. Latency to reach the location where the hidden platform was located during learning. 48 hours after the learning trials ended, the platform was removed and the activity of the animals in the pool was recorded for 90 seconds. The amount of time it took each animal to reach the old location of the platform was recorded and treatment group averages were calculated. * denotes a significant difference from the Control ($p = 0.019$). $ denotes a significant difference from the DCP-LA group ($p = 0.034$). # denotes a significant difference from the FAB+DCP-LA group ($p = 0.045$). ^ denotes a significant difference from the No Surgery group ($p = 0.011$). Data are mean +/- S.E.M.

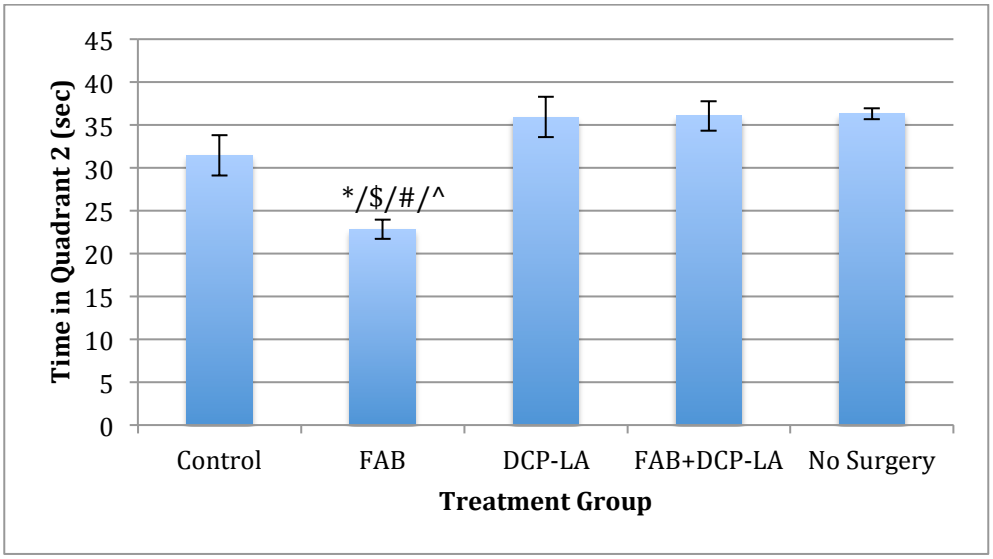


Figure 3. Time spent by each treatment group in the quadrant where the platform was located during the probe trial. 48 hours after learning trials ended, the platform was removed and the activity of the animals was recorded for 90 seconds. The total amount of time that each animal spent in the entire quadrant where the platform was located was recorded and treatment group averages were calculated. * denotes a significant difference from the Control ($p = 0.025$). $ denotes a significant difference from the DCP-LA group ($p = 0.001$). # denotes a significant difference from the FAB+DCP-LA group ($p = 0.001$). ^ denotes a significant difference from the No Surgery group ($p < 0.0005$). Data are mean +/- S.E.M.

Immunohistochemistry

Hippocampal tissue was harvested from the animals and stained using antibodies against the neuronal marker NeuN and the synaptic marker synaptophysin. The tissue was imaged using a fluorescent microscope and the mean intensity of each stain, as well as the percentage of the image covered by staining, were collected. Examples of images from each of the treatment groups can be seen in Figures 4 (for NeuN) and 5 (for synaptophysin).

NeuN Staining

The measured mean intensity of NeuN in the CA1 region was found to be significantly lower in FAB animals compared to all other treatment groups ($p < 0.0005$ vs. Control, $p < 0.0005$ vs. DCP-LA, $p = 0.002$ vs. FAB+DCP-LA) (Figure 6). These results held across all hippocampal regions tested, with FAB animals exhibiting lower mean NeuN fluorescence in both the CA3 ($p < 0.0005$ vs. all other groups) and dentate gyrus ($p < 0.0005$ vs. all other groups) compared to all other groups (Figure 6). Similarly, the percentage area covered in the CA1 region was significantly lower in FAB animals compared to all other treatment groups ($p < 0.0005$ vs. Control, $p < 0.0005$ vs. DCP-LA, $p = 0.001$ vs. FAB+DCP-LA) (Figure 6). These results held across regions, as well, with FAB animals exhibiting a lower percentage area covered in both the CA3 ($p < 0.0005$ vs. all other groups) and the dentate gyrus ($p < 0.0005$ vs. all other groups) compared to all other groups (Figure 7).

In addition, the FAB+DCP-LA animals exhibited lower mean NeuN fluorescence intensity in the CA1 and CA3 compared to Control animals ($p < 0.0005$ in CA1, $p = 0.005$ in CA3) and in all three regions compared to DCP-LA animals ($p < 0.0005$ in CA1, $p = 0.036$ in CA3, $p = 0.045$ in dentate gyrus) (Figure 6). The percentage area covered was also significantly lower in the CA1 and CA3 regions of FAB+DCP-LA animals compared to Controls ($p < 0.0005$ in both regions), but only in the CA1 compared to DCP-LA animals ($p = 0.002$) (Figure 7). No other significant differences were detected.

Synaptophysin Staining

Similarly to NeuN staining, the measured mean intensity of synaptophysin staining was found to be lower in the CA1 region of FAB animals ($p < 0.0005$ vs. all other groups) compared to all other treatment groups (Figure 8). Again, this result was consistent across hippocampal regions, with FAB animals exhibiting lower mean synaptophysin fluorescence intensity in both the CA3 ($p < 0.0005$ vs. Control and DCP-LA, $p = 0.001$ vs. FAB+DCP-LA) and dentate gyrus ($p < 0.0005$ vs. all other groups) compared to all other groups (Figure 8).

The percentage area covered by synaptophysin staining was also significantly lower in the CA1 region of FAB animals ($p < 0.0005$ vs. Control and DCP-LA, $p = 0.003$ vs. FAB+DCP-LA) compared to all other groups (Figure 9). This result was consistent with observed changes in the dentate gyrus, with FAB animals exhibiting a lower area covered by synaptophysin staining ($p < 0.0005$ vs. all other groups) compared to all other treatment groups (Figure 9). In the CA3, however, the area covered in the FAB

animals was only significantly lower than that of the DCP-LA animals ($p = 0.013$) (Figure 9).

In addition, the FAB+DCP-LA animals exhibited both a lower mean intensity ($p = 0.005$) and area covered ($p = 0.009$) in the CA1 region compared to DCP-LA animals (Figures 8 and 9). The area covered in the CA1 of FAB+DCP-LA animals was also significantly lower than that of Control animals ($p = 0.007$) (Figure 9). No other significant differences were detected.

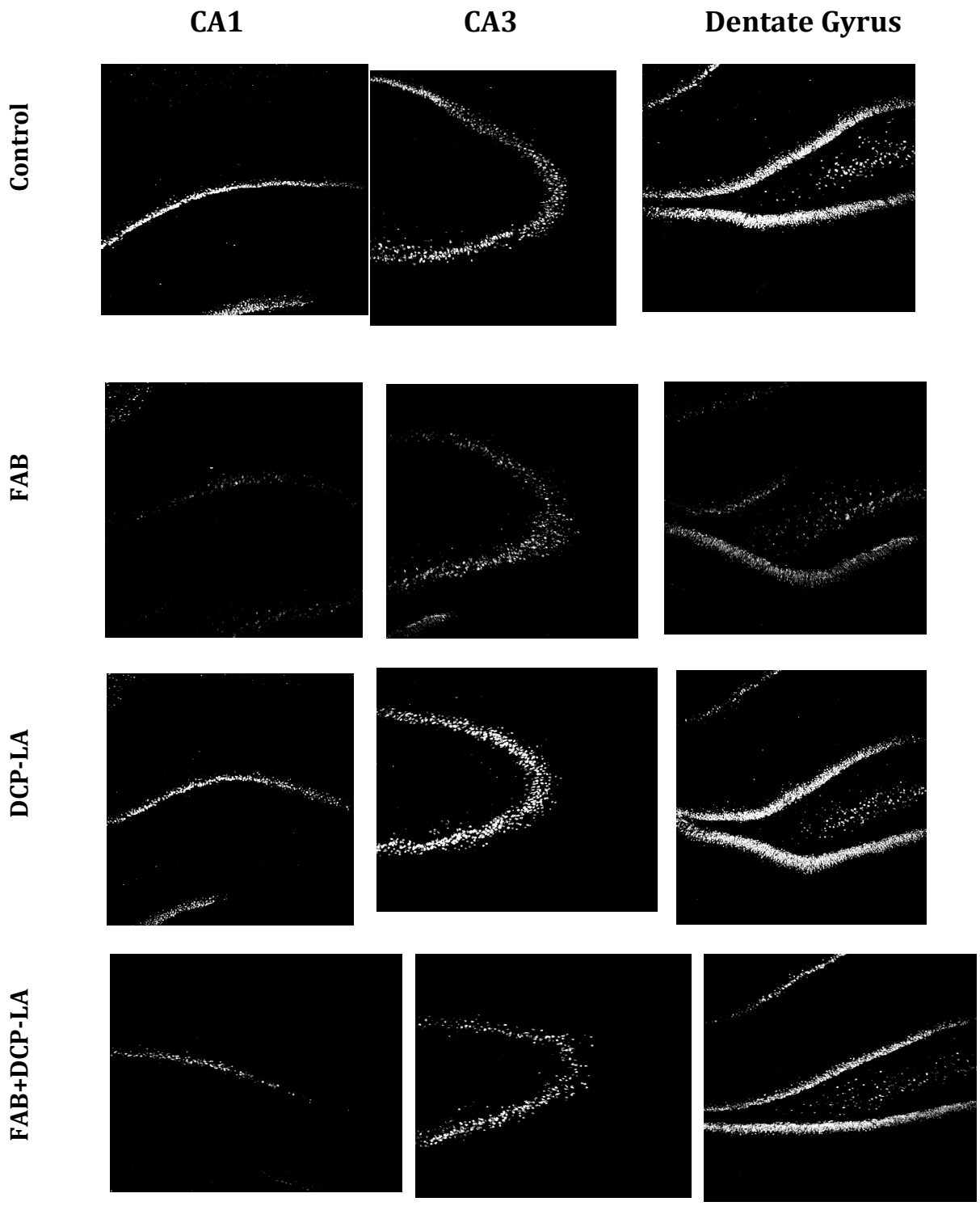


Figure 4. NeuN staining in three regions of the hippocampus.

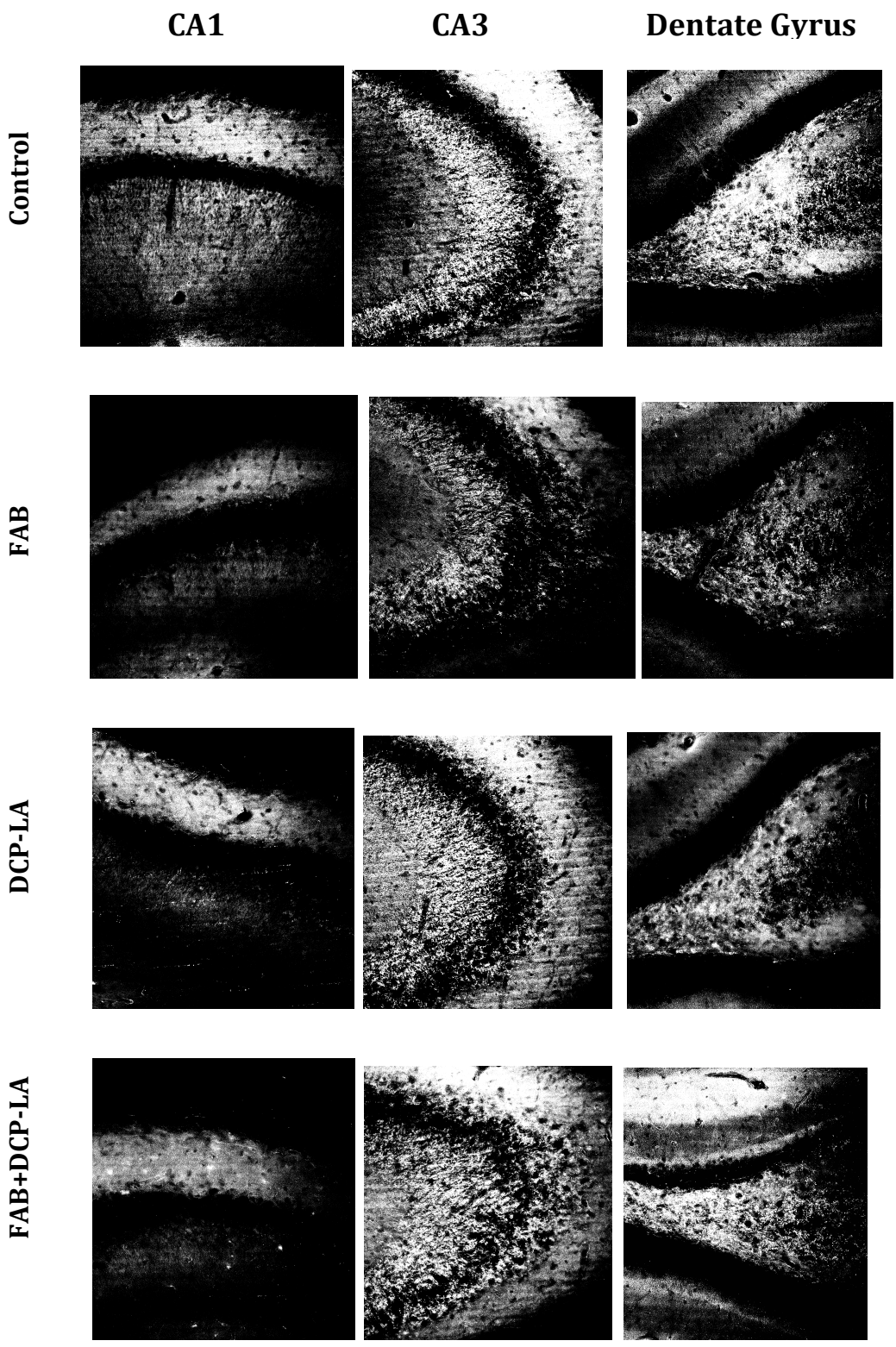


Figure 5. Synaptophysin staining in three regions of the hippocampus.

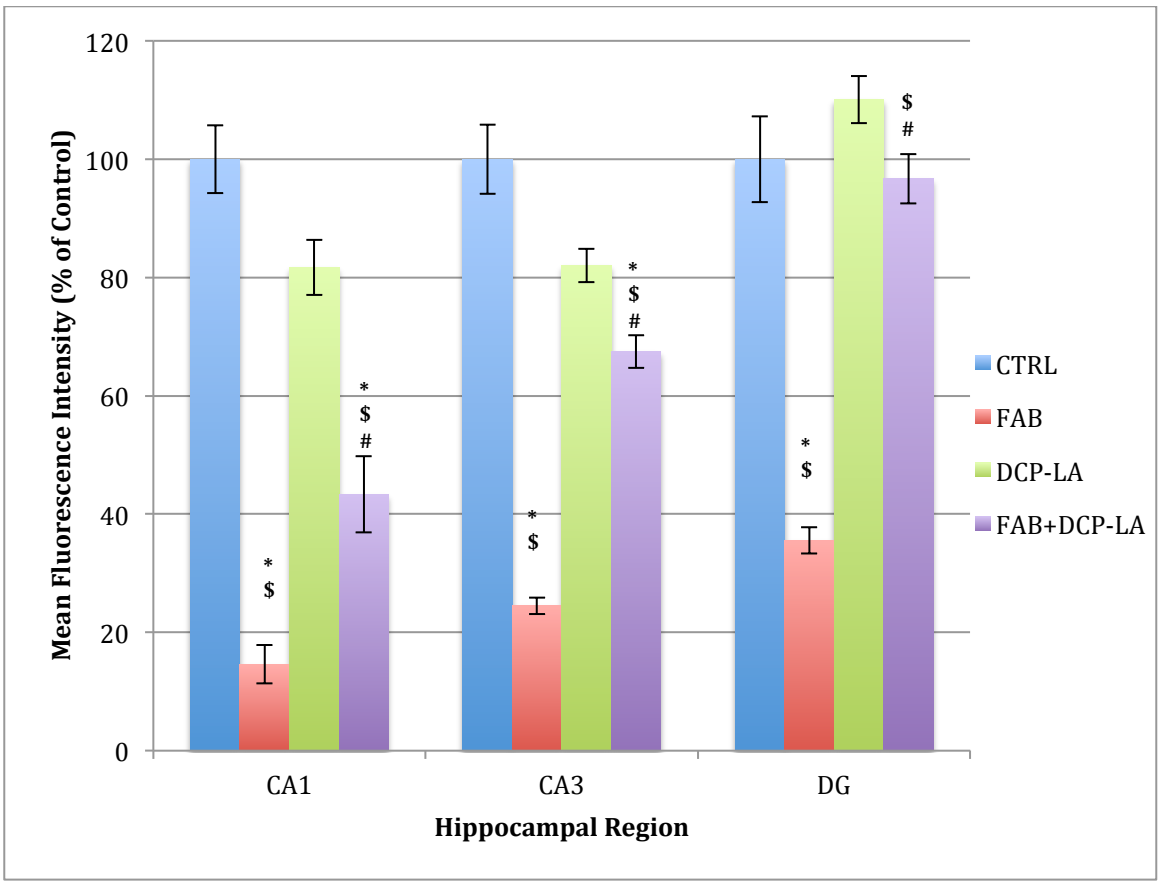


Figure 6. Mean fluorescence intensity of NeuN across treatment groups in three different regions of the hippocampus. Hippocampal sections were labeled with an antibody against NeuN. ImageJ was used to find the mean fluorescence intensity for each image and an average for each treatment group in each region was calculated. * denotes a significant difference from the Control ($p < 0.05$). $ denotes a significant difference from the DCP-LA group ($p < 0.05$). # denotes a significant difference from the FAB group ($p < 0.05$).

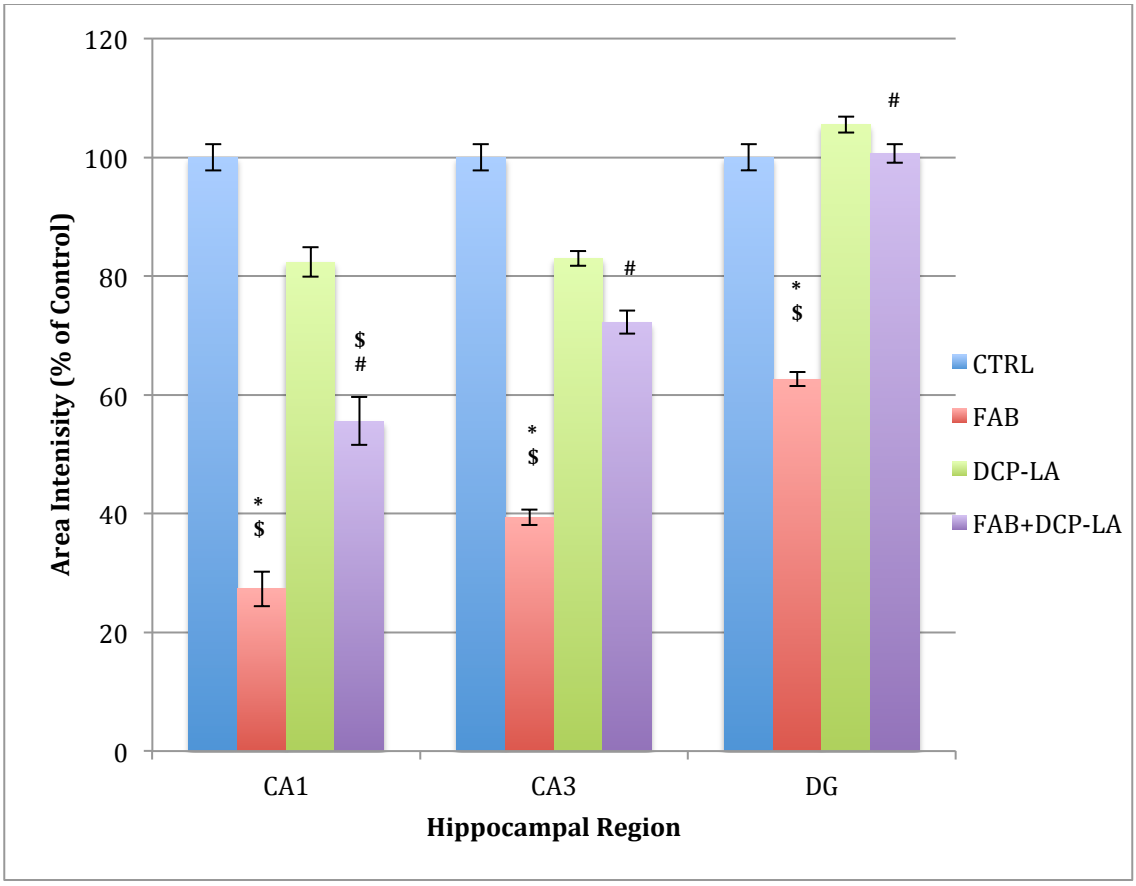


Figure 7. Percentage area covered by NeuN fluorescence across treatment groups in three different regions of the hippocampus. Hippocampal sections were labeled with an antibody against NeuN. ImageJ was used to find what percentage of a defined area was covered by fluorescence for each image and an average for each treatment group in each region was calculated. * denotes a significant difference from the Control ($p < 0.005$). $ denotes a significant difference from the DCP-LA group ($p < 0.05$). # denotes a significant difference from the FAB group ($p < 0.05$).

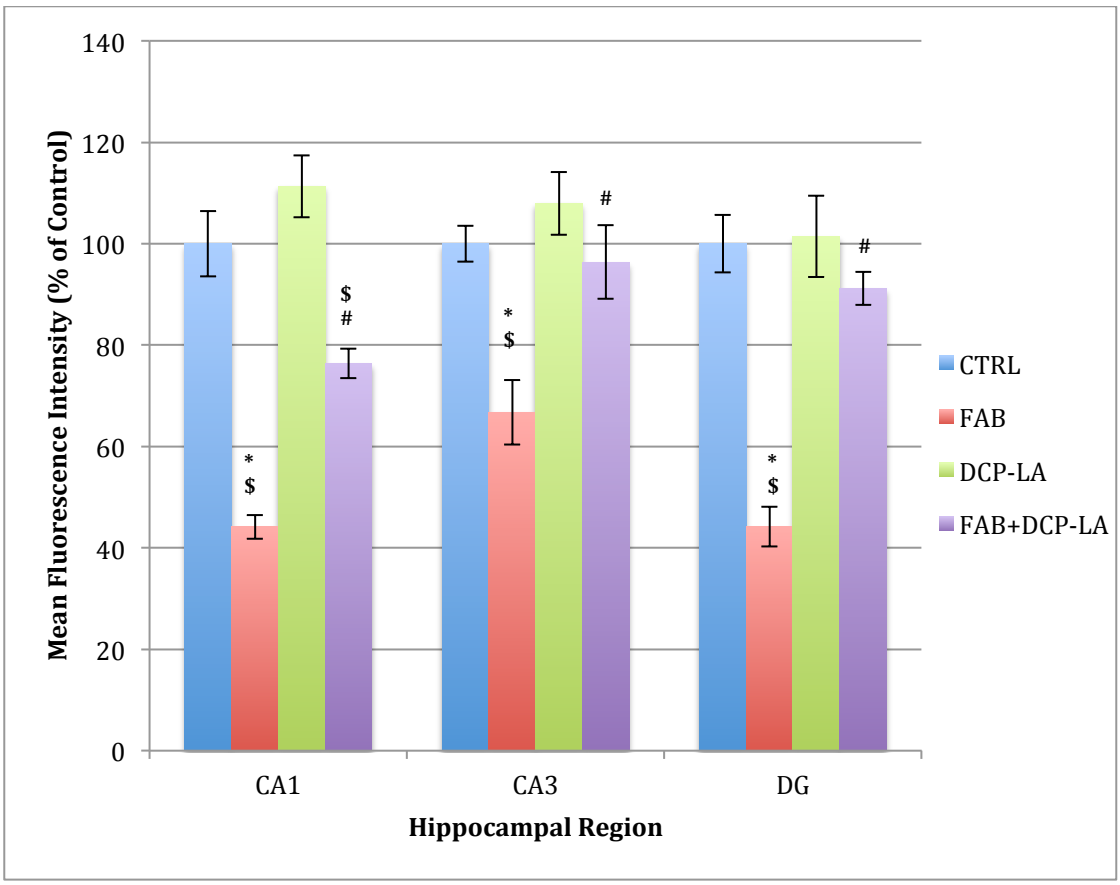


Figure 8. Mean fluorescence intensity of synaptophysin across treatment groups in three different regions of the hippocampus. Hippocampal sections were labeled with an antibody against synaptophysin. ImageJ was used to find the mean fluorescence intensity for each image and an average for each treatment group in each region was calculated. * denotes a significant difference from the Control ($p < 0.05$). $ denotes a significant difference from the DCP-LA group ($p < 0.05$). # denotes a significant difference from the FAB group ($p < 0.05$).

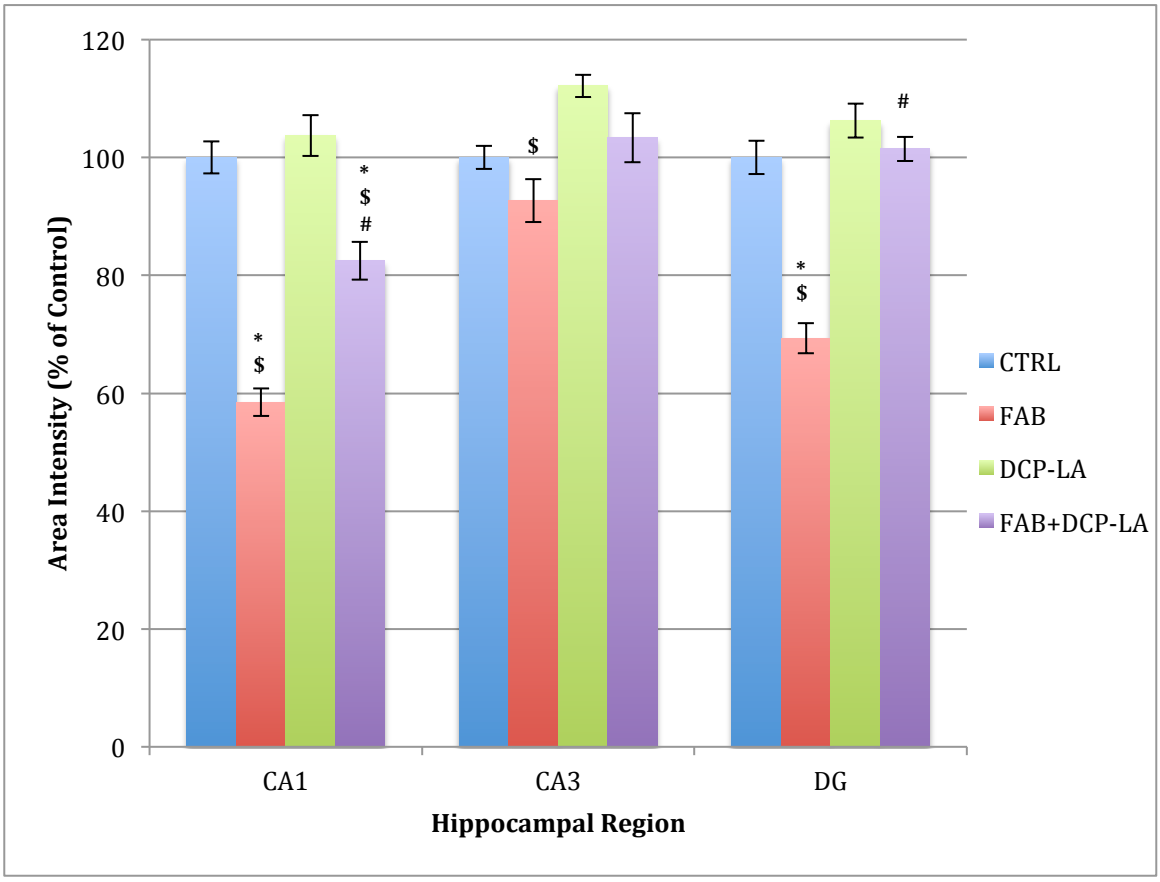


Figure 9. Percentage area covered by synaptophysin fluorescence across treatment groups in three different regions of the hippocampus. Hippocampal sections were labeled with an antibody against synaptophysin. ImageJ was used to find what percentage of a defined area was covered by fluorescence for each image and an average for each treatment group in each region was calculated. * denotes a significant difference from the Control ($p < 0.05$). $ denotes a significant difference from the DCP-LA group ($p < 0.05$). # denotes a significant difference from the FAB group ($p < 0.05$).

Discussion

The purpose of this experiment was to investigate the therapeutic potential of the PKC-ε-specific activator DCP-LA using a pharmacological rat model of AD. Although potential benefits of DCP-LA have been documented before, these studies were done in transgenic mouse models of AD, which are more closely related to the rarer, genetic form of AD. Instead, we report the effects of DCP-LA on learning, memory, synaptogenesis, and neurodegeneration in the pharmacological FAB model of AD, which is thought to be more closely related to the sporadic form of the disease. In order to do this, we infused the FAB solution ICV for 4 weeks. The animals were then trained in the Morris water maze to assess learning and memory, and their brains were removed and the hippocampi dissected out. Immunohistochemical staining was then used to quantify the expression of the neuronal marker NeuN and the synaptic marker synaptophysin.

After Morris water maze learning trials, we report a significant main effect of time. This effect indicates that amount of time required for the animals to find the platform decreased over the training period. This phenomenon is consistent with the acquisition of spatial memory, suggesting that the animals were able to utilize the visual cues set up around the maze to recognize their location relative to the platform (Morris, 1984). Specifically, the data show that the FAB animals acquired the task at a significantly slower rate than the DCP-LA, FAB+DCP-LA, and No Surgery groups, indicating impaired learning in these animals. Aside from these differences, no other groups performed statistically significantly differently from any others during the

learning trials. It is important to note, however, that this includes no significant differences between the FAB and Control groups, which highlights one of the major weaknesses of this study: sample size. One of the control animals quite clearly performed as an outlier over the first few training days, but removal of this animal from the data would have greatly impacted the statistical power of the tests and was thus undesirable.

This lack of difference among the other groups has several implications. First, the lack of difference between the No Surgery group and the Control, DCP-LA, and FAB+DCP-LA groups suggest that there was no effect of the surgical procedure itself on the ability of the animals to learn this task. This suggests that the observed effect on learning in the FAB animals is likely entirely the result of the FAB solution and not deleterious effects of the surgery. Importantly, the finding that FAB animals learned more slowly than the FAB+DCP-LA animals suggests that treatment with the drug was capable of reversing the learning deficits induced by the model, which supports our hypothesis. Interestingly, DCP-LA treatment had no effect on control animals, suggesting one of two things: either its cognitive enhancing effects may come about through mechanisms specifically related to its ability to reverse changes caused by the FAB solution, or our test was not sensitive enough to detect differences (perhaps because of the smaller sample size).

As far as the author is aware, this report of the effects of DCP-LA on learning in FAB animals is novel. The developers of the FAB model system focused on memory

deficits rather than learning, training their animals in the MWM prior to surgery and then testing platform retrieval after the 4-week infusion (Lecanu et al., 2006). At first, we attempted to replicate this study, but we found that no animals, even the No Surgery controls, had strong retention after 4 weeks (data not shown). This may reflect strain differences, as we used Sprague-Dawley rats while Lecanu et al. (2006) developed the FAB model using Long-Evans rats. Another study, conducted by Petrasek et al. (2016), also reports impaired learning of FAB rats in the MWM, but they were only investigating how the model affects spatial memory and not the effects of DCP-LA. If we compare our results to the literature using transgenic animals, however, we do see another group reporting that DCP-LA treatment improved spatial learning in the MWM (Hongpaisan et al., 2011).

Probe trials are often conducted after completion of MWM training as a means to assess memory retention (reviewed in D'Hooge and De Deyn, 2001). We observed that FAB rats had a significantly higher latency to reach the former location of the platform compared to all other groups. Similarly, they spent significantly less time in the quadrant where the platform was located compared to all other groups. These two measures combined indicate impaired recall in the FAB animals. Based upon the first task, they do not complete the original task as quickly as the other groups. The second measure suggests that the FAB rats spend more time actively searching for an escape, while the others remain in the area where they know the escape was located based upon the visual cues.

Similarly to the learning trials, the lack of any other significant differences has several implications. Again, the surgical procedure appears to have had no deleterious effects, implicating the FAB solution in the observed memory impairment. The improved performance of FAB+DCP-LA animals suggests that the drug is able to reverse the memory impairment caused by the model, which supports our hypothesis. The performance of the control animals was not enhanced by DCP-LA treatment, again suggesting that the drug's memory improving function is specifically related to its ability to reverse the memory deficits caused by the FAB model. There is also a similar lack of data to compare our findings to. Both Lecanu et al. (2006) and Petrasek et al. (2016) report that FAB rats exhibit impairments in memory, but again neither tested the ability of DCP-LA (or any other sort of drug) to reverse these impairments. One thing to keep in mind, however, is that both of these studies report a much more severe impairment than we do. As mentioned before, this may be the result of strain differences. If we compare our results to studies done in transgenic mice, Hongpaisan et al. (2011) also report improvements in memory after DCP-LA treatment.

We utilized antibody labeling of the protein NeuN to visualize and quantify the number of neurons in three regions of the hippocampus. We report a decrease in the mean NeuN fluorescence intensity in the CA1, CA3, and dentate gyrus of FAB animals compared to Control (~83%, ~77%, and 65% decrease in CA1, CA3, and dentate gyrus), DCP-LA (~65%, ~59%, and 75% decrease), and FAB+DCP-LA (~26%, ~45%, and ~61% decrease) animals. Additionally, there was a decrease in the percentage area covered by

NeuN fluorescence in all three regions of FAB hippocampi compared to Control (~77%, ~61%, and ~40% decrease in CA1, CA3, and dentate gyrus), DCP-LA (~59%, ~43%, and ~45% decrease), and FAB+DCP-LA (~34%, ~35%, and ~40% decrease) animals. These two measures together are indicative of a decrease in the density of healthy, mature neurons, which suggests large-scale neurodegeneration occurred in these animals in all three monitored regions. This is consistent with our expectation from the model, as Lecanu et al. (2006) also report neuronal loss in all three of these regions.

While the FAB+DCP-LA animals exhibit a higher mean NeuN intensity in all three regions compared to the FAB animals, the intensities and percentage areas covered that we observed in the CA1 and CA3 regions are significantly lower than those in the Control (~57% and ~33% decrease in intensity; ~43% and ~28% decrease in area covered) group. Compared to DCP-LA animals, the FAB+DCP-LA animals showed lower mean intensities in CA1 (~39% decrease), CA3 (~19% decrease), and the dentate gyrus (~17% decrease), as well as a lower area covered in the CA1 (~25% decrease). This suggests that, while DCP-LA is able to prevent some of the decrease in density of healthy cells compared to the untreated models, this rescue is incomplete. This is not *entirely* unexpected, however, considering that the animals were exposed to neuron-damaging oxidative stress for a significant length of time (4 weeks) and received only a single injection of DCP-LA *after* the infusion period had ended. It is not possible to know exactly when in this 4-week period the neuronal death would have started, but it does appear that the DCP-LA treatment was able to halt the progression of the neurodegeneration. As such,

these results do support our hypothesis, but, in the future, it may be worth trying more chronic administration (or co-administration) of DCP-LA to determine whether the damage can be completely reversed or prevented. One potential, although highly speculative, explanation for the significant decrease observed in the dentate gyrus is that treatment with DCP-LA helped to stimulate neurogenesis. A recent study (Geribaldi-Doldan et al., 2016) demonstrated that phorbol esters could induce neural progenitor proliferation in the dentate gyrus, but did not distinguish which isozyme underlies this effect. This hypothesis could be tested in the future by measuring incorporation of 5-bromo-2'-deoxyuridine after injection of DCP-LA in order to label and quantify cells undergoing cell division.

The second protein that we studied, synaptophysin, is widely used as a marker for measuring the density of synaptic connections. We observed significantly less synaptophysin fluorescence intensity in all three hippocampal regions of FAB animals compared to Control (~58%, ~35%, and ~58% decrease in CA1, CA3, and dentate gyrus), DCP-LA (~68%, ~42%, and ~59% decrease), and FAB+DCP-LA (~34%, ~33%, and ~48% decrease). This suggests that these animals have suffered synaptic loss, which is consistent with their worse performance in the MWM. Treatment with DCP-LA either restored synaptic density to control levels in all three hippocampal regions or prevented its loss, which supports our hypothesis. However, it is worth noting that the mean intensity in CA1 for FAB+DCP-LA animals was significantly less than that of the DCP-LA animals (~32% decrease). The mean intensity in the CA1 (~76% of Control) of FAB+DCP-

LA animals was overall lower than in the CA3 (~96% of Control) or dentate gyrus (~90% of Control), suggesting that the synaptic effects of DCP-LA may not have been as strong in the CA1 region. In addition to this, we report a decrease in the area covered by synaptophysin staining in the CA1 and dentate gyrus of FAB animals compared to Control (~42% and ~32% decrease in CA1 and dentate gyrus, respectively), DCP-LA (~45% and ~37% decrease), but not in the CA3, which was only significantly lower than the DCP-LA group (~20% decrease). This variable suggests that, although there was a loss of synaptic density in the CA3 of FAB animals, the overall spread of synaptic connections was not affected by the model. The significant decrease compared to DCP-LA appears to be the result of an increase in the area covered in the CA3 of DCP-LA animals compared to the control, but this difference does not reach significance. Similarly to the mean intensity, there was a significant decrease in the area covered in the FAB+DCP-LA CA1 slices compared to Control (~18% decrease) and DCP-LA (~21% decrease), but not FAB, suggesting that the synaptic effects of DCP-LA are not as extensive in the CA1 as in other hippocampal regions.

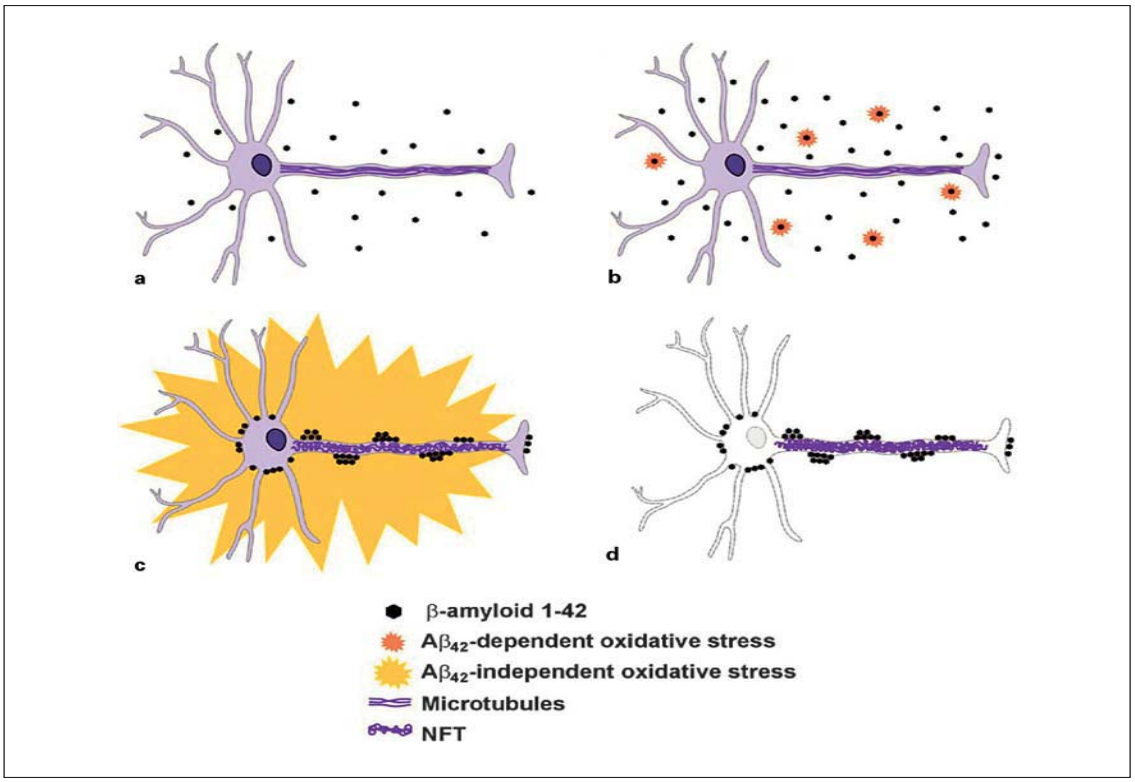
As far as the author can tell, there have been no other studies that looked specifically at synapse number in the FAB model, so in order to compare these results with the literature, we must look at studies that used other models. Hongpaisan et al. (2011) show that DCP-LA treatment resulted in an increase in the number of synapses and dendritic spines. Sen et al. (2016), while they used a model system not related to AD, showed that PKC-ε activation by DCP-LA increased synaptophysin expression in

hippocampal slices, suggesting a role for DCP-LA in synaptogenesis (although we did not see a difference compared to our control). They also show that knockdown of PKC-ε results in a decrease in synaptophysin expression, indicating that it is essential to proper synaptogenesis.

There are a number of directions that this line of research could take in the future. One would be to pursue a more complete characterization of the effects of DCP-LA in these FAB animals. One severe limitation of this study is the number of parameters we were unable to take into account. In this regard, future studies may investigate the effects of DCP-LA on plaque and tangle burden or the activation of microglia, all of which were reported to be increased in the model system (Lecanu et al., 2006). Even more basic, future studies should confirm the presence of oxidative damage by staining for 8-hydroxydeoxyguanosine or check for increased PKC-ε activity after DCP-LA treatment. In addition, different treatment courses may be considered to determine if there are any deleterious effects of long-term treatment, as well as the effective time course of DCP-LA treatment. Using an aged rat model in a future study may be beneficial to help distinguish whether or not DCP-LA can have cognitive benefit without the presence of pathology, as the aged controls would be expected to exhibit learning and memory deficits naturally, unlike the 3-month old animals we used in this study.

Ultimately, this model system was developed as an attempt to capture all of the pathology that we observe in sporadic AD. However, it may also contain an important clue for understanding the development of sporadic AD pathology. The authors report

that the model system was only generated when all three components of the solution were included (Lecanu et al., 2006). Any of them alone or in combination with a second did not produce any detectable deficits. This led the authors to propose an 'amyloidoxidative' hypothesis (see Model 4) for the development of AD pathology. In this model, the generation of ROS and/or interruption of natural antioxidant defenses create a favorable environment for the aggregation of Aβ, eventually leading to NFT formation and neuronal death. If this hypothesis is shown to have some merit, then DCP-LA may also serve as a potential preventative agent against the development of AD pathology, as data suggest that it can prevent both plaque formation (Hongpaisan et al., 2011) and oxidative damage. One study that could be done is to investigate whether DCP-LA has a protective effect against the actions of FAB solution or if it is only capable of healing the damage done. Another possibility is that DCP-LA can enhance synaptic strength, which could be tested by measuring post-synaptic currents in tissue slices after *in vivo* treatment with DCP-LA. These two mechanisms are not necessarily related to one another, but either may explain beneficial effects of DCP-LA.



Model 4. The amyloidoxidative model of AD development. This model suggests a mechanism for the development of AD molecular pathology. **A)** A healthy neuron. **B)** Increased concentrations of Aβ, which can cause oxidative stress, but is counteracted by the immune system. **C)** High levels of oxidative stress create an environment that allows for the aggregation of Aβ and formation of plaques and tangles. **D)** The combined effects of oxidative damage and Aβ kill the neuron. Image credit Lecanu et al. (2006)

In conclusion, our data suggest that treatment with the PKC-ε-specific activator DCP-LA is capable of reversing learning and memory deficits, as well as improving neuronal health and synaptogenesis in a pharmacological rat model of AD. These results are particularly valuable because they come from a novel model that was designed to more completely capture the pathology of the more common sporadic form of AD compared to the standard transgenic models. These data suggest that DCP-LA has potential as a therapeutic agent in AD, although far more testing is needed to confirm the extent of its function and safety. The model system we used also suggests an

amyloidoxidative hypothesis that may provide clues as to the mechanism that spur the onset of sporadic AD and provide a means to further test the ability of DCP-LA to act as a preventative agent, which will become more important as researchers identify biomarkers that allow for early detection of AD and the testing of preventative strategies.

References

Ahlskog JE, Geda YE, Graff-Radford NR, Petersen RC. 2011. Physical exercise as a preventative or disease-modifying treatment of dementia and brain aging. *Mayo Clinic Proceedings* 86(9):876-84.

Albert MS, DeKosky ST, Dickson D, Dubois B, Feldman HH, Fox NC, Gamst A, Holtzman DM, Jagust WJ, Petersen RC. 2011. The diagnosis of mild cognitive impairment to Alzheimer's disease: recommendations from the National Institute on Aging-Alzheimer's Association workgroups on diagnostic guidelines for Alzheimer's disease. *Alzheimers Dement* 7(3):270-79.

Alfa Cisse M, Sunyach C, Slack BE, Fisher A, Vincent B, Checler F. 2007. M1 and M3 muscarinic receptors control physiological processing of cellular prion by modulating ADAM17 phosphorylation and activity. *J Neurosci* 27:4083–4092.

Almeida CG, Tampellini D, Takahashi RH, Greengard P, Lin MT, Snyder EM, Gouras GK. 2005. Beta-amyloid accumulation in APP mutant neurons reduce PSD-95 and GluR1 in synapses. *Neurobiol Disease* 20:187-98.

Alzheimer's Association. 2006. Early-onset dementia: a national challenge, a future crisis.

Alzheimer's Association. 2016. Alzheimer's Disease Facts and Figures 2016.

Alzheimer's Disease International. 2015. World Alzheimer report 2015: the global impact of dementia.

American Psychiatric Association. 2013. *Diagnostic and statistical manual of mental disorders* (5th ed.). Arlington, VA: American Psychiatric Publishing.

Amieva H, Le Goff M, Millet X, Orgogozo JM, Peres K, Barberger-Gateau P, Jacqmin-Gadda H, Dartigues JF. Prodromal Alzheimer's disease: successive emergence of the clinical symptoms. *Ann Neurol* 64:492-98.

Anstey KJ, Cherbuin N, Budge M, Young J. 2011. Body mass index in midlife and late-life as a risk factor for dementia: a meta-analysis of prospective studies. *Obesity Reviews* 12:e426-e437.

Arrighi HM, Neumann PJ, Liederburg IM, Townsend RJ. 2010. Lethality of Alzheimer disease and its impact on nursing home placement. *Alzheimer Dis Assoc Disord* 24(1):90-5.

Balducci C, Forloni G. 2011. APP transgenic mice: their use and limitations. *NeuroMolecular Med* 13(2):117-37.

Barker WW, Luis CA, Kashuba A, Luis M, Harwood DG, Loewenstein D, Waters C, Jimison P, Shepherd E, Sevush S, et al. 2002. Relative frequencies of Alzheimer's disease, Lewy body, vascular and frontotemporal dementia, and hippocampal sclerosis in the State of Florida Brain Bank. *Alzheimer Dis Assoc Disord* 16(4):203-12.

Bats C, Groc L, Choquet D. 2007. The interaction between Stargazin and PSD-95 regulates AMPA receptor surface trafficking. *Neuron* 53:719-34.

Bertens D, Knol DL, Scheltens P, Visser PJ. 2015. Temporal evolution of biomarkers and cognitive markers in the asymptomatic, MCI, and dementia of Alzheimer's disease. *Alzheimers Dement* 11:511-22.

Beydoun MA, Beydoun H, Wang Y. 2008. Obesity and central obesity as risk factors for incident dementia and its sub-types: a systematic review and meta-analysis. *Obes Rev* 9(3):204-18.

Bornancin F, Parker PJ. 1996. Phosphorylation of threonine 638 critically controls the dephosphorylation and inactivation of protein kinase C alpha. *Current Biol* 6(9):1114-23.

Brookmeyer R, Johnson E, Ziegler-Graham K, Arrighi HM. 2007. Forecasting the global Millet Xburden of Alzheimer's disease. *Alzheimers Dement*. 3(3):186-91.

Brunnstrom HR and Englund EM. 2009. Cause of death in patients with dementia disorders. *Eur J Neurol* 16(4):488-92.

Buxbaum JD, Liu K, Luo Y, Slack JL, Stocking KL, Peschon JJ, Johnson RS, Castner BJ, Cerretti DP, Black RA. 1998. Evidence that tumor necrosis factor alpha converting enzyme is involved in regulated alpha-secretase cleavage of the Alzheimer amyloid precursor protein. *J Biol Chem* 273(43):27765-67.

Caamano-Isorna F, Corral M, Montes-Martinez A, Takkouche B. Education and dementia: a meta-analytic study. *Neuroepidemiology* 26:226-32.

Cai X, Golde TE, Younkin SG. 1993. Release of excess amyloid beta protein from a mutant amyloid beta protein precursor. *Science* 259(5094):514-516.

Cazaubon S, Bornancin F, Parker PJ. 1994. Threonine-497 is a critical site for permissive action of protein kinase C-alpha. *Biochem J* 301:443-448.

Chapman PF, White GL, Jones MW, Cooper-Blacketer D, Marhall VJ, Irizarry M, Younkin L, Good MA, Bliss TVP, Hyman BT, Younkin SG et al. 1999. Impaired synaptic plasticity and learning in aged amyloid precursor protein transgenic mice. *Nature Neuroscience* 2:271-76.

Cisse M, Braun U, Leitges M, Fisher A, Pages G, Checler F, Vincent B. 2011. ERK1-independent alpha-secretase cut of beta amyloid precursor protein via M1 muscarinic receptors and PKC-alpha/epsilon. *Mol Cell Neurosci* 47(3):223-232.

Corrada M, Hayden KM, Bullain SS, Paganini-Hill A, DeMoss J, Aguiree C, Kawas C. 2014. Age of onset of hypertension and risk of dementia in the oldest old: the 90+ study. *Alzheimers Dement* 10(4):501.

Crouch PJ, Blake R, Duce JA, Ciccotosto GD, Li Q, Barnham KJ, Curtain CC, Cherny RA, Cappai R, Dyrks T et al. 2005. Copper-dependent inhibition of human cytochrome c oxidase by a dimeric conformer of amyloid beta 1-42. *J Neurosci* 25(3):672-79.

Cummings JL, Morstorf T, Zhong K. Alzheimer's disease drug-development pipeline: few candidates, frequent failures. *Alzheimer's Research and Therapy* 6:37.

D'Hooge R, De Deyn PP. 2001. Applications of the Morris water maze in the study of learning and memory. *Brain Res Rev* 36:60-90.

Da Cruz e Silva OAB, Rebelo S, Vieira SI, Gandy S, Da Cruz e Silva EF, Greengard P. 2008. Enhanced generation of Alzheimer's amyloid beta following chronic exposure to phorbol ester correlates with differential effects on alpha and epsilon isozymes of protein kinase C. *J Neurochem* 108(2):319-30.

Dahl AK, Lopponen M, Isoaho R, Berg S, Kivela SL. 2008. Overweight and obesity in old age are not associated with greater dementia risk. *J Am Geriatr Soc* 56(12):2261-66.

Davies CA, Mann DMA, Sumpter PQ, Yates PO. 1987. A quantitative morphometric analysis of the neuronal and synaptic content of the frontal and temporal cortex in patients with Alzheimer's disease. *J Neurological Science* 78(2):151-164.

DeKosky ST, Scheff SW. 1990. Synapse loss in frontal cortex biopsies in Alzheimer's disease: correlation with cognitive severity. *Ann Neurol* 27(5):457-64.

De Roo M, Klauser P, Mendez P, Poglia L, Muller D. 2008. Activity dependent PSD formation and stabilization of newly formed spines in hippocampal slice cultures. *Cerebral Cortex* 18:151-61.

Doussau F, Augustine GJ. 2000. The actin cytoskeleton and neurotransmitter release: an overview. *Biochimie* 82(4):353-63.

Duff K, Suleman F. 2004. Transgenic mouse models of Alzheimer's disease: how useful have they been for therapeutic development. *Brief Funct Genomics* 3(1):47-59.

Dumuis A, Sebben M, Haynes L, Pin JP, Bockaert J. 1988. NMDA receptors activate the arachidonic acid cascade system is striatal neurons. *Nature* 336:68-70.

Elmore S. 2007. Apoptosis: a review of programmed cell death. *Toxicol Pathol* 35:495-516.

Evans NA, Facci L, Owen DE, Soden PE, Burbidge SA, Prinjha RK, Richardson JC, Skaper SD. 2008. Amyloid beta-42 reduces synapse number and inhibits neurite outgrowth in primary cortical and hippocampal neurons: a quantitative analysis. *J Neurosci Methods* 175(1):96-103.

Ewers M, Walsh C, Trojanowski JQ, Shaw LM, Petersen RC, Jack CR, Feldman HH, Bokde ALW, Alexander GE, Scheltens P. 2012. Predictions of conversion from mild cognitive impairment to Alzheimer's disease based upon biomarkers and neuropsychological test performance. *Neurobiol Aging* 33(7):1203-14.

Ferreiro E, Resende R, Costa R, Oliveira CR, Pereira CMF. 2006. An endoplasmic-reticulum-specific apoptotic pathway is involved in prion and amyloid beta peptide neurotoxicity. *Neurobiol Disease* 23:669-78.

Finkel T, Holbrook NJ. 2000. Oxidants, oxidative stress and the biology of ageing. *Nature* 408:239-47.

Fitzpatrick AL, Kuller LH, Lopez OL, Diehr P, O'Meara ES, Longstreth WT, Luchsinger JA. 2009. Midlife and late-life obesity and the risk of dementia: cardiovascular health study. *Arch Neurol* 66(3):336-42.

Flint AJ, Paladini RD, Koshland DE. 1990. Autophosphorylation of protein kinase C at three separated regions of its primary sequence. *Science* 240:408-11.

Friedman EM, Shih RA, Langa KM, Hurd MD. 2015. U.S. prevalence and predictors of informal caregiving for dementia. *Health Aff* 34(10):1637-41.

Games D, Adams D, Alessandrini R, Barbour R, Berthelette P, Blackwell C, Carr T, Clemens J, Donaldson T, Gillesple F et al. 1995. Alzheimer-type neuropathology in transgenic mice overexpressing V717F beta amyloid precursor protein. *Nature* 373:523-27.

Ganguli M, Snitz BE, Saxton JA. 2011. Outcomes of mild cognitive impairment by definition. *Arch Neurol* 68(6):761-67.

Gaugler JE, Kane RL, Kane RA. 2002. Family care for older adults with disabilities: Toward more targeted and interpretable research. *Int J Aging Hum Dev* 54(3):205-31.

Geribaldi-Doldan N, Flores-Giubi E, Murillo-Carretero M, Garcia-Bernal F, Carrasco M, Macias-Sanchez AJ, Dominguez-Riscart J, Verastegui C, Hernandez-Galan R, Castro C. 2016. 12-deoxyphorbols promote adult neurogenesis by inducing neural progenitor cell proliferation via PKC activation. *Int J Neuropsychopharmacol* 19(1): doi:10.1093/ijnp/pyv085.

Gudala K, Bansal D, Schifano F, Bhansali A. 2013. Diabetes mellitus and risk of dementia: a meta-analysis of prospective observational studies. *J Diabetes Invest* doi:10.1111/jsi.12087.

Gurland BJ, Wilder DE, Lantigua R, Stern Y, Chen J, Killeffer EHP, Mayeux R. 1999. Rates of dementia in three ethnoracial groups. *Int J Geriat Psychiatry* 14:481-93.

Haan MN, Mungas DM, Gonzalez HM, Ortiz TA, Acharya A, Jagust WJ. 2003. Prevalence of dementia in older Latinos: the influence of type 2 diabetes mellitus, stroke, and genetic factors. *J Am Geriat Soc* 51(2):169-177.

Hall FL, Fernyhough P, Ishii DN, Vulliet PR. 1988. Suppression of nerve growth factor-directed neurite outgrowth in PC12 cells by sphingosine, an inhibitor of protein kinase C. *J Biol Chem* 263(9):4460-66.

Hama H, Hara C, Yamaguchi K, Miyawaki A. 2004. PKC signaling mediates global enhancement of excitatory synaptogenesis in neurons triggered by local contact with astrocytes. *Neuron* 41(3):405-15.

Hebert LE, Weuve J, Scherr PA, Evans DA. 2013. Alzheimer disease in the United States (2010-2050) estimated using the 2010 census. *Neurology* 80(19):1178-83.

Holcomb L, Gordon MN, McGowan E, Yu X, Benkovic S, Jantzen P, Wright K, Saad I, Mueller R, Morgan D et al. 1998. Accelerated Alzheimer-type phenotype in transgenic mice carrying both mutant amyloid precursor protein and presenilins 1 transgenes. *Nature Medicine* 4:97-100.

Holtzman DM, Herz J, Bu G. 2012. Apolipoprotein E and apolipoprotein E receptors: normal biology and roles in Alzheimer disease. *Cold Spring Harb Perspect Med* doi:10.1101/cshperspect.a006312.

Hongpaisan J, Sun M, Alkon DL. 2011. PKC-epsilon activation prevents synaptic loss, amyloid beta elevation, and cognitive deficits in Alzheimer's disease transgenic mice. *J Neurosci* 31(2):630-43.

Hsia AY, Masliah E, McConlogue, Yu G, Tatsuno G, Hu K, Kholodenko D, Malenka RC, Nicoll RA, Mucke L. 1999. Plaque-independent disruption of neural circuits in Alzheimer's disease mouse models. *Proc Natl Acad Sci* 96(6):3228-3233.

Hsiao K, Chapman P, Nilsen S, Eckman C, Harigaya Y, Younkin S, Yang F, Cole G. 1996. Correlative memory deficits, amyloid beta elevation, and amyloid plaques in transgenic mice. *Science* 274:99-102.

Hsieh H, Boehm J, Sato C, Iwatsubo T, Tomita T, Sisodia S, Malinow R. 2006. AMPAR removal underlies amyloid-beta induced depression and dendritic spine loss. *Neuron* 52:831-43.

Hundle B, McMahon T, Dadgar J, Messing RO. 1995. Overexpression of epsilon-protein kinase C enhances nerve growth factor-induced phosphorylation of mitogen activated protein kinases and neurite outgrowth. *J Biol Chem* 270(50):30134-40.

Irizarry MC, McNamara M, Fedorchak K, Hsiao K, Hyman BT. 1997a. APPsw transgenic mice develop age-related amyloid beta deposits and neuropil abnormalities, but no neuronal loss in CA1. *J Neuropathol Exper Neurol* 56(9):965-73.

Irizarry MC, Soriano F, McNamara M, Page KJ, Schenk D, Games D, Hyman BT. 1997b. Amyloid beta is associated with neuropil changes, but not with overt neuronal loss in the human amyloid precursor protein V717F (PDAPP) transgenic mouse. *J Neurosci* 17(18):7053-59.

Iwatsubo T, Odaka A, Suzuki N, Mizusawa H, Nukina N, Ihara Y. 1994. Visualization of amyloid beta-42 and amyloid beta-40 with end-specific amyloid beta monoclonals: evidence that an initially deposited species in amyloid beta-42. *Neuron* 13(1):45-53.

Jacobsen JS, Spruyt MA, Brown AM, Sahasrabudhe SR, Blume AJ, Vitek MP, Muenkel HA, Sonnenberg-Reines J. 1994. The release of Alzheimer's disease beta amyloid peptide is reduced by phorbol treatment. *J Biol Chem* 269(11):8376-82.

James BD, Leurgans SE, Hebert LE, Scherr PA, Yaffe K, Bennett DA. 2014. Contribution of Alzheimer disease to mortality in the United States. *Neurology* 82(12):1045-50.

Johnson JE, Giorgione J, Newton AC. 2000. The C1 and C2 domains of protein kinase C are independent membrane targeting modules, with specificity for phosphatidylserine conferred by the C1 domain. *Biochemistry* 39:11360-69.

Johnson DK, Storandt M, Morris JC, Galvin JE. 2009. Longitudinal study of the transition from healthy aging to Alzheimer disease. *Arch Neurol* 66(10):1254-59.

Kanno T, Yamamoto H, Yaguchi T, Hi R, Mukasa T, Fujikawa H, Nagata T, Yamamoto S, Tanaka A, Nishizaki T. 2006. The linoleic acid derivative DCP-LAselectively activates PKC-epsilon, possibly binding to the phosphatidylserine binding site. *J Lipid Res* 47:1146-56.

Kanski J, Varadarajan S, Aksenova M, Butterfield DA. 2002. Role of glycine-33 and methionine-35 in Alzheimer's amyloid beta peptide 1-42-associated oxidative stress and neurotoxicity. *Biochimica et Biophysica Acta* 1586:190-98.

Kao S, Krichevsky AM, Kosik KS, Tsai L. 2004. BACE1 suppression by RNA interference in primary cortical neurons. *J Biol Chem* 279(3):1942-49.

Karran E, Mercken M, De Strooper B. 2011. The amyloid cascade hypothesis for Alzheimer's disease: an appraisal for the development of therapeutics. *Nature Reviews Drug Discovery* 10:698-712.

Kasahara K, Kikkawa U. 1995. Distinct effects of saturated fatty acids on protein kinase C subspecies. *J Biochem* 117:648-53.

Kasper JD, Freedman VA, Spillman BC. 2014. Disability and care needs of older Americans by dementia status: An analysis of the 2011 national health and aging trends study. U.S. Department of Health and Human Services.

Kasper JD, Freedman VA, Spillman BC, Wolff JL. 2015. The disproportionate impact of dementia on family and unpaid caregiving to older adults. *Health Aff* 34(10):1642-49.

Keranen LM, Dutli EM, Newton AC. Protein kinase C is regulated in vivo by three functionally distinct phosphorylations. *Current Biol* 5(12):1394-1403.

Kiecolt-Glaser JK, Dura JR, Speicher CE, Trask OJ, Glaser R. 1991. Spousal caregivers of dementia victims: Longitudinal changes in immunity and health. *Psychosom Med* 53:345-62.

Lacor PN, Buniel MC, Furlow PW, Clemente AS, Velasco PT, Wood M, Viola KL, Klein WL. 2007. Amyloid beta oligomer-induced aberrations in synapse composition, shape, and density provide a molecular basis for loss of connectivity in Alzheimer's disease. *J Neurosci* 27(4):796-807.

Lammich S, Kojro W, Postina R, Gilbert S, Pfeiffer R, Jasionowski M, Haass C, Fahrenholz F. 1999. Constitutive and regulated alpha-secretase cleavage of Alzheimer's amyloid precursor protein by a disintegrin metalloprotease. 96(7):3922-27.

Larson J, Lynch G, Games D, Seubert P. 1999. Alterations in synaptic transmission and long-term potentiation in hippocampal slices from young and aged PDAPP mice. *Brain Res* 840:23-35.

Lecanu L, Greeson J, Papadopoulos V. 2006. Beta amyloid and oxidative stress jointly induce neuronal death, amyloid deposits, gliosis, and memory impairment in the rat brain. *Pharmacol* 76:19-33.

Lee M, Kao S, Lemere CA, Xia W, Tseng H, Zhou Y, Neve R, Ahlijanian MK, Tsai L. 2003. APP processing is regulated by cytoplasmic phosphorylation. *J Cell Biol* 163(1):83-95.

Lee W, Boo JH, Jung MW, Park SD, Kim YH, Kim SU, Mook-Jung I. 2004. Amyloid beta peptide directly inhibits PKC activation. *Mol Cell Neurosci* 26(2):222-231.

Lemere CA, Blusztajn JK, Yamaguchi H, Wisniewski T, Saido TC, Selkoe DJ. 1996. Sequence of deposition of heterogeneous amyloid beta peptides and APO E in Down syndrome: implications for initial events in amyloid plaque formation. *Neurobiol Disease* 3:16-32.

Leu BH, Schmidt JT. 2007. Arachidonic acid as a retrograde signal controlling growth and dynamics of retinotectal arbors. *Develop Neurobiol* 68(1):18-30.

Loef M, Walach H. 2010. Midlife obesity and dementia: meta-analysis and adjusted forecast of dementia prevalence in the United States and China. *Obesity* 21(1):E51-55.

Loy CT, Schofield PR, Turner AM, Kwok JBJ. 2014. Genetics of dementia. *Lancet* 383(9919):828-40.

Lustbader JW, Cirilli M, Lin C, Xu HW, Takuma K, Wang N, Caspersen C, Chen X, Pollak S, Chaney M et al. 2004. ABAD directly links amyloid beta to mitochondrial toxicity in Alzheimer's disease. *Science* 304(5669):448-52.

Malenka RC, Madison DV, Nicoll RA. 1986. Potentiation of synaptic transmission in the hippocampus by phorbol esters. *Nature* 321:175-77.

Manczak M, Anekonda TS, Henson E, Park BS, Quinn J, Reddy PH. 2006. Mitochondria are a direct site of amyloid beta accumulation in Alzheimer's disease neurons: implications for free radical generation and oxidative damage in disease progression. *Hum Mol Genet* 15(9):1437-49.

Mangoura D, Sogos V, Dawson G. 1993. Protein kinase C-epsilon is a developmentally regulated, neuronal isoformin the chick embryo central nervous system. *J Neurosci Res* 35(5):488-98.

Mark RJ, Hensley K, Butterfield DA, Mattson MP. 1995. Amyloid beta peptide impairs ion-motive ATPase activities: evidence for a role in loss of neuronal calcium homeostasis and cell death. *J Neurosci* 15(9):6239-49.

Masters CL, Simms G, Weinman NA, Multhaup G, McDonald BL, Beyreuther K. 1985. Amyloid plaque core protein in Alzheimer disease and Down syndrome. *PNAS* 82(12):4245-49.

Matsuzaki M, Honkura N, Ellis-Davies GCR, Kasai H. 2004. Structural basis of long-term potentiation in single dendritic spines. *Nature* 429:761-66.

Mausbach BT, Chattillion EA, Roepke SK, Ziegler MG, Milic M, von Kanel R, Dimsdale JE, Mills PJ, Patterson TL, Allison MA, et al. 2012. A longitudinal analysis of the relations among stress, depressive symptoms, leisure satisfaction, and in endothelial function in caregivers. *Health Psychol* 31(4):433-40.

McGuinness B, Todd S, Passmore P, Bullock R. 2009. Blood pressure lowering in patients without prior cerebrovascular diseasefor prevention of cognitive impairment and dementia. 2009. *Cochrane Database of Systematic Reviews* doi:10.1002/14651858.

McKhann GM, Knopman DS, Chertkow H, Hyman BT, Jack CR, Kawas CH, Klunk WE, Koroshetz WJ, Manly JJ. 2011. The diagnosis of dementia due to Alzheimer's disease: recommendations from the National Institute on Aging-Alzheimer's Association workgroups on diagnostic guidelines for Alzheimer's disease. *Alzheimers Dement* 7(3):263-69.

Meng X, D'Arcy C. 2012. Education and dementia in the context of the cognitive reserve hypothesis: a systematic review with meta-analyses and qualitative analyses. PLoS ONE 7(6): e38268. doi:10.1371/journal.pone.0038268

Morris R. 1984. Developments of a water-maze procedure for studying spatial learning in the rat. *J Neurosci Methods* 11:47-60.

Nagata T, Yamamoto S, Yaguchi T, Iso H, Tanaka A, Nishizaki T. 2005. The newly synthesized linoleic acid derivative DCP-LA ameliorates memory deficits in animal models treated with amyloid beta peptide and scopolamine. *Psychogeriatrics* 5(4):122-26.

Naslund J, Haroutunian V, Mohs R, Davis KL, Davies P, Greengard P, Buxbaum JD. 2000. Correlation between elevated levels of amyloid beta peptide in the brain and cognitive decline. *J Am Med Soc* 283(12):1571-77.

Nelson TJ, Cui C, Luo Y, Alkon DL. 2009. Reduction of beta amyloid by novel protein kinase C-epsilon activators. *J Biol Chem* 284(50):34514-21.

Newton AC. 1995. Protein kinase C: structure, function, and regulation. *J Biol Chem* 270(48):28495-98.

Newton AC. 2001. Protein kinase C; structural and spatial regulation by phosphorylation, cofactors, and macromolecular interactions. *Chem Rev* 101:2353-64.

Nishizaki T, Ikeuchi Y, Matsuoka T, Sumikawa K. 1997a. Short-term depression and long-term enhancement of ACh-gated channel currents induced by linoleic and linolenic acid. *Brain Res* 751(2):253-58.

Nishizaki T, Ikeuchi Y, Matsuoka T, Sumikawa K. 1997b. Oleic acid enhances ACh receptor currents by activation of calcium/calmodulin dependent protein kinase II. *NeuroReport* 8(3):597-601.

Nishizaki T, Nomura T, Matsuoka T, Enikolopov G, Sumikawa K. 1999. Arachidonic acid induces a long-lasting facilitation of hippocampal synaptic transmission by modulating PKC activity and nicotinic ACh receptors. *Mol Brain Res* 69(2):263-72.

Oakley H, Cole SL, Logan S, Maus E, Shao P, Craft J, Guillozet-Bongaarts A, Ohno M, Disterhoft J, van Eldik L et al. 2006. Intraneuronal beta amyloid aggregate, neurodegeneration, and neuron loss in transgenic mice with five familial Alzheimer's disease mutations: potential factors in amyloid plaque formation. *J Neurosci* 26(40):10129-40.

Oddo S, Caccamo A, Shepherd JD, Murphy MP, Golde TE, Kayed R, Metherate R, Mattson MP, Akbari Y, LaFerla FM. 2003. Triple-transgenic model of Alzheimer's disease with plaques and tangles: intracellular amyloid beta and synaptic dysfunction. *Neuron* 39(3):409-21.

Orr JW, Newton AC. 1994. Requirement for negative charge on 'activation loop' of protein kinase C. *J Biol Chem* 269(44):27715-18.

Ortman JM, Velkoff VA, Hogan H. 2014. An aging nation: The older population in the United States, current population reports, P25-1140. U.S. Census Bureau, Washington, D.C.

Pastorino L, Sun A, Lu P, Zhou XZ, Balastik M, Finn G, Wulf G, Lim J, Li S, Li X et al. 2006. The prolyl isomerase Pin1 regulates amyloid precursor protein processing and amyloid beta production. *Nature* 440:528-34.

Paxinos G, Watson C. 1998. The Rat Brain in Stereotaxic Coordinates, Fourth Edition. Academic Press, San Diego, CA.

Petrasek T, Skurlova M, Maleninska K, Vojtechova I, Kristofikova Z, Matuskova H, Sirova J, Vales K, Ripova D, Stuchlik A. 2016. A rat model of Alzheimer's disease based on Abeta-42 and pro-oxidative substances exhibits cognitive deficit and alterations in glutamatergic and cholinergic neurotransmitter systems. *Front Aging Neurosci* 8(83): doi:10.3389/fnagi.2016.00083.

Pike KE, Savage G, Villemagne VL, Ng S, Moss SA, Maruff P, Mathis CA, Klunk WE, Masters CL, Rowe CC. 2007. B-amyloid imaging and memory in non-demented individuals: evidence for preclinical Alzheimer's disease. *Brain* 130(11):2837-44.

Plassman BL, Langa KM, Fisher GG, Heeringa SG, Weir DR, Ofstedal MB, Burke JR, Hurd MD, Potter GG, Rodgers WL. 2007. Prevalence of dementia in the United States: the aging, demographics, and memory study. *Neuroepidemiol.* 29(1-2):125-32.

Pogocki D, Schoneich C. 2002. Redox propertied of Met-35 in neurotoxic beta-amyloid peptide: a molecular modeling study. *Chem Res Toxicol* 15(3):408-418.

Potter GG, Plassman BL, Burke JR, Kabeto MU, Langa KM, Llewellyn DJ, Rogers MAM, Steffens DC. 2009. Cognitive performance and informant reports in the diagnosis of cognitive impairment and dementia in African Americans and whites. *Alzheimers Dement* 5(6):445-53.

Power MC, Weuve J, Gagne JJ, McQueen MB, Viswanathan A, Blacker D. 2011. The association between blood pressure and incident Alzheimer's disease: a systematic review and meta-analysis. *Epidemiology* 22(5):646-59.

Prekeris R, Mayhew MW, Cooper JB, Terrian DM. 1996. Identification and localization of an actin binding motif that is unique to the epsilon isoform of protein kinase C and participates in the regulation of synaptic function. *J Cell Biol* 132(1):77-90.

Profeno LA, Porsteinsson AP, Faraone SV. 2010. Meta-analysis of Alzheimer's disease risk with obesity, diabetes, and related disorders. *Biological Psychiatry* 67(6):505-12.

Reisberg B, Doody R, Stoffler A, Schmitt F, Ferris S, Mobius HJ. 2003. Memantine in moderate-to-severe Alzheimer's disease. *N Engl J Med* 348:1333-41.

Roivainen R, McMahon T, Messing RO. 1993. Protein kinase C isozymes hat mediate enhancement of neurite outgrowth by ethanol and phorbol esters in PC12 cells. *Brain Res* 624(1-2):85-93.

Roivainen R, Hundle B, Messing RO. 1995. Ethanol enhances growth factor activation of mitogen activated protein kinases by a protein kinase C-dependent mechanism. *Proc Natl Acad Sci* 92:1891-95.

Rolland Y, van Kan GA, Vellas B. 2008. Physical activity and Alzheimer's disease: from prevention to therapeutic perspectives. *J Am Med Dir Assoc* 9: 390-405.

Romero JP, Benito-Leon J, Mitchell AJ, Trincado R, Bermejo-Pareja F. 2014a. Under reporting of dementia deaths on death certificates using data from a population-based study. *J Alzheimers Dis* 39(4):741-8.

Romero JP, Benito-Leon J, Louis ED, Bermejo-Pareja F. 2014b. Under reporting of dementia deaths on death certificates: A systematic review of population-based cohort studies. *J Alzheimers Dis* 41(1):213-21.

Saitoh N, Hori T, Takahashi T. 2001. Activation of the epsilon isoform of protein kinase C in the mammalian nerve terminal. *Proc Natl Acad Sci* 98(24):14017-21.

Samper-Ternent R, Kuo YF, Ray LA, Ottenbacher KJ, Markides KS, Al Snih S. 2012. Prevalence of health conditions and predictors of mortality in oldest old Mexican Americans and non-Hispanic whites. *J Am Med Directors Assoc* 13(3):254-59.

Sando SB, Melquist S, Cannon A, Hutton M, Sletvold O, Saltvedt I, White LR, Lydersen S, Aasly J. 2008. Risk-reducing effect of education in Alzheimer's disease. *In J Geriatr Psychiatry* 23:1156-62.

Saunders AM, Strittmatter WJ, Schmechel D, St George-Hyslop PH, Pericak-Vance MA, Joo SH, Rosi BL, Gusella JF, Crapper-MacLachlan DR, Alberts MJ et al. 1993. Association of apolipoprotein E allele E4 with late-onset familial and sporadic Alzheimer's disease. *Neurology* 43(8):1467-72.

Schneider LS, Dagerman KS, Higgins JPT, McShane R. 2011. Lack of evidence for the efficacy of memantine in mild Alzheimer disease. *Arch Neurol* 68(8):991-98.

Schulz R and Beach SR. 1999. Caregiving as a risk factor for mortality: The caregiver health effects study. *JAMA* 282:2215-60.

Schulz R, Belle SH, Czaja SJ, McGinnis KA, Stevens A, Zhang S. 2004. Long-term care placements of dementia patients and caregiver health and well-being. *JAMA* 292(8):961-7.

Sen A, Hongpaisan J, Wang D, Nelson TJ, Alkon DL. PKC epsilon promotes synaptogenesis through membrane accumulation of the postsynaptic density protein PSD-95. *J Biol Chem* 291:16462-76.

Seshadri S, Beiser A, Kelly-Hayes M, Kase CS, Au R, Kannel WB, Wolf PA. 2006. The lifetime risk of stroke: estimates from the Framingham Study. *Stroke* 37(2):345–50.

Shapira R, Silberberg SD, Ginsburg S, Rahamimoff R. 1987. Activation of protein kinase C augments evoked transmitter release. *Nature* 325:58-60.

Shaw WS, Patterson TL, Ziegler MG, Dimsdale JE, Semple SJ, Grant I. 1999. Accelerated risk of hypertensive blood pressure recordings among Alzheimer caregivers. *J Psychosom Res* 46(3):215-27.

Sheng M, Hoogenraad CC. 2007. The postsynaptic architecture of excitatory synapses: a more quantitative view. *Annu Rev Biochem* 76:823-47.

Shirai Y, Adachi N, Saito N. 2008. Protein kinase C: function in neurons. *FEBS Journal* 275:3988-94.

Shrestha BR, Vitolo OV, Joshi P, Lordkipanidze T, Shelanski M, Dunaevsky A. 2006.

Amyloid beta peptide adversely affects spine number and motility in hippocampal neurons. *Mol Cell Neurosci* 33:274-82.

Slack BE, Nitsch RM, Livneh E, Kunz Gm, Breu J, Eldar H, Wurtman RJ. 1993. Regulation by phorbol esters of amyloid precursor protein release from Swiss 3T3 fibroblasts overexpressing protein kinase C alpha.

Snyder EM, Nong Y, Almeida CG, Paul S, Moran T, Choi EY, Nairn AC, Salter MW, Lombroso PJ, Gouras GK, et al. 2005. Regulation of NMDA receptor trafficking by amyloid beta. *Nature Neuroscience* 8:1051-58.

Sofi F, Valecchi D, Bacci D, Abbate R, Gensini GF, Casini A, Macchi C. Physical activity and risk of cognitive decline: a meta-analysis of prospective studies. *J Intern Med* 269:107-17.

Spasic D, Annaert W. 2008. Building gamma-secretase – the bits ands pieces. *J Cell Sci* 121:413-20.

Sperling RA, Aisen PS, Beckett LA, Bennett DA, Craft S, Fagan AM, Iwatsubo T, Jack CR, Kaye J, Montine TJ et al. 2011. Toward defining the preclinical stages of Alzheimer's disease: recommendations from the National Institute on Aging-Alzheimer's Association workgroups on diagnostic guidelines for Alzheimer's disease. *Alzheimers Dement* 7(3):280-92.

Srinivasan S, Avadhani NG. 2012. Cytochrome c oxidase dysfunction in oxidative stress. *Free Radic Biol Med* 53(6):1252-63.

Steinberg R, Harari OA, Lidington EL, Boyle JJ, Nohadani M, Samarel AM, Ohba M, Haskard DO, Mason JC. 2007. A protein kinase c-epsilon anti-apoptotic kinase signaling complex protects human vascular endothelial cells against apoptosis through induction of Bcl-2. *J Biol Chem* 282:32288-97.

Suh Y, Checler F. 2002. Amyloid precursor protein, presenilins, and alpha-synuclein: molecular pathogenesis and pharmacological applications in Alzheimer's disease. *Pharmacol Rev* 54:469-525.

Sultana R, Boyd-Kimball D, Poon HF, Cai J, Pierce WM, Klein JB, Markesbery WR, Zhou XZ, Lu KP, Butterfield DA. 2006. Oxidative modification and down-regulation of Pin1 in Alzheimer's disease hippocampus: a redox proteomics analysis. *Neurobiol Aging* 27:918-25.

Suzuki N, Cheung TT, Cai X, Odaka A, Otvos L, Eckman C, Golde TE, Younkin SG. 1994. An increased percentage of long amyloid beta protein secreted by familial amyloid beta protein precursor mutants. *Science* 264(5163): 1336-1340.

Tamagno E, Bardini P, Obbili A, Vitali A, Borghi R, Zaccheo D, Pronzato MA, Danni O, Smith MA, Perry G et al. 2002. Oxidative stress increases expression and activity of BACE in NT_2 neurons. *Neurobiol Disease* 10:279-88.

Tamagno E, Parola M, Bardini P, Piccini A, Borghi R, Guglielmotto M, Santoro G, Davit A, Danni O, Smith MA, et al. 2005. Beta-site APP cleaving enzyme up-regulation induced by 4-hydroxynonenal is mediated by stress-activated protein kinase pathways. *J Neurochem* 92(3):628-36.

Tanaka A, Nishizaki T. 2003. The newly synthesized linoleic acid derivative FR236924 induces a long-lasting facilitation of hippocampal neurotransmission by targeting nicotinic acetylcholine receptors. *Bioorganic Medicinal Chem Letters* 13:1037-40.

Tatarnikova OG, Orlov MA, Bobkova NV. 2015. Beta amyloid and tau protein: structure, interaction, and prion-like properties. *Biochemistry* 80(13):1800-19.

Vagelatos NT, Eslick GD. 2013. Type 2 diabetes as a risk factor for Alzheimer's disease: the confounders, interactions, and neuropathology associated with this relationship. *Epidemiol Rev* 35(1):152-60.

Vemuri P, Wiste HJ, Weigand SD, Knopman DS, Trojanowski JQ, Shaw LM, Bernstein MA, Aisen PS, Weiner M, Petersen RC et al. 2010. Serial MRI and CSF biomarkers in normal aging, MGI, and AD. *Neurology* 75(2):143-51.

Villemagne VL, Burnham S, Bourgeat P, Brown B, Ellis KA, Salvado O, Szoeke C, Macauley SL, Martins R, Maruff P et al. 2013. Amyloid β deposition, neurodegeneration, and cognitive decline in sporadic Alzheimer's disease: a prospective cohort study. *Lancet Neurol* 13:70044-49.

Vitaliano PP, Scanlan JM, Zhang J, Savage MV, Hirsch IB, Siegler I. 2002. A path model of chronic stress, the metabolic syndrome, and coronary heart disease. *Psychosom Med* 64:418-35.

von Kanel R, Dimsdale JE, Mills PJ, Ancoli-Israel S, Patterson TL, Mausbach BT, Grant I. 2006. Effect of Alzheimer caregiving stress and age on frailty markers interleukin-6, C-reactive protein, and D-dimer. *J Gerontol A Biol Sci Med Sci* 61(9):963-9.

Walsh DM, Klyubin I, Fadeeva JV, Cullen WK, Anwyl R, Wolfe MS, Rowan MJ, Selkoe DJ. 2002. Naturally secreted oligomers of amyloid beta protein potently inhibit hippocampal long-term potentiation in vivo. *Nature* 416:535-39.

Ward A, Tardiff S, Dye C, Arrighi HM. 2013. Rate of conversion from prodromal Alzheimer's disease to Alzheimer's dementia: a systematic review of the literature. *Dement Geriatr Cogn Disord* 3:320-32.

Weuve J, Hebert LE, Scherr PA, Evans DA. 2014. Deaths in the United States among persons with Alzheimer's disease (2010-2050). *Alzheimers Dement* 10(2):e40-6.

Wilson RS, Segawa E, Boyle PA, Anagnos SE, Hizel LP, Bennett DA. 2012. The natural history of cognitive decline in Alzheimer's disease. *Psychol Aging.* 27(4):1008-17.

World Health Organization. 2004. International Statistical Classification of Diseases and Related Health Problems. 10th revision. 2nd edition. Geneva, Switzerland.

World Health Organization. 2012. Dementia: a public health priority. Geneva: World Health Organization.

Xu JQ, Murphy SL, Kochanek KD, Bastian BA. 2016. Deaths: Final data for 2013. National vital statistics reports; vol 64 no 2. Hyattsville, MD: National Center for Health Statistics.

Yaguchi T, Fujikawa H, Nishizaki T. 2010. Linoleic acid derivative DCP-LA protects neurons from oxidative stress-induced apoptosis by inhibitingcaspase-3/-9 activation. *Neurochem Res* 35(5):712-17.

Yamamoto C, Higashima M, Sawada S. 1987. Quantal analysis of potentiating action of phorbol ester on synaptic transmission in the hippocampus. *Neurosci Res* 5(1):28-38.

Yao J, Du H, Yan S, Fang F, Wang C, Lue L, Guo L, Chen D, Stern DM, Gunn Moore FJ et al. 2011. Inhibition of amyloid beta peptide binding alcohol dehydrogenase-amyloid beta interaction reduces amyloid beta accumulation and improves mitochondrial function in a mouse model of Alzheimer's disease. *J Neurosci* 31(6):2313-20.

Yatin SM, Varadarajan S, Link CD, Butterfield DA. 1999. In vitro and in vivo oxidative stress associated with Alzheimer's amyloid beta peptide 1-42. *Neurobiol Aging* 20:325-30.

Zhang Y, McLaughlin R, Goodyer C, LeBlanc A. 2002. Selective cytotoxicity of intracellular amyloid beta peptide 1-42 through p53 and Bax in cultured primary human neurons. *J Cell Biol* 156(3):519-29.

Zhao D, Watson JB, Xie C. 2004. Amyloid beta prevents activation of calcium/calmodulin-dependent protein kinase II and AMPA receptor phosphorylation during hippocampal long-term potentiation. *J Neurophysiol* 92:2853-58.

Zhu G, Wang D, Lin Y, McMahon T, Koo EH, Messing RO. 2001. Protein kinase C-epsilon suppresses amyloid beta production and promotes activation of alpha secretase. *Biochem Biophes Res Comm* 285(4):997-1006.